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Amyotrophic Lateral Sclerosis
Analysis of graft survival in a trial of stem cell transplant in ALS

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Abstract

Objective: The first US Food and Drug Administration–approved clinical trial to treat amyotrophic lateral sclerosis (ALS) with neural stem cell–based therapy is in progress. The goal of the current study was to identify and assess the survival of human spinal cord–derived neural stem cells (HSSCs) transplanted into the spinal cord in patients with ALS. Methods: Spinal cords transplanted with HSSCs were examined from six autopsy cases. Homogenized tissues were interrogated for the presence of donor versus recipient DNA using real-time PCR methods (qPCR). Fluorescence in situ hybridization (FISH) was performed using DNA probes for XY chromosomes to identify male donor HSSCs in one female case, and immunohistochemistry (IHC) was used to characterize the identified donor cells. Results: Genomic DNA from donor HSSCs was identified in all cases, comprising 0.67–5.4% of total tissue DNA in patients surviving 196 to 921 days after transplantation. In the one female patient a “nest” of cells identified on H&E staining were XY-positive by FISH, confirming donor origin. A subset of XY-positive cells labeled for the neuronal marker NeuN and stem cell marker SOX2. Interpretation: This is the first study to identify human neural stem cells transplanted into a human spinal cord. Transplanted HSSCs survived up to 2.5 years posttransplant. Some cells differentiated into neurons, while others maintained their stem cell phenotype. This work is a proof of concept of the survival and differentiation of human stems cell transplanted into the spinal cord of ALS patients.

Introduction

Amyotrophic lateral sclerosis (ALS) is a rare, yet fatal neurodegenerative disease resulting from progressive degeneration of upper and lower motor neurons. ALS patients typically die within 3–5 years from diagnosis due to respiratory failure. Therapeutic options for ALS are limited to a single medication and supportive care, thus driving the search for innovative approaches to slow disease progression and improve survival.1–4 Our group is conducting the first US Food and Drug Administration–approved clinical trial to surgically transplant human spinal cord–derived stem cells (HSSCs) into the spinal cord of ALS patients. The details of the rationale, surgical methods, and phase I results of this trial have been published previously.5–8 Briefly, the injection of HSSCs into the spinal cord is safe, although the efficacy of this approach is not yet known.

Previous reports from therapeutic trials of intraspinal injection of stem cells were not able to demonstrate the presence or localization of cells in living patients due to the lack of intracellular markers that could be identified...
by imaging.9–12 Similarly, the stem cells used in our trial were not identifiable during life. This report focuses on postmortem identification of transplanted cells using qPCR and, in the one female patient autopsied to date, fluorescence in situ hybridization (FISH) using XY markers for the male donor cells.

Subjects and Methods

Subjects

The clinical trial design and initial results are published previously.5–7 This report focuses on six ALS patients who came to autopsy. Five males received injections of HSSCs into the lumbar spinal cord, and one female received injections into the cervical spinal cord.

Human neural stem cells and surgical injection into spinal cord

Details of the derivation, viability of the stem cells, and the clinical trial were described previously. Briefly, human neural stem cells (HSSCs) HSSC NSI-566RSC (Neuralstem, Inc., Rockville, MD) were derived from a single source 8-week gestation human fetal spinal cord, and serially expanded in culture. Of the six autopsy cases reported here, three patients received five unilateral injections and two patients received five bilateral injections (total 10) spaced 4 mm apart into the lumbar spinal cord at levels L2–L4. One patient received five unilateral injections into the cervical spinal cord at levels C3–C5. All injections contained a suspension of 100,000 cells in 10 μL volume.

Immunosuppression

All patients were placed on immunosuppressive therapy consisting of prednisone, basiliximab, mycophenolate mofetil, and tacrolimus.5 Tolerance of the immunosuppressive regimen was variable, and five of the six patients eventually stopped immunosuppressive medications. The period of time on immunosuppressive medications postoperatively and prior to death is presented in Table 1.

Spinal cord collection at autopsy

All patients were transported to Emory University Hospital for autopsy. The entire spinal cord was removed and the region of injection was identified by the location of the dural sutures overlying the transplantation field, as well as the matching of vascular anatomy to images taken at the time of surgery (see Fig. 2). The region of interest was cut in 0.5 cm sequential cross-section blocks (“bread loafed”) and alternate blocks were frozen on dry ice or fixed for 2–3 days in 4% paraformaldehyde. There were ~20 blocks for each spinal cord, 10 frozen and 10 fixed. The frozen blocks for each of the six patients were sampled for qPCR analysis by excising a core of anterior and lateral cord using the back end of a sterile micropipette tip, and depositing the tissue into a sterile 1.5 mL eppendorf tube. Two core samples were obtained from each block. The fixed blocks were embedded in paraffin and sectioned for routine histochemistry (hematoxylin and eosin stain, Luxol fast blue [LFB] stain), immunohistochemistry (IHC), and FISH.

Quantitative real-time PCR

Using core samples from the frozen blocks of spinal cord (each block separated by 1 cm) from six patients, the presence of the genomic DNA sequence unique to the donor HSSCs NSI-566RSC was determined by qPCR on the 7500 SDS System (Applied Biosystem, Foster City, CA). DNA from frozen tissue was extracted using the QIAamp DNA Mini Kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). During the screening test, a 96-well screening plate (#5002645; Celsera, Alameda, CA) containing two sets of 34 chimerism assays (CA001 to CA034 qPCR primers and fluorescence probes) plus an additional assay (CA999) were used for

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Gender</th>
<th>HSSC injection and region of SC</th>
<th>Number of days on FK506</th>
<th>Number of days on MMF</th>
<th>Number of days IM meds discontinued before death</th>
<th>Survival days</th>
<th>% Donor DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>U/L</td>
<td>177</td>
<td>165</td>
<td>216</td>
<td>394</td>
<td>0.06–5.40</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>Bi/L</td>
<td>107</td>
<td>503</td>
<td>67</td>
<td>572</td>
<td>0.18–0.93</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>Bi/L</td>
<td>259</td>
<td>259</td>
<td>0</td>
<td>259</td>
<td>0.03–2.39</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>U/L</td>
<td>189</td>
<td>192</td>
<td>133</td>
<td>325</td>
<td>0.07–4.20</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>U/A</td>
<td>94</td>
<td>283</td>
<td>638</td>
<td>921</td>
<td>0.14–0.67</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>U/C</td>
<td>139</td>
<td>134</td>
<td>57</td>
<td>196</td>
<td>0.06–0.96</td>
</tr>
</tbody>
</table>

HSSC, human neural stem cells; SC, spinal cord; FK506, tacrolimus; MMF, mycophenolate mofetil; IM, immunomodulatory; U, unilateral; Bi, bilateral; L, lumbar; C, cervical.
were rinsed in dH2O for 3 min. Slides were pretreated using Pretreatment Reagent (Abbott Molecular). Briefly, slides were preheated on a hot plate at 56°C overnight, deparaffinized three times 5 min each in Americalear, twice in 100% EtOH 1 min each, 0.2 N HCl for 20 min, and rinsed in dH2O for 3 min. Slides were pretreated using Pretreatment Reagent (Abbott Molecular) for 30 min at 80°C, rinsed in dH2O for 3 min, and digested in Protease 1 (Abbott Molecular) for 40 min at 37°C. Sections were rinsed in dH2O for 3 min, fixed in 10% buffered formalin for 10 min, rinsed in dH2O for 3 min, dehydrated in a series of graded ethanol (70%, 85%, 100%) 1 min each, and air dried. XY probes were added onto the sections, covered by coverslip, sealed with rubber cement, codenatured, and incubated at 37°C for 14–16 h. Rubber cement and coverslips were removed from the slides and posthybridization washes were conducted as follows: preheated 2X SSC/0.3% NP-40 at 72 ± 1°C for 2 min, air dried, DAPI II (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Abbott Molecular, Abbott Park, Illinois) was added, and sections were re-covered with coverslips. Slides were stored in the dark at −20°C overnight before imaging. Fluorescently labeled sections were analyzed using an Olympus microscope with the appropriate filters (Olympus, Melville, NY). Images were captured using CytoVision® (Leica Biosystems, Buffalo Grove, IL). For quantitative analysis, hematoxylin and eosin (H&E)-stained sections containing nests of putative stem cells were identified and outlined. Corresponding regions were marked on FISH sections and 100 cells each within these regions were counted by two independent readers. The percentage of XY- and XX-positive cells was computed from the total 200 cells counted.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded spinal cord sections (4 μm) were deparaffinized and IHC was performed on a DAKO Autostainer using antibodies for NeuN (mouse monoclonal, 1:800; Millipore, Billerica, MA), SOX2 (goat polyclonal, 1:50; R&D Systems, Minneapolis, MN), glial fibrillary acidic protein (GFAP) (mouse monoclonal, 1:100; Dako, Carpinteria, CA), OLIG2 (rabbit polyclonal, 1:100; Lifespan Biosciences, Seattle, WA), and LCA (CD45; monoclonal, 1:640; Dako, Carpinteria, CA) and costained with hematoxylin. Avidin–biotin–peroxidase complex was used to detect the antibodies using 3, 3’-diaminobenzidine (DAB) as the chromogen. Standard positive controls and normal sera without primary antibodies as negative controls were used.

**Results**

**Subject demographics**

Demographic data for the six cases, five males and one female, are presented in Table 1. Five patients received tacrolimus for 94–259 days and mycophenolate mofetil for 134–503 days posttransplant. One patient was on both immunosuppressive drugs until the time of death. Survival posttransplant surgery ranged from 196 to 921 days. Patients 1, 4, and 5 received unilateral lumbar injections, Patients 2 and 3 bilateral lumbar, and Patient 6 unilateral cervical injections.
Identification of donor DNA

To assess HSSCs graft survival, the presence of donor DNA within the recipient spinal cord was measured by qPCR. Sixteen core samples, eight from each side of the spinal cord, were analyzed from each case. In all cases, donor NSI-566RSC DNA was identified in several of the samples, with maximum percentage of donor DNA in each case ranging from 0.67% to 5.4% of total DNA (Fig. 1).

Neuropathology and localization of transplanted cells

At autopsy, localization of the site of transplant was accomplished by the presence of dural sutures and matching of the vascular anatomy between intraoperative videos and postmortem tissue. Gross inspection of the cord surface (Fig. 2A and B) and of the cross sections (Fig. 2C) did not reveal any tissue disruption, discoloration, or cavitation. Indeed, the sites of injection could not be grossly identified. Each paraffin-embedded block was sectioned through its entirety and stained with H&E. In three of the six cases we could identify one or more needle tracks corresponding to injection sites (Fig. S1). There was otherwise no tissue disruption, discoloration, or cavitation of sectioned tissue. In four cases (three male, one female), we identified “nests” of round cells with little cytoplasm that did not correspond to normal microanatomy; these cells did not stain with the GFAP or with the neuronal precursor protein doublecortin. Representative images from male Patient 4 spinal cord show histological staining with H&E (Fig. 2D and G) and LFB (Fig. 2E and H). IHC shows a lack of labeling with GFAP (Fig. 2F and I). Based on location and staining properties, these cells were interpreted to be transplanted HSSCs.

A “nest” of putative HSSCs was identified in one female patient as previously reported and shown in Figure 3A and B. This region was devoid of GFAP staining (Fig. 3C). Thus, taking advantage of the gender differences in male donor HSSCs transplanted in one female ALS patient, we targeted XY chromosomes for FISH analysis. Vysis Spectrum orange X probe and Spectrum green Y probe and counterstained with DAPI were used for FISH on two regions of the spinal cord – one region from the HSSCs injection site and one from a noninjected site that served as a negative control (Fig. S2).

Spinal cord sections from the injection site showed many XY-chromosome–positive cells within the region containing the putative HSSCs (Fig. 3D and E). XX-chromosome–positive cells were also noted in close proximity to XY-chromosome–positive cells (asterisks). Visual assessment of the DAPI labeling of XY-chromosome–positive cells showed that the nuclear morphology was intact and appeared normal with no evidence of condensation or fragmentation. Control sections from the lumbar spinal cord, distant from the injection site, showed exclusively XX-chromosome–positive cells (Fig. S2). Quantification of randomly selected areas within the injection site showed 36% XX- and 64% XY-chromosome–positive cells, while noninjection site regions had 100% XX-chromosome–positive cells. The female patient survived 196 days postsurgery demonstrating that many transplanted HSSCs survived long term. Taken together, these data demonstrate the identification and survival of intraspinal transplanted HSSCs into the spinal cord of ALS patients.

Characterization of transplanted HSSCs

Tissue sections proximate to those demonstrating the presence of donor cells by FISH were interrogated with
antibodies for various cell fate markers and standard H&E staining (Fig 3F). Sections were stained for the transcription factor SOX2, a marker of multipotent stem cells in embryos and in adults, NeuN (differentiating neurons), OLIG2 (developing and differentiated oligodendrocytes), and GFAP (astrocytes). There were many SOX2-positive cells in regions of the spinal cord corresponding to the locations of XY-positive donor cells (Fig. 3G). Visual analysis of the sections showed many more XY-chromosome-positive cells compared to SOX2-positive cells. There were also NeuN-positive cells located within regions containing XY-positive donor cells (Fig. 3H). There was no labeling of OLIG2 suggesting that the HSSCs did not take on oligodendrocyte fate (data not shown). There was also no labeling of HSSCs with LCA (Fig. S4) suggesting the absence of leukocyte infiltration to the graft region. GFAP labeling was observed throughout the spinal cord sections, but did not appear to colocalize with the XY-positive cells. Thus, there were many more XY-positive cells than NeuN-positive and SOX2-positive cells combined, suggesting that transplanted HSSCs had differentiated beyond stem cell pluripotency but not to a specific neuronal or glial population.

**Discussion**

There are three major findings from this autopsy series of ALS patients undergoing spinal cord transplantation with HSSCs. First, DNA analysis focused on the regions of transplant identified DNA from donor HSSCs in all patients up to almost 3 years following surgery. Only one of these patients tolerated full immunosuppression until the time of death, suggesting that continuous immunosuppression is not necessary for continued survival of transplanted cells,
though partial rejection cannot be excluded. Second, FISH analysis using Y-chromosome probes was able to identify and localize HSSCs in one female patient. Third, immunohistochemical labeling of the HSSCs showed evidence of neuronal differentiation with the expression of NeuN by some of the XY-positive cells. Other cells continued to express the stem cell marker SOX2, which is a prominent marker of these HSSCs prior to transplantation. We did not see labeling with oligodendrocyte marker OLIG2, and the astrocyte marker GFAP was difficult to interpret due to the diffuse expression throughout the spinal cord. However, there was a focal reduction in GFAP staining identified in areas of deposition of the transplanted cells (Figs. 2, 3). Taken together, this is the first study showing HSSCs graft survival and differentiation following transplantation into human spinal cord.

A critical component to the success of this clinical trial is the survival of transplanted HSSCs in the spinal cord.

Figure 3. Donor HSSC localization and characterization using XY chromosome FISH and IHC, respectively, in a female ALS patient. H&E staining shows nests of cells in the female spinal cord (A) (circle). High-power image corresponding to the nest of cells outlined in (A) is shown in (B). Proximal sections stained with GFAP show lack of labeling of nest of cells (C). FISH labeling shows numerous X (red) Y (green)-positive cells counterstained with DAPI (blue) (D). Asterisks shows XX-positive recipient cells in the surrounding regions. Inset image from (D) is shown in (E). Donor HSSCs are positive for XY (solid arrow). H&E labeling of HSSCs graft (arrow) (F) label with SOX2 and (G) and NeuN (H). Scale bars: 1 mm (A), 50 μm (B–D), 10 μm (E), 100 μm (F–H). HSSC, human spinal cord-derived stem cell; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; ALS, amyotrophic lateral sclerosis; GFAP, glial fibrillary acidic protein.
Our data show that donor HSSC DNA was present 196–921 days posttransplant survival. Because this is a first-in-human trial, it is not clear whether immunosuppressive therapy is necessary for long-term survival of the transplanted cells. Interestingly, donor DNA was detected by qPCR 57–638 days posttransplant after the discontinuation of immunosuppression therapy, and FISH analysis identified donor cells in situ at 196 days posttransplant. In this instance, the subject had not been on immunosuppressive drugs for 57 days prior to death. We found no correlation of DNA content to survival period after discontinuation of immunosuppressant medications. These data demonstrate that transplanted HSSCs can survive for a prolonged period, even in the absence of immunosuppression, and raise the interesting question of how long, if at all, a subject requires immunosuppressive medication following HSSC transplantation.

The presence of donor DNA using qPCR methods complimented the histological assessments of HSSC survival after transplantation. Our H&E staining identified non-native nests of cells near the injection site in the spinal cord of three males and one female, which we suspected to be of donor origin. In the female patient, XY-chromosome–positive labeling with FISH confirmed that the non-native nests of cells were the transplanted HSSCs. Due to the fact that the H&E labeling identified similar nests of cells in the male patients, we suspect that all transplanted ALS patients had successful HSSCs graft survival. Five of six patients were males, thus limiting our ability to use FISH analysis to distinguish HSSCs based on gender. Taken together, this is the first therapeutic trial localizing HSSCs following intraspinal injection. This finding is important as it demonstrates that human spinal cord provides a permissive microenvironment for allogenic fetal-derived transplant and the feasibility of FISH analysis for future clinical trials.

While the current approach demonstrated the survival of graft cells, it is difficult to rigorously quantify the percentage of grafted cells surviving. Such a measure is relevant to the question of how much immunosuppression to use and for how long. It is also relevant to assessing the effectiveness of the graft. That is, one would expect that the number of surviving cells would directly impact the therapeutic potential of the treatment. We have currently begun using iron oxide nanoparticle loading of donor cells prior to transplantation into large animals. This approach appears not to perturb cell health or differentiation in vitro. It also allows for visualization of the grafts to assess surgical accuracy in the immediate postoperative period. Finally, postmortem iron staining allows for quantification of graft survival, distribution, and accuracy (N. N. Boulis, pers. comm. 2014).

The therapeutic concept of cell transplantation into the spinal cord is based on the idea that these cells may survive, possibly differentiate, and provide trophic support, acting as “nurse cells” for endogenous motor neurons. This concept is supported by preclinical studies in animals showing that these HSSCs survive, differentiate, and integrate into the recipient spinal cord environment. In the SOD1 rat model of ALS these stem cells differentiated into glial cells and interneurons that functionally integrated into preexisting neural circuitry. In these studies, double labeling with IHC showed 70.4% of the human nuclear protein (HNU) colabeled with class III β-tubulin (TUJ1), 19.2% with stem cell marker Nestin, and 1.3% with GFAP suggesting extensive neuronal differentiation. In addition, these HSSCs produced glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factors (BDNF), which may also provide local trophic support for motor neurons. In each of these experimental paradigms there was a positive effect of spinal cord stem cell transplantation on animal survival.

In this first-in-human study, characterization of HSSCs transplanted in human ALS spinal cord showed evidence of neuronal differentiation and maintenance of stem cell markers. Pretransplant examination of cytopsin prepared donor cells and stained with ICC (immunocytochemistry) revealed all were positive for SOX2, very few were positive for OLIG2, and no cells labeled with NeuN (Fig. S3). This finding is similar to previous work characterizing this HSSC cell line as 100% SOX1, 93.8% Nestin, 8% βIII-tubulin, and 0.75% OLIG2-positive cells. The one female patient where donor cells could be identified by FISH showed populations of transplanted cells that were labeled with SOX2, suggesting maintenance of the stem cell properties, and NeuN demonstrating differentiation into neuronal lineage after transplantation, which is consistent with the data from animal models. Future studies in human tissue will address the integration of donor cells into the spinal cord, and their effects on the environment of endogenous neurons.

In conclusion, this demonstration of survival and differentiation of transplanted HSSCs in ALS patients is an essential positive step to test the potential for therapeutic efficacy of using HSSCs as neuroprotective and/or neurorestorative treatment for ALS and possibly other neurodegenerative disorders.

Acknowledgment

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Author Contributions


Conflict of Interest

N. M. B.: personal fees, NeuralStem Inc., outside the current study; patents, licensed to NeuralStem Inc. T. H.: reports works for NeuralStem Inc. patents issued NeuralStem Inc. C. H.: personal fees from Roche Molecular Systems, Inc., unrelated to the current study. K. J.: personal fees from Neuralstem, Inc., during the study; personal fees and other from Neuralstem, Inc., outside the current study, patent issued Neuralstem, Inc. J. D. G.: grants from Neuralstem Inc.

Dr. Boulis reports personal fees from NeuralStem Inc, Outside the submitted work; in addition, Dr. Boulis has a patent floating cannula licensed to NeuralStem Inc, and a patent Spinal Platform licensed to NeuralStem Inc. Dr. Feldman reports grants from ALS Association, grants from NINDS. Dr. Glass reports grants from Neuralstem Inc, grants from NINDS. Dr. Hazel has a patent Transplantation of human neural cells for treatment of amyotrophic lateral sclerosis (ALS) issued. Dr. Hill reports personal fees from Roche Molecular Systems.

Dr. Johe reports personal fees from Neuralstem, Inc., during the conduct of the study; personal fees and other from Neuralstem, Inc., outside the submitted work; In addition, Dr. Johe has a patent Neuralstem, Inc. issued.

References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. H&E staining of a spinal cord section from Patient 6. Representative images show low and high


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power of needle tracks (arrows) corresponding to the injection site (A and B).

**Figure S2.** Fluorescence in situ hybridization (FISH) staining using DNA probes CEP X Spectrum orange (X chromosome) and Y Spectrum green (Y chromosome) in a spinal cord section of a female patient. A “noninjected” lumbar spinal cord section of the same female patient that received cervical injections shown in Figure 3 served as a negative control. H&E staining of the lumbar cord shows an absence of “nest” of cells (A and B). FISH staining shows exclusively red XX chromosome labeling counterstained with DAPI in all the cells (C, inset i & ii). Cervical spinal cord section from the “injected” region shows cells from the central canal with H&E (D), FISH (E), and a blood vessel (F) with XX chromosome labeling. These images show the specificity of the XY probe labeling shown in Figure 3. Scale bars: 1 mm (A), 50 μm (B–F), 10 μm (i, ii).

**Figure S3.** Immunocytochemistry staining of cyto spin prepared HSSC. All the HSSCs show positive staining for SOX2 (A). There are very few cells (circles) that have positive staining for OLIG2 (B). There are no NeuN-positive cells (C).

**Figure S4.** IHC staining of a cervical spinal cord section from a female patient. There is no LCA staining in the “nest” of putative HSSCs (A and B). In the same section, a blood vessel shows numerous LCA-positive labeling demonstrating the specificity of the antibody (C).
Notch Signaling Regulates Motor Neuron Differentiation of Human Embryonic Stem Cells

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Key Words. Neural differentiation • Notch • Human Embryonic stem cells • Progenitor cells

ABSTRACT

In the pMN domain of the spinal cord, Notch signaling regulates the balance between motor neuron differentiation and maintenance of the progenitor state for later oligodendrocyte differentiation. Here, we sought to study the role of Notch signaling in regulation of the switch from the pMN progenitor state to differentiated motor neurons in a human model system. Human embryonic stem cells (hESCs) were directed to differentiate to pMN-like progenitor cells by the inductive action of retinoic acid and a Shh agonist, purmorphamine. We found that the expression of the Notch signaling effector Hes5 was induced in hESC-derived pMN-like progenitors and remained highly expressed when they were cultured under conditions favoring motor neuron differentiation. Inhibition of Notch signaling by a γ-secretase inhibitor in the differentiating pMN-like progenitor cells decreased Hes5 expression and enhanced the differentiation toward motor neurons. Conversely, over-expression of Hes5 in pMN-like progenitor cells during the differentiation interfered with retinoic acid- and purmorphamine-induced motor neuron differentiation and inhibited the emergence of motor neurons. Inhibition of Notch signaling had a permissive rather than an inductive effect on motor neuron differentiation. Our results indicate that Notch signaling has a regulatory role in the switch from the pMN progenitor to the differentiated motor neuron state. Inhibition of Notch signaling can be harnessed to enhance the differentiation of hESCs toward motor neurons. STEM CELLS 2015;33:403–415

INTRODUCTION

The Notch signaling pathway plays an essential role in maintenance of progenitor cell populations and in preventing their differentiation into mature progenies. Notch signaling is initiated when Notch receptor on one cell is activated by a ligand expressed on a neighboring cell. Upon activation, the Notch receptor intracellular domain is cleaved by Presenilin proteases of the γ-secretase complex and translocates to the nucleus to form a complex with CBF1/RBPj, Su(H), Lag-1 (CSL) and Master-mind (Maml) proteins [1–4]. This complex then activates expression of the Hes (Hes1 and Hes5) and Hey transcription factors, which repress the expression of proneural genes such as Neurogenin 1/2 and Ascl1, thereby inhibiting neuronal differentiation and maintaining neural progenitor cells [5].

In the developing spinal cord, Notch signaling has a prominent role both in maintenance of neural and glial progenitor cells and in regulation of specific neuronal fate decisions. Specific progenitor cells with distinct identities and fates are organized along the dorso-ventral axis of the neural tube in five domains, termed p0–p3 and pMN. Recently it was shown that the transcription factor Nkx6.1 plays an active role in inducing the expression of the Notch ligand Dll1 in both the pMN and p2 domains and the resulting Notch signaling maintains the progenitor state in the distinct domains [6]. Accordingly, conditional knockout of Notch1 receptor results in a reduction of all neural progenitor subtypes in the ventral spinal cord [7].

Progenitor cells in the p0–p3 domains generate different classes of ventral interneurons, named V0–V3, respectively, whereas pMN progenitor cells at early stages of development appear to be committed to generate motor neurons (MNs). Later in development, they switch to produce oligodendrocytes [8–11]. pMN progenitor cells selectively express the bHLH protein Olig2, which is required to specify both motor neuron and oligodendrocyte cell identities [12, 13]. Olig2 primes pMN progenitor cells to become motor neurons by triggering the expression of Neurogenin 2 (Ngn2) bHLH protein. Coexpression of Olig2 and Ngn2 directs the pMN progenitors to leave the cell cycle and to become motor neurons, while progenitors in which Ngn2 expression is low remain as proliferative progenitors which are specified to an oligodendrocyte fate [13–15]. Ngn2 is repressed by Notch signaling, as was shown by the upregulation of Ngn2 expression in the ventral spinal cord of Notch1
expression levels of the Notch downstream effector Hes5 and controls differentiation of hESCs into motor neurons, the study elaborates in neural progenitors and is upregulated upon their differentiation into motor neurons during the neurogenic phase [16, 17].

It was shown that loss of the Notch signaling in the pMN domain increased motor neuron differentiation and results in a progressive depletion of the pMN progenitors over time. Conversely, activation of Notch signaling resulted in a reduction in motor neurons [6, 17]. In light of the potential role of Notch signaling during motor neurons development in animal models, we sought to study its role in human motor neuron development using human embryonic stem cells (hESCs) as a model system.

Human ESCs have been reported to generate spinal motor neurons in a pathway that recapitulates the steps of motor neuron differentiation in vivo. After initial neuralization, caudalization is induced by retinoic acid (RA) and ventralization by Shh morphogen [18–21]. In response to the Shh, hESCs-derived pMN progenitors express Pax6, Nkx6.1, and Olig2 transcription factors similar to their in vivo counterparts, and can be further differentiated into early Hb9 expressing and to mature ChAT-producing spinal motor neurons.

Using BAC transgenic reporter lines, the Notch components Hes5 and Dil1 have been shown to be dynamically expressed during the differentiation of hESCs into motor neurons [22]. Hes5 is highly expressed in hESCs-derived neural progenitors and is downregulated during their differentiation into motor neurons. Conversely, Dil1 is expressed at low levels in neural progenitors and is upregulated upon their differentiation, being expressed in Hb9-positive motor neurons.

To better understand how the activity of Notch signaling controls differentiation of hESCs into motor neurons, the study reports here tested the functional relationship between the expression levels of the Notch downstream effectors Hes5 and motor neuron differentiation. We show that in response to RA and the Shh agonist purmorphamine (PUR), hESC-derived neural progenitor cells are specified to generate pMN-like progenitor cells characterized by the expression of Olig2 and Ngn2. The neuralization and subsequent specification to pMN-like progenitors are concomitant with the induction of the expression of Hes5. However, further differentiation of the pMN-like progenitor cells into motor neurons is low, raising the possibility that Notch signaling inhibits their differentiation. Using the γ-secretase inhibitor DAPT to inhibit Notch signaling in differentiating pMN-like progenitor cells, we found that inhibition of Notch signaling downregulates Hes5 expression and enhances the differentiation of pMN-like progenitor cells into motor neurons. Conversely, over-expression of Hes5 in differentiating pMN-like progenitors inhibits subsequent differentiation into motor neurons. Still, in the absence of RA and PUR, inhibition of Notch signaling was not sufficient to direct the differentiation of the pMN progenitors toward motor neural fate, indicating a permissive rather than instructive role of Notch signaling in the process of differentiation toward spinal motor neurons.

For differentiation, hESC colonies were picked up by means of collagenase IV (1 mg/ml; Gibco-BRL, Gaithersburg, MD, www.lifetecnologies.com), triturated into small, 50–100 cell clumps, and placed into ultralow adherent culture dishes (Thermo Scientific Nunc HydroCell, www.thermoscientific.com). For the first 4 days, cells were grown in neural stem cells (NSC) media, consisting of Dulbecco’s modified Eagle’s medium (DMEM)/nutrient mixture F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA, www.lifetecnologies.com) and 2% B27 supplement with 20 ng/ml FGF2 (PeproTech, Inc., Rocky Hill, NJ) and 5 μM SB431542 (SB; Selleck Chemicals LLC, Houston, TX, www.selleckchem.com). At day 14, neural spheres were switched to medium consisting of DMEM/F-12 and 1% N2 supplement with 1 μM all-trans RA (Sigma-Aldrich, Saint Louis, MO, www.sigma-aldrich.com) and 1 μM dibutyryl cAMP (Sigma). At day 21, the spheres were cultured in medium consisting of Neurobasal (Invitrogen) and N2 supplement with 1 μM RA, 0.5 μM PUR (Cayman Chemical, Ann Arbor, MI, www.caymanchem.com), and 1 μM dibutyryl cAMP for a 3-week period. For differentiation, spheres were cut into small clusters and plated on poly-lysine/faminin-coated cover glasses for 1 week, in Neurobasal medium with 1% N2 supplement containing 0.25 μM or 0.5 μM RA, 0.125 μM, or 0.25 μM PUR, 10 ng/ml each brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1) (PeproTech, Inc., www.peprotech.com), and 1 μM dibutyryl cAMP. DAPT, N-[3,5-difluorophenacetyl]-l-alanyl-S-phenylglycine t-butyler ester, dissolved in dimethyl sulfoxide (DMSO) (Sigma), was used at a final concentration of 1 μM. DMSO was used as vehicle control.

**Lentiviral Constructs and Transduction**

Human Hes5 (aa 1–167) was amplified using Phusion Hot Start Flex DNA polymerase (New England Biolabs, Inc., Ipswich, MA, www.neb.com) and cloned into pFLAG-CMV-2. FLAG-tagged Hes5 was cloned to SIN18.Cppt.hEF1a.PRE lentiviral vector. Empty lentiviral vector was used as control. Concentrated lentiviral stocks were prepared as described [23]. Neural spheres treated with 1 μM RA and 0.5 μM PUR for 19 days were cut into small clusters and incubated overnight with the concentrated viral supernatant, which was then replaced with fresh Neurobasal medium supplemented with RA and PUR. Two days later, the transduced cells were plated for differentiation as described above.

**Immunocytochemistry**

Cells were fixed in 4% Paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained at room temperature with primary antibodies. Primary antibodies used in this study included antibodies against Ngn2 (Santa Cruz Biotechnology, Inc., Dallas, TX, www.scbt.com, 1:75), Goat Olig2 (R&D System, Inc., Minneapolis, MN, www.rndsystems.com, 1:75), mouse Olig2 (clone 211F1.1, Millipore Corporation, www.emdchem.com, 1:150), Islet-1 (Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA, www.dshb.biology.uiowa.edu, 1:50), Lim3 (DSHB, 1:200), MNR2 or Hb9 (DSHB, 1:50), ChAT (R&D System, Inc., 1:300), FLAG (Sigma-Aldrich, 1:1,000), and DYKDDDDK Tag (Cell Signaling Technology, Inc., Danver, MA, www.cellsignal.com, 1:500). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, www.vectorlabs.com). Quantification was performed using ImageJ software (NIH, public domain).
domain software) by measuring positive stained area relative to total DAPI. Quantifications are represented as a mean percentage of total DAPI ± SD or SEM and are from at least 15 random fields captured in three or more independent experiments.

PCR Analysis
Total RNA was extracted from cells at different stages along the differentiation into motor neurons, by means of TRizol (Invitrogen). cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and random primers, according to the manufacturer’s instructions (Promega Corporation, Madison, WI, www.promega.com). RT-PCR was performed with Taq DNA Polymerase (Promega Corporation). Primers used are given in Supporting Information.

For quantitative real-time PCR, TaqMan Assays-on-Demand Gene Expression Products (Supporting Information data), TaqMan Universal PCR Master Mix, and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, www.appliedbiosystems.com) were used. Large ribosomal protein P0 (RPLP0) was used as an internal reference for normalization.

Statistical Analysis
All experiments were performed at least three times unless otherwise indicated. Data are presented as means ± SD or SEM. Statistical significance was calculated using GraphPad Instat software using one-tailed unpaired Student’s t test for comparison between two groups. A p-value of <.05 was considered significant.

RESULTS

Derivation and Characterization of pMN-Like Progenitor Cells from hESCs
Induction of hESC differentiation toward motor neurons was previously described to progress through three sequential steps: neuralization, generation of caudal-ventral pMN progenitors, and differentiation into motor neurons. Based on similar principles, we used a modified four-stage protocol (Supporting Information Fig. S1) [18–20].

In stage 1, hESCs were induced to differentiate to neuroectoderm by culturing hESC clusters in the presence of SB431542 (activin receptor inhibitor) and FGF2 for 4 days followed by 10 days culturing in the presence of FGF2 only. At the end of this culture period, the clusters acquired a round morphology typical of neural spheres and were highly enriched for neural precursor cells expressing PSA-NCAM [25].

In stage 2, specification of these neural precursors toward a caudal fate was induced by treating the spheres with RA (1 μM). The spheres diameter were maintained below 300 μm by gentle trituration to allow the penetration of RA. Following 1 week of treatment with RA, the neural precursors acquired a caudal fate as indicated by the upregulation of Fox genes expression (Fig. 1A). The expression of Hox-c genes suggested that the neural progenitors acquired a rostral–cervical spinal cord identity [26].

It was previously shown that motor neurons differentiate from ventral progenitors of the pMN domain that coexpress Olig2 and Ngn2 [14, 15, 27–29]. In the developing spinal cord, the expression of Olig2 and Ngn2 is induced by Shh secreted from the floor plate and the notochord [26, 30]. To induce ventralization of the caudal progenitors generated upon RA treatment, in stage 3 the spheres were cultured for 3 weeks in the presence of the Shh agonist PUR (0.5 μM) in addition to RA. QRT-PCR analysis revealed that following 1 week caudalization with RA, Olig2 and Ngn2 expression levels in the progenitors were induced by 2.5- and 17-fold, respectively, while addition of PUR further induced both Olig2 and Ngn2 by 13- and 3.5-fold, respectively (Fig. 1B, 1C). Immunofluorescence staining revealed that upon the first week of RA treatment only few cells were immunoreactive with either anti-Olig2 (Fig. 1D) or -Ngn2 (Fig. 1E), while at the end of 3 weeks treatment with RA and PUR, 57% of the progenitors were stained positive for Olig2 (Fig. 1F) and 46% were stained positive for Ngn2 (Fig. 1G). Forty-four percentage of the cells coexpressed Olig2 and Ngn2 as demonstrated by communostaining for these markers (Supporting Information Fig. S2). The expression of both Olig2 and Ngn2 is indicative of ventral pMN-like progenitors.

Analysis of motor neuron markers expression by qRT-PCR revealed that Hb9 gene was upregulated by 17-fold upon RA treatment and its expression was slightly increased (24-fold) in pMN-like progenitor cells upon addition of PUR (Fig. 1H). Isl1 gene expression was induced by 1.6-fold in the caudal progenitors, and its expression was mildly increased in the pMN-like progenitors (Fig. 1I). However, communostaining of the pMN-like progenitors for Hb9 and either Olig2 or Ngn2 revealed that while the majority of the cells expressed Olig2 or Ngn2, only a few cells were immunoreactive with anti-Hb9 (Fig. 1J, 1K). This observation suggested that at that stage the cells did not yet differentiate into motor neurons but remained as pMN-like progenitors that have the potential to differentiate into motor neurons.

Differentiation of pMN-Like Progenitor Cells Toward Motor Neurons
In stage 4, the pMN-like progenitors were induced to differentiate into early motor neurons [Hb9(+), ChAT(–)] by plating on laminin for 1 week in differentiation medium (0.5-RP) that was supplemented with halved concentrations of RA and PUR (0.5 μM and 0.25 μM, respectively). The medium was further supplemented with dibutyryl cAMP and the neurotrophic factors BDNF, GDNF, and IGF-1. As a control, the progenitors were differentiated in the presence of DMSO vehicle combined with neurotrophic factors and dibutyryl cAMP.

Following 1 week of differentiation, the cells were analyzed for the expression of motor neuron markers Isl1, Lhx3, and Hb9. The transcriptional levels of Isl1 and Lhx3 were not significantly changed compared with the levels in the pMN progenitors (Fig. 2A, 2B). The expression level of Hb9 was significantly (p = .01) increased by 9-fold in control cells treated with DMSO and by 12-fold in cells treated with RA and PUR (Fig. 2C). It appears that plating control cells on laminin promoted MN differentiation that was further mildly augmented by RA and PUR. The increase in Hb9 expression may be attributed to its activation by the upregulated expression of Ngn2 in pMN-like progenitors [31].

Immunofluorescence staining of the differentiating progenitors revealed 17% Lhx3 and 12% Isl1 positive cells in the presence of RA and PUR compared with 10% Lhx3 and 5% Isl1-positive cells in the control (p < .01) (Fig. 2D, 2D, 2E, 2E, 2G). Moreover, in the presence of RA and PUR, 22% of the cells were Hb9 positive compared with 5% positive cells in the control (p < .001) (Fig. 2F, 2F, 2G). Still at this stage of differentiation 36% and 18% of the cells expressed Olig2 and Ngn2, respectively.
Taken together, these data suggest that RA and PUR promoted the differentiation of the pMN-like progenitors toward MN fate, albeit a proportion of the cells remained in the pMN-like progenitor state.

The Notch Downstream Effector Hes5 Is Upregulated Upon Differentiation of hESCs into Motor Neurons

It is well established that Notch signaling plays a key role in the maintenance of undifferentiated neural progenitors while...
Figure 2. Expression of motor neurons markers upon differentiation of ventral pMN-like progenitor cells. Ventral pMN-like progenitor cells (3W RAP) were differentiated for 1 week in the presence of 0.5 μM retinoic acid (RA) and 0.25 μM purmorphamine (PUR) (0.5-RP) or in the presence of DMSO vehicle control. (A–C): QRT-PCR analysis of the expression of Isl1 (A), Lhx3 (B), and Hb9 (C) genes in progenitor cells differentiating in the absence (DMSO) or presence of RA and PUR (0.5-RP) relative to the expression levels in ventral pMN-like progenitor cells (3W RAP). Expression level in pMN-like progenitor cells was set as 1. Fold activation over control is derived from three experiments. Data are presented as mean ± SD. *, *p < .01; **, p < .05 by Student’s t test.

(D–G): Immunofluorescence images demonstrating the expression of Lhx3 (D, D'), Isl1 (E, E'), and Hb9 (F, F') in cells differentiated in the presence of DMSO (D–F) or RA and PUR (0.5-RP, D'–F'). Blue indicates DAPI counter stained nuclei. Scale bars = 100 μm. (G) Quantification of the percentage of Lhx3, Isl1, and Hb9 positive cells following differentiation in the presence of DMSO or 0.5-RP. Data derived from three experiments are represented as mean ± SD. *, *p < .01; **, p < .05 by Student’s t test.

(H, I): Notch signaling is active along the differentiation of neural progenitor cells into motor neurons. (H): RT-PCR analysis of the expression of Notch receptors and Hes genes in undifferentiated human embryonic stem cell (HES), NP, caudal progenitor cells (RA), ventral pMN-like progenitor cells (RAP), and progenitors differentiating in the presence of DMSO or 0.5-RP. Data derived from three experiments are represented as mean ± SD. *, *p < .01; **, p < .05 by Student’s t test. (I): QRT-PCR analysis of the expression of Hes5 gene in undifferentiated hESCs, NP, caudal progenitor cells (1W RA), ventral pMN-like progenitor cells (3W RAP), progenitors differentiating in the presence of RA and PUR (0.5-RP). GAPDH levels were used as quantitative reference. (I): QRT-PCR analysis of the expression of Hes5 gene in undifferentiated hESCs, NP, caudal progenitor cells (1W RA), ventral pMN-like progenitor cells (3W RAP), progenitors differentiating in the presence of RA and PUR (0.5-RP) and progenitors differentiating in the presence of RA, PUR, and DAPT (0.5-RPD). Expression level in undifferentiated hESCs was set as 1. Fold activation over undifferentiated hESCs is derived from four experiments. Data are presented as mean ± SD. *, *p < .01; **, p < .05 by Student’s t test. Abbreviations: NP, neural progenitor cells; DAPI, 4,6-diamidino-2-phenylindole.
preventing premature differentiation. To test whether Notch signaling prevents pMN-like progenitors to differentiate into postmitotic MNs, we tested whether Notch signaling is active in the differentiating pMN-like progenitors. We first assessed the expression of Notch receptors in hESCs and their differentiating progeny through the stages of the differentiation protocol. RT-PCR analysis showed that Notch receptors (Notch1, Notch2, and Notch3) were expressed at similar levels in hESCs as well as in neural precursors, caudal, and pMN-like progenitors and in the progenitors that were induced to differentiate (Fig. 2H). In contrast, low expression levels of the Notch downstream effectors, Hes1 and Hes5, were observed in hESCs and their expression was significantly induced upon differentiation into neural precursors, caudal, and pMN-like progenitors as well as differentiating progenitors (Fig. 2H).

Both Hes1 and Hes5 were shown to be expressed in the ventricular zone of the spinal cord in complementary domains. However, in Notch1−/− mice decreased expression of Hes5 but not Hes1 was observed in the ventral spinal cord [7, 32], suggesting that in the ventral spinal cord Hes5 is a direct target of Notch signaling while Hes1 could be regulated by other signaling pathways. Thus, we focused our analysis on Hes5 and tested its expression throughout the four differentiation stages by qRT-PCR.

Hes5 was dramatically induced (100-fold) upon differentiation of hESCs into neural precursors and a similar level of expression was maintained upon further differentiation to caudal progenitors. Following RA and PUR treatment and differentiation into pMN-like progenitors, a fourfold increase in Hes5 expression was observed. This expression level was maintained after 1 week of plating the progenitors for differentiation into MNs, in line with the maintenance of pMN-like progenitor state by a proportion of the cells (Fig. 2I). Collectively, these data suggest that Notch signaling is activated upon differentiation of hESCs toward pMN-like progenitors and that Hes5 may regulate the maintenance of pMN-like progenitors and the prevention of their differentiation.

Inhibition of Notch Signaling Enhances the Differentiation of pMN-Like Progenitor Cells into Motor Neurons

To study whether Notch signaling prevents the differentiation of the pMN-like progenitors into motor neurons we tested whether its inhibition promotes motor neuron differentiation of the pMN-like progenitors. Notch signaling can be blocked pharmacologically by DAPT, which inhibits γ-secretase activity and prevents Notch receptor cleavage [33, 34]. To test whether DAPT inhibits Notch signaling during the differentiation of the pMN-like progenitors toward motor neurons, the progenitors were allowed to differentiate as before in the presence of RA and PUR combined DMSO or with DAPT (0.5-REP or 0.5-RPD, respectively). In the presence of DAPT, an eightfold decrease in Hes5 expression level was observed in the differentiating pMN-like progenitors, indicating that Notch signaling was inhibited (Fig. 2I).

Next, we tested whether the decrease in Hes5 expression was concomitant with an increased expression of motor neuronal markers. It was previously shown that specification of pMN progenitors into motor neuron fate is regulated by Ngn2 and Olig2, where progenitors that express both Olig2 and Ngn2 develop as motor neurons while those expressing only Olig2 become oligodendrocytes [14, 15, 27, 29]. Neither increase nor decrease in Olig2 expression was detected in DAPT-treated cells, while a 1.5-fold increase in Ngn2 expression was observed (Fig. 3A). Similarly, immunostaining showed comparable levels of Olig2 either in the absence or presence of DAPT (Fig. 3B, 3B’) while the expression of Ngn2 was increased in the presence of DAPT (Fig. 3C, 3C’). These results suggest that Notch inhibition promoted the differentiation of the pMN-like progenitors toward the motor neuron fate. In DAPT-treated cultures of differentiating pMN-like progenitors, the expression levels of Isl1 and Lhx3 were increased by 3- and 1.7-fold, respectively (Fig. 3D), indicating that inhibition of Notch signaling promoted the differentiation of the pMN-like progenitors toward motor neurons.

Ngn2 synergizes with Isl1 and Lhx3 to activate the expression of Hb9, which is specifically expressed in motor neurons [35–39]. In accordance with the increased expression of Ngn2, Isl1, and Lhx3, a threefold increase in Hb9 expression level was observed in DAPT-treated cells (Fig. 3E).

Immunostaining showed that in the presence of DAPT, Isl1 and Lhx3 expressions were detected in 30% of the differentiating cells compared with 12% and 17%, respectively, in the absence of DAPT (Fig. 3F, 3F’, 3G, 3G’, 3J). Hb9 expression was detected in 31% of the cells differentiating in the presence of DAPT compared with 20% in its absence (Fig. 3H, 3H’, 3I, 3I’).

The early motor neurons could be further matured into motor neurons by continuous culturing in suspension. Following 4–5 weeks culturing period in the presence of DAPT, the differentiated cells no longer expressed Hb9 while a substantial percent of the cells were stained positive to choline acetyltransferase (Chat), which is exclusively expressed in motor neurons of the ventral spinal cord (Fig. 3I, 3I’).

Moreover, when treatment with DAPT was combined with further lowering the concentrations of RA and PUR (0.25-RPD medium containing 0.25 μM RA, 0.125 μM PUR, and 1 μM DAPT), 40% of the differentiating cells expressed Hb9 compared with 20% in the absence of DAPT (Fig. 3K, 3K’, 3L). Therefore, in subsequent experiments, we used the 0.25-RPD medium for differentiation of the pMN-like progenitors. Collectively, these data suggested that inhibition of Notch signaling in differentiating pMN-like progenitors resulted in an increased expression of motor neuron markers.

When the cells were allowed to differentiate in the presence of DAPT without RA and PUR, only 15% of the differentiating cells expressed Hb9 (data not shown). This finding suggested an instructive role of RA and PUR in directing the differentiation of the progenitors toward motor neurons, which was augmented when Notch signaling was inhibited.

Over-Expression of Hes5 Inhibits the Differentiation of pMN-Like Progenitor Cells Toward Motor Neurons

Previous studies have identified Hes5 as a Notch target in the developing spinal cord, where it represses proneural genes such as Ngn2 [7, 40, 41]. To identify the downstream targets of Hes5 in the differentiating motor neurons, we used a lentiviral vector to over-express Flag-tagged Hes5 (FL-Hes5) in pMN-like progenitors just prior to their differentiation into motor neurons. Upon transduction of pMN-like progenitors with FL-Hes5 vector followed by differentiation in the presence of RA and PUR, an eightfold increase in Hes5 expression level was observed compared with control cells that were
transduced with a vector that did not include Hes5 (Vector, Fig. 4A). As was expected, FL-Hes5 transduced expression was not affected by DAPT (Fig. 4A), indicating a constitutive Hes5 expression in differentiating motor neurons, similar to constitutively active Notch signaling. To test the effect of constitutive Hes5 expression on motor neuron differentiation, we analyzed the expression levels of motor neuron markers in FL-Hes5 over-expressing cells compared with vector-only expressing cells.

Ngn2 was previously shown to be a direct target for Hes5 repression [41]; accordingly in FL-Hes5 transduced cells, Ngn2 was no longer induced by RA and PUR compared with a 1.9-
fold induction in vector-only transduced cells (Fig. 4B, 0.25-RP). Moreover, in FL-Hes5 transduced cells Ngn2 expression was not induced in the presence of DAPT (Fig. 4B, 0.25-RPD), indicating a constitutive repression of Ngn2 expression in the differentiating pMN-like progenitors.

In vector-only transduced cells, RA and PUR induced a fourfold increase in Hb9 expression level, while only a twofold increase was observed in FL-Hes5 transduced cells (Fig. 4C, 0.25-RP). The expression level of Lhx3 in vector-only transduced cells was induced by 7.9-fold in the presence of RA and PUR, while only a twofold increase was observed in FL-Hes5 transduced cells (Fig. 4D, 0.25-RP). These data suggest that constitutively active Notch signaling interferes with RA and PUR to induce differentiation of pMN-like progenitors toward motor neuron fate. In the presence of DAPT, FL-Hes5 over-expression resulted in a 2.5-fold reduction in both Hb9 and Lhx3 expression levels compared with the expression levels in vector-only transduced cells (Fig. 4C, 4D; 0.25-RPD).

In contrast, in the presence of RA and PUR, Isl1 expression was similarly induced in both FL-Hes5 and vector-only transduced cells (1.7-fold) (Fig. 4E, 0.25-RP). In the presence of DAPT comparable expression levels of Isl1 (fourfold) were observed in either FL-Hes5 or vector-only expressing cells (Fig. 4E, 0.25-RPD). These results suggest that Hes5 does not directly regulate Isl1.

In support with the RNA expression data, immunostaining of FL-Hes5 transduced cells for the motor neuron markers revealed a dramatic decrease in differentiation of the pMN-like progenitors into motor neurons. In the absence of DAPT (Fig. 5A–5D), as low as 3%–5% of the FL-Hes5 transduced cells were stained positive for Hb9, Isl1, or Lhx3 compared with 10%–15% positive cells in vector-only transduced cells (5i–5K, 0.25-RP). In the presence of DAPT (Fig. 5E–5H, 0.25-RPD), 4%–6% of the FL-Hes5 transduced cells were stained positive compared with 20%–30% positive cells in vector-only transduced cells (Fig. 5i–5K, 0.25-RPD). Immunostaining for FL-Hes5 and Hb9 showed that Flag expressing cells did not express Hb9 (Supporting Information Fig. S4). Interestingly, while the level of Isl1 mRNA transcripts was not reduced by FL-Hes5 over expression (Fig. 4E), immunostaining analysis showed reduction in the percentage of Isl1 positive cells (Fig. 5D′, 5H′, 5K) suggesting post-transcriptional regulation of Isl1 as previously reported [42, 43].

Our data suggest that constitutive expression of FL-Hes5 in differentiating pMN-like progenitors inhibited their differentiation into motor neurons. These results suggest that Notch signaling maintains pMN progenitors and prevents them from differentiation toward postmitotic motor neurons.

**Inhibition of Notch Signaling Enhances Motor Neuron Fate Specified by RA and PUR**

Lhx3 expression is not exclusive to motor neurons but is also characteristic to progenitors in the p2 domain of the developing spinal cord. The p2 progenitors express the Chx10 transcription factor in addition to Lhx3 and give rise to V2 interneurons [39]. To assess whether the pMN-like progenitors differentiate to a mixed population of motor neurons and interneurons, the differentiating cells were co-transduced with FL-Hes5 and either Lhx3 or Hb9. Strikingly, in the presence of RA and PUR, Chx10-expressing cells were not demonstrated in the absence or presence of DAPT (Fig. 6A–6B′), while at the same time cells expressing Lhx3 (Fig. 6A, 6A′) or Hb9 (Fig. 6B, 6B′) were observed as expected. However, when the cells were allowed to differentiate in the presence of DAPT without RA and PUR, we observed two cell populations: one coexpressed Chx10 and Lhx3 (Fig. 6C) and the other expressed either Hb9 or Chx10 (Fig. 6D), indicating differentiation into a mixed population of both interneurons and motor neurons. In the presence of DMSO vehicle, only a low percentage of the cells expressed Chx10, Lhx3, or Hb9 (Fig. 6C′, 6D′). These results suggest that RA and PUR specify the pMN-like progenitors toward the motor neural fate while at the same time they prevent the differentiation toward the interneural fate. Moreover, inhibition of Notch signaling enhances the motor neural-directed differentiation induced by RA and PUR.

**DISCUSSION**

In this study, we show that Notch signaling has a role in motor neuron differentiation of hESCs. We demonstrate that Notch signaling is active during the differentiation of pMN-like progenitors inhibiting their maturation into motor neurons. Inhibition of Notch signaling at the stage of pMN-like progenitors differentiation by DAPT significantly enhances the
Figure 4. Over-expression of FL-Hes5 reduces the differentiation of pMN-like progenitor cells into motor neurons. pMN-like progenitor cells infected with FL-Hes5 lentiviral vector (FL-Hes5) or with an empty lentiviral vector (Vector) were differentiated for 1 week in the presence of DMSO vehicle (DMSO) or 0.25 μM retinoic acid (RA) and 0.125 μM purmorphamine (PUR) combined with DMSO vehicle (0.25-RP) or with DAPT (0.25-RPD). (A–E): QRT-PCR analysis of the expression of Hes5 (A), Ngn2 (B), Hb9 (C), Lhx3 (D), and Isl1 (E) genes in vector-only (Vector) and FL-Hes5 (FL-Hes5) infected progenitor cells differentiating in the presence of DMSO vehicle (DMSO; white bars) or in the presence of RA and PUR combined with DMSO vehicle (0.25-RP; blue bars) or with DAPT (0.25-RPD; black bars). Expression level in vector-only infected cells differentiating in the presence DMSO vehicle (DMSO) was set as 1. Fold activation is derived from four experiments. Data are presented as mean ± SEM. *, p < .01; **, p < .05 by Student’s t test.
emergence of motor neurons, while forced expression of the Notch effector Hes5 inhibits motor neuron differentiation. Our data indicate that RA and Shh specify differentiating pMN-like progenitors to become motor neurons, and that inhibition of Notch signaling provides a permissive signal for the differentiation of the specified progenitors toward motor neuron fate.

In line with previous publications [2, 44] we show that Notch signaling is activated upon early neural differentiation of hESCs as evidenced by induction of expression of its
Intriguingly, we demonstrate that the expression level of Hes5 is further increased following specification of early neural progenitors into pMN-like progenitors by RA and the Shh agonist PUR. Moreover, it remains expressed at comparable levels in cultures of pMN-like progenitors under conditions favoring differentiation into motor neurons.

We provide evidence that Notch signaling inhibits the differentiation of the pMN-like progenitors toward motor neurons. Interfering with Notch signaling during the stage of their differentiation by DAPT leads to a marked decrease in Hes5 expression levels and enhances differentiation to motor neurons. Conversely, over-expression of Hes5 in differentiating pMN-like progenitors largely inhibits motor neuron differentiation.

The role of Notch signaling in the pMN domain of the developing spinal cord was previously studied in various animal models. Notch signaling was shown to preserve the undifferentiated state of pMN progenitors during the period of motor neuron generation for later differentiation to oligodendrocytes [16, 17]. Here, we used hESCs to efficiently generate pMN-like progenitors and study, for the first time, the role of Notch signaling during their differentiation into motor neurons in a humanized model system in vitro. We found that over-expression of Hes5 in the differentiating progenitors led to reduced expression levels of Ngn2, while the inhibition of Notch signaling in these progenitors resulted in an increase in the expression of Ngn2. These observations are consistent with a previous report of Ngn2 as a direct target for repression by Notch signaling [41]. Our data suggest that the inhibition of Notch signaling upregulates the expression of Ngn2 in differentiating pMN-like progenitors and promotes their differentiation into motor neurons.

Previously, it has been reported that inhibition of Notch signaling in mouse embryoid bodies (EBs) derived from ptc1 null mutated ESCs, in which Shh is constitutively active, resulted in precocious loss of ventral neuronal precursors to enhanced neuronal differentiation [45]. However, in these EBs, the inhibition of Notch signaling resulted in reduced expression of Olig2 and Nkx2.2, while in our pMN-like progenitors DAPT had no significant effect on Olig2 expression but rather increased the expression of Ngn2. Moreover, treating ptc1 null EBs with Shh and DAPT increased Isl1/2 expression but had no effect on Hb9 and Lhx3 expression. Hence, while precocious neuronal differentiation was observed in the absence of Notch signaling, motor neuron differentiation was not specifically augmented as observed in our results. Species-specific variation or differences in the methodology used may explain the dissimilar observations.

In a previous study, it was reported that in conditional Notch1 receptor null mice, more V2 interneurons are generated at the expense of earlier born motor neurons [7]. Our data indicate that the inhibition of Notch signaling per se in vitro is not sufficient to promote differentiation into a specific neuronal subtype. Inhibition of Notch signaling in the absence of RA and PUR resulted in differentiation of the pMN-like progenitors to a mixed population of motor neurons expressing Hb9 and V2 interneurons expressing Chx10 with no significantly enhanced differentiation to motor neurons or to V2 interneurons. This observation indicates that inhibition of Notch signaling provides pMN-like progenitors with a permissive rather than instructive signal for neuronal differentiation, while RA and Shh specify the progenitors toward motor neuron fate and block the differentiation into V2 interneurons. Furthermore, over-expression of Hes5 blocked the induction of Ngn2 expression and reduced the activation of motor neuron-specific genes by RA and PUR. Thus, it is possible that Notch signaling inhibits the differentiation of the pMN-like progenitors by interfering with RA and PUR in specification of the motor neuron fate.

We demonstrated that in addition to downregulating Ngn2 expression, over-expression of Hes5 in the differentiating pMN-like progenitors also reduced the expression levels of

![Image](image_url)
Lhx3 and Hb9 genes. Previously, it was shown that Ngn2 is repressed directly by Hes5 [41]. However, whether Hes5 regulates the expression of Hb9 or Lhx3 through direct binding to their regulatory regions is currently unknown.

Previously it was shown that the MN enhancer, which binds both bHLH proteins (Ngn2 and NeuroM) and Lim-HD proteins (IsI1 and Lhx3), regulates the expression of Hb9 gene [35, 36]. The bHLH proteins bind the two E-box consensus elements (CANNTG) included in MN and synergize with the Lim-HD proteins to activate Hb9 expression. Thus, it is possible that Hes5 affects the expression of Hb9 indirectly by downregulating the expression of Ngn2.

Alternatively, it is possible that Hes5 targets the Hb9 enhancer directly. In support of this assumption, we identified within the MN enhancer a N-box consensus element (CAC-NAG) which was reported as a specific binding site for Hes5 [46]. Future analysis may indicate whether Hes5 binds directly to the MN enhancer and regulates Hb9 expression. Recently it was reported that the expression of human Lhx3 gene is regulated by multiple enhancers, but no N-box element was identified in these regulatory elements, arguing against direct binding of Lhx3 regulatory regions by Hes5 [47].

The promotion of motor neuron differentiation by inhibition of Notch signaling may be used to enhance the yield of motor neuron differentiation protocols. Based on the inductive effect of RA and Shh signaling, our yield of motor neurons was 20%. Inhibition of Notch signaling doubled the yield of motor neurons. Inhibition of Notch signaling and improving the efficiency of motor neuron generation in vitro may be highly valuable for disease modeling, and high throughput screening assays for molecules that may have therapeutic value in motor neuron diseases [48].

CONCLUSIONS

In conclusion, our data provide evidence for Notch signaling as an important mechanism in controlling the differentiation of pMN progenitor cells into motor neurons. By upregulating the expression levels of Hes5, Notch signaling reduces the expression of Ngn2 as well as Hb9 and Lhx3, which are induced by RA and PUR and are required for directing the pMN progenitor cells toward motor neuron fate. The results argue for a model in which Notch signaling inhibits the differentiation of pMN progenitor cells by blocking the capacity of RA and PUR to induce the expression of key transcription factors that are required for specification and differentiation of the progenitor cells to motor neurons.

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AUTHOR CONTRIBUTIONS

E.B.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; E.F.: financial support, manuscript writing, and final approval of manuscript; B.E.F.: conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

Benjamin Reubinoff is a founder, holds shares and is the Chief Scientific Officer (CSO) of CellCure Neuroscience Ltd. The focus of the company is the development of human embryonic stem cells for transplantation therapy in neurological and retinal degeneration disorders. The company does not fund the study presented in this manuscript and currently the company has no interest in the results of the study.


Histological Bulbar Manifestations in the ALS Rat

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Abstract
Background: Almost all patients with amyotrophic lateral sclerosis (ALS) develop bulbar symptoms; therefore, it is important to have valid animal models that accurately reflect these features. While the SOD1-G93A rat is extensively used as an ALS model, bulbar symptoms in this model are not well characterized. Objective: In the present study, we aimed to better characterize bulbar dysfunction in terms of histology to determine whether the SOD1-G93A rat is a useful model for bulbar-onset ALS. Methods: Sixty-day-old SOD1-G93A rats on a Sprague-Dawley background and age-matched wild-type controls were assessed weekly for global motor function, facial nerve function, and vagal nerve function. The study endpoint was determined when an SOD1-G93A rat could not right itself within 30 s of being placed on its side. At that point, neuronal counts were assessed in different brainstem cranial nerve nuclei. In addition, the masseter muscle, posterior belly of the digastric muscle, and tongue muscle were evaluated for intact neuromuscular junctions. Results: Our data demonstrate decreases in the number of motor neurons in the trigeminal, facial, and hypoglossal nuclei, as well as compromised neuromuscular junction integrity in the muscles they innervate. Conclusion: These findings suggest that, from a histological standpoint, the SOD1-G93A rat is a valid model of ALS bulbar symptoms.

Introduction

Characterized by muscle weakness and paralysis resulting from the progressive degeneration of both upper and lower motor neurons (MNs), amyotrophic lateral sclerosis (ALS) is an invariably fatal neurodegenerative disorder, with 50% of patients dying within 3 years of onset [1]. Almost all ALS patients develop some bulbar dysfunction attributed to degeneration of brainstem motor nuclei of cranial nerves V, VII, IX, X, and XII [2-5]. This degeneration results in functional deficits in muscles of the head, neck, and face that affect speech, swallowing, tongue coordination, and vocal cord function. Notably, bulbar-onset ALS is also associated with a more rapid progression and poorer prognosis than limb-onset ALS [6]. Thus, given the predominance and implication of bulbar symptoms in ALS, an adequate animal model that reflects these findings is crucial.

The SOD1-G93A transgenic mouse, based on the familial ALS-associated mutation in the Cu2+/Zn2+ superoxide dismutase (SOD1) gene, is routinely used as an animal model for ALS [2, 7]. This model develops weakness at 100 days of age followed by paralysis and death at 140
days of age [8]. Moreover, various studies indicate that these mice are adequate models for bulbar-onset ALS, with decreased lick and mastication rates, and decreased pharyngeal muscle strength when compared to controls [2]. Recently, the SOD1-G93A rat has also gained popularity due its larger size, making it more amenable to testing novel surgical therapeutic options. These rats become symptomatic between 95 and 125 days of age and exhibit a short symptomatic phase, with death typically occurring between day 118 and 146; however, despite the fact that SOD1-G93A rats are an established ALS model, bulbar motor dysfunction is not as well characterized [9]. Therefore, in the current study, we sought to better characterize the bulbar features in SOD1-G93A rats to determine whether they are a robust histological model for bulbar symptoms present in ALS patients.

### Materials and Methods

All animal work was approved by the University of Michigan Institutional Animal Care and Use Committee. Hemizygous 60-day-old SOD1-G93A male rats on a Sprague-Dawley background were obtained from Taconic (Hudson, N.Y., USA) and age-matched wild-type Sprague-Dawley rats were used as controls.

All rats were taken through a multitude of weekly assessments, including the walking grid test to examine global motor function, vibrissae observation and blinking reflex observation to test facial nerve function, and laryngoscopies to test vagal nerve function. Briefly, the walking grid test involves placing a rat on an open grid and evaluating the number of times the animal slips off the grid. Each rat was taken through this test 3 times and the average value from all attempts was recorded. Observations of each animal’s vibrissae responses to hand-clapping and blinking reflexes in response to a puff of air were performed following the method previously described by de Faria et al. [10]. Finally, laryngoscopies involved visualizing and examining abduction and adduction of the vocal cords using our established methods, where all results were evaluated by an otolaryngologist [11].

The study endpoint occurred when SOD1-G93A rats were unable to right themselves within 30 s of being placed on their side. The endpoint for controls coincided with the age the last SOD1-G93A rat was sacrificed at 231 days. Terminal body weight and tongue weight were obtained prior to harvest. At that point, brainstem, masseter muscle, posterior belly of the digastric muscle, and tongue muscle were dissected, fixed in 4% paraformaldehyde, embedded in OCT, and cut into 14-μm transverse sections for staining.

To count neurons in the motor trigeminal, facial, and hypoglossal nuclei as well as in the dorsal vagal nucleus and nucleus ambiguus, brainstems from 3 rats were treated with Nissl’s stain as previously described [12]. These nuclei are primarily motor nuclei; therefore, the total number of stained neurons is representative of the number of MNs. Cranial nerve nuclei were evaluated bilaterally in 2 slices for a total of 4 values per rat, and counts were averaged to establish an estimate of the number of MNs in each specific nucleus.

To evaluate the integrity of neuromuscular junctions (NMJs), nonconsecutive muscle sections were immunohistochemically stained with acetylcholinesterase and neurofilament to label postsynaptic terminals and presynaptic nerve fibers, respectively, as previously described [13]. Briefly, the total number of NMJs was found by counting the number of acetylcholine-stained clusters in a specific muscle. Next, the number of intact NMJs was determined by counting NMJs that were contacted by neurofilament-stained nerve fibers, and NMJ integrity then was determined by dividing the percentage of intact NMJs by the total number of NMJs. NMJ integrity was quantified on 3 slices of each muscle from 6 SOD1-G93A rats and 3–6 control rats.

### Results

**Lifespan, Body Weights, Tongue Weights, and Functional Testing**

The average lifespan for SOD1-G93A rats was 189 ± 30.07 days, with a range of 161–231 days, and at the study endpoint, the average body weight of SOD1-G93A rats was significantly less than that of wild-type controls (248.3 vs. 460.5 g, respectively; p < 0.05; data not shown); however, no difference in tongue weight was observed (data not shown). The SOD1-G93A rats utilized in our study had a decreased ability to ambulate that was evident from the first walking grid test at 68 days throughout the entire period analyzed, with the exception of the evaluation on day 74 (data not shown). At 117 days, however, the SOD1-G93A rats were no longer able to move from their starting positions and exhibited a complete inability to perform this test; thus, further walking grid testing was halted at that time. Facial nerve testing by observing the animals’ blinking reflexes and vibrissae responses weekly did not demonstrate any difference between SOD1-G93A rats and controls, even in end-stage animals (data not shown). Likewise, weekly laryngoscopies continued to show full abduction/adduction of the vocal cords, even in end-stage SOD1-G93A rats (data not shown).

**Cranial Nuclei MN Number and NMJ Integrity**

The number of MNs in facial, hypoglossal, and trigeminal nuclei was significantly decreased in SOD1-G93A rats compared to controls (p < 0.05; fig. 1b–d). Likewise, the dorsal motor nucleus of the vagus exhibited a decreased number of neurons; however, the decrease was not statistically significant (fig. 1a). In contrast, the total neuron number in the nucleus ambiguus was increased in the SOD1-G93A rats compared to controls (fig. 1e), although again, this did not reach statistical significance. Determination of the percentage of NMJs contacted by a neural element revealed significantly decreased NMJ integrity in...
the masseter (p < 0.05; fig. 2a), posterior belly of digastric (p < 0.05; fig. 2b), and tongue muscles (p < 0.05; fig. 2c). Representative images of the NMJ integrity assessment from a control rat and an SOD1-G93A rat are shown in figure 2d, e. These histological findings support the SOD1-G93A rat as a useful model for bulbar ALS NMJ integrity.

Discussion

In this study, we demonstrate that the SOD1-G93A rat can be used as a histological model for bulbar features of ALS. This is reflected by both decreases in the total number of neurons in the facial, hypoglossal, and trigeminal nuclei as well as by decreases in NMJ integrity in the masseter, posterior belly of the digastic, and tongue muscles. Of note, however, this deterioration is not accompanied by any observable loss of function with regard to vibrissae observation, blinking reflex observation, or laryngoscopies.

The lack of bulbar functional impairment in the SOD1-G93A rats, even with severe motor deficits, is surprising and unlike what is seen in humans where bulbar involvement usually accompanies a similarly overt motor impairment. This could be due to many reasons. First, the SOD1-G93A rats may have a larger neuronal ‘reserve’
than humans, requiring a larger degree of cranial neuronal loss before the deficits become phenotypically apparent. Alternatively, there may be inherent differences between disease symptomology in SOD1-G93A rats relative to human ALS subjects. Of note, however, tongue motility and functioning, which exhibited deficits in a separate study [14], were not tested here. Lastly, our SOD1-G93A rats exhibited motor deficits earlier than what is described in the literature and died later than expected; therefore, the longer, slower progression suggests that these rats might have had a less aggressive form of disease that mitigated the development of overt facial motor function deficits. Regardless of the reason, the current findings demonstrate that SOD1-G93A rats likely cannot be used as a functional model for bulbar ALS features.

Importantly, however, our histological findings do align well with known disease findings in human ALS subjects [2–4]. In the current study, we observed a decreased
number of neurons in the facial, hypoglossal, and trigeminal nuclei. In addition, there was a trend toward a decreased number of neurons in the SOD1-G93A rat in the dorsal motor nucleus, but this was not statistically significant. Furthermore, an interesting finding of unknown etiology is the lack of a decrease in the number of neurons in the nucleus ambiguus in the SOD1-G93A rat. This result is incongruent with the effects of the disease and is thought to be an anomaly, which we anticipate would diminish with an increased sample size. Although the previous literature on the histological features of ALS in SOD1-G93A rats is sparse (Table 1), our results are supported by a previous study describing facial nucleus MN loss in SOD1-G93A rats [15] but contrast with another study that demonstrates no significant MN loss in facial or hypoglossal nuclei and only a modest decline in trigeminal nuclei [16]. This latter finding may be due to the rapid demise of the animals used in that study, such that there was not enough time for MNs in cranial nerve nuclei to undergo degeneration; the authors did, however, observe vacuolar changes in hypoglossal and facial nuclei [16]. Similarly, in another study the hypoglossal nucleus had no decrease in activity [14]. Furthermore, the reported heterogeneity in MN counts across studies can likely be explained by the susceptibility of SOD1-G93A rats to genetic drift [9]. Based on the current and reported findings, however, we assume that above a certain transgene copy number level, the SOD1-G93A rat represents a valid model of bulbar ALS in terms of brainstem MN histological characteristics.

With regard to the NMJ, a decrease in the total number of neurons in the facial, hypoglossal, and trigeminal nuclei was seen in the SOD1-G93A rats when compared to controls. While this conflicts with the study by Smittkamp et al. [14] that did not show denervation in tongue muscle of the SOD1-G93A rat, they did report denervation in the sternomastoid muscle, which we did not evaluate in the current study. Moreover, it should be noted that our current study utilized a relatively small sample size (n = 3–6). This may have impacted the significance of our findings for both NMJ integrity and MN counts alike, although given the current results, we believe this study adds important information to the literature in terms of establishing a definite histological change in the number of MNs and NMJ integrity, despite the lack of observable functional deficits in the SOD1-G93A rats. Overall, these measures suggest that the SOD1-G93A rat is an adequate histological model for ALS.

Conclusions

Although the SOD1-G93A rat model did not mimic the functional bulbar impairments commonly seen in humans, our study demonstrating attenuated cranial nerve MN number and loss of NMJ integrity supports the SOD1-G93A rat as a valid histological model for bulbar ALS.

Acknowledgments

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Disclosure Statement

The authors have no conflicts of interest to report pertaining to the materials or methods used in this study or the findings specified in this article.

Table 1. Summary of available literature on bulbar histology in SOD1-G93A rats

<table>
<thead>
<tr>
<th>Study</th>
<th>MN pathology</th>
<th>Muscle/NMJ histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smittkamp et al. [14]</td>
<td>no difference in hypoglossal nucleus activity</td>
<td>denervation in sternomastoid muscle, but not tongue muscle</td>
</tr>
<tr>
<td>Llado et al. [16]</td>
<td>– no significant loss in number of MNs in facial and hypoglossal nuclei; however, vacuolar changes were seen in cranial nerve nuclei – modest decline in MNs of trigeminal nuclei</td>
<td>N/A</td>
</tr>
<tr>
<td>Storkebaum et al. [15]</td>
<td>facial nucleus MN loss</td>
<td>N/A</td>
</tr>
<tr>
<td>Current study</td>
<td>decreased total neuronal number in facial, hypoglossal, and trigeminal nuclei</td>
<td>decreased NMJ integrity in masseter, posterior belly of digastric, and tongue muscles</td>
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References

The Spectrum of Motor Neuron Diseases: From Childhood Spinal Muscular Atrophy to Adult Amyotrophic Lateral Sclerosis

Stacey A. Sakowski1 · Eva L. Feldman1,2

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Key Words Amyotrophic lateral sclerosis · Motor neuron · Motor neuron disease · Spinal muscular atrophy

Since the topic of motor neuron diseases was last covered in Neurotherapeutics in 2008, increasing insight into genetic causes and underlying disease mechanisms has fueled several advances that have paved the way for new therapeutic strategies that are currently in development or being tested in clinical trials. In this special issue, we present reviews by leading investigators in the field that detail these advances for spinal muscular atrophy (SMA), an inherited neuromuscular disease associated with motor neuron degeneration and muscle atrophy that presents early in life, and amyotrophic lateral sclerosis (ALS), a fatal adult-onset neuromuscular disease also characterized by loss of motor neurons. The papers are divided into 4 main sections that cover 1) SMA advances, 2) ALS challenges and recent biological advances, 3) ALS clinical trial design considerations and outcome measures, and 4) emerging ALS treatments. Together, these articles offer a timely and comprehensive look into the recent research advances, current clinical considerations, and therapeutic prospects currently in development or being tested for motor neuron diseases.

The issue begins with 2 papers focused on SMA. In the first article, Drs. Michelle Farrar and Matthew Kiernan [1] provide a detailed overview of the genetic basis of SMA forms and variants and present common pathophysiological mechanisms, including discussion of the function of the survival motor neuron protein. They then discuss considerations for therapeutic development and testing before reviewing potential molecular biomarkers for outcome assessments and trial design. Next, Drs. Constantin d’Ydewalle and Charlotte Sumner [2] present an eloquent synopsis of promising SMA treatments currently in advanced stages of development, a discussion that is prefaced by an overview of the available in vivo mouse SMA models. The treatment strategies include some originally designed for applications in ALS, approaches to induce survival motor neuron, and new treatment paradigms harnessing epigenetic targets, antisense oligonucleotides, and gene therapy approaches. Notably, both articles also discuss important issues that remain to be addressed for current progress, such as how well preclinical models recapitulate the human phenotype, how to factor in disease heterogeneity and identify and define potential therapeutic windows, and how to best analyze effects on disease progression. We believe the recent novel insights and current advances bode well for continued progress and the identification of much needed treatment options in SMA.

The second section of the issue begins with a review by Drs. Jeffrey Rosenfeld and Michael Strong [3], which specifies the current challenges underlying therapeutic development that accompany the recent expansions in our understanding of the complex, multifaceted nature of ALS. Briefly, establishing firm diagnostic and prognostic criteria, determining what role frontotemporal dysfunction plays in the classification of ALS, identifying an accepted biomarker, determining how multidisciplinary care affects phenotypic variability, and understanding how the genetic basis of ALS and gene–environment interactions contribute to pathogenesis are all key issues that need to be addressed. Along these lines, this section continues with articles highlighting the most recent genetic and biological advances in ALS. In the article by Cooper-Knock et al. [4], we hear about the advances gained from the discovery of the recent identification of a hexanucleotide repeat expansion in C9orf72, and the authors emphasize the associated phenotypes, proposed disease mechanisms related
to the repeat expansion, similarities to other disorders with repeat expansions, premise for genetic screening, and most promising therapeutic developments. Dr. Sami Barmada [5] then discusses data supporting a converging mechanism in ALS centered on RNA dysregulation, presenting the current evidence depicting abnormalities in RNA processing and metabolism, mislocalization or aggregation of RNA binding proteins, and mutations in RNA processing enzymes. He also identifies potential therapeutic targets based on these mechanisms that may foster novel treatment strategies. Next, the pathways associating TAR DNA-binding protein 43 mutations and pathological inclusions observed in ALS with disease pathogenesis are reviewed by Drs. Emma Scotter, Han-Jou Chen, and Christopher Shaw [6], and the potential of TAR DNA-binding protein 43-positive protein inclusions as therapeutic targets is also discussed. Finally, Drs. Kristopher Gray Hooten, David Beers, Weihua Zhou, and Stanley Appel [7] introduce the noncell-autonomous nature of ALS, specifically detailing the dual role of the immune system in disease onset and progression. They also discuss the current therapeutic strategies in development to enhance neuroprotection and combat toxic neuroinflammation. Collectively, the papers in this section present a detailed view of some of the most recent discoveries in the last decade regarding ALS pathogenesis, and also portray the implications of these advances and current resulting progress towards therapeutic development.

While enhancing our understanding of disease pathogenesis is of paramount importance to therapeutic development, also equally critical, as mentioned above, is the need to overcome the challenges that accompany clinical translation and testing of newly discovered therapeutic options in patients with ALS. The third section of this issue therefore includes a collection of articles detailing these challenges and trial design considerations that are important for continued progress. First, Drs. Katharine Nicholson, Merit Cudkowicz, and James Berry [8] present an overview of how improvements in clinical trial design are affecting the current therapeutic testing landscape. They emphasize the need to consider treatment delivery paradigms, subgroups among the heterogeneous ALS patient population, and potential trial outcome measures in ALS clinical research. They also review how the development of diagnostic, prognostic, and pharmacodynamic biomarkers, and the increasing application of adaptive trial designs are setting the stage for exciting progress in the clinical ALS research arena. The question of potential outcome measures is then outlined by Dr. Seward Rutkove [9]. He offers insight into the currently used approaches to assess disease phenotypes and ways to account for inherent intersubject variability, and further discusses recommendations and considerations for assessing clinical outcomes in future clinical trials. Likewise, Dr. Zachary Simmons [10] addresses the value of determining patient-perceived quality of life in addition to traditional physical outcome measures in ALS clinical trials. Specifically, he introduces the various available tools to assess subject well-being, and offers recommendations as to which tools are best fit for certain clinical testing paradigms to capture the true effect of the interventions being tested. Novel advances in neuroimaging are then reviewed by Drs. Esther Verstraete and Bradley Foerster [11], who present the clinical value of the various advanced neuroimaging modalities utilized to study ALS, discuss the potential of imaging to offer diagnostic and prognostic biomarkers, and introduce the remaining challenges underlying the translation and utility of neuroimaging in clinical applications and therapeutic trials. To end this section, Dr. Neta Zach et al. [12] highlight the tremendous benefits of the Pooled Resource Open-access ALS Clinical Trials (PRO-ACT) database, a platform that currently contains data on the thousands of patients with ALS participating in 17 completed clinical trials, and incorporation of new patient and trial data is ongoing. Briefly, this resource has enabled important research regarding clinical trial recruitment, enrollment, and outcome assessment considerations, and also promoted an opportunity to gain insight into ALS pathogenesis across the heterogeneous patient population. Together, the articles in this section lend support to the continued clinical treatment advances that are possible with improved trial designs and an enhanced understanding of patient classifications and clinical outcomes.

Finally, in the last section we present 2 papers that review emerging treatment strategies that are rapidly making their way through the clinical translation pipeline. We begin with an article by Drs. Linga Reddy and Timothy Miller [13] that details the recent advances in RNA-based therapeutics for ALS and emphasizes the current progress in the preclinical and early clinical applications of antisense oligonucleotides and small interfering RNAs. The final paper, by our research group, then reviews the current progress in the development and application of stem cell-based therapies in ALS [14]. Briefly, we present preclinical data examining the therapeutic utility of various stem cell types and an overview of the paradigms that have progressed to early clinical trials; we include a brief update on the recent progress of our own clinical trial examining intraspinal transplantation of neural progenitor cells in patients with ALS. Overall, while most of the studies are small and not designed to determine efficacy, the results of the preclinical and early clinical studies support the continued development of stem cell therapies for ALS. We believe that with continued attention to issues such as selecting correct patient subpopulations, overcoming diagnostic delays, determining informative outcome measures, and improving trial design, we are poised for continued progress in both RNA-based and cellular therapies in ALS.

In summary, the emerging mechanistic themes amongst SMA and ALS, including alterations in RNA metabolism, loss of connectivity along the neuromuscular axis, and defects in axonal transport, are facilitating an enhanced understanding
of the pathogenesis underlying these diseases affecting motor neurons, as well as the identification of novel therapeutic targets and treatment strategies. Likewise, continuing improvements in our ability to diagnose, characterize, and assess patient well-being are enhancing our ability to design informative, powerful clinical trials. Although questions still remain regarding potential therapeutic windows and how to address the multifaceted treatment demands of the complex and heterogeneous nature of motor neuron diseases, recent years have brought a wave of advances in our ability to design well-planned and informative clinical trials that are fueling an incredible pace of therapeutic discovery for SMA and ALS. The current articles provide an excellent evaluation of these advances, and we greatly appreciate the time and effort of both the authors and reviewers, as their collective efforts and contributions have resulted in a spectacular, informative issue.

We would also like to thank Dr. Maral Mouradian, the Editor-in-Chief of Neurotherapeutics, Linda Powell, the Editorial Manager of Neurotherapeutics, and Saraswathi Sabapathy, from the Springer Journal Editorial Office, for the invitation to oversee this exciting issue and for their assistance and support.

References

Recent Advances and the Future of Stem Cell Therapies in Amyotrophic Lateral Sclerosis

Stephen A. Goutman · Kevin S. Chen · Eva L. Feldman

Abstract
Amyotrophic lateral sclerosis is a progressive neurodegenerative disease of the motor neurons without a known cure. Based on the possibility of cellular neuroprotection and early preclinical results, stem cells have gained widespread enthusiasm as a potential treatment strategy. Preclinical models demonstrate a protective role of engrafted stem cells and provided the basis for human trials carried out using various types of stem cells, as well as a range of cell delivery methods. To date, no trial has demonstrated a clear therapeutic benefit; however, results remain encouraging and are the basis for ongoing studies. In addition, stem cell technology continues to improve, and induced pluripotent stem cells may offer additional therapeutic options in the future. Improved disease models and clinical trials will be essential in order to validate stem cells as a beneficial therapy.

Key words
Amyotrophic lateral sclerosis · Stem cell therapy · Cell transplantation · Neural progenitor cell · Mesenchymal stem cell · Granulocyte-colony stimulating factor · Clinical trials

Introduction
Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder affecting motor neurons (MNs) in the cortex, brainstem, and spinal cord that causes weakness and atrophy of skeletal muscles [1]. While traditionally considered a purely motor disease, neuronal abnormalities in the prefrontal and temporal cortex may also lead to frontal executive dysfunction, with about 15% of patients manifesting frontotemporal dementia [2]. The worldwide incidence of ALS is 2–4 cases per 100,000 persons, although there is some ethnic variation [3]. The disease is sporadic in about 85% of cases and is familial in about 15% of cases [4]. The average survival is 3–5 years from symptom onset [1]. Riluzole, the only Food and Drug Administration-approved medication for ALS, has at best modest effects [5]. Owing to the relentless nature of the disease, many therapeutics have been tested; however, most have been without success [6, 7]. Thus, interest in the potential of stem cell-based therapies has been increasing considerably in recent years.

The initial proposed use of stem cells as a therapy for ALS stemmed from the possibility of MN replacement and considered several stem cell types. All stem cells possess the capacity for self-renewal and undergo asymmetric division to give rise to a daughter cell that is capable of developing a phenotype other than that of the parent cell. Embryonic stem cells are totipotent and able to generate all cell types, whereas pluripotent stem cells give rise to a particular subset of cells [8]. Neural progenitor cells (NPCs) are pluripotent stem cells that possess an ability to achieve characteristics of neurons or glia in daughter cells [8, 9]. Given the versatility of embryonic and pluripotent stem cells, an opportunity arose to harness stem cells for the generation of new MNs for a disease like ALS with selective MN loss. Early attempts at MN replacement using NPCs and embryonic stem cells, however, were fraught with difficulty [10–12]. Although NPCs can successfully
recapitulate normal MN development, stem cell-derived MNs must survive in a potentially diseased microenvironment, integrate into descending and local circuits of motor control, grow projection axons that travel over a meter in some cases, and form functional neuromuscular junctions [10, 12]. Thus, present studies have redirected focus away from MN replacement to a “neighbourhood theory”, where stem cells offer a local neuroprotective role to prevent the degeneration of existing MNs.

Mechanisms by which stem cells may provide neuroprotective support include the paracrine expression of neurotrophic factors, differentiation into nondiseased supporting non-neuronal cells, including astrocytes and microglia, and differentiation into modulatory neurons that synapse on diseased MNs [13]. Sources of stem cells that continue to generate interest for therapeutic potential in ALS are embryonic stem cells, NPC lines derived from fetal or adult tissues, and non-neural progenitor cells that may moderate the MN micro-environment [14]. This has effectively translated into several human therapeutic trials, which have employed the induction of peripheral blood stem cells (PBSCs) by granulocyte colony-stimulating factor (G-CSF) treatment, autologous transplantation of mesenchymal stem cells (MSCs) derived from the bone marrow, transplantation of olfactory ensheathing cells (OECs), and, most recently, transplantation of fetal-derived human spinal cord stem cells (HSSCs) and human fetal cortex-derived NPCs modified to secrete glial-derived neurotrophic factor (GDNF).

This review will briefly touch on preclinical studies (Table 1) relevant to stem cell-based paradigms that have been successfully translated to clinical trials (Table 2). While the preclinical literature is vast regarding stem cells and their application in ALS, the relative paucity of clinical trials underscores both the challenge of our current in vitro and animal models, as well as the difficulty in conducting well-designed clinical trials for this disease. Still, many novel strategies are gaining traction and significant achievements in stem cell therapy for ALS are on the horizon.

Transitioning from Early Preclinical Studies to Current Transplantation Paradigms

The mutant Cu²⁺/Zn²⁺ superoxide dismutase (SOD1)-G93A transgenic mouse and rat have served as the basis for much of the preclinical work in ALS stem cell therapy. These animals are based on the first identified gene underlying familial ALS [92], and recapitulate the progressive weakness and muscle wasting associated with selective MN loss characteristic of the disease. In the earliest studies, a survival benefit was demonstrated in irradiated SOD1-G93A mice treated with human umbilical cord blood [93, 94]. Follow-up studies also showed that transplanted human cord blood along with immunosuppression with cyclosporine delayed disease progression and that the transplanted cells were detected in the brain and spinal cord [25, 26]. From these beginnings arose many strategies to harness the potential of stem cells for ALS.

Given that the goal of early stem cell therapies for ALS were directed at MN replacement, the finding that MNs derived from mouse embryonic stem cells could be grafted into a chick spinal cord and synapse with muscles was exciting [95]; however, results of similar studies in rodent models of ALS were not met with the same success, likely related to features of both ALS, as well as the challenges of reconstructing the motor system as mentioned above. For example, SOD1-G93A rats that underwent grafting of mouse embryonic stem cells into the spinal cord only exhibited a transient motor improvement that may have been due to trophic support provided by the grafted MNs to the degenerating endogenous MNs [11]. Thus, this transient improvement instead served as a springboard for studies focused on the neighborhood theory, which promotes beneficial neuronal synaptic interactions and the creation of a microenvironment that is supportive of existing MNs [10, 12]. This concept is important, especially considering the notion that ALS may not be a cell autonomous disorder, and that, in at least the mutant SOD1 form of the disease, toxicity is not limited to the MNs but also affects surrounding microglia and astrocytes, which can be manipulated with stem cell therapy [30, 96–98].

Evidence supporting the potential therapeutic benefit of altering the MN microenvironment was the focus of a number of studies that aimed to modify non-neuronal cells and offer neurotrophic factor support. Injections of hematopoietic stem cells into a mouse model that undergoes selective MN degeneration did not result in the formation of primary neural tissue, but did result in functional improvements thought to be related to a neuroprotective effect from GDNF produced by the grafted cells [22]. Further support of the microenvironment was demonstrated in chimeric mice produced by injecting wild-type embryonic stem cells into SOD1-G85R or G37R mutant mouse blastocysts. In this chimeric model, it was discovered that mutant SOD1-expressing MNs exhibited prolonged survival when surrounded by wild-type non-neuronal cells [99]. Other studies have focused on specific non-neuronal cells. In a study of astrocytes, SOD1-G37R mice with reduced mutant SOD1 expression in astrocytes exhibited delayed microglial activation that resulted in slowed disease progression [100]. Further animal models supporting the therapeutic benefit of wild-type astrocytes involved the transplantation of glial-restricted precursors into the cervical spine of SOD1-G93A mice. The result of this intervention was prolonged survival with reduced MN loss and slowed progression of motor functional declines [44]. Benefits in survival are also noted when cells modified to secrete GDNF are injected in mutant SOD1 rodent models. Interestingly, implantation of human MSCs engineered to secrete GDNF into skeletal

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<table>
<thead>
<tr>
<th>Reference</th>
<th>Donor spec.</th>
<th>Cell type</th>
<th>Modification</th>
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<th>Delivery method</th>
<th>Treatment age</th>
<th>Adjunct therapy</th>
<th>Functional improvement</th>
<th>Survival benefit</th>
<th>Histologic change</th>
<th>Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lopez-Gonzales (2009)</td>
<td>Mouse ES</td>
<td>MN</td>
<td>Differentiated in vitro; GFP expressing</td>
<td>SOD1-G93A rat</td>
<td>1 × 10^5 cells, intraspinal</td>
<td>10 weeks</td>
<td>Ciclosporin A</td>
<td>Delay in motor decline by Rotarod</td>
<td>None</td>
<td>n/a</td>
<td>Degeneration of grafted cells at endstage</td>
</tr>
<tr>
<td>Deshpande (2006)</td>
<td>Mouse ES</td>
<td>MN</td>
<td>Differentiated in vitro; GFP expressing</td>
<td>Neuroadapted Sindbis virus-induced motor neuron death, rat</td>
<td>6 × 10^4 cells, intraspinal</td>
<td>5–7 weeks</td>
<td>Ciclosporin A; dibutyryl cyclic adenosine monophosphate, GDNF and or rolipram</td>
<td>Recovery of grip strength in animals receiving all supplemental treatments</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
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<tr>
<td>Harper (2004)</td>
<td>Mouse ES</td>
<td>MN</td>
<td>Differentiated in vitro; GFP expressing</td>
<td>Neuroadapted Sindbis virus-induced motor neuron death, rat</td>
<td>6 × 10^4 cells, intraspinal</td>
<td>5–7 weeks</td>
<td>Ciclosporin A; Rho kinase inhibitor (Y27632) or dibutyryl cyclic adenosine monophosphate</td>
<td>None</td>
<td>n/a</td>
<td>Axonal growth facilitated by dibutyryl cyclic adenosine monophosphate</td>
<td></td>
</tr>
<tr>
<td>Kerr (2003)</td>
<td>Human ES</td>
<td>None</td>
<td></td>
<td>Neuroadapted Sindbis virus-induced motor neuron death, rat</td>
<td>3 × 10^5 cells, intrathecal</td>
<td>3–4 weeks</td>
<td>Ciclosporin A or FK-506</td>
<td>Recovery of motor function by BBB and grip strength testing</td>
<td>None</td>
<td>Increased motor neuron survival</td>
<td>Possibly mediated via TGF-α or BDNF</td>
</tr>
<tr>
<td>Corti (2004)</td>
<td>Mouse BM</td>
<td>None</td>
<td></td>
<td>SOD1-G93A mouse</td>
<td>3 × 10^7 cells, intraperitoneal</td>
<td>4 weeks</td>
<td>Irradiation, 800 md</td>
<td>Delay in motor decline by Rotarod</td>
<td>10–13 days</td>
<td>Increased motor neurons and ventral root axons at 100 days</td>
<td>A large proportion of microglial cells formed</td>
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<tr>
<td>Solomon (2006)</td>
<td>Mouse BM</td>
<td>GFP expressing</td>
<td></td>
<td>SOD1-G93A mouse</td>
<td>5 × 10^6 cells, intravenous</td>
<td>6 weeks</td>
<td>Irradiation, 950–1100 rads</td>
<td>None</td>
<td>None</td>
<td>Large proportion of transplanted cells with microglial markers integrated into spinal cord</td>
<td></td>
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<tr>
<td>Ohnishi (2009)</td>
<td>Mouse BM</td>
<td>GFP expressing</td>
<td></td>
<td>SOD1-G93A mouse</td>
<td>6 × 10^7 cells, intra-bone marrow</td>
<td>87.7–89 days</td>
<td>Irradiation, 6 Gy × 2 doses</td>
<td>Delay in motor decline by grip strength meter</td>
<td>10–13 days</td>
<td>Increased motor neurons and ventral root axons in both eGFP and mutant SOD1 BM transplanted animals over wild type</td>
<td>Microglial markers noted in transplanted cells</td>
</tr>
<tr>
<td>Pastor (2013)</td>
<td>Mouse BM</td>
<td>GFP expressing or GDNF knockout</td>
<td>mdf mouse</td>
<td>1 × 10^6 cells, intramuscular</td>
<td>10 weeks</td>
<td>None</td>
<td>Improvement in motor function by rotarod and treadmill testing</td>
<td>n/a</td>
<td>Improvement in neuromuscular junction and muscle histology; increased motor neuron survival on the side of treated limb</td>
<td>Increased GDNF expression noted in spinal cord and corresponding cortex</td>
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<tr>
<td>Cabanes (2007)</td>
<td>Mouse BM</td>
<td>CD117+, GFP expressing</td>
<td>mdf mouse</td>
<td>3 × 10^5 cells, intraspinal</td>
<td>6 weeks</td>
<td>n/a</td>
<td>Improvement by footprint testing</td>
<td>n/a</td>
<td>Increase motor neuron survival</td>
<td>GDNF levels higher in grafted mice</td>
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<tr>
<td>Corti (2010) [23]</td>
<td>Mouse BM</td>
<td>Lin-c-kit+</td>
<td>SOD1-G93A mouse</td>
<td>1×10^6 cells intravenous</td>
<td>70 days</td>
<td>n/a</td>
<td>Delay in motor decline by Rotarod</td>
<td>16–17 days</td>
<td>Increased motor neuron survival (~50%) with preserved ventral nerve root axons</td>
<td>Mechanism of protection possibly mediated by expression of GLT1 and elaboration of VEGF and angiopoietin 2</td>
<td></td>
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<tr>
<td>Pastor (2012) [24]</td>
<td>Mouse BM, MSC</td>
<td>GFP expressing or GDNF knockout</td>
<td>mdx mouse</td>
<td>0.5–1×10^6 cells, intraspinal</td>
<td>n/a</td>
<td>None</td>
<td>Greater improvements seen in BM transplants over MSC transplants</td>
<td>n/a</td>
<td>Transplanted BM proliferate and retain bone marrow phenotype; MSCs underwent apoptosis</td>
<td>Increased GDNF expression in BM transplanted animals, functional improvements abolished in GDNF knockout BM transplant</td>
<td></td>
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<tr>
<td>Garbuzova-Davis (2008) [25]</td>
<td>Human UCB</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>1–5×10^7 cells intravenous</td>
<td>7–8 weeks</td>
<td>Ciclosporin A</td>
<td>Delay and improvement in hindlimb extension and Rotarod</td>
<td>~13 days (2.5×10^7 cell group)</td>
<td>Reduced microglial density in spinal cord</td>
<td>n/a</td>
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<tr>
<td>Garbuzova-Davis (2003) [26]</td>
<td>Human UCB</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>1×10^6 cells intravenous</td>
<td>9 weeks</td>
<td>Ciclosporin A</td>
<td>Delay in motor decline by extension reflex and footprint testing</td>
<td>None</td>
<td>n/a</td>
<td>Distributed throughout CNS and formed cells with astrocyte and neuronal markers</td>
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<tr>
<td>Souayah (2012) [27]</td>
<td>Human UCB</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>1×10^8 cells, intravenous</td>
<td>5–6 weeks</td>
<td>None</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Improved neuromuscular transmission by electrophysiological testing</td>
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<tr>
<td>Bigini (2011) [28]</td>
<td>Human UCB</td>
<td>None</td>
<td>SOD1-G93A mouse; Wobbler mouse</td>
<td>5×10^5 cells, intraventricular</td>
<td>10 weeks (SOD1); 4 weeks (Wobbler)</td>
<td>Ciclosporin A</td>
<td>Slowed decline by stride length and Rotarod (SOD1); slowed decline by running speed and grip strength (Wobbler)</td>
<td>18 days</td>
<td>No difference in MN survival (SOD1); increased MN survival (Wobbler)</td>
<td>n/a</td>
<td></td>
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<tr>
<td>Knippenberg (2012) [29]</td>
<td>Human UCB</td>
<td>CD34+</td>
<td>SOD1-G93A mouse</td>
<td>2×10^5 cells, intraspinal</td>
<td>40 or 90 days</td>
<td>Ciclosporin A</td>
<td>Improvement in motor function by rotarod, stride length, and footprint analysis</td>
<td>6 days (12 days in females)</td>
<td>Increased motor neuron survival (~50%)</td>
<td>No detected changes in growth factor production</td>
<td></td>
</tr>
<tr>
<td>Rizvanov (2011) [30]</td>
<td>Human UCB</td>
<td>VEGF and FGF2 expressing</td>
<td>SOD1-G93A mouse</td>
<td>1×10^6 cells intravenous</td>
<td>24–28 weeks</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>VEGF–FGF2 expressing cells demonstrated astrocyte markers</td>
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<tr>
<td>Rizvanov (2008) [31]</td>
<td>Human UCB</td>
<td>VEGF and LICAM expressing</td>
<td>SOD1-G93A mouse</td>
<td>1×10^6 cells, intravenous</td>
<td>22–25 weeks</td>
<td>None</td>
<td>n/a</td>
<td>n/a</td>
<td>Transplanted cells formed endothelial cells</td>
<td>n/a</td>
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<tr>
<td>Reference</td>
<td>Donor spec.</td>
<td>Cell type</td>
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<td>Experimental model</td>
<td>Delivery method</td>
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<tr>
<td>Habisch (2007) [32]</td>
<td>Human MSC, UCB</td>
<td>Neuroectodermal derivatives of each also used</td>
<td>SOD1-G93A mouse</td>
<td>1 x 10^5 cells, intrathecal</td>
<td>45 days</td>
<td>Ciclosporin A</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Improved intraparenchymal incorporation with bone marrow-derived cells</td>
<td>None</td>
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<tr>
<td>Uccelli (2012) [33]</td>
<td>Mouse MSC</td>
<td>Luciferase expressing</td>
<td>SOD1-G93A mouse</td>
<td>1 x 10^6 cells, intravenous</td>
<td>90 days</td>
<td>None</td>
<td>Improvement by rotarod, extension reflex, and motor score</td>
<td>17 days</td>
<td>Reducing ubiquitin inclusions</td>
<td>Decreased activated astrocyte and microglial cells, improvement in profile of oxidative stress/antioxidant enzyme expression</td>
<td></td>
</tr>
<tr>
<td>Forostyak (2011) [34]</td>
<td>Rat MSC</td>
<td>GFP expressing</td>
<td>SOD1-G93A rat</td>
<td>1 x 10^5 cells intraspinal, 2 x 10^5 intravenous</td>
<td>16 weeks</td>
<td>Ciclosporin A</td>
<td>Slowed decline in motor function by BBB and grip strength testing</td>
<td>11 days</td>
<td>Increased motor neuron survival in treated group</td>
<td>None</td>
<td></td>
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<tr>
<td>Boucherie (2009) [35]</td>
<td>Rat MSC</td>
<td>None</td>
<td>SOD1-G93A rat</td>
<td>2 x 10^5 cells, intrathecal</td>
<td>90 days</td>
<td>None</td>
<td>Delayed disease onset by motor score</td>
<td>16 days</td>
<td>Increased motor neuron survival and significant proportion formed astrocyte-like cells, with decreased microglia</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Zhao (2007) [36]</td>
<td>Human MSC</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>3 x 10^5 cells, intravenous</td>
<td>8 weeks</td>
<td>Irradiation, 6 Gy</td>
<td>Improved motor function by rotarod testing</td>
<td>18 days</td>
<td>Increased motor neuron survival at 16 and 20 weeks, improved CMAP amplitudes</td>
<td>None</td>
<td></td>
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<tr>
<td>Vercelli (2008) [37]</td>
<td>Human MSC</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>1 x 10^5 cells, intraspinal</td>
<td>28 weeks</td>
<td>None</td>
<td>Improved motor function by rotarod testing</td>
<td>11 days</td>
<td>Increased motor neuron survival and decreased microglial activation</td>
<td>None</td>
<td></td>
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<tr>
<td>Kim (2010) [38]</td>
<td>Human MSC</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>0.1, 2.0, and 10 x 10^5 cells, intrathecal</td>
<td>60 days</td>
<td>Ciclosporin A</td>
<td>Slowed decline by rotarod testing (10 x 10^5 cell group)</td>
<td>~6-8 days (2 x 10^5 and 10 x 10^5 cell groups)</td>
<td>Increased motor neuron survival</td>
<td>None</td>
<td></td>
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<tr>
<td>Suzuki (2008) [39]</td>
<td>Human MSC</td>
<td>GFP, GDNF expressing</td>
<td>SOD1-G93A rat</td>
<td>3.6 x 10^5 cells, intramuscular split in 3 doses</td>
<td>80 days</td>
<td>Bupivacaine; ciclosporin A</td>
<td>Delay in motor dysfunction by BBB rating</td>
<td>18-28 days</td>
<td>Preservation of neuromuscular junctions and corresponding motor neurons</td>
<td>None</td>
<td></td>
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<tr>
<td>Knippenberg (2012) [40]</td>
<td>Human MSC</td>
<td>Glucagon-like peptide 1 expressing, alginate matrix embedded</td>
<td>SOD1-G93A mouse</td>
<td>30 alginate capsules</td>
<td>40 days</td>
<td>None</td>
<td>Improvement in motor function by rotarod and footprint analysis</td>
<td>13 days</td>
<td>No difference in MN, reduction in reactive astrocytes and microglia</td>
<td>Increased expression of heat shock protein 70 in treated mice</td>
<td></td>
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<tr>
<td>Choi (2010) [41]</td>
<td>Human MSC</td>
<td>Retroviral transduction of Neurogenin 1</td>
<td>SOD1-G93A mouse</td>
<td>1 x 10^6 cells intravenous</td>
<td>8, 14-16, or 13 and 15 weeks</td>
<td>Ciclosporin A</td>
<td>Delay in motor decline by rotarod</td>
<td>n/a</td>
<td>Increased motor neuron survival</td>
<td>None</td>
<td></td>
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<tr>
<td>Reference</td>
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<tr>
<td>Morita (2008) [42]</td>
<td>Mouse/ Rat</td>
<td>OEC, MSC</td>
<td>GFP expressing</td>
<td>Lue1 26delTT mouse</td>
<td>3–4 × 10^5 cells, intraventricular</td>
<td>13–14 weeks</td>
<td>FK-506</td>
<td>None</td>
<td>Difference seen only in MSC-treated females</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>Martin (2007) [43]</td>
<td>Mouse</td>
<td>OEC</td>
<td>GFP expressing</td>
<td>SOD1-G93A mouse</td>
<td>0.4–120 × 10^5 cells, intrapinal</td>
<td>70 days</td>
<td>None</td>
<td>Improved motor function by running wheel activity and inclined plane test</td>
<td>22.3 days</td>
<td>Differentiated cells show markers for MNs, astrocytes, and oligodendrocytes</td>
<td>Axons seen to project to peripheral nerve but fail to make neuromuscular junctions</td>
</tr>
<tr>
<td>Lepore (2008) [44]</td>
<td>Rat</td>
<td>Glial restricted precursor</td>
<td>GLT1-overexpressing, also GLT1-null cells</td>
<td>SOD1-G93A rat</td>
<td>9 × 10^5 cells, intrapinal</td>
<td>90 days</td>
<td>Ciclosporin A</td>
<td>Delay in forelimb (site of transplantation) but not hindlimb function; slowed decline of phrenic nerve peak CMAP amplitudes</td>
<td>16.9 days</td>
<td>Increased motor neuron survival (47% increase)</td>
<td>Majority astrocyte differentiation, may be mediated by GLT1 expression</td>
</tr>
<tr>
<td>Lepore (2011) [45]</td>
<td>Human</td>
<td>Glial restricted precursor</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>2–6 × 10^5 cells, intrapinal</td>
<td>50–60 days</td>
<td>Ciclosporin A or FK-506/ rapamycin</td>
<td>None</td>
<td>No difference</td>
<td>n/a</td>
<td></td>
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<tr>
<td>Corti (2007) [46]</td>
<td>Mouse</td>
<td>Primary NPC</td>
<td>GFP expressing; Lex1X++; CXCR4+</td>
<td>SOD1-G93A mouse</td>
<td>2 × 10^4 cells intrapinal</td>
<td>70 days</td>
<td>n/a</td>
<td>Motor decline delayed, no change in slope of disease progression</td>
<td>22–23 days</td>
<td>Increased MN survival and preservation of ventral nerve roots at 110 days but not at end stage</td>
<td>A large proportion of neuronal cells formed, with a subset of motor neurons. Neuroprotection may be mediated by VEGF and IGF-1</td>
</tr>
<tr>
<td>Mitrecic (2010) [47]</td>
<td>Rat</td>
<td>Primary NPC</td>
<td>GFP expressing</td>
<td>SOD1-G93A rat</td>
<td>1 × 10^7 cells intravenous</td>
<td>14 and 26 weeks</td>
<td>Nitroprusside; TNF in half of treatment group</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>Hwang (2009) [48]</td>
<td>Human</td>
<td>NPC</td>
<td>VEGF expressing</td>
<td>SOD1-G93A mouse</td>
<td>1 × 10^5 cells, intrathecal</td>
<td>70 days</td>
<td>n/a</td>
<td>Delay in motor decline by norex, paw grip endurance and extension reflex testing</td>
<td>12 days</td>
<td>n/a</td>
<td>Downregulation of apoptotic proteins in treated animals</td>
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<tr>
<td>Park (2009) [49]</td>
<td>Human</td>
<td>NPC</td>
<td>GFP with GDNF or multiple growth factors (BDNF, IGF-1, VEGF, NT-3, GDNF) expressing</td>
<td>SOD1-G93A mouse</td>
<td>Intrathecal</td>
<td>75 days</td>
<td>Ciclosporin A</td>
<td>None</td>
<td>Survival decreased in treatment group</td>
<td>Increased motor neuron survival with GDNF expressing cells</td>
<td>Sexual dimorphism noted, with female animals with worse outcomes</td>
</tr>
<tr>
<td>Reference</td>
<td>Donor spec.</td>
<td>Cell type</td>
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</tr>
<tr>
<td>Xu (2006)</td>
<td>Human NPC</td>
<td>None</td>
<td>None</td>
<td>SOD1-G93A rat</td>
<td>4×10^5 cells, intraspinal</td>
<td>62 day</td>
<td>FK-506</td>
<td>Slowed progression by BBB tests</td>
<td>11 days</td>
<td>Increased motor neuron survival (nearly 200 %)</td>
<td>Increased GDNF secretion in grafted animals</td>
</tr>
<tr>
<td>Xu (2009)</td>
<td>Human NPC</td>
<td>None</td>
<td>None</td>
<td>SOD1-G93A rat</td>
<td>1.6×10^5 cells, intraspinal</td>
<td>56 day</td>
<td>FK-506</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Yan (2006)</td>
<td>Human NPC</td>
<td>None</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>8×10^5 cells, intraspinal</td>
<td>8 weeks</td>
<td>FK-506 and/or mycophenolate mofetil, or anti CD4 antibodies</td>
<td>Improved motor function by modified Wrathall scale with combination immunosuppressants</td>
<td>~3 week benefit with combination immunosuppressants</td>
<td>Prominently neuronal phenotype formed</td>
<td>Combination immunosuppression promotes survival of grafted cells, which imports functional and survival benefit</td>
</tr>
<tr>
<td>Klein (2005)</td>
<td>Human NPC</td>
<td>GDNF expressing</td>
<td>SOD1-G93A rat</td>
<td>4.8×10^5 cells, intraspinal</td>
<td>90 days</td>
<td>Ciclosporin A</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>n/a</td>
</tr>
<tr>
<td>Suzuki (2007)</td>
<td>Human NPC</td>
<td>GDNF expressing</td>
<td>SOD1-G93A rat</td>
<td>4.8×7.2×10^5 cells, intraspinal</td>
<td>65 days</td>
<td>Ciclosporin A</td>
<td>none</td>
<td>none</td>
<td>Increased motor neuron survival by grafted cells; no change in neuromuscular junctions</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Garbuzova-Davis (2002)</td>
<td>Human NPC</td>
<td>None</td>
<td>SOD1-G93A mouse</td>
<td>7.5×10^4 cells, intraspinal</td>
<td>53 days</td>
<td>Ciclosporin A</td>
<td>Suggestion of delayed motor decline by staircase testing, beam balance, open field and footprint testing</td>
<td>None</td>
<td>None</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Gao (2005)</td>
<td>Human NPC</td>
<td>MN differentiated in vitro; GFP expressing</td>
<td>Neonatal sciotic axotomy rat</td>
<td>1×10^5 cells, intraspinal</td>
<td>2 months</td>
<td>Ciclosporin A</td>
<td>Improvement by sciatic function index</td>
<td>n/a</td>
<td>51 % transplanted cells with MN markers, and 19 % taking up retrograde marker from muscle</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Hwang (2009)</td>
<td>Human NPC</td>
<td>VEGF expressing</td>
<td>SOD1-G93A mouse</td>
<td>1×10^5 cells, intrathecal</td>
<td>70 days</td>
<td>None</td>
<td>Delayed disease onset by rotarod, paw grip endurance and extension reflex testing</td>
<td>12 days</td>
<td>Some transplanted cells differentiated into MN-like cells</td>
<td>Reduced proapoptotic proteins and elevated antiapoptotic proteins</td>
<td></td>
</tr>
<tr>
<td>Xu (2011)</td>
<td>Human NPC</td>
<td>None</td>
<td>None</td>
<td>SOD1-G93A rat</td>
<td>2.4×10^5 cells, intraspinal</td>
<td>63 days</td>
<td>FK-506</td>
<td>Delayed motor decline by BBB and inclined plane testing</td>
<td>17 days</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Helferian (2012)</td>
<td>Human NPC</td>
<td>None</td>
<td>SOD1-G93A rat</td>
<td>1.8–2.6×10^5 cells, intraspinal</td>
<td>60–65 days</td>
<td>FK-506 and mycophenolate mofetil</td>
<td>Improvement in motor function by BBB scale and electrophysiological testing</td>
<td>None</td>
<td>Increased motor neuron survival (~43 %); decreased reactive astrocyte and microglial populations</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Popescu (2013)</td>
<td>Human iPSC-NPC</td>
<td>None</td>
<td>SOD1-G93A rat</td>
<td>1×10^5 cells, intraspinal</td>
<td>3 months</td>
<td>Ciclosporin A</td>
<td>n/a</td>
<td>n/a</td>
<td>Cells formed neuronal phenotype, with MN-like morphology</td>
<td>n/a</td>
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muscle were also able to support MN survival in the spinal cord [39], with synergistic effects attributed to vascular endothelial growth factor (VEGF) production [101]. Therefore, the ability of surrounding non-neuronal cells in the spinal cord and at the neuromuscular junction appears to play an important role in ALS pathogenesis, and the properties of stem cells are thereby ideally suited to achieve the goal of modulating the MN microenvironment.

Several stem cell types have since been examined for their potential efficacy in preclinical ALS models, including stem cells obtained directly from bone marrow. Direct implantation of nondiseased bone marrow into bone marrow of SOD1-G93A mice improved survival, an effect linked to the presence of non-neuronal cells derived from the transplanted tissue in the spinal cord [20]. Likewise, intraperitoneal transplantation of wild-type bone marrow into SOD1-G93A mice resulted in extensive incorporation of transplanted cells as microglia with improved animal survival [18]. Enriching the population of transplanted cells for c-kit+ stem cells allowed the infusion of cells peripherally into SOD1-G93A mice, with again improved function and survival associated with non-neuronal cells that migrated to the spinal cord [23]. Similarly, crossing SOD1-G93A mice with mice unable to generate myeloid or lymphoid cells slowed the disease course following subsequent bone marrow transplantation with wild-type SOD1-expressing cells, and this study further demonstrated that microglia in the mice receiving wild-type bone marrow transplants produced less superoxide, nitrite, and nitrate, and were therefore less neurotoxic [102]. Thus, these experiments again showed that MN pathology is not necessarily intrinsic to the MN, but may additionally rely on interactions with surrounding glial tissue.

Another potential source of stem cells are OECs isolated from the olfactory bulb, as they represent a relatively accessible source of endogenous NPCs. These cells showed promise in early models of spinal cord injury, where they promoted axonal regrowth and remyelination [103, 104]. Interestingly, transplantation of OECs isolated from the olfactory bulb of green fluorescent protein-expressing C57BL/6 mice into SOD1-G93A animals prolonged survival, and this effect was not associated with the formation of neuromuscular junctions in SOD1-G93A mice [43], suggesting again that the formation of interneurons, astrocytes, and even oligodendroglia may provide support for diseased MNs.

Studies using NPCs derived from elsewhere in the nervous system have lent further credence to a strategy of supporting existing MNs via trophic support. In one study, mouse-derived NPCs selected for those expressing the Lewis X surface marker and the chemokine receptor CXCR4 supported native MNs via VEGF and insulin-like growth factor-I (IGF-I)-mediated neuroprotective pathways [46]. Subsequent use of human NPCs modified to overexpress VEGF also favored antiapoptotic pathways over proapoptotic pathways in native

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<tr>
<td><strong>Reference</strong></td>
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<tr>
<td>Nizzardo (2014)</td>
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<td>Stem cell type</td>
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</tr>
<tr>
<td>PBSC</td>
</tr>
<tr>
<td>G-CSF 5 μg/kg/day SC×4 days repeated at months 0, 3, 6, 9</td>
</tr>
<tr>
<td>G-CSF 5 μg/kg/day SC every 12 h×4 days at months 0, 3, 6, 9 with 125 ml 18 % mannitol IV4 times per day×5 days starting on day 3 of G-CSF</td>
</tr>
<tr>
<td>IV</td>
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<tr>
<td>Intrathecal</td>
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<tr>
<td>IV</td>
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<tr>
<td>Intracortical</td>
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<td>Intracortical</td>
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<tr>
<td>Stem cell type</td>
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</tr>
<tr>
<td>Bone marrow derived MSCs</td>
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<tr>
<td>Intramuscular, Intrathecal</td>
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<tr>
<td>Intraventricular</td>
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<td>Intraspinal</td>
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<td>Intraspinal</td>
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<tr>
<td>Intraspinal</td>
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<tr>
<td>Intraarterial</td>
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<tr>
<td>Stem cell type</td>
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</tr>
<tr>
<td>OECs</td>
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<td></td>
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<tr>
<td>NPCs</td>
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MN s [48], and human NPCs modified to secret GDNF were able to confer protection when transplanted into the spinal cords of SOD1-G93A rats [53], although this did not result in continued muscle innervation [54]. Similarly, experiments with transplanted GDNF or IGF-1-secreting human NPCs rescued MNs but again did not result in improvement of motor performance or lifespan in SOD1-G93A rats [49]. Taken together, these experiments demonstrated that NPCs are able to rescue native MNs via local trophic signaling rather than replacing MNs, although more work to attain a more robust effect will be required to observe functional benefit.

Along the lines of creating a neuroprotective neighborhood, the human fetal spinal cord stem cell line NSI-566RSC has been studied for use in cellular therapy [14]. This cell line was derived from an 8-week fetal spinal cord and has the advantage of reduced teratoma formation risk compared with embryonic stem cells, as they are partially differentiated NPCs. When grafted into the SOD1-G93A rat spinal cord, these cells form both neurons and glia, synapse with native MNs, and elaborate a range of neurotrophic factors [14]. Furthermore, grafting of these cells resulted in a delay in onset and progression of disease symptoms and increased the lifespan of experimental animals [50]. Notably, the grafted cells formed gamma-aminobutyric acid-ergic neurons that synapsed with neighboring MNs in the ventral horn, although they did not form synaptic connections outside the spinal cord [51, 58]. Therefore, with the ability to form glial cells and a diversity of neuronal synaptic contacts around diseased MNs, NPCs such as NSI-566RSC have the advantage of being able to tackle the multifaceted local physiologic derangements seen in ALS.

Overall, the preclinical studies (Table 1; reviewed in [105]) underscore the potential for stem cells to modulate the microenvironment in ALS. Along with the many detailed mechanistic studies that have been performed using the various cell types described above, these preclinical data have formed the foundation for early clinical trials that seek to harness the unique attributes of stem cells to rescue diseased MNs.

**Clinical Studies**

Translation of stem cell therapy from the laboratory to the clinical realm requires 1) propagation of an easily accessible source of progenitor cells; 2) efficient delivery of cells to the affected areas; and 3) the ability of the cells to survive and integrate into local circuits, such that degenerating cell populations can be replaced or aberrant physiology reversed. In terms of the first requirement for suitable stem cell sources, recent studies have focused on the use of G-CSF-induced PBSCs, bone marrow-derived MSCs, OECs, and NPCs. The second point has also been addressed by a number of techniques for the collection and delivery for each stem cell population, with delivery methods including intravenous, intra-arterial, intrathecal, intracerebral, and intraspinal routes. And, finally, to the third requirement, stem cell integration must be quantifiable on a clinical scale. For ALS, this includes measurements of function such as the ALS Functional Rating Scale-Revised (ALSFRS-R), respiratory parameters, and survival, as well as the enrollment of adequate numbers to detect improvements in comparison with control treatments. To date, most studies are proof-of-concept or safety trials, and, as such, are conducted without placebo control groups. Likewise, the study sizes are small and are not powered to determine clinical efficacy. Thus, none of the reviewed studies make firm conclusions regarding improvements in the ALS disease course; however, these clinical studies represent an important first step in the development of stem cell therapies for ALS. In the following sections, the published studies involving the application of stem cells for the treatment of ALS in humans (summarized in Table 2) will be reviewed.

Granulocyte-Colony Stimulating Factor and Peripheral Blood Stem Cells

G-CSF is a hematopoietic growth factor that can mobilize CD34+ hematopoietic stem cells from the bone marrow to the peripheral blood, resulting in a population of PBSCs that can be collected for later use [63, 65, 106]. These CD34+ PBSCs were initially identified after cancer patients received hematotoxic chemotherapy [106], and it was later shown that these circulating hematopoietic progenitor cells could migrate into the central nervous system (CNS) and provide support for diseased MNs [107]. G-CSF itself is suggested to have a neuroprotective effect [108]. Thus, a number of strategies were implemented using G-CSF to mobilize or collect and redistribute PBSCs to the CNS in ALS.

Based on the presumption that PBSCs can migrate into the CNS, subcutaneous G-CSF was given to 13 patients, resulting in a slowing of disease progression evidenced by ALSFRS-R, as well as maintenance of compound muscle action potential amplitudes [61]. Another study in 17 patients comparing subcutaneous G-CSF with a placebo control arm showed elevated CD34+ PBSCs, but no difference in disease progression [62]. Similarly, the STEMALS trial utilized subcutaneous G-CSF along with a 5-day course of mannitol with the hopes of increasing permeability across the blood–brain barrier in 26 patients [63, 64]. Again, there was an increase in circulating CD34+ PBSCs, and in this trial a decrease in the proinflammatory cytokines monocyte chemotactic protein-1 and interleukin-17, but no change in ALSFRS-R. Enriching the number of circulating PBSCs by first collecting and then readministering CD34+ cells induced by G-CSF has also been attempted. One study utilized subcutaneous G-CSF in 8 patients prior to PBSC isolation and peripheral infusion, and while no adverse effects were reported, clinical and imaging

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measures did not seem to be significantly affected [65]. Finally, an aggressive strategy was attempted in 6 patients with ALS who received total body radiation followed by peripheral infusion of G-CSF-primed PBSCs from human leukocyte antigen-matched siblings [67]. Patients were immunosuppressed with methotrexate and tacrolimus, and subsequent graft-versus-host disease occurred in half of the patients. Although donor hematopoietic stem cells entered the CNS at sites of MN degeneration and engrafted as immunomodulatory cells, no clinical benefit was detected and the study was halted owing to a lack of benefit and impaired quality of life.

In contrast to the above G-CSF studies, others have examined more invasive procedures for PBSC delivery in order to bypass the blood–brain barrier. An early study tested 3 patients using a protocol of subcutaneous G-CSF therapy and isolation of CD34+ stem cells followed by intrathecal administration of the collected stem cells and showed minimal adverse effects [66]. Alternatively, a separate group focused on CD133+ cells mobilized by G-CSF followed by direct injection of the cells into cortical motor areas of the brain utilizing a frame-based or frame-lessly stereotactically guided needle [68, 69]. In the initial study, 10 patients were enrolled and compared with 10 control patients not accepting treatment or who applied after the study period [68]. One patient died within 10 days of surgery from a myocardial infarction. The treatment group showed an improvement in baseline ALSFRS-R scores; however, the control group had a higher ALSFRS-R score at baseline limiting a comparison. An additional 67 patients were then evaluated after having undergone the same procedure [69]. Two postoperative deaths were reported, and no outcome data were reported, but these serious adverse events suggest that further use of frontal cortex injection should be approached cautiously to minimize patient risk.

Overall, these G-CSF studies appear to demonstrate clinical safety, with a suggestion of clinical efficacy in some cases as well, and, based on preclinical models, this is an area that demonstrates therapeutic potential. It is clear, however, that these trials struggle with the technique of stem cell delivery, balancing widespread yet inefficient distribution of blood-borne PBSCs against expedient yet risky surgical methods. Hence, moving forward, this method of therapy will benefit from some agreement on G-CSF delivery strategies, as well as good clinical trial design utilizing large numbers of well-defined patient populations and standardized outcome measures.

Bone Marrow-Derived Hematopoietic Progenitor Stem Cells

Bone marrow-derived MSCs are another source of therapeutic potential in ALS [74]. MSCs may exert neuroprotective effects via paracrine, or “bystander”, mechanisms, such as the release of anti-inflammatory, anti apoptotic, and neurotrophic factors, and by influencing other cell types to take on a protective phenotype [109, 110]. MSCs further offer the advantages of 1) being an easily obtainable source; 2) possessing the ability for expansion in vitro; 3) lacking a requirement for immunosuppressive therapy to prevent rejections; and 4) having a reduced risk of malignant transformation [70]. Of note, it has also been suggested that MSCs are able to differentiate into neuron-like [111, 112] and glia-like [112, 113] lineages, although some have questioned this ability [114].

A number of studies have attempted to harness the potential of MSCs as a treatment for ALS. One study intrathecally administered MSCs obtained by bone marrow aspiration in 19 patients, and while all patients received MSCs via intrathecal lumbar puncture, 9 of the 19 patients also received intravenous MSCs [70]. No serious adverse events and a 6-month period of disease stability was reported following the procedure. In another study, 10 patients underwent isolation of bone marrow-derived MSCs, which were then administered intrathecally via lumbar puncture [71]. Again, the procedure was without serious adverse events; however, while some patients demonstrated stability of ALSFRS-R scores, others showed a decline from baseline. In addition, bone marrow-derived MSCs are the focus of an ongoing phase II clinical trial at the Mayo Clinic, Massachusetts General Hospital, and University of Massachusetts sponsored by Brainstorm-cell Therapeutics (Clinic Trial NCT02017912). In this study, patients are randomized to receive an intramuscular and intrathecal injection of autologous bone marrow-derived MSCs that are propagated ex vivo and induced to secrete neurotrophic factors. The study plans to enroll 48 patients and will evaluate safety and efficacy of the intervention.

Many other groups have also attempted more invasive methods of introducing MSCs in to the CNS. A case in which an Ommaya reservoir was utilized for intraventricular delivery of bone marrow-derived MSCs reported no adverse events [72]. Consecutive phase I studies in Italy in which bone marrow was obtained from the iliac crest and expanded in vitro prior to direct surgical implantation of the cells into the dorsal spinal cord involved 9 patients initially [73, 74], followed by an additional 10 patients [76], for a total of 19 patients [75]. In these studies, the procedure was well-tolerated and led to a slowed disease course in 6 patients; 4 of these patients were the youngest in the trials, and 2 subjects had a lower MN-predominant form of the disease. Thus, the authors suggested that slowing of the disease course in these subjects may have been a reflection of the disease phenotype and not the therapy. Another group, in Spain, evaluated 11 patients following injection of autologous bone marrow-derived MSCs into the spinal cord [77, 78]. No serious adverse events were reported nor were there any reported changes in the disease course; however, MNs near the areas of grafting showed fewer degenerative signs on histopathology. Finally,
a study in Turkey assessed patients following various routes of bone marrow-derived MSC administration and indicated that the procedure was safe, and in some cases motor improvement was also reported. [79].

Alternatively, the combination of MSCs from the bone marrow combined with T-cell vaccination has also been studied [80]. In this procedure, MSCs that were obtained from 7 patients were differentiated into NPCs and infused intrarterially 48 h after a third T-cell vaccination dose. No serious adverse events were reported and some patients experienced a transient improvement in symptoms.

In summary, bone marrow-derived MSCs remain a viable source of cells that confer the advantage of easy expansion and manipulation in vitro for subsequent autologous transplantation. While the currently reported approaches for grafting bone marrow-derived MSCs have tended to be more invasive, these strategies circumvent some of the limitations of intravenous administration seen in the G-CSF studies. Moving forward, however, it will be important to determine therapeutic differences between G-CSF-induced PBSCs and bone marrow-derived MSCs, as well as to determine the optimum collection and delivery procedures.

OECs

OECs have been studied in China based on the preclinical evidence studying NPCs obtained from olfactory tissue [84]. In 1 study, fetal OECs were injected into the bilateral corona radiata in a nonrandomized, nonblinded cohort of patients [84]. At 4 months, reporting by the patients or caregivers indicated a reduction in disease progression rates. In a larger study involving 507 patients receiving intraspinal and intracortical injections (with many undergoing multiple intracortical injections), short- and long-term outcomes were reported [85, 86], reflecting a statistically significant increase in ALSFRS-R scores following the injections, but no improvement in pulmonary function tests. Notably, 7 Dutch patients with ALS who underwent the procedure in China were evaluated at their local institution and showed no improvement in symptoms [81]. Similarly, a patient followed in the USA who underwent the procedure had an acceleration in disease progression and also suffered from a possible brain hemorrhage and vasogenic edema at the injection site [82]. Moreover, a postmortem study examining brain tissue from 2 patients who underwent the procedure showed graft encaement and did not show evidence of axonal regeneration, neuronal differentiation, or myelination to suggest an alteration of ALS neuropathology [83].

Based on preclinical models, while the studies involving OECs may deserve further attention, these studies have not utilized good clinical study design, especially given the large number of patients enrolled. More objective measures aside from the ALSFRS-R did not show improvements and these nonblinded studies may be influenced by a number of types of bias. Thus, this therapy should continue to be evaluated with close scrutiny, with a need for further support from well-designed clinical studies.

NPCs

Based on the promising preclinical data mentioned above, a phase I, first-in-human, Food and Drug Administration-approved clinical trial utilizing NSI-566RSC has recently been completed [14, 87–89, 91]. In this study, 12 patients underwent unilateral or bilateral lumbar intraspinal transplantation surgeries and 6 patients received unilateral cervical intraspinal transplantation surgeries following a risk-escalation design. Of note, 3 patients received both lumbar and cervical transplants; therefore, the phase I study involved a total of 15 patients (Table 3). The study demonstrated safety

| Table 3 | Phase I and II trial design for the first-in-human, Food and Drug Administration-approved clinical trial utilizing NSI-566RSC in patients with amyotrophic lateral sclerosis |
|---------|
| **Phase I trial design** | | | |
| **Group** | **Number of patients** | **Subject details** | **Injection target** | **Injection details** | **Final cell dose** |
| A1 | 3 | Nonambulatory | Lumbar | 5 unilateral | $5 \times 10^5$ |
| A2 | 3 | Nonambulatory | Lumbar | 10 bilateral | $1 \times 10^6$ |
| B | 3 | Ambulatory | Lumbar | 5 unilateral | $5 \times 10^5$ |
| C/E | 3 | Ambulatory | Lumbar | 10 bilateral | $1 \times 10^6$ |
| D | 3 | Ambulatory | Cervical | 5 unilateral | $5 \times 10^5$ |
| **Phase II trial design** | | | | |
| **Group** | **Number of patients** | **Injection target** | **Number of injections** | **Final cell dose** |
| A | 3 | Cervical | 10 bilateral | $2 \times 10^6$ |
| B | 3 | Cervical | 20 bilateral | $4 \times 10^6$ |
| C | 3 | Cervical | 20 bilateral | $6 \times 10^6$ |
| D | 3 | Cervical | 20 bilateral | $8 \times 10^6$ |
| E | 3 | Lumbar and cervical | 20 bilateral for each target | $16 \times 10^6$ |
and tolerability of the surgical procedure using a customized injection platform with a floating cannula designed to reduce risk of injury to the spinal cord given cardiorespiratory motion [89, 91], and anatomical injection accuracy to the ventral horn was determined using presurgical magnetic resonance imaging evaluation (Fig. 1; [10]). As of the final outcome reporting in early 2014 [91], 6 patients died owing to disease progression, and 1 died from an unrelated congenital heart defect. Moreover, while the study was not designed to evaluate efficacy, preliminary analysis of disease monitoring in a majority of patients demonstrated slowed disease progression in multiple clinical measures, with the greatest effect on disease progression seen in those patients who received the highest number of injections/cells (Fig. 2; [91]). Briefly, ALSFRS-R measurements for the cohort of patients receiving both lumbar and cervical injections (upper panel, Fig. 2a) were converted into data points reflecting the change in ALSFRS-R per year for various 9-month windows (lower panel, Fig. 2a). For example, the presurgical window reflected disease progression rates prior to the initial lumbar surgery (green window), and windows following the transplantation surgery reflect changes in ALSFRS-R over set time frames postsurgery (see representative blue windows). Of note, the timing of the second transplantation surgery in which cells were delivered into the cervical targets are indicated by the vertical dashed lines. Taken together, this analysis reflects an improvement in the rate of decline in ALSFRS-R following both the lumbar and cervical stem cell transplants [as demonstrated by positive slope peaks (Fig. 2a)]; however, this benefit decreases over time [as noted by the trough in the plot (Fig. 2a)], suggesting that there are apparent windows of benefit, which result following cellular transplantation (Fig. 2b).

The promise of this trial has been further underscored by the recent in-depth postmortem analyses available for 6 patients [90]. DNA from transplanted cells was detected in the spinal cord of all samples near the injection sites, and nests of stem cells could also be visualized on histology (Fig. 3; [90]). Notably, the 5 patients who demonstrated a slowed progression or stabilization of disease in this phase I trial were all treated within approximately 2 years of symptom onset and had no bulbar features, suggesting that early intervention may provide a better response to this modality of stem cell treatment [91]. Again, this highlights the potential for stem cells to rescue native MNs, although the window for neuroprotection closes as the disease progresses.

Given the safety and feasibility established in the phase I trial, a phase II study began in September 2013 and ended in July 2014. The phase II trial was designed to identify the maximum tolerated dose of stem cells coupled with the

**Fig. 1** Accurate anatomical targeting of stem cell delivery. **a.** T2-weighted magnetic resonance imaging scan showing a sagittal view of the spinal cord and the position of the conus medullaris and lumbar enlargement. **b.** Axial view of the spinal cord at the level of T12. **c.** Precise needle placement into the ventral horn of the spinal cord is calculated from a magnified image of part b. Estimated measurements of spinal cord diameter (6.02 mm) and distance from the dorsal root entry zone to the ventral horn (4.08 mm) are shown. Scale: 1 cm per grid division. **d.** Schematic of targeted injection of stem cells into the spinal cord. Reproduced from Boulis et al., Nat Rev Neurol 2011;8:172–6, [10]
maximum tolerated number of cervical and lumbar injections (Table 3). There were 5 treatment groups with 3 patients per group, whereby Group A received a dose of 2 million cells via 5 bilateral cervical injections (10 total), Group B progressed to 4 million cells injected in 10 bilateral cervical injections (20 total), and Groups C and D received 6 and 8 million cells, respectively, in 20 cervical injections. The final group, group E, then received 20 injections of 8 million cells into both cervical and lumbar regions, for a total of 16 million cells. All surgeries have been completed and final outcome monitoring and data review are underway; it is anticipated that an initial safety report is forthcoming, and planning is ongoing for future trials with this therapy to assess efficacy.

Induced Pluripotent Stem Cells

The ability to reprogram differentiated cells into an embryonic-like state, thus generating induced pluripotent stem (iPS) cells, was first demonstrated in 2006 [115], and has opened the door for attractive disease modeling and therapeutic strategies in ALS. Notably, the possibility of generating pluripotent iPS cells as a therapeutic option eliminates ethical concerns, as well as the risk of tissue rejection. Early preclinical studies demonstrated the successful grafting of human iPS cells into rat spinal cords, which gave rise to NPCs and, in particular, to astrocyte-like cells [116]. Given the relatively early state of this technology, however, no clinical

Fig. 2 Preliminary analysis of potential windows of human spinal stem cell (HSSC) biological activity in subjects 10–12. To identify the most biologically active period of the injected HSSCs, postsurgery data points for group E subjects were divided into a series of 9-month windows, beginning each month postsurgery, and slopes were calculated across each window. Slopes were also calculated using Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R) data points for the presurgical window. a The top panel demonstrates ALSFRS-R scores for group E subjects during the presurgical period (green) and representative ranges associated with the various sliding postsurgical 9-month windows (dark blue). The bottom panel demonstrates the slopes obtained for each sliding window, with the x-axis corresponding to the first month for each 9-month window (i.e., window 1 corresponds to months 1–10 postsurgery, window 2 corresponds to months 2–11 postsurgery, window 3 corresponds to months 3–12 postsurgery, etc.). The first plotted slope for each subject corresponds to their presurgical progression rate. Slope values higher than the presurgical slope at baseline represent improved or attenuated progression rates during the designated window. Note that the starting month of the final sliding window for each patient coincides with the dates of the second surgery, which occur at 17.5, 19.0, and 16.6 months after the initial cohort C surgery (time 0) for subjects 10, 11, and 12, respectively. (b) The presurgical slope and postsurgical slopes associated with the window correlating to the peak benefit windows for both the lumbar and cervical postsurgery time frames are summarized. Reproduced from Feldman et al., Ann Neurol 2014;75:363–73 [91]
trials using iPS cells are underway, although it is likely that iPS cells will play an increasing role in ALS research in the near future. Additionally, as it has been shown that iPS cells can be generated from human dermal fibroblasts [117, 118] and many groups are capitalizing on this chance to produce embryonic-like iPS cells from patients with MN disease such as ALS [119–122]. Thus, the clinical potential of patient-derived iPS lies in the ability for patient-specific disease modeling, high-throughput drug screening, and perhaps eventually gene editing and cell replacement therapy [122, 123].

Conclusions and Future Directions

The results of preclinical studies supported the utilization of stem cell therapies as a means to modify the disease course in ALS. As previously mentioned, although early goals were set on MN replacement, preclinical models now suggest that the mechanistic benefits of stem cells in ALS favor a neighborhood theory, where secreted factors from stem cells support diseased MNs. Many results from animal models, however, have yet to be confirmed in human studies, and in order to realize the full potential of stem cells, further advancements are needed in terms of both therapeutic optimization and clinical trial design.

Moving forward, while stem cell therapeutic trials in humans are currently using a variety of cell types, state-of-the-art advances in disease modeling will advance our ability to determine which strategies represent viable treatment strategies. Notably, the ability to model ALS using iPS cells is a promising frontier because, at this time, our preclinical models mainly focus on animal models based on SOD1-G93A. Given that mutations in SOD1 only represent a very small fraction of all ALS cases, and that pathologic features may vary from familial and more frequent sporadic forms of the disease, SOD1-G93A models may not allow us to understand the full risks and benefits of potential stem cell therapies. By establishing iPS cell

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Fig. 3 Gross and histological analysis of male amyotrophic lateral sclerosis (ALS) spinal cord. Gross image of the spinal cord shows the cord surface at the site of human spinal cord stem cell (HSSC) transplant (a, b). The vascular anatomy between intraoperative videos (a) corresponds to the postmortem tissue (b). Cross-section of the cord shows no visible tissue disruption (e). Histological staining with hematoxylin and eosin (d, g), Luxol fast blue (e, h), and immunohistochemistry for glial fibrillary acidic protein (f, i) of 8-μm spinal cord sections from patient 4 are shown. Nest of putative HSSCs are outlined in (d–f). Scale bars: 1 mm (d–f); 50 μm (g–i). Reproduced from Tadesse et al., *Ann Clin Transl Neurol*, in press [90]
lines derived directly from patients with ALS, physiologically relevant and high-throughput in vitro evaluation of treatments will allow us to further characterize ALS disease mechanisms and further understand how stem cells act as modifiers.

Next, although unrelated to stem cell therapies directly, the diagnostic delays of around 1 year typical of ALS also hinder therapeutic windows [124]. It is likely that the microenvironment is already compromised at the time symptoms develop and even more so when ALS is diagnosed; therefore, improving the time to diagnosis as a means to achieve earlier institution of therapy is essential in order to confer the best possible opportunity for MN rescue. The development of more robust biomarkers, including electrodiagnostic testing, transcranial magnetic stimulation, and advanced imaging techniques will complement advances in stem cell therapy by allowing earlier detection of disease, as well as more detailed assessments of therapeutic benefits [125, 126].

Notably, the selection of optimal outcome measures is also a challenge not unique to stem cell studies. As highlighted above and in Table 2, most studies to date, including our own, have focused on the safety and tolerability of the stem cell therapy, with efficacy remaining a secondary end point. As these trials mature and safety is established, efficacy outcomes will be expected. Assessment of MNs, graft survival, and migration of stem cells in vivo, however, is currently not directly possible, making surrogate endpoints essential. The topic of disease measurements has been the subject of a recent review [125], and is also covered elsewhere in this issue of Neurotherapeutics. Hence, while a full discussion of outcome measures is outside the scope of this review, a few points deserve attention. First, unlike drug trials, pharmacokinetic and pharmacodynamic markers are not available. Some groups have assessed the presence of circulating cells in the blood, although this type of evaluation is not applicable to all stem cell delivery mechanisms. In upcoming trials with cellular therapies enhanced to secrete growth factors, functional measures of these cells may be appropriate. As in many ALS trials, ALSFRS-R and survival remain viable endpoints but do not have specificity for evaluating therapy-specific efficacy measures. In other words, while ALSFRS-R can provide a good measure of patient function, it does not provide specific insight into the MN microenvironment. This highlights the importance of an autopsy to assess, in appropriate cases, graft survival and pathologic alterations in response to the therapy. The drawback is that an analysis of the native MN survival may only reflect those MNs that would have survived regardless of the intervention. Some groups have also tried to demonstrate stem cell migration via tagging procedures, and while this can show the location of these stem cells, it does not prove efficacy. As a result, we must still rely on surrogate markers. In our trial we are particularly excited about the potential insight that spinal cord MRI could provide, and certainly the modalities of diffusion tensor imaging and MR spectroscopy may be useful for other varying stem cell delivery methods as well.

Finally, good clinical trial designs will be essential to fully understand the effects and mechanisms of stem cell therapies. To date, only rare stem cell trials have advanced to a point where control groups are integrated into the study design or efficacy is being evaluated. Double-blinded, randomized, placebo-controlled studies may be possible with certain paradigms, but, with increasing invasiveness of procedures, the ethics and utility of sham procedures/surgeries becomes problematic. Still, for stem cell therapy to remain a legitimate treatment avenue, rigorous adherence to principles of clinical trial design must remain paramount. Now is a time when scientific innovation, bioengineering technologies, and medical expertise have reached a threshold, thus allowing stem cell therapy for ALS to be realized. Opportunities for new discovery remain close, and a time when stem cell therapy may turn the tide against ALS hopefully remains just over the horizon.

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Autocrine Production of IGF-I Increases Stem Cell-Mediated Neuroprotection

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Key Words. Key Words. Insulin-like growth factor-I • Growth factor • Human spinal stem cell • Amyotrophic lateral sclerosis • Cellular therapy • Stem cell • Neuroprotection

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder resulting in motor neuron (MN) loss. There are currently no effective therapies; however, cellular therapies using neural progenitor cells protect MNs and attenuate disease progression in G93A-SOD1 ALS rats. Recently, we completed a phase I clinical trial examining intraspinal human spinal stem cell (HSSC) transplantation in ALS patients which demonstrated our approach was safe and feasible, supporting the phase II trial currently in progress. In parallel, efforts focused on understanding the mechanisms underlying the preclinical benefit of HSSCs in vitro and in animal models of ALS led us to investigate how insulin-like growth factor-I (IGF-I) production contributes to cellular therapy neuroprotection. IGF-I is a potent growth factor with proven efficacy in preclinical ALS studies, and we contend that autocrine IGF-I production may enhance the salutary effects of HSSCs. By comparing the biological properties of HSSCs to HSSCs expressing sixfold higher levels of IGF-I, we demonstrate that IGF-I production augments the production of glial-derived neurotrophic factor and accelerates neurite outgrowth without adversely affecting HSSC proliferation or terminal differentiation. Furthermore, we demonstrate that increased IGF-I induces more potent MN protection from excitotoxicity via both indirect and direct mechanisms, as demonstrated using hanging inserts with primary MNs or by culturing with organotypic spinal cord slices, respectively. These findings support our theory that combining autocrine growth factor production with HSSC transplantation may offer a novel means to achieve additive neuroprotection in ALS. Stem Cells 2015;33:1480–1489

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disease resulting in upper and lower motor neuron (MN) loss. There are no effective treatments and death typically occurs within 3–5 years of onset [1]. One obstacle facing therapeutic development is the complex, unclear etiology and several mechanisms have been proposed, including oxidative stress, mitochondrial dysfunction, a toxic microenvironment, astrocytic and glial dysfunction, loss of distal neuromuscular junctions, and a breakdown in local neurocircuitry within the spinal cord [2]. In addition, reductions in trophic factors within the spinal cord microenvironment in ALS further enhance MN susceptibility to the disease process [3–5]. Given this complexity, it is likely that a multifaceted treatment approaches may be warranted.

Cellular therapies are a new therapeutic avenue for ALS; they provide de novo neural tissue to support neurocircuitry and represent a source for in situ production of neuroprotective growth factors [6, 7]. Recently, we completed a phase I clinical trial examining intraspinal transplantation of human spinal stem cells (HSSCs) in 15 ALS patients, validating the feasibility and safety of this cellular therapy approach [8–13]. Phase II of the trial examining dosing and efficacy is ongoing, and in parallel, investigations in the laboratory are focused on understanding the mechanisms underlying how HSSCs support MNs in ALS. Previous work in G93A-SOD1 ALS rats confirmed that HSSCs integrate into the spinal cord, form synapses with host tissue, maintain MN numbers, and produce several neuroprotective growth factors, including glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and insulin-like growth factor-I (IGF-I) following intraspinal transplantation [14–18]. Of these growth factors, IGF-I is the most highly expressed, suggesting it may contribute to HSSC-mediated neuroprotection.

IGF-I has potent neurotrophic and neuroprotective effects and extensive preclinical evidence supports the attenuation of MN loss and maintenance of neuronal synapses and neuromuscular junctions by IGF-I [19]. We and others have shown that viral-mediated IGF-I delivery
increases MN numbers, improves grip strength, delays progression, and prolongs survival in ALS rodents [20–23]. A phase III trial investigating subcutaneous IGF-I in ALS patients, however, did not successfully demonstrate therapeutic efficacy, although this was likely due to failure of IGF-I to reach the spinal cord and confer continuous protection to diseased MNs [24]. The ability of cellular therapies to provide localized neurotrophic support directly to MNs within the spinal cord microenvironment may circumvent the issues encountered in earlier IGF-I trials.

In this study, we developed a novel stem cell line expressing increased IGF-I levels, HSSC:IGF-I, to examine the potential contributions of autocrine and paracrine IGF-I production to neuroprotection within the neuromuscular axis. Specifically, we assess the differences between HSSCs expressing lower levels of IGF-I and HSSC:IGF-I, focusing on growth factor production and the potential actions of IGF-I on cell proliferation, migration, and differentiation. We further explore the additive role of IGF-I in neuroprotection by examining the ability of HSSC:IGF-I to provide MN protection against excitotoxicity compared to HSSCs. We anticipate these studies will demonstrate how autocrine and paracrine growth factor production contributes to cellular therapy neuroprotection and lend further support to our contention that HSSCs confer multifaceted therapeutic benefits in ALS.

MATERIALS AND METHODS

HSSC and HSSC:IGF-I Culture

HSSCs (NSI-566RSC) were prepared from spinal cord tissue obtained from a single 8-week human fetus following an elective abortion as described previously [17, 25]. To generate HSSC:IGF-I, HSSCs were exposed to replication-defective recombinant lentivirus engineered to overexpress a preproisoform of human IGF-I cDNA driven by the human ubiquitin C (Ubc) promoter. The resulting cells were propagated as a single cell line without further selection and named NSI-566RSC.Ubc_IGF-I. Using a control construct expressing green fluorescent protein (GFP) under the same promoter, approximately 90%–95% of the proliferating cells were GFP-positive.

Cell culture followed established protocols [18, 26, 27] using culture reagents obtained from Sigma (St. Louis, MO, www.sigmaaldrich.com), unless otherwise noted. Briefly, culture vessels were coated with 100 μg/ml poly(lysine) (PDL; Millipore, Billerica, MA, www.millipore.com) in HEPES buffer and incubated for 24 hours at room temperature. Surfaces were then washed three times with sterile water and allowed to completely dry under the hood before coating with 25 μg/ml fibronectin in phosphate buffered saline (PBS) for 1 hour. Fibronectin solution was aspirated and vessels were immediately without drying. HSSC and HSSC:IGF-I were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Gibco, Life Technologies, Carlsbad, CA, www.lifetechnologies.com/us/en/home/brands/gibco.html) supplemented with 100 μg/ml human plasma apo-transferrin, 25 mg/ml recombinant human insulin, 1.56 g/l glucose, 20 nM progesterone, 100 μM putrescine, and 30 nM sodium selenite. For maintenance in a progenitor state, 10 ng/ml fibroblast growth factor (FGF) was added to the growth media. For differentiation, cells were cultured in differentiation media comprised of DMEM without FGF, supplemented with 4 mM l-glutamine, 20 μM l-lysine, 6 μM l-asparagine, 67 μM l-proline, 250 nM vitamin B12, 25 mg/l insulin, 100 mg/l transferrin, 20 nM progesterone, 100 μM putrescine, and 30 nM sodium selenite, for 7 days, with a 50% media change every other day. Cells were analyzed in their undifferentiated state (D0) or after 3 or 7 days (D3 or D7, respectively) of differentiation.

ELISA, Dot Blot Analysis, and Western Blotting

To confirm that lentiviral transduction led to increased stable expression of IGF-I, conditioned media were collected after 10 passages and analyzed by ELISA. To collect conditioned media, flasks were washed with PBS, 10 ml growth (D0) or differentiation (D3, D6, and D7) media without insulin was added, and cells were cultured for 24 hours prior to media collection. Conditioned media were concentrated 10-fold to 1 ml using Centricon filters (3 kDa cut off; Millipore) following the manufactures’ guidelines and IGF-I levels at D0 and D6 were quantified using a human-specific IGF-I ELISA (R&D Systems, Minneapolis, MN, www.rndsystems.com). For dot blotting, concentrated media (200 μl) from D0 and D7 cultures were applied to nitrocellulose membranes using a Whatman Schleicher & Schuell Minifold I filtration manifold (Sigma) and exposed to Kodak BioMax XAR film (Sigma).

Western blotting on D0, D3, and D7 cell lysates was performed as previously described [27, 28]. Equal amounts of protein were loaded on either 10% or 12%, polyacrylamide gels, dependent on the size of the protein of interest. Polyvinylidene difluoride membranes were incubated with primary antibody overnight at 4°C and with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Antibody binding was developed with LumilGLO Reagent and Peroxide (Cell Signaling, Danvers, MA, www.cellsignal.com) and exposed to Kodak BioMax XAR film (Sigma).

Quantitative Real-Time RT-PCR

Total RNA was extracted from HSSC and HSSC:IGF-I at D0 and D7 using an RNeasy Kit (Qiagen, Valencia, CA, www.qiagen.com/us) following the manufacturers’ instructions. Reverse transcription was performed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, www.bio-rad.com). Quantitative real-time RT-PCR (QPCR) analysis was performed using Power SYBR Green PCR Master Mix and a StepOnePlus thermal cycler (Applied Biosystems, Life Technologies, Grand Island, NY, www.lifetechnologies.com/us/en/website-overview/ab-welcome.html) using primers for IGF-IR, VEGF, GDNF, BDNF, vGlut1, vGlut3, GluR2, and GAPDH (Millipore). Antibody binding was visualized as described above.
coated glass coverslips in 24-well plates for 0, 3, or 7 days, conjugated secondary antibodies (Jackson ImmunoResearch, experiments.

chambers and transwell inserts containing HSSC or HSSC:IGF-I differentiation media plus 10% fetal bovine serum with or without an Olympus BX-51 microscope equipped with a digital camera. Images were collected using an Olympus BX-51 microscope. To


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Edina, MN, www.neuromics.com), GAD65/67 (Millipore), 0.1% Triton/PBS. Primary antibodies for TUJ1 (Neuromics, Triton/PBS, and then blocked in 5% normal donkey serum/

[27, 29]. HSSC/HSSC:IGF-I were grown on PDL and fibronectin-

Immunocytochemistry and Neural Index Assays

Immunocytochemistry was performed as previously described [27, 29]. HSSC/HSSC:IGF-I were grown on PDL and fibronectin-coated glass coverslips in 24-well plates for 0, 3, or 7 days, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton/PBS, and then blocked in 5% normal donkey serum/0.1% Triton/PBS. Primary antibodies for TUJ1 (Neuromics, Edina, MN, www.neuromics.com), GAD65/67 (Millipore), VGLUT2 (Millipore), or IGF-IR/β (Sigma) were incubated overnight at 4°C. Following incubation with Cy3, Cy5, or FITC-conjugated secondary antibodies (Jackson ImmunoResearch, Westgrove, PA, www.jacksonimmuno.com), coverslips were mounted on glass slides using ProLong Gold with DAPI (Molecular Probes, Life Technologies, Carlsbad, CA, www.molecularprobes.com). Images were collected using an Olympus BX-51 microscope. To measure the extent of neural differentiation, TUJ1 and DAPI-labeled images were analyzed following our published neural index protocol [27]. Briefly, TUJ1-labeled and corresponding DAPI images were opened in MetaMorph (Molecular Devices, Sunnyvale, CA, www.moleculardevices.com). Cell number was counted on DAPI images using the “count nuclei” plug-in, with manual adjustments to correct for any miscounted cells. Thresholds on TUJ1 images were then adjusted and the area of neurite coverage was measured using region statistics. The neural index was expressed as neurite area (cell$^2$/cell).

Primary MN Coculture for Excitotoxicity Assays

Primary embryonic rat MNs were isolated according to our previously published protocol [27, 30] and cultured on PDL-coated glass coverslips in a 24-well plate. After 24 hours, the MNs were fed with 250 µl differentiation media for coculturing with HSSC or HSSC:IGF-I, which were seeded onto PDL-coated 3 µm pore transwell inserts (Corning, Tewksbury, MA, www.corning.com/lifesciences/worldwide.aspx) and differentiated for 4 days prior to coculturing. Cocultures were left for 3 days prior to beginning the excitotoxicity assay. All culture reagents were purchased from Gibco, Life Technologies unless otherwise indicated.

Excitotoxic stress was induced by adding 100 µM glutamate to the coculture media for 24 hours. The contribution of paracrine IGF-I production to protection was assessed by adding the IGF-IR inhibitor, NVPAEW541 (1 µM), 2 hours prior to glutamate. After glutamate treatment, MNs were fixed in 4% paraformaldehyde for 10 minutes and TUNEL was used to detect DNA fragmentation [27, 30]. Samples were labeled with digoxigenin-dUTP and stained with horseradish peroxidase-conjugated antidigoxigenin antibody using the ApopTagPlus In Situ Apoptosis Peroxidase Detection Kit (Chemicon). Alternatively, fluorescent TUNEL processing was carried out as described above with a Fluorescein-labeled conjugate using the ApopTagPlus In Situ Apoptosis Fluorescein Detection Kit (Chemicon). Fluorescent signal was detected and recorded using an Olympus BX-51 microscope. TUNEL-positive cells were counted in at least 10 representative fields per condition by a blinded investigator, per our published protocol, for an average total of approximately 1,000–2,000 MNs per condition [27].

Organotypic Spinal Cord Slice Cultures for Excitotoxicity Assays

Organotypic spinal cord slice cultures were prepared from P5–8 rat pups [31]. Briefly, lumbar spinal cords were isolated, membranes were removed, and spinal cord tissue was mounted in 7% agarose for Vibrotome sectioning. Sections (300 µm) were collected in ice cold PBS and three spinal cord slices were added to each Millicell Cell Culture Insert (0.4 µm pore, 300 mm diameter; Millipore) in a six-well plate. Wells were flooded with slice growth media containing 50% minimal essential medium, 25 mM HEPES, 25% heat-inactivated horse serum, and 25% Hanks’ balanced saline solution, supplemented with 25.6 mg/ml d-glucose and 2 mM glutamine, at a final pH of 7.2. Cultures were incubated at 37°C in 5% CO₂ for 2 weeks, with media changes every 2 days. For coculture experiments, 2 µl of HSSC or HSSC:IGF-I cell suspension (6 × 10³ cells per milliliter) was placed over the ventral horn of the slices 2 days after plating.

Table 1. Quantitative real-time RT-PCR primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>IGF-IR</td>
<td>CAATAAGGTCTGTCACAGAGACC</td>
<td>CTCCTTTTCTGGATATTGCTGT</td>
</tr>
<tr>
<td>VEGF</td>
<td>ATGGAGAGAGGAGAGG</td>
<td>AGTTGGGGCCTAGTCGCGG</td>
</tr>
<tr>
<td>GDNF</td>
<td>CTGACTTTGGCTCGGGAATG</td>
<td>TTGTCACTCAACGGCTTCTATT</td>
</tr>
<tr>
<td>BDNF</td>
<td>CCAAGCGAGTTCAAGAGG</td>
<td>TCCAGCAAGAAGAAAGAAGGA</td>
</tr>
<tr>
<td>vlGlut1</td>
<td>AAGTCCTGAGGTTGCGGTAGG</td>
<td>ACCAGTAACATACGTGAGGTGTT</td>
</tr>
<tr>
<td>vlGlut3</td>
<td>GGGGTTGTTGGTGGCAGCTCATT</td>
<td>CCCCCCTCTGATCGTCTCATT</td>
</tr>
<tr>
<td>GAD67</td>
<td>GCCAGACAGCAGATTGTG</td>
<td>CGATTTCCAGGCAATTTGATG</td>
</tr>
</tbody>
</table>

Abbreviations: BDNF, brain-derived neurotrophic factor; GAD67, glutamate decarboxylase; GDNF, glial cell-derived neurotrophic factor; IGF-IR, insulin-like growth factor-I receptor; VEGF, vascular endothelial growth factor; vGlut1, vesicular glutamate transporter 1; vGlut3, vesicular glutamate transporter 3.
To induce excitotoxic stress, culture media were supplemented at day 7 with 100 μM threo-hydroxyaspartate (THA; Sigma) to inhibit glutamate transport until day 14. Slices were fixed in 4% PFA and processed for standard immunocytochemistry as described above, using SMI-32/ChAT (Millipore) to label large alpha MNs and HuNu (Millipore) to confirm HSSC and HSSC:IGF-I placement on the spinal slices. Fluorescent images were captured using an Olympus BX-51 microscope and SMI-32/ChAT-labeled MNs were quantified in the ventral horns of each slice. The total remaining MNs were quantified from nine slices per condition from three independent experiments.

**Statistical Analyses**

All results are representative of at least three independent experiments. Statistical significance was determined using one-way ANOVA followed by Tukey’s multiple comparison test or linear regression analysis (GraphPad Prism, La Jolla, CA, www.graphpad.com).

**Results**

**Characterization of Growth Factor Production by HSSC:IGF-I**

To examine the contribution of IGF-I production to HSSC neuroprotection, a lentivirus encoding full length human IGF-I was used to generate HSSC:IGF-I. ELISA on conditioned media from HSSC and HSSC:IGF-I cultures demonstrated that HSSCs produce low basal levels of 0.2 fg/cell per day IGF-I, a level that was consistent throughout the first week of differentiation (Fig. 1A). HSSC:IGF-I exhibited a sixfold increase in IGF-I production, averaging approximately 1.2 fg/cell per day (Fig. 1A), and this increased IGF-I production was maintained as the cells underwent differentiation. We next assessed the levels of several growth factors typically expressed by HSSCs using dot blot analysis on conditioned media from undifferentiated cells (D0) and cells after 7 days (D7) of differentiation. VEGF secretion was comparable in HSSC and HSSC:IGF-I and decreased similarly as both cell lines differentiated, while BDNF levels were consistent between cell lines and time points (Fig. 1B). GDNF levels were also comparable at D0; however, at D7, higher levels of GDNF were detected in conditioned media from HSSC:IGF-I compared to that from HSSCs (Fig. 1B). As previous studies have shown that HSSCs respond to IGF-I signaling, we also explored how growth factor receptor levels are regulated by paracrine IGF-I expression. IGF-IR protein levels in both cell lines throughout differentiation were reduced in HSSC:IGF-I relative to HSSCs at each time point examined (Fig. 1C). Given the association between IGF-I expression and VEGF signaling, we also investigated the effect of increased IGF-I expression on VEGF signaling levels. VEGF is only expressed by HSSC and HSSC:IGF-I at D0; however, basal levels of VEGF were considerably lower in HSSC:IGF-I compared to HSSC (Fig. 1C). Finally, to validate our protein expression results, we performed QPCR and similarly observed no measurable difference in the expression levels of IGF-IR (Fig. 1D), VEGF (Fig. 1E), or BDNF (Fig. 1G) between cell lines at the time points examined, although we did detect a significant increase in D7 HSSC:IGF-I GDNF transcript levels relative to levels in D7 HSSCs (Fig. 1F), a finding consistent with our protein expression analyses. Together, these results demonstrate significantly increased IGF-I production by HSSC:IGF-I that is maintained through 7 days of differentiation and is also accompanied by reduced early VEGF expression and increased D7 GDNF expression.

**Effects of IGF-I on Cellular Behavior and Differentiation in HSSC:IGF-I**

IGF-I can modify cell behaviors such as proliferation and migration [32, 33]; therefore, we measured EdU incorporation to assess the effect of enhanced IGF-I production on HSSC:IGF-I proliferation rates. There were no significant differences in the proliferation rates of undifferentiated D0 HSSC:IGF-I compared to HSSC (Fig. 2A–2C) and no differences were observed between the cell lines at D3 of differentiation (Fig. 2A, 2D, 2E), suggesting that IGF-I does not maintain proliferation in HSSC:IGF-I. Alternatively, examination of the effect of IGF-I expression on the migratory potential of undifferentiated D0 HSSC:IGF-I revealed that HSSC:IGF-I had approximately double the migratory potential of HSSCs (Fig. 2F); however, no significant migration was detected in either cell line at D7 of differentiation (data not shown).

Previously, we demonstrated that exogenous IGF-I treatment enhanced HSSC neural differentiation [27]; therefore, we next examined how autocrine production of IGF-I affected HSSC:IGF-I differentiation. We first analyzed differentiation using our published neural index pipeline, which accounts for both neurite number and length relative to the number of cells present [27], and observed a twofold increase in the neural index of HSSC:IGF-I compared to HSSCs (52 μm²/cell vs. 24 μm²/cell, respectively; Fig. 3A), a difference that is clearly visible in the representative TUJ1 images (Fig. 3B, 3C). Next, we evaluated the expression of vGlut1, vGlut3, GluR2, and GAD67 using QPCR to characterize terminal differentiation, as HSSCs exhibit terminal phenotypes including both glutamatergic and GABAergic neurons by D7 of differentiation [18]. While no significant differences in the total percentage of differentiated cells were observed for either lineage, a significant increase in vGlut1 levels was present in D7 HSSC:IGF-I compared to HSSC (Fig. 3D). Additionally, decreased vGlut3 expression was seen in D7 HSSC:IGF-I compared to HSSCs (Fig. 3E), and GluR2 and GAD67 expression level differences between the two cell lines were not significant (Fig. 3F, 3G). These data demonstrate that increased IGF-I expression enhances the early migratory potential and neuronal differentiation of the cells, but does not significantly alter HSSC proliferation or behavioral properties following differentiation, thus maintaining the potential safety profile of HSSC:IGF-I for future translational applications.

**Neuroprotective Effects of HSSC:IGF-I**

To determine how increased IGF-I expression affects HSSC neuroprotection, we first modeled glutamate-induced excitotoxicity associated with ALS by exposing primary rat embryonic MNs to glutamate in vitro, a treatment that induces approximately 77% cell death (Fig. 4A). To assess the benefits of only secreted factors, we cocultured HSSCs or HSSC:IGF-I in a compartment adjacent to MNs. While HSSCs significantly reduced glutamate-associated MN death to 42%, HSSC:IGF-I further reduced cell death to under 20%, a level comparable...
to that of the nontreated control group (Fig. 4A; data not shown). Furthermore, the addition of the IGF-IR inhibitor NVPAEW541 prevented the additional neuroprotection conferred by HSSC:IGF-I, reverting protection back to 39% death, a level comparable to that of HSSCs (Fig. 4A). These findings indicate that there is a measurable contribution of IGF-I to HSSC neuroprotection. Second, we analyzed the impact of secreted and cell contact-mediated HSSC and HSSC:IGF-I protection by plating the cells directly over spinal cord organotypic cultures subjected to ALS-associated excitotoxic stress. Cell placement over the spinal cord ventral horn was confirmed by stem cell-specific HuNu staining (Fig. 4C). Induction of glutamate toxicity using THA resulted in the survival of only 26% of the MNs in the spinal cord slices (Fig. 4B, 4E) relative to untreated controls; however, the addition of HSSCs increased MN survival to 50% in the presence of THA (Fig. 4B, 4F) and the addition of HSSC:IGF-I significantly increased MN survival beyond that observed with HSSCs, resulting in 78% of MNs per ventral horn surviving THA treatment (Fig. 4B, 4F, 4G). Overall, these studies demonstrate that IGF-I expression offers additive benefits to the therapeutic potential of HSSCs.
ALS is a fatal, progressive neurodegenerative disease with no effective treatment. Characterized by a complex and elusive etiology that may not lend itself to conventional targeted drug development strategies, multifaceted treatments may be necessary to achieve meaningful outcomes. Stem cell-based therapies have gained momentum as a potential ALS therapy, as they offer a novel means to confer multifaceted protection [8, 12]. We recently completed a phase I clinical trial establishing the safety and feasibility of intraspinal HSSC transplantation in ALS patients, and the resulting phase II clinical trial assessing therapeutic dosing and efficacy is underway [9–13]. As we continue through the phases of this trial to establish stem cell-based therapies as a viable ALS treatment option, parallel efforts are focused on understanding the mechanisms underlying cellular therapy efficacy. This study describes the development and characterization of HSSC:IGF-I, a cell line that has the potential to offer the additional neurotrophic and neuroprotective benefits of the growth factor IGF-I. We demonstrate that HSSC:IGF-I produce robust levels of IGF-I, that this modification does not induce any detrimental alterations in cell behavior, and that IGF-I expression enhances HSSC neuroprotection against ALS insults relative to unmodified HSSCs. Together, these data support an additive role for autocrine and paracrine growth factor signaling in cellular therapy neuroprotection in ALS and suggest that the continued development of cellular therapies producing growth factors, including IGF-I, is warranted.

We contend that IGF-I production by HSSCs will confer additive benefits to the salutary effects of HSSC transplantation for ALS, as both IGF-I and HSSCs have proven efficacy in preclinical studies. HSSCs naturally produce an array of growth factors, including BDNF, GDNF, VEGF, and IGF-I, and upon intraspinal transplantation into G93A-SOD1 ALS rats, they differentiate into glutamatergic and GABAergic neurons, synapse with host MNs, and attenuate disease progression in ALS rodent models, as evidenced by delayed onset and progression rates and improved survival, especially when transplanted into multiple spinal cord regions [14–18]. Positive effects include a 27% increase in MN protection in the vicinity of HSSC grafts and transient functional improvements; however, no long-term effects on the disease course and little efficacy in regions away from the grafts, reflected in the approximate 50% decrease in total MN numbers compared to control rats and loss of descending motor tract conduction, were noted in a subsequent study [14]. Thus, despite the localized benefits of HSSC transplantation, there is a critical need to maintain neurocircuitry along the entire neuromuscular and corticospinal axis in order to achieve functional outcomes in ALS; a feat that could be achieved by autocrine and paracrine neurotrophic and neuroprotective growth factor production by cellular therapies targeting upper MNs.

Growth factors have long been considered a potential therapy for ALS, and extensive preclinical and clinical data support the utility of IGF-I in ALS [19]. In vitro studies demonstrate that IGF-I treatment activates the neuroprotective p44/42 MAPK and PI3K/Akt signaling pathways to attenuate glutamate-induced cell death in primary embryonic MN cultures [30], and viral-mediated IGF-I transfection of SHSY-5Y cells protects not only transfected cells against glutamate...
toxicity but also neighboring cells, suggesting that growth factor production is capable of both autocrine and paracrine effects [28]. Numerous in vivo studies also provide further justification for IGF-I treatment in ALS, with IGF-I exerting beneficial effects on MNs and at the level of the neuromuscular junction and spinal cord microenvironment [19–22]. Furthermore, the potential clinical utility of IGF-I is further supported by the significantly reduced levels of IGF-I observed in the cerebrospinal fluid of ALS patients [4]. The lack of significant efficacy in the phase III clinical trial assessing subcutaneous IGF-I in ALS patients, however, was likely attributable to failure of IGF-I to reach vulnerable MNs following the used subcutaneous delivery approach [24, 34–36]. Notably, this limitation can be overcome using cellular therapy strategies to deliver growth factor-producing cells directly into the brain or spinal cord milieu.

Figure 3. HSSC and HSSC:IGF-I differentiation. (A–C): Representative images of TUJ1 and DAPI-labeled HSSC (B) and HSSC:IGF-I (C) for calculation of the neural index (A) at D7. (D–G): Quantitative real-time RT-PCR (QPCR) analysis of phenotypic markers of differentiated neurons including vGlut1 (D), vGlut3 (E), GluR2 (F), and GAD (G). QPCR data were normalized to GAPDH and presented as a fold-change from D0 HSSC. Data are shown as mean ± SD (*, p < .05) or are representative images (B, C, scale bar = 100 μm) of at least three independent experiments. Abbreviations: HSSC, human spinal stem cell; IGF-I, insulin-like growth factor-I.
In this study, we engineered HSSCs to produce increased levels of IGF-I to establish HSSC:IGF-I as a novel tool to evaluate the potential effects of IGF-I expression in neuroprotection. These cells exhibit a sixfold increase in IGF-I in both their progenitor cell state and following differentiation, a finding consistent with previous reports demonstrating 6.4-fold increases in IGF-I production following lentiviral transfection of postnatal subventricular zone neural progenitor cells [37]. In addition to increasing IGF-I expression, we also identified increased levels of GDNF following differentiation in HSSC:IGF-I, an observation supporting the premise that neurotrophic factor production may offer additive benefits to HSSC efficacy. While we have previously demonstrated that HSSCs are an incredibly safe cell line [9, 11, 13, 14], IGF-I has the potential to increase cell proliferation and impact migration [37–41]; therefore, we verified that enhancing IGF-I expression in HSSCs does not adversely alter cell behavior. In line with our previous studies assessing the impact of exogenous IGF-I on HSSC behavior, this data indicate that while HSSC:IGF-I express the IGF-IR, IGF-I does not act as a mitogen for HSSC, thus confirming the inherent safety profile of HSSC:IGF-I for future translational applications. Specifically, we see no significant increases in HSSC:IGF-I proliferation at any time point, a finding that may likely be attributed to the reduced expression of IGF-IR observed in HSSC:IGF-I relative to HSSCs. In addition, we see no notable migration of HSSC:IGF-I following differentiation. This is consistent with our in vivo experiences demonstrating that HSSCs only migrate to a minimal extent following intraspinal transplantation, with the majority of cells remaining in a large bolus at the injection site [14, 42, 43]. For HSSC:IGF-I cells, however, the observed increase in migratory potential prior to differentiation may be advantageous, whereby lower numbers of intraspinal injections may be sufficient achieve adequate coverage across the desired spinal cord segments [16, 43, 44]. Together, these behavioral HSSC:IGF-I characteristics support their utilization to assess the potential additive contributions of IGF-I production to HSSC-mediated neuroprotection.

Growth factor expression alters the differentiation profile of umbilical cord stem cells [45]; therefore, we examined how IGF-I affects terminal differentiation in HSSCs. In line with previous reports and our initial studies using exogenous IGF-I [17, 18, 27], HSSC:IGF-I have a higher neural index than HSSCs, but the majority of terminally differentiated cell types

Figure 4. Neuroprotective potential of HSSCs and HSSC:IGF-I. (A): Quantification of MN death using the TUNEL assay following exposure to glutamate (100 μM) in the absence or presence of HSSCs or HSSC:IGF-I cocultured using transwell inserts. IGF-IR inhibition by NVPAEW541 (1 μM) was used to determine the contribution of paracrine IGF-I production to neuroprotection. Data are presented as mean ± SD (*, p < .05 for HSSC vs. HSSC:IGF-I). (B–G): Quantification of MN survival (B) in rat spinal cord organotypic slices directly cocultured with HSSCs or HSSC:IGF-I following treatment with THA (100 μM) to induce toxicity. Representative images confirming of the presence of HuNu-positive HSSCs cultured on top of spinal slices (C; dashed line delineates the outline of the ventral horn, solid line delineates the extent of the white matter) and of ChAT/SMI-32 MN staining in the ventral horns of a nontreated control slice (D), a THA-treated slice (E), a THA-treated slice cocultured with HSSCs (F), and a THA-treated slice cocultured with HSSC:IGF-I (G). Arrows depict representative MNs within the spinal cord slices. Data are presented as mean ± SD (*, p < .05 vs. slice + THA) or are representative images (C, scale bar = 100 μm; D, scale bar = 50 μm) of at least three independent experiments. Abbreviations: HSSC, human spinal stem cell; IGF-I, insulin-like growth factor-I; MN, motor neuron; THA, threo-hydroxyaspartate.
remain similar between the lines. These findings correlate with other studies demonstrating increased TUN1 expression in response to IGF-I overexpression in differentiating neural progenitor cells [37]. Furthermore, no differences in astrocyte or oligodendrocyte ratios were seen between cell lines, and both glutamatergic and GABAergic neurons were predominately represented. Given the reported loss of inhibitory interneurons and the critical need to maintain neurocircuitry along the entire neuromuscular axis [14, 46], the generation of glutamatergic and GABAergic neurons further validates the therapeutic potential of HSSCs in ALS. Thus, the observed differentiation profile of HSSC:IGF-I maintains that IGF-I production by HSSCs can support MNs and also provide critical infrastructure to spinal cord neurocircuitry.

As further shown by these studies, HSSC:IGF-I confer neuroprotection via both direct and indirect mechanisms, suggesting that IGF-I may enhance HSSC cell contact-mediated neuroprotection as well as elicit paracrine effects on MNs and surrounding cells within the spinal cord microenvironment through secreted factors. Notably, the increased levels of IGF-I likely underlie the enhanced protection conferred by HSSC:IGF-I, as evidenced by both the increased protective capacity of HSSC:IGF-I relative to HSSCs, as well as the attenuated protection in the presence of IGF-IR inhibition. Similar to this approach, gene therapy has been used to develop a number of stem cell-based combinatorial cellular therapy modalities expressing neuroprotective factors for ALS [12]. Intracerebroventricular injection of mesenchymal stem cells expressing the antioxidant protein GLP-1 into G93A-SOD1 mice improved disease onset, motor performance, and survival [47], and modifying mesenchymal stem cells to express GDNF and VEGF provided neuroprotection in G93A-SOD1 rats [48, 49]. GDNF-expressing glial progenitors also protected MNs following intraspinal transplantation in G93A-SOD1 rats; however, protection was not seen with unmodified cells [50]. These studies support a similar role for autocrine and paracrine IGF-I production as a means to augment the therapeutic potential of HSSCs in ALS [14, 16, 27, 48].

CONCLUSIONS

Extensive preclinical data justify the therapeutic use of HSSCs and IGF-I individually [8, 12, 19] and the safety of both individual treatment strategies has been established in clinical trials [11, 24]. Most recently, the successful completion of our phase I trial examining intraspinal HSC transplantation in ALS patients has paved the way for the ongoing phase II trial assessing the therapeutic dosing and initial efficacy of this groundbreaking treatment. In parallel, our quest to elucidate how IGF-I expression influences cellular therapy-mediated neuroprotection in ALS prompted the derivation of HSSC:IGF-I. We demonstrate that IGF-I expression does not enhance cell proliferation, but does increase the early migratory capacity, neural differentiation, and neuroprotective potential of HSSC:IGF-I. These data support IGF-I-mediated neurotrophism as a means to achieve additive neuroprotection, and suggest that transplantation of HSSC:IGF-I in addition to this HSSC therapeutic approach may offer further MN protection in ALS. Notably, transplantation of HSSC:IGF-I into the brain has the potential to confer protection to upper MNs by harnessing the stable, permanent expression of diffusible IGF-I within the central nervous system, thus achieving critical neuroprotection at multiple sites along the neuromuscular axis. With continued progress through our ongoing clinical trial using HSSCs, along with the completion of future studies establishing the feasibility and efficacy of growth factor-producing cellular therapies in ALS models, we have incredible potential to establish an effective, multifaceted MN protection strategy for ALS, and potentially for other neurological disease applications as well.

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AUTHOR CONTRIBUTIONS

J.S.L.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; S.A.S.: data analysis and interpretation, manuscript writing, and final approval of manuscript; L.M.M.: collection and/or assembly of data, data analysis and interpretation, and final approval of manuscript; C.P.: collection and/or assembly of data and final approval of manuscript; T.G.H. and K.J.: provision of study material or patients and final approval of manuscript; E.L.F.: conception and design, financial support, and final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

T.G.H. and K.J. are employees of Neuralistem, Inc. E.L.F. is an unpaid consultant to Neuralistem, Inc. All other authors have nothing to declare.

REFERENCES


Review

The dual roles of immunity in ALS: Injury overrides protection

Benjamin J. Murdock, Diane E. Bender, Benjamin M. Segal, Eva L. Feldman

Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disease affecting motor neurons. Disease progression is accompanied by a multi-phased immune response, and recent studies indicate that the immune system is not simply a bystander during disease, but plays an active role in shaping ALS pathology. The role of the immune system during ALS progression is highly complex, however, as it has been found to have a role in both enhancing neurodegeneration as well as protecting the central nervous system. Previous reports have established that the immune response can therefore be separated into two distinct phases: a protective Type 2 response followed by a neurotoxic Type 1 response. This review will address the two phases of the immune response in ALS and describe their roles during disease progression. More importantly, it will also examine the likely sources of immune polarization that are responsible for shifting immunity from the protective T2 phase to the neurotoxic T1 phase.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating adult-onset illness characterized by degeneration of the motor neurons at all levels of the motor system. Depending on the site of disease onset, initial symptoms can include dysarthria, dysphagia, palsy, and proximal or distal weakness. As the disease progresses, patients can experience muscle atrophy, spasticity, hyperreflexia, paralysis, and eventually failure of the respiratory system (Mitchell and Borasio, 2007). Following diagnosis, the expected lifespan of patients is 3–5 years, with a prevalence of 3.9 per 100,000 in the United States (Mehta et al., 2014). There is currently no cure for ALS, and treatment options are limited.

The cause – or causes – of ALS are unknown. Most cases of ALS arise spontaneously, but even in ALS with a known mutation the exact etiology of the disease remains elusive. Neuroinflammation is a hallmark of ALS in both human patients and mouse models (Zhao et al., 2013). While clinical symptoms result from degeneration of the motor neurons, it is becoming increasingly clear that the immune system plays a key role in pathology (Zhao et al., 2013). This review will examine the role of the immune system in both driving and protecting against neuronal damage during ALS. It will also attempt to compile the existing data into a coherent narrative explaining how and why the immune system plays both a beneficial and detrimental role in disease.

Involvement of the immune system in ALS

One of the consistent themes underlying ALS is the concept of damage. This may seem obvious given the neurodegenerative pathology of ALS, but neuronal damage can arise from a wide number of sources that result in identical clinical presentation. Along with the well-known mutations in Cu, Zn superoxide dismutase (SOD1) (Gurney et al., 1994; Takazawa et al., 2010), TAR DNA binding protein 43 (TDP-43) (Gitcho et al., 2008; Beers et al., 2006; Lino et al., 2002; Pramatarova et al., 2001; Wang et al., 2008). Moreover, amelioration of a number of mSOD1-related defects, such as protein aggregation or mitochondrial damage, fails to prevent ALS in mSOD1 mice, indicating that other factors are involved in disease progression (Arnold et al., 2013; Parone et al., 2013). In contrast, there is accumulating evidence that the immune system plays a critical role in the pathology of ALS.

Despite its reputation as an immune privileged site, leukocytes readily cross the blood–brain barrier (BBB) to inflict damage to neurons and glial cells in multiple sclerosis and other neuroinflammatory diseases. In contrast, in a number of animal models, microglia, the CNS resident myeloid cells, and/or peripheral leukocytes contain and repair CNS damage (Kettenmann et al., 2013; London et al., 2013; Napoli and Neumann, 2009). The immune system can therefore be a double-edged sword: it can cause great damage, but may also be necessary for repair (Table 1). Early glimpses at ALS immunity provided evidence that the immune system was altered during ALS but failed to provide evidence of direct immune involvement in disease, either in a pathogenic or protective capacity. These reports showed that proinflammatory factors were upregulated in the spinal cord of mSOD1 mice during early stages of ALS (Alexianu et al., 2001), that monocytes and dendritic cells (DC) can be detected in the CNS of mSOD1 mice (Henkel et al., 2004), that microglia are activated in the cerebrum of ALS patients (Turner et al., 2004), and that the IgG taken from some human ALS patients can induce apoptosis of motor neuron-like hybrid cells in vitro (Alexianu et al., 1994; Smith et al., 1994). Subsequent studies provided more direct evidence of immune involvement: microglia expressing mSOD1 were shown to produce more nitric oxide (NO) and cause more damage to motor neurons than wild type (WT) microglia (Xiao et al., 2007), mSOD1 microglia produce higher levels of monocyte chemoattractant protein 1 (MCP-1), also known as chemokine (C–C motif) ligand 2 (CCL2), and have an inflammatory phenotype (Sargsyan et al., 2009), and depleting monocytes in mSOD1 mice delays disease onset and extends survival (Butovsky et al., 2012). The immune system, however, does not simply exacerbate disease in the animal model. Mice lacking T cells have accelerated disease, but reconstitution with regulatory T cells or effector T cells ameliorates disease (Banerjee et al., 2008; Beers et al., 2008). Additionally, microglia can also be neuroprotective during ALS in the correct context (Liao et al., 2012). Thus, the immune system plays a complex role in both inhibiting and driving ALS pathology, and it is the common factor regardless of the source of neuronal damage. The immune system may therefore serve as a funnel of sorts through which all different forms of ALS ultimately flow.

Understanding the immune system in ALS is particularly important within the context of drug design and clinical trials. The complex role of the immune system during ALS progression complicates therapeutic design and may explain the previous failures of immune-based treatments utilized in clinical trials. Anti-inflammatory compounds such as celecoxib, pentoxifylline, minocycline, and glatiramer acetate have all been used to treat ALS (Cudkowicz et al., 2006; Gordon et al., 2007; Meining et al., 2006, 2009), and all have been ineffective (Cudkowicz et al., 2006; Meining et al., 2009) or worse have exacerbated disease (Gordon et al., 2007; Meining et al., 2006). These results are likely explained by the dual nature of the immune system during ALS. While suppression of the immune system may potentially reduce damage caused to neurons, it also dampens any of the protective immune effects also seen during ALS pathology. In addition, the immune system in ALS likely has a significant effect in other clinical trials as well. Treatments utilizing stem cells to repair or replace damaged neurons (Lunn et al., 2014) may be affected, as the ability of...
stem cells to properly differentiate is significantly affected by the local immune microenvironment (Christou et al., 2013; Kardashamy et al., 2014; Mitrecic et al., 2010; Nicaise et al., 2011; Rolls et al., 2007). As such, an understanding of the role of the immune system as well as the potential mechanisms driving both pathogenic and protective immunity is of the utmost importance in designing future therapeutics.

### Immune phases of ALS

The inflammatory response can be broadly defined based on the cellular composition of the cellular infiltrate, the phenotype of the infiltrating cells, and the cytokines associated with inflammation. During the adaptive immune response, the polarization of T helper (Th) cells can result in different immune responses, with Th1, Th2, and Th17 being the most well-known. T cells are not the only cells capable of being polarized, however, and the Th-based nomenclature fails to take into account innate cells which are also capable of polarization. Moreover, innate cell polarization can occur in the absence of T cells, making the term “Th” inaccurate. For the purposes of this review, and for the sake of simplicity, we will broadly define the immune response as Type I (T1) or Type II (T2). T1 responses, which can be destructive, are characterized by the presence of classically activated macrophages, Th1 cells, and T1 cytokines such as interferon-gamma (IFN-γ). T2 responses, which can counter-regulate T1 responses, consist of alternatively activated macrophages, Th2 cells, and T2 cytokines such as interleukin (IL)-4. Additionally, protective regulatory cells and cytokines such as regulatory T cells (Treg) and IL-10 are frequently associated with T2 response, even though they are not strictly T2.

Zhou et al. convincingly argue in their 2013 ALS review that the immune response to disease pathology should be viewed as two distinct phases: an early, protective T2 phase characterized by stable disease, and a late T1 phase characterized by rapid disease progression (Zhao et al., 2013). In the following discussion, we will address two important questions. First, what role is the immune response playing in each phase of ALS? Second, what causes the transition from the protective T2 phase to the destructive T1 phase? Answering both of these questions is the key to elucidating the pathology of ALS and to designing potential new treatments.

Based on published data, it is likely that the protective T2 phase consists of immune cells attempting to clear debris from dying neurons. It is also quite likely that the immune system is playing an active role in repair of the CNS, though this possibility has not been explored at great length. On the other hand, the transition to the destructive T1 phase is more complicated and may result from numerous, and not mutually exclusive, sources. Aggregated or misfolded proteins, toll-like receptor signaling, a transition from apoptosis to necroptosis, reactive oxygen species (ROS), and disrupted barrier function may all facilitate the transition from T2 to T1 immunity.

### The T2 immune phase (pre-symptomatic or stable disease)

#### Debris clearance

During early ALS, neuronal survival signals are downregulated (Warita et al., 2001) and motor neurons show increased susceptibility to apoptosis (Raoul et al., 2002). The early T2 response during ALS is likely driven by the accumulation of apoptotic signals and cellular debris. Clearance of apoptotic cells and cellular debris is normally performed by phagocytes, which specialize in uptake of pathogens and extracellular components, and in the CNS microglia are responsible for clearance of dying neurons (Neumann et al., 2009; Pope and Voigt, 2014). Degenerating neurons induce microglial activity by producing growth factors which act as chemoattractants and enhance phagocytosis of debris (Noda et al., 2014). Whereas T1 signals drive phagocytosis of pathogens, the T2 response is associated with phagocytosis of apoptotic cells (Gordon, 2003; Neumann et al., 2009). In mSOD1 mice, an increase in CNS phagocytic cells can be detected early during disease (Fig. 1), and inflammatory markers continue to rise during the course of disease progression (Henkel et al., 2006).

Uptake of debris by myeloid cells can be mediated by Toll-like receptors (TLRs) (Sanjuan et al., 2007). TLRs recognize structurally conserved molecules and are used by innate immune cells to sense and respond to

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**Table 1**
The immune system's pathogenic and protective role in ALS.

<table>
<thead>
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<th>Role of immunity</th>
<th>Specific finding</th>
<th>Cellular source</th>
<th>Ref.</th>
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<tr>
<td>Pathogenic</td>
<td>Neuronal damage alone is not is not enough to induce ALS</td>
<td>Mouse</td>
<td>Beers et al. (2006); Lino et al. (2002); Pramatarova et al. (2001); Wang et al. (2008)</td>
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<td></td>
<td>Amelioration of specific cellular mSOD1 defects does not prevent ALS</td>
<td>Mouse</td>
<td>Arnold et al. (2013); Parone et al. (2013)</td>
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<td></td>
<td>Proinflammatory markers are upregulated early in ALS</td>
<td>Mouse</td>
<td>Alexianu et al. (2001)</td>
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<td></td>
<td>Spinal cords</td>
<td>Human TC</td>
<td>Alexianu et al. (1994); Smith et al. (1994)</td>
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<td></td>
<td>Antibodies from ALS patients induce motor neuron apoptosis</td>
<td>Human</td>
<td>Turner et al. (2004)</td>
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<tr>
<td></td>
<td>Microglia are activated in motor neurons during ALS</td>
<td>Mouse TC</td>
<td>Liao et al. (2012); Sargsyan et al. (2009); Xiao et al. (2007)</td>
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<tr>
<td></td>
<td>Monocyte and dendritic cell RNA transcripts can be detected in the CNS during ALS; transcripts are higher during rapid ALS progression</td>
<td>Human</td>
<td>Henkel et al. (2004)</td>
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<td></td>
<td>Monocyte depletion extends survival during ALS</td>
<td>Mouse</td>
<td>Butovsky et al. (2012)</td>
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<td></td>
<td>CD8 T cell levels are elevated in the blood during ALS, and CD8 T cells accumulate in the CNS</td>
<td>Human</td>
<td>Chiu et al. (2008); Engelhardt et al. (1993); Kawamata et al. (1992); Rentzos et al. (2012)</td>
</tr>
<tr>
<td>Protective</td>
<td>Microglia and peripheral leukocytes repair numerous types of CNS damage</td>
<td>Mouse</td>
<td>Kettenmann et al. (2013); London et al. (2013); Napoli and Neumann (2009)</td>
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<td></td>
<td>Antibodies against mSOD1 can extend survival during ALS mSOD1-expressing microglia isolated during early ALS are neuroprotective</td>
<td>Mouse</td>
<td>Patel et al. (2014); Urushitani et al. (2007)</td>
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<td></td>
<td>Effector and regulatory T cells ameliorate ALS; lack of T cell signaling accelerates disease</td>
<td>Mouse</td>
<td>Banerjee et al. (2008); Beers et al. (2008); Chiu et al. (2008)</td>
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<td></td>
<td>Regulatory T cell levels are upregulated in early ALS but are gradually lost over the course of disease</td>
<td>Mouse</td>
<td>Beers et al. (2011a,b); Zhao et al. (2012)</td>
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<td></td>
<td>Decreased TGF-β expression increases ALS susceptibility</td>
<td>Human</td>
<td>Iida et al. (2011)</td>
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</table>
their environment. TLRs, particularly TLR4, have recently been shown to play a central role in phagocytosis of degenerating neurons by microglia (Rajbhandari et al., 2014). Numerous components of the TLR signaling pathway are upregulated in patients with ALS, including TLR2 and TLR4 (Casula et al., 2011), and mSOD1 can directly stimulate microglia activation via TLR2, TLR4, or CD14 (Zhao et al., 2010). While TLR signaling can make microglia more cytotoxic to neurons (Zhao et al., 2010), Simard and Rivest showed that microglia lacking MyD88, an intracellular adaptor protein necessary for TLR2 and TLR4 signaling, were associated with enhanced neurodegeneration following brain injury (Simard and Rivest, 2007). This group also showed that SOD1<sup>G37R</sup> mice deficient in expression of MyD88 had a modest, though non-significant, reduction in survival time (Kang and Rivest, 2007). It is unclear, however, whether clearance of debris in ALS is restricted solely to microglia or whether other cells are involved as well. This same study generated chimeric SOD1<sup>G37R</sup> mice reconstituted with MyD88-deficient bone marrow cells so that circulating myeloid cells, but not microglia, are MyD88 deficient. The lifespan of the chimeric mice was reduced by greater than ten percent compared to mice reconstituted with mSOD1<sup>+/+</sup> MyD88<sup>++/+</sup> bone marrow (Kang and Rivest, 2007).

Studies involving other neurological disorders have found that infiltrating bone-marrow derived cells are protective. Bone marrow-derived dendritic cells are essential for the clearance of plaques in animal models of Alzheimer's disease (Butovsky et al., 2007; Simard et al., 2006), and infiltrating immune cells can have a protective effect during CNS injury via the secretion of anti-inflammatory IL-10 (Shechter et al., 2009). IL-10 is upregulated during the early stages of ALS in mice; however, the source of IL-10 production was not examined (Beers et al., 2011b). In both mouse and human studies, there is insufficient data to conclusively say whether bone marrow-derived cells infiltrate the CNS in significant numbers during the early T2 phase of disease. Expression of MCP-1 is increased in the CNS of mSOD1 mice compared to WT mice as early as two weeks of age (Henkel et al., 2006) and is increased 10-fold in both the T2 and T1 phases (Beers et al., 2011b;
Kawaguchi-Niida et al., 2013). MCP-1 levels are elevated in the plasma of human patients as well (Zhang et al., 2006). On the other hand, Butovsky et al. showed that during the early phase of disease, myeloid cells in the CNS of mSOD1 mice are comprised primarily of resident microglia, while bone marrow-derived monocytes do not accumulate until later (Butovsky et al., 2012). It is therefore currently unclear what role bone marrow-derived cells play during the T2 phase of ALS.

**CNS Repair**

The clearance of debris in the CNS in response to damage is often accompanied by a repair response, and the early immune T2 phase during ALS is consistent with such a response. The T2 phase is characterized by enhanced IL-10 expression in the spinal cord and accumulation of regulatory T cells (Treg) in the lymph nodes and blood of mSOD1 mice (Beers et al., 2011a). In vivo depletion of CD4 T cells accelerates disease. This is consistent with a role of Treg cells in maintaining tolerance to self-antigens during health, disease, and subsequent repair (Schwartz et al., 2009). In addition, mice with mutant TDP-43 fail to recover from sciatric nerve crush due to a defective repair mechanism (Swarup et al., 2012). Further, the repair response is defective in ALS model mice. In ALS, the initial repair response is gradually lost over the course of disease (Miyazaki et al., 2009), and growth factors are upregulated in the CNS of mSOD1 mice during disease progression (Xie et al., 2004). Augmentation of the repair response using insulin-like growth factor-1 (IGF-I) in mice and human patients slows the decline of motor function (Nagano et al., 2005a,b). In addition, cells other than motor neurons, such as oligodendrocytes, degrade in mSOD1 mice, increasing the need for CNS repair (Kang et al., 2013).

In some animal models of CNS traumatic or toxic injury, infiltrating CD11b+ myeloid cells, which include microglia, monocytes, bone marrow-derived dendritic cells, and neutrophils, promote neuronal survival, nerve regeneration, and/or stem cell proliferation and differentiation (Barrette et al., 2008; Pineau et al., 2010). Monocyte-derived macrophages increase retinal ganglion cell survival and retinal progenitor cell renewal after glutamate eye intoxication (London et al., 2011), and in the injured spinal cord, hematopoietic cells suppress local inflammation via IL-10 production (Shechter et al., 2009). These cells seem to dampen the immune response by recirculating from the CNS to the lymph nodes where they inhibit T cell activation (Cone et al., 2010; Mohammad et al., 2014). As mentioned above, it is unclear in ALS which cell population may be participating in the CNS repair process. While microglia certainly play a central role in ALS, recruited bone marrow-derived monocytes have not been detected in high numbers during the T2 phase of ALS. However, these cells differentiate into mature dendritic cells, proliferate, and express higher levels of Ly6C accumulated over the course of disease while the other population declined. The authors attributed the difference in Ly6C expression to different populations of monocytes (Butovsky et al., 2012). Based on the staining characteristics of the cells expressing lower levels of Ly6C however, these cells could be neutrophils. The subpopulation expressing slightly lower levels of Ly6C arises early in the lifespan of the mouse and is almost completely absent during progressive disease. Furthermore, in our studies of human ALS patients, we observe differences in both the total number of neutrophils as well as the phenotype of these cells (unpublished data). Collectively, these data support the idea that neutrophils may play a role during the early repair mechanism.

**The T1 immune phase (progressive disease)**

**Innate immunity**

While an early T2 immune response appears to be neuroprotective, the delayed T1 response during the progressive phase of disease is destructive. In mouse models, the later phase of immunity in ALS is characterized by a rapid disease progression and production of T1 cytokines such as IFN-γ and IL-6 (Beers et al., 2011a; Zhao et al., 2013). Similar results are observed in the spinal cords of human ALS patients (Aebischer et al., 2012), particularly those with rapid disease progression (Henkel et al., 2013). In ALS, microglia can have a protective or pathogenic role (Liao et al., 2012) and likely serve as the fulcrum of the T2 to T1 transition (the immune polarization of the CNS will be discussed below). Monocytes, monocyte-derived dendritic cells, and Th1 cells, however, are likely the primary drivers of the established T1 inflammatory response. MCP-1 levels and the MCP-1/vascular endothelial growth factor (VEGF) ratio are elevated in the cerebrospinal fluid (CSF) of ALS patients in contrast to healthy controls (Nagata et al., 2007), and numerous monocyte transcripts are increased in spinal cord tissue from recently deceased ALS patients (Henkel et al., 2004). Similar patterns are seen in mSOD1 mice (Kawaguchi-Niida et al., 2013), and blocking Ly6C+ monocytes results in delayed disease symptoms and extended lifespan (Butovsky et al., 2012). This creates something of a paradox, however. We have already established that bone marrow-derived cells play a protective role in animal models of traumatic and toxic CNS injury and possibly in early stages of ALS, but how then do we explain the role of monocytes in driving neurodegeneration? The answer is that monocytes infiltrating the CNS are plastic and highly responsive to local environmental cues. They can differentiate along different lineages within the target organ, which in turn might determine their ability to activate either effector or regulatory T cells (Deshpande et al., 2007; Ifergan et al., 2008). Much of this process can be learned from mouse models of multiple sclerosis. In the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis, monocyte-derived dendritic cells can exacerbate disease (Ge et al., 2013; Sagar et al., 2012; Zhang et al., 2005) or can be protective (Li et al., 2008; Perruche et al., 2008; Zhu et al., 2011). In the absence of an inflammatory signal or in the presence of regulatory conditions, infiltrating monocytes retain an immature phenotype and transit from the CNS to the lymph nodes to suppress the immune system (Mohammad et al., 2014). If the local environment lacks anti-inflammatory signals and contains proinflammatory signals, however, these cells differentiate into mature dendritic cells, proliferate, and accumulate in the CNS (Lauar et al., 2008). A similar phenomenon is observed in mSOD1 mice. During early disease progression, markers of monocyte infiltration can be detected, but CD11c, a marker of myeloid maturity in mice, is not observed until the onset of progressive disease (Beers et al., 2011b; Chiu et al., 2008; Henkel et al., 2006). Moreover, in patients with familial ALS, there is a significant upregulation in the expression of the high-affinity IgF receptor (FcERI) gene (Butovsky et al., 2012); FcERI is one of the primary markers used to identify bone marrow-derived inflammatory dendritic cells (Hammad et al., 2010). By depleting bone marrow-derived...
monocytes in mSOD1 mice, the authors significantly prolonged survival and delayed onset of disease. This is important because T1-differentiated myeloid cells are essential for the defense against certain pathogens and are necessary for the initiation of the adaptive immune response (Banchereau and Steinman, 1998). This may explain why effector T cells are primarily detected in the later stages of disease. In the case of ALS it is unclear, however, if myeloid cells accumulating in the CNS are exclusively dendritic cells, or if some of the monocytes differentiate into macrophages as well. Both can arise from bone marrow-derived monocytes and can have very similar characteristics (Havenith et al., 1993). In other forms CNS injury, axonal dieback is facilitated by bone marrow-derived macrophages, although it is unclear if these cells are macrophages or dendritic cells (Evans et al., 2014). Given the relatively mild T cell response in ALS, it is possible that the CD11c⁺ cells accumulating in the CNS are macrophages rather than dendritic cells.

Adaptive immunity

The paucity of CNS-infiltrating T cells in ALS when compared to diseases such as multiple sclerosis may reflect the fact that ALS is not an autoimmune disease and does not involve an adaptive response targeting self-antigens in the CNS. Conversely, there is some evidence that antibodies specific for self-antigens are produced in some individuals with ALS and they correlate with progressive disease (Pagani et al., 2011). One recent study, however, suggests that autoantibodies to neuronal antigen are found in only a small subset of patients with ALS (Coban et al., 2013), while another study found a modest increase in the levels of certain self-antibodies, most of which were non-neuronal (May et al., 2014). Alternatively, one recent study found that IgG, IgM, and IgA are not increased in ALS patients and that their levels correlate with progressive disease (Pagani et al., 2015). Beers et al. observed no significant increase in the expression of IL-17 during the disease course in SOD1G93A mice (Beers et al., 2011b), while on the other hand, several studies found increases in IL-17 levels in the CSF (Rentzos et al., 2010) or the percentage of CD4 T cells that produce IL-17 in the blood of human patients (Saresella et al., 2013). Another study reported elevated IL-17 in the serum and CNS of ALS patients; however, there was no significant correlation with disease score and expression seemed restricted to CD8 T cells (Fiala et al., 2010). In all of these studies, the increase in IL-17 levels was modest, especially when compared to known autoimmune diseases such as multiple sclerosis or rheumatoid arthritis. Moreover, autoimmunity is driven by the adaptive arm of the immune response, and in ALS, CD4 T cells appear to play more of a protective role in disease progression. The stable T2 phase of ALS may be maintained, in part, by regulatory T cells, and it is the loss of this regulation which results in a transition to a destructive T1 phase of disease (Henkel et al., 2013; Rentzos et al., 2012; Zhao et al., 2012). Genetically engineered mice that lack T cells or T cell receptors (Chiu et al., 2008) exhibit an accelerated disease course but can be rescued via reconstitution with immunocompetent bone marrow cells (Beers et al., 2008). It is entirely possible, of course, that CD4 T cells serve a protective role initially followed by a pathogenic role similar to recruited monocytes; however, there is no definitive evidence as yet to suggest that CD4 T cells play a role in driving neurodegeneration, and B cells, which are responsible for production of autoantibodies, are not altered in the majority of ALS patients nor seem necessary for disease pathology (Naor et al., 2009). Therefore, there is no conclusive evidence that an autoimmune response is occurring in ALS.

What role then is the T1 response playing in ALS? The most likely answer is that as the damage to the neurons becomes increasingly severe, the T2 response becomes unable to compensate. As a result, the immune system shifts to the more destructive T1 response to cope with neurons that are undergoing apoptosis without clearance or are failing to undergo apoptosis (Re et al., 2014). CD8 T cells could participate in a T1 response to eliminate damaged cells. Consistent with this, the frequency of CD8 T cells is elevated in the CNS of mSOD1 mice (Chiu et al., 2008). Similarly the percentage of CD8 T cells and natural killer cells is elevated in the blood of human patients (Rentzos et al., 2012). Depending on the timing, these CD8 levels may also drop, indicating recruitment to the CNS (Mantovani et al., 2009). Moreover, several older studies have detected CD8 T cells clustering in the post-mortem CNS tissue from human patients (Engelhardt et al., 1993; Kawamata et al., 1992), and we have seen differences in CD8 T cell levels and phenotype in the blood of human patients compared to healthy controls (unpublished data). Finally, in studies where T cells have been depleted from mSOD1 mice, the benefits gained from reduction in pro-inflammatory CD8 T cells could be offset by the loss of regulatory T cells (Beers et al., 2008).

Immune polarization in ALS

In many ways the T2 to T1 transition observed in ALS is an inversion of the typical immune pattern seen in response to trauma and infections. In a normal immune response, there is an aggressive T1 response which clears the tissue of pathogens followed by a T2 response which removes debris and initiates repair mechanisms. In ALS, it is as if the immune system is being forced backwards through its normal program as damage occurs and debris accumulates. It should be noted, however, that this shift from T2 to T1 is a gradual process which takes several months in mice and may take several years in human patients. Moreover, as is frequently the case in diseases with complex immune responses (Murdock et al., 2011), the transition from one phase to another is messy or incomplete: even mice in the final stages of disease express significantly elevated levels of T2-associated cytokines and have significantly more regulatory T cells in circulation (Beers et al., 2011a). The progressive polarization of the immune cells in the CNS, particularly the microglia (Liao et al., 2012), likely accounts for this T2 to T1 transition in the CNS and is likely driven by a number of microenvironmental factors. Below are several potential, although not necessarily exclusive, sources of immune polarization.

TLR ligands

TLRs are used by cells to sense their environment. Within the human CNS, most cells express TLRs (Bsibsi et al., 2002), and these receptors can play a variety of roles, both in neurodegeneration and repair (van Noort and Bsibsi, 2009). During ALS, many TLRs are upregulated (Casula et al., 2011). The most obvious role for these TLRs during the T1 phase is the induction of neurodegeneration, as stimulating astrocytes or microglia with TLR ligands in neuronal co-cultures results in significantly increased levels of T2-associated cytokines and have significantly more regulatory T cells in circulation (Beers et al., 2011a). Aggregated mSOD1 can induce a pro-inflammatory response in the mononuclear cells of ALS patients (Fiala et al., 2010), and mSOD1 activates microglia via TLR2, TLR4, and the MyD88 pathway (Kang and Rivest, 2007; Zhao et al., 2010) (Fig. 2). Moreover, microglia can be sensitized to TLR signaling by local cytokines (Parajuli et al., 2012), and astrocytes can be sensitized by previous TLR stimulation (Henn et al., 2011), leading to enhanced activation and a more proinflammatory cytokine environment. As mentioned previously, however, TLR signaling in the CNS is also important for clearance of debris and the initiation of nerve regeneration (Bovin et al., 2007; Kang and Rivest, 2007; Simard and Rivest, 2007). Although disruption of TLR4 hinders the microglial ability to phagocytize neuronal debris in vivo (Rajbhandari et al., 2014). Furthermore, TLR2 and TLR4 signaling have opposing roles in modulating adult neurogenesis in the brain, despite both signaling through the MyD88 pathway, with TLR2 signaling promoting neurogenesis and TLR4 inhibiting neural progenitor cells (Rolls et al., 2015).
Therefore, while TLRs certainly play a role in neurodegeneration, they also play an even greater role in protecting the CNS during ALS. Given the dual role of TLR signaling in ALS, it is likely that changes in TLR ligands in the CNS drive changes in immune polarization. The ligands driving this process, particularly those driving the T1 response, may be different depending on what protein is altered or mutated. In the case of the SOD1 mutation, cytotoxicity is due to mutant SOD1 signaling through the TLRs (Kang and Rivest, 2007), as extracellular mutant SOD1 is not toxic to neurons on its own (Zhao et al., 2010). Interestingly, the immune system is able to discriminate between the WT and the mutant form of the protein, and how the immune system detects these inflammatory proteins may therefore be at the heart of immune polarization. Accumulation of protein aggregates is one of the hallmarks of ALS and is observed in both human patients and non-mutant SOD1 mouse models (Blokhuis et al., 2013). Previous reports have shown that aggregated proteins can trigger a pro-inflammatory response (Gsponer and Vendruscolo, 2006), with some via TLR2, TLR4, and the complement system (Joubert et al., 2012). Therefore, it is possible that the accumulation of extracellular protein aggregates may induce a T1 response via TLRs, and previous studies have detected enhanced levels of SOD1 in the CSF of ALS patients (Winer et al., 2013). Intracellular protein aggregates do not seem to have this effect in ALS, as overexpression of heat shock protein to prevent SOD1 aggregation showed no delay in onset or extension of survival (Liu et al., 2005). Note, however, this study affected only intracellular mSOD1.
whereas therapeutic studies using vaccines or antibodies against extra-cellular mSOD1 have extended the survival of ALS mice (Patel et al., 2014; Urushtiani et al., 2007). Blocking mSOD1 with antibody treatment may be effective because mSOD1, but not WT SOD1, is actively secreted by neurons (Urushtiani et al., 2006), and secreted mSOD1 would be able to bind to TLRs expressed on immune cells, whereas intracellular mSOD1 would not. There is evidence to suggest that an initial buildup of intracellular mSOD1 contributes to disease pathology: mSOD1 mice missing the unfolded protein response have accelerated disease onset and reduced lifespan compared to standard mSOD1 mice (Wang et al., 2011). Similarly, autophagy is upregulated in mSOD1 mice and continues to rise as the disease progresses (Morimoto et al., 2007; Tian et al., 2011). Thus, mSOD1 may play a dual role by damaging neurons from within while simultaneously polarizing the immune system.

One can therefore envision a pathway where mutant SOD1 or misfolded WT SOD1 (Bosco et al., 2010) accumulates in the neurons and the cell responds by attempting to neutralize the improperly folded proteins via the unfolded protein response and autophagy. When the generation of misfolded proteins begins to outpace these cellular mechanisms and these proteins begin to accumulate, the cell then begins expelling the misfolded protein in an attempt to reduce protein levels in the cell or as a distress signal, with the resulting extracellular mSOD1 activating immune TLRs. This in turn upregulates the T1 response and enables a shift away from the protective T2 immune response observed early in disease. This proposed mechanism would of course be specific to ALS caused by SOD1 mutations, and these cases of familial ALS make up a small fraction of the total ALS cases. It is possible though that similar mechanisms may be at work in other forms of ALS, as protein misfolding and protein aggregation are still observed. Alternatively, there is evidence to suggest that misfolded SOD1 plays a central role even in sporadic ALS (Rotunno and Bosco, 2013), meaning that the proposed mechanism would be applicable to all forms of ALS.

Apoptosis vs. necroptosis

Along a similar vein, it is possible that neuron death over the course of ALS changes from apoptosis to necroptosis. Previous studies have shown that neurons in ALS are more susceptible to apoptosis (Raoul et al., 2002), and therapeutic treatment to reduce neuronal apoptosis ameliorates ALS (Huang et al., 2013; Ohta et al., 2008); however, a recent study by Re et al. demonstrated that neurons in both familial and sporadic ALS eventually succumb to necroptosis (Re et al., 2014). The difference between each type of cell death is significant, as each triggers a vastly different immune response. In the case of apoptosis, cells are rapidly cleared by macrophages, which in turn generate protective cytokines such as TGF-β and IL-10 (Peng et al., 2007). Apoptotic cells can also induce a pro-tolerance phenotype in bone marrow-derived dendritic cells that can prevent autoimmunity (Zhou et al., 2013). This response to apoptotic cells is consistent with the protective T2 response observed early in ALS pathogenesis. On the other hand, non-apoptotic mechanisms of cell death such necroptosis, which is a controlled form of necrosis, can drive an aggressive T1 inflammatory response similar to that seen in the T1 phase of ALS (Elliott and Ravichandran, 2010; Kaczmarek et al., 2013; Rovere-Querini et al., 2004). During this period, danger signals such as High Mobility Group Box 1 (HMGB1) are released by damaged and necrotic cells (Gao et al., 2012; Rovere-Querini et al., 2004; Scaffidi et al., 2002), and studies have shown upregulation and release of HMGB1 as ALS progresses in msSOD1 mice (Lo Coco et al., 2007) and human patients (Casula et al., 2011). Furthermore, HMGB1 stimulates the immune system via TLR2 and TLR4 and results in, among other things, the release of MCP-1 (Kim et al., 2013; Yu et al., 2006). The presence of HMGB1 or other danger signals could therefore trigger a signaling cascade, as HMGB1 itself can induce apoptosis via TLR 4 (Ding et al., 2013), and perpetuate myeloid inflammation (Bonaldti et al., 2003).

Reactive oxygen species

Reactive oxygen species (ROS) are neurotoxic (Kuhn, 2014) and play a central role in ALS (Barber and Shaw, 2010). Increased ROS production can be observed in the spinal cords of mSOD1 mice (Naumenko et al., 2011), mutant mSOD1 microglia do greater ROS-mediated damage to motor neurons than WT microglia (Xiao et al., 2007), and inhibiting ROS has been shown to slow ALS progression in a number of studies (Tanaka et al., 2011, 2014). The role of ROS, however, is more than simply destructive: ROS can have a profound effect on the immune response through indirect or direct means. Indirectly, higher levels of ROS increase neuronal damage and potentially create more debris or necrotic cells (Barber and Shaw, 2010; Pollari et al., 2014; Tanaka et al., 2008) that can drive the T1-mediated inflammatory response. The damage caused by ROS can then potentiate even further damage by inhibiting DNA repair (Jaiswal et al., 2000, 2001) and increasing motor neuron susceptibility to apoptosis (Raoul et al., 2002). More directly, ROS can affect activation, differentiation, and survival of cells in the environment, including immune cells. Nitric oxide (NO), which is produced by activated myeloid cells, in particular may skew the polarization of the immune system under inflammatory conditions by inducing the differentiation of Th1 cells (Niedbala et al., 2002) and suppressing other forms of adaptive immunity (Niedbala et al., 2013; Yang et al., 2013). NO can stimulate dendritic cell maturation under both baseline or inflammatory conditions (Fernandez-Ruiz et al., 2004, 2005; Paolucci et al., 2003), and hydrogen peroxide, another ROS, stimulates DC activation, enhancing their ability to activate T cells (Rutault et al., 1999).

ROS play a role in the differentiation of other cell populations as well. For example, blood-borne CD34+ progenitor cells can also give rise to dendritic cells with similar function to monocyte-derived dendritic cells (Herbst et al., 1997) but are induced to mature and proliferate by NO (Shami and Weinberg, 1996). Additionally, TR2 ligands can induce these cells towards a pro-T1 phenotype (Sioud and Fioiand, 2007), and the resulting dendritic cells are superior at stimulating CD8 T cells in long-term co-cultures (Ferlazzo et al., 1999). ROS, however, can protect against inflammation as well. NO has been shown to induce the formation of Tregs (Kraaij et al., 2010; Niedbala et al., 2007), and the Treg transcription factor forkhead box P3 (Foxp3) is upregulated in the CNS of mSOD1 during the T2 phase of ALS (Beers et al., 2011b). Moreover, despite being pro-inflammatory, ROS and especially NO can induce apoptosis within myeloid cell populations (Taylor et al., 2003). Thus, ROS levels may control the balance of immune activation and stem cell differentiation in the CNS during ALS.

Barrier function

Breakdown of the blood brain barrier (BBB) and the blood-spinal cord barrier (BSCB) may play a role in ALS progression. Numerous mouse studies have found that disruptions occur in the BBB and BSCB during both the early and the late stages of ALS (Garbuszova-Davis et al., 2007; Miyazaki et al., 2011; Nicaise et al., 2009; Winkler et al., 2013, 2014; Zhong et al., 2008), and there is evidence to suggest similar disruptions occur in human patients (Henkel et al., 2009; Miyazaki et al., 2011). This breakdown may facilitate the infiltration of leukocytes, toxins, and molecular signals that may enable or accelerate ALS (Rodrigues et al., 2012). Changes in the BBB and BSCB lead to an altered and potentially pro-inflammatory environment within the CNS that can augment the immune response in the CNS. However, it is unclear if the disruption to barrier tight junctions contributes to the immune pathology seen in ALS or whether cellular accumulation would occur in the absence of disruption. In mutant SOD1 models, disruption of the BBB and BSCB occurs at all stages of disease, including prior to neurodegeneration (Garbuszova-Davis et al., 2007; Miyazaki et al., 2011), and breakdown of barrier function contributes directly to early degeneration of the motor neurons (Winkler et al., 2014). In addition to driving
neurodegeneration and the likely subsequent inflammatory response, barrier disruption also aids in the accumulation of immune cells in the CNS (Kierdorf et al., 2013). In the case of ALS, this may result in accumulating cells that can drive a T2 response, a T1 response, or both. Allowing the infiltration of monocytes into the CNS may skew the immune response by altering the balance of T cells and Ly6C+ monocytes (Zhu et al., 2011). As the number of monocytes increases, it is possible that the T cell to monocyte ratio drops, resulting in enhanced ROS production and the suppression of T cells via NO. This could account for the progressive loss of Tregs seen over the course of disease (Beers et al., 2011a; Zhao et al., 2012) and explain why both Tregs and activated effector T cells can both be used to ameliorate ALS (Banerjee et al., 2008; Beers et al., 2008). This, however, is speculation, and as yet there is no direct evidence linking BBB and BSCB function to the T1 polarization of the immune response in ALS.

Concluding remarks

Accumulating evidence indicates that the immune system is not a bystander in ALS but plays an active role in pathology. The exact role of the immune response, however, has remained unclear and research has been hampered by results that are often contradictory. Recent research has demonstrated that the immune response in ALS occurs in two separate phases, one protective and one progressive, which could explain the discrepancies that have been previously reported. This knowledge has enabled us to reevaluate existing data and try to create a cohesive narrative explaining not only how the immune response is contributing to the various stages of ALS, but why. The information presented in this review represents an attempt to explain the role of the immune system, both positive and negative, in influencing ALS pathology. Our goal is therefore to spark ideas for new avenues of research that will ultimately yield novel therapies for this devastating illness.

Conflict of interest

The authors have no conflict of interest to disclose.

Author contributions

All authors contributed to the conception, discussion, writing, and editing of this manuscript.

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Amyotrophic lateral sclerosis: mechanisms and therapeutics in the epigenomic era

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Abstract | Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease of the motor neurons, which results in weakness and atrophy of voluntary skeletal muscles. Treatments do not modify the disease trajectory effectively, and only modestly improve survival. A complex interaction between genes, environmental exposure and impaired molecular pathways contributes to pathology in patients with ALS. Epigenetic mechanisms control the hereditary and reversible regulation of gene expression without altering the basic genetic code. Aberrant epigenetic patterns—including abnormal microRNA (miRNA) biogenesis and function, DNA modifications, histone remodeling, and RNA editing—are acquired throughout life and are influenced by environmental factors. Thus, understanding the molecular processes that lead to epigenetic dysregulation in patients with ALS might facilitate the discovery of novel therapeutic targets and biomarkers that could reduce diagnostic delay. These achievements could prove crucial for successful disease modification in patients with ALS. We review the latest findings regarding the role of miRNA modifications and other epigenetic mechanisms in ALS, and discuss their potential as therapeutic targets.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that affects motor neurons in the brain, brainstem and spinal cord, resulting in progressive weakness and atrophy of voluntary skeletal muscles. Treatments are modestly effective at best, and the majority of patients die within 3–5 years of diagnosis—often from respiratory failure—although survival outside this timeframe can vary with certain clinical presentations. The disease exhibits phenotypic heterogeneity, and although ALS was once considered a single disorder, newer theories suggest that different disorders might share a common final phenotype. As many as 50% of patients with ALS also have cognitive impairment, with a subset of patients (~15%) exhibiting characteristics of frontotemporal dementia (FTD), frontotemporal lobar degeneration (FTLD), and progressive social, behavioural and/or language dysfunction. Several ALS-related mutations, discussed below, are also common in FTD, leading FTD and ALS to be considered part of a continuum of neurodegenerative disorders.

The majority of ALS is sporadic with unknown aetiology, whereas ~15% is familial with dominant inheritance. Sporadic and familial ALS are clinically indistinguishable and share several pathogenetic pathways. A number of ALS-related genes are known; however, genetic mutations do not solely account for neurodegeneration, and they cannot explain the existence of the large number of idiopathic cases. Interestingly, sporadic ALS has been linked to many environmental factors, including heavy metal toxicity and exposure to pesticides and fertilizers. A simple view would predict that genetic variation and environmental exposure contribute to ALS, yet it is also likely that environmental exposures influence epigenetic mechanisms—which reversibly regulate gene expression—to promote the onset and progression of ALS.

Unravelling the complex mechanisms behind the pathogenesis of ALS is crucial for the development of novel diagnostic and therapeutic approaches, and years of evidence support the existence of unique, pathological, epigenetic processes in ALS. Here, we briefly review what is known about the underlying mechanisms and genetic causes of ALS, and then discuss the environmental influences and epigenetic basis of this disease. We also discuss how the main epigenetic mechanisms interact in neurodegeneration, providing insight into potential therapeutic strategies.

Proposed disease mechanisms in ALS

The pathogenetic mechanisms proposed for ALS involve a plethora of alterations to the motor neuron microenvironment, including accumulation of protein aggregates, defective RNA processing, oxidative stress, glutamate excitotoxicity, glial dysfunction, neuroinflammation, apoptosis, mitochondrial dysfunction, fragmentation of the Golgi apparatus, and metal imbalances. At very early disease stages, many molecular changes in the neuromuscular junction precede motor neuron death. The presence of intraneuronal aggregates of...
The pathogenesis of amyotrophic lateral sclerosis (ALS) is mediated by genetic variation, environmental exposure and epigenetic regulation. Epigenetic mechanisms might explain how gene expression and function are controlled, and how gene–gene and gene–environmental interactions are mediated. DNA methylation, histone remodeling, RNA editing, and microRNA (miRNA) modifications are epigenetic mechanisms that are dysregulated in ALS models and in patients. miRNAs modulate many physiological processes through an intricate network. miRNA pathway disruptions could be a cause or consequence of altered RNA and protein metabolism, the inflammatory response, cytotoxicity and/or neuromuscular junction impairments, all of which underlie ALS pathology. miRNAs have great potential as novel biomarkers and therapeutic targets for ALS and other neurodegenerative diseases.

Key points

- The pathogenesis of amyotrophic lateral sclerosis (ALS) is mediated by genetic variation, environmental exposure and epigenetic regulation.
- Epigenetic mechanisms might explain how gene expression and function are controlled, and how gene–gene and gene–environmental interactions are mediated.
- DNA methylation, histone remodeling, RNA editing, and microRNA (miRNA) modifications are epigenetic mechanisms that are dysregulated in ALS models and in patients.
- miRNAs modulate many physiological processes through an intricate network of fine-tuning robustness and complexity of the transcriptome and proteome.
- miRNA pathway disruptions could be a cause or consequence of altered RNA and protein metabolism, the inflammatory response, cytotoxicity and/or neuromuscular junction impairments, all of which underlie ALS pathology.
- miRNAs have great potential as novel biomarkers and therapeutic targets for ALS and other neurodegenerative diseases.

SOD1

Dominant mutations in the superoxide dismutase 1 (SOD1) gene account for one-fifth of familial ALS cases,\(^1\) and in vitro and in vivo models based on SOD1 mutations are widely used to investigate disease mechanisms. SOD1 protein is an antioxidant found in the cytosol and mitochondrial intermembrane space, and converts superoxide radicals to hydrogen peroxide. Over 170 SOD1 missense mutations are known, which yield a toxic gain of (unknown) function.\(^2\) Although reductions in enzymatic dismutase activity were originally thought to promote oxidative stress and excitotoxicity in motor neurons,\(^3,4,5\) degeneration might rather be caused by noxious mutant SOD1 protein aggregation followed by aberrant association with mitochondria.\(^6,7,8\) SOD1 aggregates are recruited to spinal mitochondria, and crosslinked onto integral membrane components.\(^9,10,11\) SOD1 has prion-like properties, with an ability to sequence wild-type proteins and seed their aggregation or promote misfolding.\(^12\) Additionally, mutations in SOD1 cause misfolding of the nascent SOD1 polypeptide, which—in association with other molecules—can lead to the formation of aggregates, especially during oxidative stress.\(^13\) Interestingly, expression of mutant SOD1 within motor neurons modulates disease onset, though it is not always sufficient to prompt disease.\(^14\)

TARDBP and FUS

TAR DNA-binding protein 43 (TDP-43) and RNA-binding protein FUS (previously known as fused in sarcoma or translocated in liposarcoma) are members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. TDP-43 and FUS are both nuclear RNA binding proteins that shuttle between the nucleus and cytoplasm, and are involved in transcription, splicing and messenger RNP transport.\(^15,16,17\) Over 40 different missense mutations in TARDBP, which encodes the TDP-43 protein, have been linked to familial ALS.\(^18,19\) Most of these mutations occur in the unstructured, glycine-rich C-terminal domain, which binds to hnRNP A/B complexes;\(^20\) these C-terminal regions are commonly observed in ALS and FTD inclusions.\(^21,22\) ALS-associated mutations in FUS occur within a linkage region on chromosome 16 and affect the protein C-terminus.\(^23,24\) FUS mutations account for ~5% of familial ALS, and most patients with FUS-associated ALS have FUS-inmunoreactive cytoplasmic inclusions but the typical phosphorylated TDP-43-positive inclusions are absent.\(^25,26\) Mutations in FUS can also share common pathways with mutant TDP-43 that lead to neurodegeneration,\(^27,28\) or can yield axonal defects via interaction and aggregation with survival of motor neuron protein (SMN).\(^29,30\) SMN is required for spliceosome assembly, and reduced levels of SMN underlie the childhood neuromuscular disease spinal muscular atrophy,\(^31\) which has similar features to ALS.

In ALS, TDP-43 and FUS can interact with other proteins that regulate RNA metabolism. Under pathological conditions, such as cellular stress, these associations between mutant TDP-43, FUS and mRNAs can lead to abnormal phosphorylation, ubiquitination and aggregation of proteins, as well as aberrations in their normal cellular functions,\(^32,33\) and the formation of stress granules.\(^34,35\)

Stress granules are composed of repressed translation complexes, where mRNA-binding proteins consolidate stalled mRNA. These classically transient structures enable a cell to adapt to a stressful response; however, in neurodegenerative diseases they can become pathologically persistent.\(^36,37\) It is feasible that aggregated RNA-binding proteins become sequestered in stress granules, which in turn leads to the formation of cytoplasmic inclusions. TDP-43 is a principal component of the hallmark ubiquitinated inclusions seen in many patients with ALS,\(^38,39,40\) and several investigator groups have observed co-localization between TDP-43 and stress granule markers in tissues from patients with ALS or FTD.\(^41,42,43\) Notably, in most patients with familial ALS, TARDBP...
is not mutated, yet TDP-43 aggregates may be present (except in cases caused by SOD1 or FUS mutations), which disrupt RNA processing and could be one trigger of degeneration.29

**C9orf72**

The most common genetic cause of ALS and FTD is linked to chromosome 9p21, where a founder haplotype occurs in the so-called ALS–FTD locus—which includes the chromosome 9 open reading frame 72 (C9orf72) gene—and is associated with autosomal dominant inheritance.59 In the affected haplotype, expansions in a large noncoding hexanucleotide (GGGGCC) repeat in the first intron of C9orf72 can reach hundreds of copies in patients presenting an FTD/ALS phenotype and TDP-43 pathology, as well as in presymptomatic carriers.59,60 Interestingly, although FTD and ALS are related disorders and can sometimes be detected within the same family harbouring mutations in the ALS–FTD locus (9p21), ALS is rarely accompanied by FTD in SOD1, TARDBP or FUS mutations.60–63

The exact mechanism by which the C9orf72 repeat expansion triggers disease is under active investigation, and a number of possible explanations currently exist. First, the repeat expansion could potentially alter the expression of the mutant allele: a decrease in mRNA expression of C9orf72 transcripts has been observed in patients with ALS and/or FTD,60,64 providing evidence for a loss of function. A more recent study, however, only detected a trend towards decreased expression.65 Alternatively, accumulation of repeat RNA transcripts within nuclear foci in the brain and spinal cord of patients with ALS and/or FTD has also been observed.66 These repeat transcripts undergo unconventional, bidirectional repeat associated non-ATG (or ‘RAN’) translation,66,67 thus generating simple peptides that accumulate into distinct foci in patients with ALS and FTD related to C9orf72.66,68 Two of these peptides affect transcription and translation, interfering with both mRNA splicing and the biogenesis of ribosomal RNA.69 These later observations suggest the repeat expansion leads to neurodegeneration via a gain-of-function mechanism.65

**Other mutations**

Several other genes have also been linked to ALS etiology. Mutations in ubiquilin 2 (UBQLN2), which encodes a protein that targets abnormal proteins for degradation, yield X-linked FTD or ALS with TDP-43-positive neuropathology.70 Mutant forms of proteins with a role in RNA and DNA binding, such as the protein encoded by matrin 3 (MATR3), have also been associated with ALS.71 The RNA-binding proteins hnRNPA1 and hnRNPA2B1 have a prion-like domain that influences mRNA metabolism and transport,72 and can interact directly with the C-terminal region of TDP-43.46,72,73 Mutant forms of these proteins have been reported in one patient with dominantly inherited ALS,74 and in animal models, the mutant hnRNPA1 and hnRNPA2 were found to be prone to fibrillation and incorporation into stress granules and cytoplasmic inclusions.74

Mutations in senataxin (SETX), which codes for a DNA/RNA helicase involved in RNA processing, have been observed in patients with ALS and patients with progressive motor neuropathy.75 ALS and/or FTD phenotypes have also been associated with mutations in the non-structured N-terminal region or in the α helix of the coiled-coil-helix-coiled-coil-helix-domain-containing 10 (CHCHD10) gene, which encodes a protein located in the mitochondrial intermembrane space.76,77 Additional associated genes include GRN—one of the major causes of FTLD with TDP-43 pathology—ANG,78 CHMP2B,80 PFN1,81 OPTN, VCP82 and others.5,79 Notably, many of these genes are also involved in RNA processing, as are TARDBP, FUS, and C9orf72, and are mutated in other neurodegenerative diseases. These observations categorically link ALS to a group of pathologies that exhibit altered RNA processing and protein homeostasis.11

**Environmental factors**

Although there is a clear genetic predisposition to ALS, other pathogenetic factors are likely to be environmentally determined,6,83 and recently, a multistep model was proposed that suggests a series of events underlie ALS initiation.44 Moreover, sporadic ALS is linked to toxic exposures, diet, inflammatory cytokines, acquired gene mutations, and other factors—some of which act synergistically to foster neurodegeneration. Many of these factors are involved in molecular mechanisms that mediate epigenetic changes.

Lead is the most studied metal in patients with ALS, and though a link between exposure to lead and ALS remains inconclusive, multiple studies have reported elevated levels of lead in several different tissues taken from patients with ALS.45–49 Furthermore, a recent meta-analysis estimated a 5% increase in the risk of developing ALS after previous lead exposure.50 Accumulation of mercury—a neurotoxin that inhibits SOD1 activity—in motor neurons damages the cytoskeletal components and impairs axonal transport, causing phenotypes similar to ALS.91 However, a direct correlation with the disease has not been established.

High levels of aluminium are also associated with the development of symptoms similar to motor neuron disease. Regionally increased incidences of ALS have been recorded in a western Pacific region known for excessive amounts of aluminium in the soil and drinking water.92 Also, mice fed diets high in aluminium develop ALS-like characteristics.93 Although the literature indicates that metal exposures alone are insufficient to explain ALS pathogenesis, the ubiquitous nature of neurotoxic environmental metals demands further investigation through large-scale epidemiological studies with rigorous designs.91

The risk of ALS has also been linked with exposure to fertilizers, insecticides and herbicides.94–96 Occupational and home exposure to pesticides and fertilizers tend to be higher in patients with ALS than in controls matched for age and sex,97 and these exposures are also associated with increased risk for developing Parkinson disease (PD).98,99
Several groups have detected associations between cigarette smoking and an elevated risk of ALS, as well as reduced survival. One case–control study identified trends for a threefold higher risk of ALS in current smokers compared with nonsmokers, and two other studies also found that smoking is independently associated with an increased risk of ALS. However, a meta-analysis detected only a moderate association of ALS with current smokers, and a prospective cohort study found an increased risk for ALS and worse prognosis only in female smokers, and a case–control study found no correlation at all. Inconsistencies in results are probably due to limitations in experimental design, small sample sizes and the use of relatives and/or friends as controls; therefore, additional studies are required. Finally, the physical exertion and trauma, especially head injuries, experienced by war veterans and athletes could be associated with increased risk of ALS.

Epigenetics

Epigenetics represents a potential convergence between genetic predisposition and environmental exposures. Although controversy has surrounded the definition of epigenetics (Box 1), in this Review we identify epigenetics as “the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states.” Epigenetic mechanisms include DNA methylation, histone remodelling, RNA editing, and noncoding RNAs such as microRNAs (miRNAs). They coordinate neural development, plasticity and ageing—and are essential for cellular homeostasis—by responding to stimuli beyond the inherited genetic blueprint. Epigenetic programming through previous experience might also allow the brain to adapt to a chronically stressful environment. Dysregulation of epigenetic mechanisms, however, could be driven by the same long-term environmental influences that contribute to ALS risk, suggesting that the accumulation of some epigenetic patterns throughout life drives the onset and progression of ALS. The reversible nature of epigenetic marks also makes this mechanism an attractive pharmacological target for ALS therapy development. In the following sections, we discuss what is known regarding the contribution of some of the major epigenetic mechanisms to ALS pathogenesis. These findings are also summarized in Table 1.

DNA methylation

DNA methylation involves the post-transcriptional covalent addition of a methyl group to cytosine residues in DNA, leading to 5-methylcytosine (5mC) formation. This addition modifies transcriptional protein–DNA interactions by changing chromatin structure and transcription rates. The DNA-(cytosine-5)-methyltransferase (DNMT) enzymes DNMT1, DNMT3a and DNMT3b catalyse the covalent transfer of methyl groups from S-adenosyl-methionine to the 5′ carbon position of a substrate cytosine residue. Reversal of DNA methylation is a complex process and not fully understood.

DNA methylation commonly occurs in genomic regulatory regions, such as promoter elements or CpG dinucleotides, which are clustered in CpG islands or CpG island shores. In promoter regions, 5mC modifications serve as docking sites for chromatin-remodeling proteins, and are associated with negative regulation of gene expression through interference with transcription factor binding or by providing a binding site for transcriptional repressors. By contrast, intragenic 5mC modifications facilitate gene expression.

The establishment and maintenance of DNA methylation patterns during brain development, learning and memory follow complex processes involving the interaction between DNMTs and methyl-CpG binding domain proteins (MBDs). In addition, the ten-eleven translocation (TET; also known as methylcytosine dioxygenase) family of enzymes oxidizes 5mC to generate an alternatively modified cytosine residue, 5-hydroxymethylcytosine (5hmC), which inhibits MBD protein binding and opposes 5mC function. Thus, DNA methylation is a powerful molecular strategy to regulate gene expression, and it might further serve as an epigenetic biomarker and therapeutic target because methylation is stable, easily detectable and reversible.

Supporting a role for reversible DNA methylation in ALS, DNMT3A is present in the brains and spinal cords of patients with ALS, and its overexpression prompts cell death in motor-neuron-like cells in vitro. Blood and neural tissue from patients with ALS also exhibit enhanced global DNA 5mC and 5hmC, and sites of gene methylation in brain and spinal cord tissue from patients with ALS have been identified. For example, genome-wide methylation arrays have revealed hypomethylation of the ALS-related gene OPTN. Additionally, loss of function of the protein arginine N-methyltransferase 1

Box 1 | What is epigenetics?

Epigenetics (‘above genetics’) was first described by the British developmental biologist and geneticist Conrad H. Waddington (1905–1975) to explain the interplay between genetics and external forces during development that define the phenotype of an organism. The use of the term epigenetics has been controversial, as the boundaries of the definition have been ambiguous from the very beginning.

After Waddington first described epigenetics, two distinct interpretations of the concept emerged. One described epigenetics as the study of “mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence.” Central requirements of this definition are that DNA methylation and histone modifications are the sole mechanisms that maintain the epigenome, and that phenotypic changes resulting from epigenetic memory are carried on by transgenerational inheritance.

The second interpretation is more global, and more akin to Waddington’s concept of epigenetics (Box 1), in this Review we identify epigenetic predisposition and environmental exposures.

A different epigenetic target for ALS therapy development. In the following sections, we discuss what is known regarding the contribution of some of the major epigenetic mechanisms to ALS pathogenesis. These findings are also summarized in Table 1.

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—leads to changes in the histone code.\textsuperscript{118} Blood and neural tissue from patients with ALS and ALS models exhibit enhanced global 5mC and 5hmC.\textsuperscript{119,120} Redistribution of FUS to the cytoplasm yields loss-of-function in PRMT1, altering the histone code.\textsuperscript{121} However, redistribution of FUS to the cytoplasm yields loss-of-function in PRMT1, altering the histone code.\textsuperscript{121}

**Epigenetic mechanisms and findings in ALS**

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**Abbreviations:** 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; ADAR2, adenosine deaminase RNA-specific 2; ALS, amyotrophic lateral sclerosis; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; C9orf72, chromosome 9 open reading frame 72; DNMT, DNA-(cytosine-5)-methyltransferase; ELP3, RNA polymerase II elongator complex protein 3; HAT, histone acetyltransferase; HDAC, histone deacetylase; miRNA, microRNA; OPTN, optineurin; PRMT1, protein arginine N-methyltransferase 1; SS18L1, synovial sarcoma translocation gene on chromosome 18 like 1; TDP-43, TAR DNA-binding protein 43.

**Histone modifications and chromatin remodelling**

Post-translational modifications of H3 and H4 histone tails via acetylation and methylation allow chromatin to dynamically change from a highly packaged, transcriptionally inactive conformation (heterochromatin) to a transcriptionally active state (euchromatin).\textsuperscript{107,125} Whereas acetylation directly changes chromatin structure, histone methylation—as with DNA methylation—serves as a docking site for transcriptional-regulation proteins. The acetylation of histone tails by the histone acetyltransferases promotes transcription, whereas removal of acetyl groups by histone deacetylases (HDACs) represses gene expression.\textsuperscript{126} Methylation of histone tails is catalysed by histone transferases; monomethylation is associated with euchromatization, and dimethylation and trimethylation are associated with heterochromatization.\textsuperscript{127}

HDAC overexpression can be detrimental to the CNS, and reduced histone acetylation is a common observation in models of neurodegenerative disease.\textsuperscript{125} Forced expression of HDAC3 causes degeneration of rat neurons and cultured HT22 hippocampal cells,\textsuperscript{128} and a postmortem analysis reported an increase in HDAC2 mRNA and a reduction in HDAC11 mRNA in spinal cord and brain tissue from patients with ALS.\textsuperscript{129} Conversely, a more recent study using two SOD1 mouse models found an increase in HDAC11 mRNA,\textsuperscript{130} although these results are yet to be corroborated by subsequent studies. Similarly, a single point mutation in SS18L1, a component of the
chromatin-remodelling complex, ablates the interaction with histone acetyltransferase machinery and modifies the histone acetyltransferase catalytic subunit in the RNA polymerase II elongator complex protein 3 (ELP3), which regulates histone acetylation and methylation and is important for motor neuron axonal homeostasis. C9orf72 repeat-expansion carriers with ALS and/or FTD also exhibit reduced brain C9orf72 mRNA expression, owing to an altered histone methylation pattern featuring trimethylation at four lysine residues. This methylation pattern alone might be sufficient to produce neuropathological alterations. Thus, DNA methylation and histone modifications might be important therapeutic targets.

RNA editing
RNA editing enzymatically changes nucleotide sequences so that they differ from those encoded by the original genomic DNA. The most common change in patients with ALS is the deamination of adenosine to inosine. This modification might underlie the excitotoxic effects of variants of the AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor in ALS, whereby reductions in the enzyme adenosine deaminase RNA-specific 2 (ADAR2; also known as double-stranded RNA-specific editase 1) limits the conversion of adenosine to inosine at the GluA2 (Glu/Arg) site of the AMPA receptor pore-lining domain, and promote an abnormal AMPA variant in ALS. The consequences of altered RNA editing in this case are further supported by the observation that motor neurons in patients with ALS are particularly susceptible to RNA-editing deficiencies, as ADAR2-deficient motor neurons undergo slow death in conditional ADAR2 knockout mice. Furthermore, this enzymatic deficiency causes mislocalization and aggregation of TDP-43. These defects can be reversed in conditional ADAR2-knockout mice with injection of AAV9-ADAR2, which enhances the expression of exogenous ADAR2 in the CNS, and prevents progressive motor dysfunction. These results suggest that delivery of ADAR2 via gene therapy also correct defects in patients with ALS.

miRNA
miRNAs are evolutionarily conserved noncoding RNAs, about 22 nucleotides long, that can individually regulate several hundred targets via RNA-dependent post-transcriptional silencing mechanisms. Conversely, miRNA transcripts can be regulated by many miRNAs. These noncoding molecules can be intergenic or intragenic, and some miRNAs can be cotranscribed as polycistronic clusters. The human genome harbours at least 1,881 precursor miRNAs capable of modulating many physiological processes—including neuronal homeostasis and cell differentiation—by intricate fine-tuning of the transcriptome and proteome. miRNAs are also hypothesized to contribute to genetic networks controlled by feed-forward loops, and they are temporally and differentially expressed among different tissues; for example, the miRNA miR-138 is restricted to the CNS whereas its precursor is ubiquitously expressed. An accelerated turnover rate in evolutionarily conserved miR-124 affects neuronal differentiation of mesenchymal stem cells, and miR-9 exhibits abundant neuron-specific expression.

miRNAs undergo a detailed maturation process that occurs in the nucleus and cytoplasm. In the canonical biogenesis pathway (Figure 1a), primary miRNA transcripts (pri-miRNAs) are transcribed in the nucleus and processed by Drosha (also called ribonuclease 3) to produce precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm and processed by endoribonuclease Dicer to become mature double-stranded short miRNAs. Next, argonaute (AGO) proteins associate with miRNAs to prompt the separation of the double-stranded molecule and form the RNA-induced silencing complex (RISC). The miRNAs then guide the RISC complex toward the 3' untranslated region of specific mRNA targets to epigenetically control gene expression by either translational repression or transcript degradation. In mammals, only a single member of the AGO family, AGO2, harbours the catalytic activity required for miRNA cleavage. Cleavage is initiated in the context of adequate miRNA and target complementarity; however, complementarity is limited for most animal miRNAs and only a short ‘seed sequence’ of 2-8 nucleotides at the 5' end is essential for determining specificity. Alternatively, mammalian miRNA biogenesis can also occur via non-canonical pathways. In the mirtron pathway, miRNA synthesis does not require processing by Drosha, and direct pre-miRNA loading onto AGO2 does not require Dicer. Some nuclear miRNAs also target noncoding RNAs via AGO2-mediated cleavage, in which circular noncoding antisense RNA stabilizes a sense transcript, whereas destabilization of the antisense transcript occurs by nuclear miRNA.

For the vast majority of diseases, differentially expressed miRNAs have been found between diseased and normal tissue, and miRNAs are usually subject to change during the pathogenetic course. Thus, the miRNA-mediated alteration of biological pathways could explain, at least partly, complex diseases like ALS. In neurodegenerative diseases, the implications of miRNA dysregulation have not been fully elucidated, and it is crucial to determine whether dysregulation occurs via transcriptional or post-transcriptional mechanisms, or both. Notably, the dysfunction of components in miRNA biogenesis can have severe consequences. Loss of Dicer1 induces symptoms similar to spinal muscular atrophy in rodent motor neurons and also contributes to amyloid-β accumulation and dopamine loss. Similarly, decreases in TDP-43 can impact miRNA processing via interactions with Drosha-containing protein complexes (Figure 1b). In addition to forming a complex with DGCR8, Drosha can interact with several polypeptides—including TDP-43, AGO2, and some nuclear primary miRNAs or cytoplasmic pre-miRNAs—to establish an alternative complex with limited processing function. Whereas miRNAs are generally stable, long-lived molecules, high complementarity between miRNAs and their targets can further lead to trimming and destabilization of miRNAs. An accelerated turnover rate in...
neuronal miRNAs suggests a major role for this pathway in neuronal physiology.166 Several studies have examined miRNAs in ALS models and in patient biopsy samples. In one study, the expression of certain miRNAs previously shown to bind to TDP-43 in vitro was altered in cerebrospinal fluid (CSF) and serum from patients with sporadic ALS.168 This study further determined that some miRNAs are normally found in serum, whereas others, such as miR-9-5p, miR-132-5p, and miR-558-3p, are more abundant in CSF, indicating that changes in serum do not necessarily reflect CSF miRNA levels.169 Moreover, strongly regulated miRNAs were identified in lymphoblast cell lines from patients with familial ALS. Specifically, both strands of miR-143 were downregulated, whereas miR-558-3p was unaffected. Suppression of miR-132-5p/3p and miR-574-5p/3p was also evident in all patients except those with SOD1 mutations (that is, those with mutations in TARDDBP, FUS, or C9orf72). Furthermore, miR-663a and miR-9-5p were exclusively downregulated in patients with FUS mutations, and let-7b was altered in both FUS and C9orf72 mutation carriers.169 Overall, the prevalence of mostly downregulated TDP-43-binding miRNAs suggests that a general defect in RNA metabolism may be present in patients with ALS.

miRNA alterations that impact the proposed pathogenetic mechanisms of ALS have also been observed. A disease-specific, more than twofold upregulation of miR-338-3p is present in blood leukocytes, CSF, serum and spinal cords of patients with sporadic ALS relative to controls, and in situ hybridization verified that miR-338-3p accumulates in lumbar dorsal horn grey matter.170 Interestingly, miR-338-3p is also enriched in distal axons where it modulates mitochondrial function and is involved in apoptosis, neurodegeneration and glutamate clearance, presumably via interactions with targets such as the glutamate transporter SLC1A2 and apoptosis-associated tyrosine kinase.171 Expression of excitatory amino acid transporter 2 (EAAT2) is indirectly increased by neuronal miRNAs, such as miR-124a, and EAAT2 packaged in exosomes is internalized by astrocytes.172 Decreases in miR-124a are seen in the spinal cord of mutant SOD1 mice, suggesting that dysregulated glutamate transport pathways probably contribute to the exacerbation of ALS pathology.171
Increased expression of miR-23a, miR-29b, miR-206, and miR-455 has been observed in skeletal muscle samples from patients with ALS. miR-23a suppresses the activity of the peroxisome proliferator-activated receptor-γ co-activator (PGC)-1α, a protein involved in mitochondrial biogenesis and function, which might indicate that specific miRNAs represent therapeutic targets. Similarly, miR-206 is increased in skeletal muscles of patients with ALS and in symmetric and mutantom SOD1 mice, possibly owing to the effects of denervation. The loss of miR-206 accelerates disease progression in mice, probably because of its involvement in skeletal muscle development, synaptic plasticity, neuromuscular junction regeneration, and nerve and muscle communication. Finally, one group examined the expression of numerous miRNAs in spinal cords from patients with ALS, and found that downregulations accounted for the vast majority of significant differences in miRNA expression. Two additional studies from the same group determined that downregulation of miR-b1336 and miR-b2403, which normally stabilize NF-L, leads to the loss of NF-L mRNA and subsequent neuromuscular junction pathology. This study also revealed that miR-146a*, miR-524-5p, and miR-582-3p, which have miRNA recognition elements within the human NF-L mRNA 3' untranslated region, are also dysregulated in the spinal cords of patients with ALS.

Inflammation and the immune system are also implicated in ALS pathology and disease progression: microglial activation, T-cell-independent macrophage activation, monocyte recruitment to diseased tissue, and dysregulation of immune-related genes are all present in patients with ALS. Moreover, unique pro-inflammatory miRNA and gene profiles are present in the blood of patients with ALS and in microglia and LY6C+ peripheral monocytes of mutant SOD1 mice, including increased expression of miR-27a, miR-155, miR-146a, miR-451, miR-223, miR-142-5p, let-7a/b, and miR-532-3p, among others. The TGF-β1-targeting miRNAs miR-21 and miR-106b are upregulated in patients with ALS. miR-155, which reduces TGF-β1 production, promotes macrophage inflammatory responses, and increases pro-inflammatory cytokine secretion, is also upregulated in the spinal cords and peripheral blood cells of mutant SOD1 mice and patients with ALS. Furthermore, upregulation of additional immune-related miRNAs, including miR-22, miR-125b, miR-146b, and miR-365, are present within activated microglia in the brains of SOD1-Gly93AΔa mice. The pro-inflammatory and anti-inflammatory cytokine interleukin 6 (IL-6) is normally negatively regulated by miR-365. In microglia of patients with ALS, miRNA-365 is upregulated, which is accompanied by a corresponding significant reduction in IL-6. On the other hand, miR-125b targets signal transducer and activator of transcription 3 (STAT3), and downregulation of these two signalling pathways favours pro-inflammatory signals by increasing expression of tumour necrosis factor (TNF). Table 2 summarizes the miRNAs with known importance to ALS pathology.

Converging pathways
Given the highly tuned epigenetic regulation of gene expression, neuronal homeostasis is most likely orchestrated by complex molecular networks directed by dynamic crosstalk between multiple epigenetic mechanisms. Therefore, alterations to epigenetic players or pathways can cause deleterious downstream effects. For example, miRNA function goes beyond that of RNA interference by directly interacting with gene promoters, thereby driving epigenetic regulation of DNA methylation and histone modification. The control of miRNAs is similar to that of protein-coding genes with double-negative feedback loops. At least one identified miRNA directs chromatin remodelling of a promoter region, and synthetically produced miRNA-like molecules can similarly cause gene silencing. Acceleration of ALS disease progression is also associated with HDAC4: elevated levels were found in muscle biopsy samples from a patient with ALS who had poor functional outcome and low capacity for reinnervation. miR-206 regulates HDAC4 expression and is likewise upregulated in muscle samples from patients with ALS; however, expression did not correlate with reinnervation.

Mutations in ALS-related genes can also have a modulatory effect on miRNAs. FUS mutations—particularly FUS Gly48Ala, which causes increased FUS protein expression—are found in the 3' untranslated region and are complemented by miR-141 and miR-200a, thus linking FUS and these miRNAs by a feed-forward regulatory loop. TDP-43 and FUS also interact, functioning in a biochemical complex to modulate HDAC6 mRNA production, whereas SOD1 acts independently of this complex. Moreover, a polymorphism in the GRN 3' untranslated region hinders miR-659 binding, resulting in decreased expression of progranulin and a higher risk of FTD–ALS spectrum disorders. miR-29b can also directly regulate progranulin levels, thus supporting the induction of miR-29 as a potential therapeutic strategy to increase proper protein translation. Moreover, TDP-43 knockdown in human cultured cells alters miRNAs that bind TDP-43, including downregulation of let-7b and upregulation of miR-663. Also binding to TDP-43 in the nucleus are primary miR-132 (highly enriched in neurons), primary miR-143, primary miR-558, and primary miR-574. These findings support the contention that TDP-43 is a nuclear Drosha-associated protein with post-transcriptional involvement in miRNA biogenesis (Figure 1b).

Although it is unclear how the activity of mature miRNAs is regulated in the cytoplasm, we hypothesize that the dysregulation of key miRNAs could potentially be caused by their sequestration within protein aggregates, namely TDP-43 aggregates formed through cellular stress, or overexpression of or mutations in TARDBP (Figure 1c). Prior evidence shows that TDP-43 negatively regulates the miR-1 family (miR-1 and miR-206) by limiting their bioavailability to RNA-induced silencing complexes. Additionlly, overexpression of TDP-43 increases levels of HDAC4, a miR-1–family target, and this dysregulation of HDAC4 is correlated with ALS.
progression.166 Three members of the miR-132 cluster are significantly downregulated in the brains of patients with FTD who present with TDP-43 inclusions,183 and most TDP-43-binding miRNAs are generally downregulated as well.166 Furthermore, FUS C-terminal mutants are mislocalized in the cytoplasm, and are recruited to stress granules along with wild-type FUS and other RNA-binding proteins to form large aggregates, thus disrupting RNA metabolism.72 Similarly, aggregated TDP-43 is capable of sequestering miRNAs as well as other small molecules, such as miRNAs.

Therapeutics
There is no effective treatment for ALS, and riluzole—the lone FDA-approved disease-modifying drug—only modestly slows disease progression.166 However, alternative strategies are under investigation. A phase 1 clinical trial of an antisense oligonucleotide (ISIS 333611) that targets SOD1 mRNA to halt the production of the mutant protein has proven effective and safe when delivered to the CSF of patients with SOD1-related familial ALS.188 A similar drug that targets the sense strand of the C9orf72 hexanucleotide repeat mitigated toxicity by suppressing RNA foci formation in vitro65,68 and in vivo169 without reducing RNA levels. These results support the development of antisense oligonucleotides to treat C9orf72-linked and SOD1-linked ALS. On the other hand, understanding the contribution to ALS made by dysregulated miRNAs and their targets might help identify new pathways involved in neurodegeneration, thus offering novel opportunities for targeted intervention.

There are two approaches to the development of miRNA-based therapeutics. miRNA antagonists inhibit endogenous miRNAs that have a toxic gain-of-function in diseased tissues, and involve the use of an anti-miR—a chemically modified antisense RNA—to knockdown miRNA. The resulting miRNA duplex is not active, thus countering the miRNA’s negative regulatory effects.

---

### Table 2 | Key miRNAs differentially expressed in ALS

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Function</th>
<th>Importance to ALS</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-206</td>
<td>Human skeletal-muscle-specific; affects neuro-muscle interactions</td>
<td>Increased in patients with ALS; Absence causes delay in muscle reinervation via lack of a local signalling</td>
<td>Williams et al. (2009)173, Tolivenen et al. (2014)184</td>
</tr>
<tr>
<td>miR-143</td>
<td>Possibly involved in cardiac morphogenesis</td>
<td>Downregulated in ALS</td>
<td>Freischmidt et al. (2013)158</td>
</tr>
<tr>
<td>miR-365</td>
<td>Inhibitory effect on IL-6 3' untranslated region</td>
<td>Negative regulator of IL-6 in microglia, augmented in ALS</td>
<td>Parisi et al. (2014)176</td>
</tr>
<tr>
<td>miR-9</td>
<td>Highly involved in brain neuronal development, regulates axon growth direct regulation of MAP1B mRNA</td>
<td>Elevated in patients with ALS harbouring TARDBP mutations; miR-9-5p is decreased in patients with FUS mutations</td>
<td>Zhang et al. (2013)177, Kye and Goncalves (2014)158, Freischmidt et al. (2013)158</td>
</tr>
<tr>
<td>miR-132p</td>
<td>Highly enriched in neurons, Promotes neuronal outgrowth by reducing p250GAP levels</td>
<td>Cytoplasmic TDP-43 aggregates hinder the biogenesis of miR-132p</td>
<td>Kawahara et al. (2012)187</td>
</tr>
<tr>
<td>miR-124a</td>
<td>Negatively regulates the expression of the glutamate transporters EAAT2</td>
<td>Decreased expression in spinal cords of SOD1 mutant mice</td>
<td>Morel et al. (2013)171</td>
</tr>
<tr>
<td>miR-23a</td>
<td>Suppresses PGC-1α translation in a manner dependent on the 3' untranslated region</td>
<td>Elevated in skeletal muscles of patients with ALS; Might explain mitochondrial dysfunction</td>
<td>Russell et al. (2013)172</td>
</tr>
<tr>
<td>miR-29b and miR-455</td>
<td>miR-29b has a role in muscle regeneration; miR-455 is involved in muscle wasting</td>
<td>Elevated in skeletal muscle tissues from patients with ALS</td>
<td>Russell et al. (2013)172</td>
</tr>
<tr>
<td>miR-21 and miR-106b</td>
<td>Target TGF-β1 expression</td>
<td>Upregulated in patients with ALS; Reduce TGF-β1 levels</td>
<td>Butovsky et al. (2012)175</td>
</tr>
<tr>
<td>miR-155</td>
<td>Targets TGF-β1, promotes macrophage inflammatory responses, and increases pro-inflammatory cytokine secretion</td>
<td>Upregulated in spinal cords and blood of patients with ALS, and in SOD1-mutant mice; Treatment by anti-miR-155 delays SOD1-mouse mortality</td>
<td>Koval et al. (2013)159, Butovsky et al. (2012)175</td>
</tr>
<tr>
<td>miR-365 and miR-129b</td>
<td>Regulates the pro-inflammatory and anti-inflammatory cytokine IL-6 in familial ALS (miR-365); targets STAT3 (miR-129b)</td>
<td>Downregulated in patients with ALS, favouring pro-inflammatory signals by increasing TNF</td>
<td>Parisi et al. (2013)176</td>
</tr>
<tr>
<td>miR-1336 and miR-2403</td>
<td>Stabilize NF-L at the neuromuscular junction</td>
<td>Differentially expressed in spinal cords of patients with sporadic ALS</td>
<td>Ishilaq et al. (2014)27</td>
</tr>
<tr>
<td>miR-146a*, miR-524-5p, and miR-582-3p</td>
<td>Target human NF-L mRNA</td>
<td>Dysregulated in spinal cords of patients with ALS</td>
<td>Campos-Melo et al. (2013)158</td>
</tr>
</tbody>
</table>

Abbreviations: ALS, amyotrophic lateral sclerosis; EAAT2, excitatory amino acid transporter 2; FUS, fused in sarcoma; MAP1B, microtubule-associated protein 1β; miRNA, microRNA; NF-L, neurofilament light polypeptide; SOD1, superoxide dismutase; p250GAP, Rho GTPase-activating protein 32; STAT3, signal transducer and activator of transcription 3; TARDBP, TAR DNA binding protein; TDP-43, TAR DNA-binding protein 43; TGF-β1, transforming growth factor beta 1; TNF, tumour necrosis factor.
Cytotoxicity
Schwann cell
Skeletal muscle
Axon
Figure 2

consequence of ALS pathogenesis. miRNA, microRNA; TGF
associated with ALS pathology. Abbreviations: ALS, amyotrophic lateral sclerosis;
cellular pathways that cause the downstream effects ending in the neurodegeneration
also suggests that the dysregulation of key miRNAs triggers the alterations in these
transmission of wrongful cues due to disrupted signalling at the neuromuscular
protein complex metabolism is possibly altered by cytoplasmic protein inclusions,
occur through the dysregulation of various cellular pathways. Specifically, miRNA–
NATURE REVIEWS | RNA and protein
Protein
metabolism
| Dysregulation of miRNA biogenesis and function may be a cause and/or
consequence of ALS pathogenesis. Impairments in miRNA biogenesis or function
occur through the dysregulation of various cellular pathways. Specifically, miRNA–
protein complex metabolism is possibly altered by cytoplasmic protein inclusions,
transmission of wrongful cues due to disrupted signalling at the neuromuscular
junction, hampering of cell homeostasis due to cytotoxicity associated with faulty
glutamate clearance, and an overactive inflammatory response. Conversely, evidence
also suggests that the dysregulation of key miRNAs triggers the alterations in these
cellular pathways that cause the downstream effects ending in the neurodegeneration
associated with ALS pathology. Abbreviations: ALS, amyotrophic lateral sclerosis;
miRNA, microRNA; TGFβ1, transforming growth factor β1.

In one of the first attempts to use this strategy in ALS, delivery of anti-miR-155 to SOD1 Gly93Ala mice via ventricular osmotic pumps successfully delayed mortality. A pitfall associated with this approach is the potential for nonspecific binding to other RNAs. Alternatively, the second approach to miRNA therapeutics involves miRNA mimics and miRNA replacement therapies, which can reintroduce miRNAs into cells exhibiting downregulation, thereby reactivating key pathways. Notably, injection of artificial miR-124a oligonucleotides can counteract the increase in EAAT2 expression associated with decreased miR-124a that is observed in mutant SOD1 murine spinal cords.

The first miRNA-based therapeutic (currently being evaluated in phase II clinical trials) is the miR-122 antagonist SPC3649, which targets the hepatitis C virus. So far, this agent has exhibited no adverse effects. Although miRNA-targeted therapeutics are still in infancy, continued, rapid technological progress has the potential to facilitate the translation of these approaches to future human clinical trials.

In recent years, therapies that target histone modifications have been successful in fighting neurodegeneration and cancer. The HDAC inhibitor sodium phenylbutyrate (NaPB) upregulates astrocytic neurotrophin expression in the CNS of AD-model mice, thus improving cognition, and in SOD1 Gly93Ala mice, riluzole and NaPB synergistically improve survival and phenotype. Likewise, a human clinical trial assessing NaPB further demonstrated that it is safe and tolerable, and does not alter blood riluzole levels. In addition, certain DNMT and HDAC inhibitors are already approved by the FDA for use in cancer, and these agents might possibly reverse other aberrant epigenetic changes in the CNS as well. Thus, the identification of epigenetic biomarkers for sporadic ALS could elucidate pathological molecular mechanisms, aid in diagnosis, and reveal therapeutic targets.

Conclusions

The mechanisms leading to ALS are complex, and despite extensive research into the genetic causes and environmental risk factors, the vulnerability of motor neurons to neurodegeneration cannot be explained by the individual involvement of these factors. The joint genetic and environmental contributions involving posttranscriptional changes or epigenetic mechanisms, however, are probably crucial to understanding ALS pathogenesis, and establishing a path to treatment. Furthermore, disruptions to miRNA pathways are a likely cause and/or consequence of mechanisms that lead to ALS pathology (Figure 2), including altered RNA and protein metabolism, inflammatory responses, cytotoxicity, and impaired structure and signalling at the neuromuscular junction. Thus, epigenetics offers new scientific approaches and tools for uncovering the pathophysiology of neurodegeneration. Furthermore, the reversible nature of epigenetic mechanisms positions them as attractive targets for therapeutic development. In fact, some molecules and environmental manipulations have successfully reversed certain epigenetic marks in both laboratory and clinical settings, supporting the potential of therapeutic agents based on epigenetic mechanisms. Further investigation of proteins involved in miRNA biogenesis and disruption, and identification of targets that could restore altered cellular pathways, such as those mediated by TDP-43 and FUS, might also open new therapeutic avenues.

Review criteria

References were identified from English language publications in PubMed through January 2015, and from the authors’ collections of scientific literature. Combinations of the following key words were used: “amyotrophic lateral sclerosis”, “ALS”, “frontotemporal dementia”, “neurodegeneration”, “Alzheimer’s disease”, “Parkinson’s disease”, “pathology”, “neuroinflammation”, “protein aggregates”, “disease modifier”, “genetics”, “C9orf72”, “TDP-43”, “FUS/TLS”, “epigenetics”, “miRNA”, “posttranslational modifications”, “DNA modifications”, “chromatin remodeling”, “noncoding RNA”, “RNA editing”, “motor neuron disease”, “environment”, “exposure”, “risk factor”, “heavy metals”, “biomarkers”, “therapeutics”. Publications from the past five years were prioritized.


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Clinical Trials of Therapies for Amyotrophic Lateral Sclerosis
One Size Does Not Fit All

The French neurologist Jean-Marie Charcot (1825-1893) is acclaimed for his studies on the motor system and unifying previously disparate entities into 1 disorder, amyotrophic lateral sclerosis (ALS). His careful work correlating neurological signs with anatomy led to the concept that a spectrum of neurological disorders, including progressive muscular atrophy, progressive bulbar palsy, and primary lateral sclerosis, represented a single entity, ALS. Charcot summarized his ideas in a frequently cited review in 1874 that has dominated medical nosology for 140 years.1

Today in clinical practice, neurologists easily recognize the various subtypes of ALS, which, in addition to the previously mentioned, include the flail arm and leg variants.2 We now question whether ALS is 1 disorder, as Charcot would contend,3 or if the clock has turned back. Are these subtypes of 1 disease or multiple different diseases, with 1 final common anatomical and clinical end point?

The rapid advancement in genetic discoveries points to multiple different disorders, with ALS being much more a syndrome than a single distinct entity. For example, the C9orf72 hexanucleotide expansion, the genetic mutation currently most commonly associated with familial and sporadic ALS, represents a clinical spectrum, with the extremes representing ALS or frontotemporal dementia, and the middle region representing patients having classic symptoms of ALS combined with executive dysfunction and reduced survival. However, phenotypes in SOD1, the first ALS gene identified, range from a short and rapid course to a prolonged clinical course, depending on the specific mutation. Fused in sarcoma affects younger persons and has a more rapid progression.2

We contend that the persistence in considering ALS as a single disorder is a major reason why many previous therapeutic drug trials have failed. These drugs may benefit some ALS subtypes but have no effect on others. Furthermore, reliance on the SOD1 mouse model for drug development may not accurately assess all ALS disease mechanisms and it is possible that effective drugs may not even be tested in patients if these drugs do not show efficacy in mouse models. A more rational approach is in defining ALS subtypes based on both clinical presentation and, when applicable, genetic variability. With the understanding that ALS is not a single disorder, the ALS research community needs to move away from the idea that there is 1 ideal clinical trial end point. We agree that the application of new efficacy outcome measures combined with pharmacodynamic markers will improve the ability to detect meaningful clinical outcomes and that these end points may need to be stratified by disease heterogeneity.4 Similar views have recently been provided by the US Food and Drug Administration.5 In response to a February 2013 hearing on drug treatment for ALS, the US Food and Drug Administration agreed that disease heterogeneity may affect clinical trial outcomes and that it would support methods that can identify these subgroups for an efficacy analysis. Furthermore, the US Food and Drug Administration indicated that it is open to alternative clinical trial end points and novel study designs that apply end points other than the more traditional survival and functional scale outcomes.

Patients with ALS also recognize the importance of incorporating the heterogeneity of the disease and wish to work with the ALS research community to use these differences in the development of clinical trials. Josh von Schaumburg, BA (email communication, September 20, 2014), a member of the ALS Emergency Treatment Fund, questioned whether subgroup analyses in prior clinical trials would identify an effective treatment for a family member with young bulbar-onset disease. Considering the enthusiasm of patients with ALS participating in clinical trials at all stages of their disease, the use of disease phenotypic variability combined with new biomarkers and genotyping may aid in the collection of phase 2 outcomes unique from outcomes for phase 3 studies so that phase 2 studies do not simply represent underpowered phase 3 trials.

We believe that the proposed mechanism underlying therapeutic intervention must guide the choice of clinical end points. We completed a phase 1 and phase 2 trial of intraspinal stem cell transplantation in ALS.6 Our final cohort of 3 patients in the phase 2 trial underwent both lumbar and cervical transplantation and received a total of 16 million neural stem cells. The therapeutic rationale underlying transplantation was the preservation of motor neurons in and around the transplanted regions.7 Our proposed therapy would not benefit patients with bulbar disease because the stem cells cannot be delivered to the most affected regions nor would they benefit those with more advanced disease stages because the stem cells do not replace lost motor neurons. From a clinical trial design perspective, this same group of patients was at a stage in their disease where current outcome measures may not have been robust enough to detect a treatment effect. As investigators, we recognize patients’ desires to participate in these studies regardless of disease severity. Participation in clinical research brings patients hope and also provides a means for them to feel that they are helping to advance ALS knowledge. As we design the next phase

[Note: The full text continues with further discussion and references.]
2/phase 3 trial, we are encountering the difficulties and conundrums of previous clinical trials that have had to grapple with ALS as not 1 disease but a syndrome. While we aim to use novel end points to assess the efficacy of stem cells, we may need to rely on more established measures in a group of patients to demonstrate a statistically meaningful disease modification.

If we are to make therapeutic progress, we need to reassess our position as a neurological community and come to terms with the idea that one size does not fit all when we approach clinical trial designs in ALS. This will not be without its own challenges, especially in clinical trial recruitment and the powering of studies. Yet without a rational and more strategic approach, future ALS clinical trials will fail just like their predecessors. The nagging question remains: have previous therapies failed because of our lack of defining disease subtypes and the selection of the wrong clinical end points? More importantly, as we shift from the medical nosology of Charcot 140 years ago to a nosology based on a combination of rapidly advancing genetics and imaging, can our increased understanding of ALS as a syndrome lead to improved trial designs with relevant clinical outcomes? As a neurological community we need to readdress our diagnostic and therapeutic approaches, because when it comes to ALS, one size does not fit all.

REFERENCES

Diabetic Neuropathy
Chapter 22

Therapy for diabetic neuropathy: an overview

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INTRODUCTION

Diabetes is the most common cause of peripheral neuropathy worldwide (Smith et al., 2005). Diabetic peripheral neuropathy (DPN) represents a heterogeneous group of disorders, which includes distal symmetric polyneuropathy, mononeuropathy, diabetic amyotrophy, autonomic neuropathy, mononeuritis multiplex, radiculopathy, and cranial neuropathy. Pirart et al. followed a cohort of 4400 patients with diabetes and showed that almost 50% of these patients developed neuropathy after 25 years of disease (Pirart 1977a, b, c). There is strong evidence that patients with impaired glucose tolerance may also develop complications of diabetes and end stage organ complications (Novella et al., 2001; Singleton et al., 2001; Gong et al., 2011). In this chapter, we will focus on therapy for this common complication of diabetes.

TREATMENT STRATEGIES FOR PREVENTING NEUROPATHY

Glycemic control

The Diabetes Control and Complications Trial (DCCT) (1983–1993), a multicenter, randomized, controlled clinical trial, demonstrated reduced development of long-term microvascular complications in type 1 diabetic patients after intensive glycemic therapy (Diabetes Control and Complications Trial Research Group, 1993). Intensive therapy consisted of 3 or more injections of insulin per day compared with conventional therapy, 1–2 injections per day. The intensive treatment group showed significantly lower incidences of neuropathy, retinopathy, nephropathy, and limb amputations due to diabetes compared to the conventional treatment group. Furthermore, nerve conduction velocities were improved after intensive treatment. This large trial also showed that strict glycemic control can prevent autonomic neuropathy. In fact, the incidence of cardiovascular autonomic neuropathy was reduced in the intensive treatment group by 53% compared to the conventional treatment group. The subsequent EDIC trial demonstrated that the benefit of enhanced glucose control continued for an additional 8 years after the intervention was stopped despite similar glycemic control between the two groups (Martin et al., 2006).

The UKPDS Group examined intensive treatment (with either sulfonylurea or insulin) compared to conventional treatment in patients with type 2 diabetes between 1977 and 1991 (UKPDS Group, 1998). Compared with the conventional treatment group, the risk in the intensive group was 12% lower for any diabetes-related end point. There was also evidence of a mild reduction in the risk of neuropathy as measured by a biothesiometer at 15 years (relative risk 0.60, 95% CI 0.39–0.94). The disadvantages of intensive treatment were weight gain and an increased risk of hypoglycemia. However, a Cochrane review of several studies investigating enhanced glucose control in patients with type 2 diabetes failed to demonstrate a statistically significant effect on the prevention of neuropathy (Callaghan et al., 2012).

SYMPTOMATIC TREATMENT STRATEGIES

Diabetic polyneuropathy

Neuropathic pain

Neuropathic pain often evolves spontaneously, but can be stimulus-dependent. Patients often describe this pain as burning, aching, or stabbing. In the peripheral nervous system, small, pain-mediating C and Aδ fibers become

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sensitized by damage, thus enhancing nerve excitability. Spontaneous discharges, ectopic discharges from the dorsal root ganglia, and ephaptic transmission also occur. Altered Na channel expression also contributes to the development of neuropathic pain (Waxman et al., 2000). Voltage-gated Na channels can be divided into tetrodotoxin (TTX)-resistant and -sensitive channels on primary sensory neurons, including the dorsal root ganglia. In injured peripheral nerves, the TTX-resistant channels decrease, whereas the TTX-sensitive channels increase on small fiber neurons. These changes contribute to hyperexcitability and subsequent emergence of neuropathic pain (Suzuki and Dickenson, 2000; Waxman et al., 2000). Furthermore, cytokines such as TNF-α and mediators like prostaglandin E2, bradykinin, or epinephrine are released and are involved in neuropathic pain development. N-methyl-D-aspartate (NMDA)-receptors play an important role in central sensitization, binding glutamate (Woolf and Thompson, 1991). This results in a persistent state of hyperexcitability of dorsal horn neurons. Concurrently, antinociceptive GABAergic, opiate, and glycine pathways are inhibited. Excessive downregulation or defective upregulation of these inhibitory systems may sustain neuropathic pain. A study in rats by Woolf et al. (1992) suggested that central terminals of axotomized dorsal root ganglia neurons sprout into lamina II of the dorsal horn. Lamina II usually receives C fiber nociceptive input (Woolf et al., 1992). When non-nociceptive Aβ fibers sprout into lamina II, a painful response to a nonpainful stimulus can occur. However, another study (Hughes et al., 2003) showed a lack of evidence for this hypothesis, because this sprouting could not be demonstrated.

**DIAGNOSIS OF DIABETIC PAINFUL NEUROPATHY**

Pain is often the dominant symptom of diabetic neuropathy but is not specific and other causes for painful neuropathies have to be excluded. Electrodiagnostic studies are limited to large fibers, so this test may fail to diagnose a small fiber-predominant or autonomic neuropathy. However, the majority of painful neuropathies involve large fibers as well and electrodiagnostic assessment is often diagnostic of the underlying neuropathy. Specific tests for small fiber neuropathy include quantitative sensory testing (QST), quantitative sudomotor axon reflex testing (QSART), and thermoregulatory sweat testing. Skin biopsy to examine epidermal nerve fiber density and morphology can also be performed. A more detailed review of the challenges in the diagnosis of painful diabetic neuropathy is also provided in Chapters 5 and 35.

Once the diagnosis has been confirmed, two main categories of treatment can be considered: a symptomatic treatment to alleviate pain and a treatment of the underlying pathophysiologic cause.

**Pharmacotherapies for painful diabetic neuropathies**

(Table 22.1)

**ANTIDEPRESSANTS**

*Tricyclic and tetracyclic antidepressants* (TCAs) have been found to be efficacious for several types of painful neuropathies. TCAs are serotonin/norepinephrine reuptake inhibitors. The different subtypes do not differ significantly in their efficacy. The effects include blocking Na channels in the peripheral nervous system (PNS) and alteration of serotonin and norepinephrine activity in the central nervous system (CNS) nociceptive-modulation system. Many studies have shown the efficacy of TCAs compared to placebo (Sindrup et al., 2005).

The analgesic effect of TCAs seems to occur at the synaptic level by increasing the bioavailability of serotonin and norepinephrine, which are able to inhibit effects of the nociceptive pathway. These neurotransmitters are involved in two descending inhibitory pathways located in the brainstem and spinal cord.

Amitriptyline was evaluated in a double-blind, randomized crossover trial by Max et al. (1992) in 29 patients with painful diabetic neuropathy (Max et al., 1992). The patients received 25 mg amitriptyline at bedtime, titrated to the maximum tolerated dose (150 mg/day) over 3 weeks. The final dose was maintained for another 3 weeks. Amitriptyline was compared against an active placebo, a combination of diazepam and benzatropine to mimic the adverse effects. Amitriptyline (150 mg/day) showed a benefit compared to placebo and the pain reduction was not correlated with improvement in mood. The most common side-effects were dry mouth, sedation, dizziness, and constipation. Of note, many patients benefit from doses ranging from 50–100 mg daily.

Desipramine (111 mg/day) is better tolerated than amitriptyline and showed the same positive effect (Max et al., 1992). Furthermore, it has the least anticholinergic and sedating side-effects compared with the first generation tricyclic antidepressants. Desipramine could be an alternative for patients unable to tolerate amitriptyline.

While TCAs have the lowest number-needed-to-treat compared to other neuropathic pain medications, their use is limited by their side-effects. Both serotonin and norepinephrine have important activities in other areas of the body. For example, serotonin regulates gastrointestinal motility and vasoconstriction in splanchnic, renal, and pulmonary tissue. Platelets utilize serotonin, stored in granules, to aid in coagulation. Norepinephrine is the main sympathetic postganglionic neurotransmitter.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of patients</th>
<th>Daily Doses</th>
<th>Duration</th>
<th>Outcome</th>
<th>NNT</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TCAs</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Amitriptyline</td>
<td>29</td>
<td>≤ 150 mg</td>
<td>Crossover, 2 × 6 weeks</td>
<td>Amitriptyline &gt; placebo</td>
<td>2.1</td>
<td>(Max et al., 1987)</td>
</tr>
<tr>
<td>Desipramine</td>
<td>20</td>
<td>Average 201 mg</td>
<td>Crossover, 2 × 6 weeks</td>
<td>Desipramine &gt; placebo</td>
<td>2.2</td>
<td>(Max et al., 1992)</td>
</tr>
<tr>
<td>Imipramine</td>
<td>19</td>
<td>25–350 mg</td>
<td>Crossover, 3 × 2 weeks</td>
<td>Imipramine &gt; placebo</td>
<td>4</td>
<td>(Sindrup et al., 1990b)</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>18</td>
<td>50–200 mg</td>
<td>Crossover, 3 × 2 weeks</td>
<td>Clomipramine &gt; placebo</td>
<td>4.5</td>
<td>(Sindrup et al., 1990c)</td>
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<tr>
<td><strong>SSRIs</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Paroxetine</td>
<td>26</td>
<td>40 mg</td>
<td>Crossover, 3 × 2 weeks</td>
<td>Paroxetine &gt; placebo</td>
<td>5</td>
<td>(Sindrup et al., 1990a)</td>
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<tr>
<td>Citalopram</td>
<td>18</td>
<td>40 mg</td>
<td>Crossover, 3 × 2 weeks</td>
<td>Citalopram &gt; placebo</td>
<td>3</td>
<td>(Sindrup et al., 1992)</td>
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<tr>
<td>Fluoxetine</td>
<td>46</td>
<td>40 mg</td>
<td>Crossover, 2 × 6 weeks</td>
<td>Fluoxetine = placebo</td>
<td>NA</td>
<td>(Max et al., 1992)</td>
</tr>
<tr>
<td><strong>SNRIs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>244</td>
<td>150–225 mg</td>
<td>Parallel, 6 weeks</td>
<td>Venlafaxine &gt; placebo</td>
<td>4.5</td>
<td>(Rowbotham et al., 2004)</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>457</td>
<td>20, 60, 120 mg</td>
<td>Parallel, 12 weeks</td>
<td>Duloxetine (60 mg, 120 mg) &gt; placebo</td>
<td>60 mg:4.3 120 mg:3.8</td>
<td>(Goldstein et al., 2005)</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>348</td>
<td>60 mg, 120 mg</td>
<td>Parallel, 12 weeks</td>
<td>Duloxetine (60 mg, 120 mg) &gt; placebo</td>
<td>60 mg:11 120 mg:5</td>
<td>(Raskin et al., 2005)</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>334</td>
<td>60 mg, 120 mg</td>
<td>Parallel, 12 weeks</td>
<td>Duloxetine (60 mg, 120 mg) &gt; placebo</td>
<td>60 mg:6.3 120 mg:3.8</td>
<td>(Wernicke et al., 2006)</td>
</tr>
<tr>
<td><strong>Anticonvulsants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>40</td>
<td>400 mg</td>
<td>Cross over, 2 weeks</td>
<td>Carbamazepine &gt; placebo</td>
<td>2.3</td>
<td>(Rull et al., 1996)</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>59</td>
<td>&lt;400 mg</td>
<td>Parallel, 8 weeks</td>
<td>Lamotrigine &gt; placebo</td>
<td>4</td>
<td>(Eisenberg et al., 2001)</td>
</tr>
<tr>
<td>Oxcarbazepine</td>
<td>146</td>
<td>&lt;1800 mg</td>
<td>Parallel, 16 weeks</td>
<td>Oxcarbazepine &gt; placebo</td>
<td>6</td>
<td>(Dogra et al., 2005)</td>
</tr>
<tr>
<td>Topiramate</td>
<td>323</td>
<td>&lt;400 mg</td>
<td>Parallel, 12 weeks</td>
<td>Topiramate &gt; placebo</td>
<td>7.4</td>
<td>(Raskin et al., 2004)</td>
</tr>
<tr>
<td><strong>Calcium channel α2-δ agonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gabapentin</td>
<td>165</td>
<td>&lt;3600 mg</td>
<td>Parallel, 8 weeks</td>
<td>Gabapentin &gt; placebo</td>
<td>4</td>
<td>(Backonja et al., 1998)</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>146</td>
<td>300 mg</td>
<td>Parallel, 8 weeks</td>
<td>Pregabalin &gt; placebo</td>
<td>3.9</td>
<td>(Rosenstock et al., 2004)</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>338</td>
<td>75, 300, 600 mg</td>
<td>Parallel, 5 weeks</td>
<td>Pregabalin (300, 600 mg) &gt; placebo</td>
<td>300 mg:3.6 600 mg:3.3</td>
<td>(Lesser et al., 2004)</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>246</td>
<td>150, 600 mg</td>
<td>Parallel, 6 weeks</td>
<td>Pregabalin (600 mg) &gt; placebo</td>
<td>600 mg:4.2</td>
<td>(Richter et al., 2005)</td>
</tr>
<tr>
<td><strong>μ Receptor agonists</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tramadol</td>
<td>127</td>
<td>100–400 mg</td>
<td>Parallel, 6 weeks</td>
<td>Tramadol &gt; placebo</td>
<td>3.1</td>
<td>(Harati et al., 1998)</td>
</tr>
<tr>
<td>Oxycodone CR</td>
<td>159</td>
<td>10–100 mg</td>
<td>Parallel, 6 weeks</td>
<td>Oxycodone &gt; placebo</td>
<td>NA</td>
<td>(Gimbel et al., 2003)</td>
</tr>
<tr>
<td><strong>NMDA antagonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>19</td>
<td>400 mg</td>
<td>Crossover, 9 weeks</td>
<td>Dextromethorphan &gt; placebo</td>
<td>3.2</td>
<td>(Sang et al., 2002)</td>
</tr>
<tr>
<td>Memantine</td>
<td>19</td>
<td>55 mg</td>
<td>Crossover, 9 weeks</td>
<td>Memantine = placebo</td>
<td>NS</td>
<td>(Sang et al., 2002)</td>
</tr>
<tr>
<td><strong>Topical agents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsaicin cream</td>
<td>252</td>
<td>0.075% q.i.d.</td>
<td>8 weeks</td>
<td>Capsaicin &gt; placebo</td>
<td>NA</td>
<td>(Capsaicin Study Group, 1992)</td>
</tr>
</tbody>
</table>

TCA, tricyclic and tetracyclic antidepressants; SSRIs, selective serotonin reuptake inhibitors; SNRIs, serotonin-norepinephrine reuptake inhibitors; NNT, number needed to treat; NA, not available; NS, not significant; q.i.d.: four times a day.
and thus stimulates a variety of sympathetic effects. Anticholinergic adverse effects are thus common and include dry mouth, constipation, orthostatic hypotension, and urinary retention. Because of cardiac toxicity, the use of TCAs should be considered very carefully in patients with ischemic cardiac disease and ventricular conduction abnormalities. Patients older than 40 years should obtain a screening ECG. TCAs can also induce sedation since serotonin and norepinephrine are involved in sleep–wake rhythm control.

Selective serotonin/norepinephrine reuptake inhibitors (SSNRIs) have also been studied for painful neuropathies.

Duloxetine at a dose of 60 mg and 120 mg per day showed a significant effect in decreasing neuropathic pain. Three large randomized placebo-controlled studies have reported its efficacy, which were pooled in a recent analysis (Goldstein et al., 2005; Raskin et al., 2005; Wernicke et al., 2006). All studies evaluated 60 mg of duloxetine administered daily or twice daily and showed a significant benefit in reducing the weekly mean of the 24 h average pain score compared with placebo, starting after the first week of therapy. This effect was sustained for the entirety of the trials (Kajdasz et al., 2007). Duloxetine is usually well tolerated, with fewer gastrointestinal adverse effects than other SSNRIs.

Venlafaxine has to be used carefully in patients with cardiac disease and should be titrated slowly. Nevertheless, venlafaxine, demonstrated benefit at a dose of 150–225 mg/day in a study of 244 patients and was clearly superior to placebo (Rowbotham et al., 2004). ECG monitoring is recommended with this medication.

Selective serotonin reuptake inhibitors (SSRIs) have been rather disappointing in the treatment of neuropathic pain. In fact, it is still controversial whether SSRIs are successful in pain relief.

Two small trials (Sindrup et al., 1990a, b, c, 1992) indicated a small but significant pain reduction with citalopram and paroxetine. Paroxetine has been more successful in treating pain at a dose of 40 mg/day, but less effective than imipramine. Citalopram 40 mg/day performed better than placebo. Escitalopram at a dose of 20 mg daily (Otto et al., 2008) was found to reduce pain only in a few patients. Furthermore, fluoxetine (40 mg/day) was not more effective than placebo (Max et al., 1992). Based on these studies, a recommendation for the use of SSRIs as a standard treatment for neuropathic pain cannot be given.

Antiepileptics

Antiepileptics regulate neuronal excitability at the synaptic level; this became the impetus to explore other indications for their use beyond epilepsy.

The calcium channel α2-δ ligands gabapentin and pregabalin are well-tolerated medications with few pharmacologic interactions. Several double-blind randomized controlled studies in diabetic neuropathy have demonstrated efficacy. They bind to voltage-gated calcium channels at the α2-δ subunits and inhibit release of neurotransmitters. Both agents require dose adjustments in patients with renal dysfunction; furthermore they can cause dizziness, sedation, and pedal edema. The first well-designed study (Backonja et al., 1998) demonstrated an analgesic effect of gabapentin compared to placebo, titrated from 900 to 3600 mg/day. Gabapentin at a dose of 2400 mg/day or less is more effective than amitriptyline (90 mg/day). However, another study (Morello et al., 1999) found no difference between gabapentin (900–1800 mg/day) and amitriptyline (25–75 mg/day). The advantage of pregabalin is better GI absorption. Due to the linear pharmacokinetics of pregabalin, the onset of pain reduction is much quicker. Four trials have evaluated the efficacy of pregabalin in relieving pain in DPN (Lesser et al., 2004; Rosenstock et al., 2004; Freynhagen et al., 2005; Richter et al., 2005). Pregabalin at a dose between 300 and 600 mg per day showed significant improvement compared to placebo.

Another double-blind, randomized crossover trial (Gilron et al., 2009) attempted to demonstrate whether gabapentin and nortriptyline in combination provide more pain relief than either single agent in DPN and postherpetic neuralgia. Combined gabapentin and nortriptyline were more effective in lowering daily pain intensity. Interestingly, combination therapy was superior to nortriptyline alone in regards to improvement of mood. Consequently, the authors of this study recommend combination therapy for patients who do not experience pain relief with one agent alone. In contrast, the COMBO-DN trial compared the efficacy of duloxetine and gabapentin combination therapy with higher dose monotherapy and found no difference (Tesfaye et al., 2013). Clearly, further studies are necessary to examine appropriate use of combination treatment.

Phenytoin was one of the first nonsedating, sodium channel antagonists available for treating epilepsy. Phenytoin also has the ability to block L-type mediated Ca current, inhibit NMDA response, depress basal intraneuronal levels of cyclic guanosine monophosphate, and increase neuronal GABA concentration. During the 1970s, phenytoin was tested as a treatment in painful DPN (Saudek et al., 1977; Chadda and Mathur, 1978). Lack of efficacy and severe side-effects, however, prevent phenytoin from being used as a first line option for treatment of DPN.

Carbamazepine blocks voltage-gated Na channels and is also able to inhibit L-type Ca and NMDA currents.
Furthermore, carbamazepine causes increased release of serotonin and antagonizes adenosine A1 receptors, which are important in nociceptive transmission. Compared to nortriptyline in a 30 day crossover trial, no difference in pain reduction was observed (Gomez-Perez et al., 1996). This agent is used very successfully in treatment of neuropathic pain at doses between 200 and 600 mg/day. But due to its side-effects and the availability of better alternatives, carbamazepine should not be used as first line therapy. Of note, carbamazepine is still the first line treatment for trigeminal neuralgia.

Oxcarbazepine, a ketoanalog of carbamazepine, blocks sodium channels. Although oxcarbazepine is well tolerated, one large trial failed to show benefit at a dose between 1200 and 1800 mg/day compared to placebo (Grosskopf et al., 2006). However, in another smaller study a significant decrease of pain was seen. The role of oxcarbazepine for neuropathic pain requires more studies to clarify its impact.

Sodium valproate is thought to increase GABA synthesis and release in the CNS, as well as inhibit T-type calcium channels and increase potassium inward currents. It is well-tolerated in the short term and decreases pain (500 mg/day), but is limited by long term adverse effects such as hair loss, hepatic dysfunction, weight gain, tremor, and bone marrow suppression (Kochar et al., 2004).

Lamotrigine stabilizes the slow inactivated conformation of a subtype of a Na channel, inhibits glutamate release, and increases GABA levels in the brain. Lamotrigine is well tolerated but has to be titrated very slowly. Two randomized double-blind, placebo-controlled trials (Vinik et al., 2007) showed an effect in relieving pain at a dose of 400 mg per day after 19 weeks. But at the end of the trial there was no difference between lamotrigine and placebo. Thus, the role of lamotrigine in neuropathic pain is not clear.

Topiramate, successfully used in migraine prevention, blocks activity-dependent voltage-gated sodium channels, L-type voltage-gated calcium channels, and postsynaptic AMPA excitatory amino acid receptors. A 12 week randomized, double-blind multicenter study (Raskin et al., 2004) showed pain reduction at a dose of 400 mg per day in 50% of topiramate-treated patients and 34% of placebo-treated patients. Topiramate was able to reduce the worst pain intensity and sleep disturbance significantly. Side-effects include diarrhea, loss of appetite, somnolence, and nausea.

No significant effect has been shown for zonisamide, which blocks voltage-dependent sodium channels and T-type calcium channels (Atli and Dogra, 2005). A dose of 540 mg per day for 6 weeks did not show an improvement in pain. Furthermore, its use is limited by side-effects such as headache, weight loss, restlessness, and GI discomfort.

Lacosamide, the newest of this drug class, enhances slow inactivation of the voltage-gated sodium channel. Reduction of pain was demonstrated in a recent double-blind, randomized, placebo-controlled trial, but the results were not statistically significant (Ziegler et al., 2010).

Levetiracetam is thought to act by inhibiting presynaptic neurotransmitter release by binding to the synaptic vesicle protein 2A in the brain and spinal cord, and has also recently been tried in neuropathic pain. Two recent trials (Finnerup, 2008; Vilholm et al., 2008) failed to find an effect in postmastectomy neuropathic pain and in spinal cord injury pain. Three case studies have reported that levetiracetam alleviates pain, however.

Opioids

Opioids play a role as adjunct therapy in neuropathic pain when other treatments are not enough. Treatment with opiates should start with either hydrocodone or oxycodone, with slow titration. The fear of physical dependence or addiction is reasonable, but addiction does not occur frequently. A study with diabetic patients using opiates for pain relief reported low rates of dependence and addiction (Porter and Jick, 1980). Oxycodone has also been studied in placebo-controlled trials. Oxycodone is a µ- and κ-opioid agonist. A randomized, placebo-controlled, double-blind trial (Watson et al., 2003) demonstrated a significant lower mean daily pain intensity in patients treated with oxycodone compared to the placebo group receiving benztrapine over 4 weeks. The mean daily dose of oxycodone added up to 40 mg. Side-effects such as nausea, constipation, sweating, and dizziness were more frequent in the oxycodone group than in the placebo group.

Another opioid that is commonly used for neuropathic pain control is methadone. Methadone is a µ-agonist with the ability to inhibit N-methyl-D-aspartate (NMDA) receptor activation in the central nervous system. NMDA receptor activation is an essential component for the development of chronic pain, morphine tolerance, and NMDA-induced hyperalgesia. Methadone also inhibits the reuptake of norepinephrine and serotonin (Foley, 2003). In chronic noncancer pain management, Sandoval et al. reviewed 21 clinical trials and reported that methadone treatment results in a 66% response rate for patients who are unresponsive to other opioid treatment (Sandoval et al., 2005). Unfortunately, only a few small studies of methadone treatment for painful diabetic neuropathy are available (Foley, 2003; Gagnon et al., 2003; Morley et al., 2003). In these reports, methadone does decrease neuropathic pain, but larger studies are needed. Methadone shares similar...
side-effects with other opioids. In addition, methadone has been reported to induce tachyarrhythmias and should be avoided for patients with similar cardiac conditions. Furthermore, frequent monitoring for the development of drug tolerance, addiction, side-effects, and potential drug–drug interactions are essential steps to achieve the best results with this therapy.

Tramadol

Tramadol, as a µ-receptor agonist, can also stimulate the serotonergic and noradrenergic systems. In a randomized, double-blind, placebo-controlled study (Sindrup et al., 1999), tramadol at a dose between 200 and 400 mg per day over 6 weeks reduced pain, paresthesia and touch-evoked pain in DPN compared to placebo. Another trial (Freeman, 2007), examining the benefit of a combination therapy with 37.5 mg tramadol and 325 mg acetaminophen (APAP) over 66 days, reported an improvement compared to placebo. Tramadol may be an alternative to other opioids. Nevertheless, its use is limited by its side-effects such as nausea, constipation, headache, or dyspepsia.

Nonsteroidal antiinflammatory drugs

Inflammatory responses accompany peripheral nerve injury, including the production of prostaglandins. Prostaglandin synthesis depends on cyclooxygenase (COX)-1 or COX-2. The prostaglandin-mediated pain is the result of increased sodium currents and calcium influx in peripheral nociceptive nerve cells. In addition, this reaction provides a release of central neurotransmitters and depolarization of second-order nociceptive neurons. Nonsteroidal antiinflammatory drugs (NSAID) inhibit cyclooxygenase, and therefore prostaglandin-mediated inflammation. Usually NSAIDs are not recommended for neuropathic pain therapy due to their side-effects (GI tract, renal, cardiac) and their questionable effect in neuropathic pain reduction. A review of NSAID use in neuropathic pain (Vo et al., 2009) revealed a discrepancy in the widespread use of NSAIDs by patients with neuropathic pain and the belief among pain specialists of the lack of efficacy. There have been studies indicating efficacy of NSAID therapy in neuropathic pain in animal models, but not in human conditions. However, other studies have failed to demonstrate a difference between NSAIDs and placebo. Currently there is insufficient evidence to recommend NSAIDs for the treatment of neuropathic pain and NSAID use may not be appropriate for those patients with diabetic neuropathy.

N-Methyl-d-aspartate antagonists (NMDA antagonists)

Glutamate is the most common excitatory neurotransmitter in the CNS and binds to NMDA and AMPA-receptors. Li et al. (1999) demonstrated that a higher density of NMDA and AMPA receptors was present in obese diabetic ob/ob mice than in lean mice (Li et al., 1999). Altered binding affinities for AMPA and especially NMDA were observed in the obese mice. NMDA plays an important role in central sensitization in neuropathic pain. Increased glutamatergic input and NMDA receptor activity contribute to central sensitization of painful diabetic neuropathy. Persistent overstimulation of NMDA receptors causes a plastic change in the spinal dorsal horn. Animal studies demonstrated that NMDA receptor antagonists were able to reduce pain after nerve injury (Sang et al., 2002). Four randomized trials reported efficacy of dextromethorphan in diabetic neuropathy, but not in postherpetic neuralgia (PHN) (Nelson et al., 1997). Memantine has not shown an effect in either PHN or in neuropathic pain after amputation or surgery (Eisenberg et al., 1998). A randomized, placebo-controlled, double-blind study (Sang et al., 2002) reported pain relief of 35% for dextromethorphan at a full dose compared to placebo (lorazepam). But there was no statistically significant difference between dextromethorphan and placebo at the primary end point (efficacy). A 2 week period of titration was followed by a 2 week maintenance period for dextromethorphan, memantine, or lorazepam, the active placebo. This study showed that dextromethorphan at a high dose is effective in reducing pain with a similar number needed to treat (3.2) as gabapentin, tramadol, and tricyclic antidepressants. In contrast, memantine at maximally tolerated doses did not perform better than placebo. Dextromethorphan is metabolized by the P450 2D6 isoenzyme, which can cause difficulties in dosing due to its multiple genetic variants. Furthermore, it is also limited by side-effects such as dry mouth, gastrointestinal distress, or sedation. Due to the fact that NMDA receptors are expressed in the brain, blocking these receptors may cause deterioration of cognition, mood, and abnormal movements.

Topical agents

A potential advantage of topical agents is the avoidance of systemic side-effects. However, the analgesic effects are also highly localized. Lidocaine, capsaicin, and salicylate have been tested in many trials. Lidocaine 5% patches have been proven in several trials to relieve pain in DPN. In an open label study (Barbano et al., 2004), lidocaine patches reduced pain...
significantly and improved quality of life ratings. They were also well tolerated, and no side-effects or systemic accumulation was found with the regimen of 4 patches per 18 hours. Another randomized, open-label controlled trial (Baron et al., 2009) compared 5% lidocaine patches and pregabalin in patients with painful DPN and postherpetic neuralgia. After 4 weeks of treatment, similar positive effects were achieved in both treatment groups. The adverse effects were less frequent with lidocaine than with pregabalin. Mild skin irritation can occur but is rare.

Capsaicin is a natural alkaloid extracted from chili peppers. Capsaicin binds the vanilloid TRPV-1 receptor in lamina II of the spinal cord dorsal horn. A study of inhibitory neurotransmission in the spinal dorsal horn (Ferrini et al., 2007) demonstrated a release of substance P in primary afferent fibers after TRPV-1 receptor activation. Subsequently, substance P, binding the NK-1 receptor, stimulates inhibitory neurotransmitters in laminae I, III, and IV. This leads to increased release of GABA and glycine. One large multicenter, placebo-controlled study performed by the Capsaicin Study Group in 1991 demonstrated pain relief in patients with DPN (Capsaicin Study Group, 1992). Recent Cochrane reviews revealed that low dose topical capsaicin does not reduce neuropathic pain but that high dose capsaicin does (Derry and Moore, 2012; Derry et al., 2013). The main disadvantage of capsaicin is the transient burning pain felt initially upon application of the agent to the skin. Optimal pain relief can be gained after three to four times daily application for up to 6–8 weeks.

A pilot study (Yuen et al., 2002) examined the effect of isosorbide dinitrate (ISDN), a NO donor with vasodilating potential, in DPN. ISDN was able to reduce pain; 50% of the tested patients reported pain relief and decided to continue the use of the ISDN spray. ISDN is believed to cause improvements in microvascular blood flow by vasodilation. There were no serious side-effects aside from two cases of mild transient headache.

**Botulinum toxin**

A double-blind crossover trial (Yuan et al., 2009) with botulinum toxin type A (BoNT/A) was administered intraepidermally in 18 patients with painful DPN. This study was performed based on experimental evidence that BoNT/A not only inhibits the release of acetylcholine, but also may modify afferent sensory fiber firing (Chuang et al., 2004; Aoki, 2005). In formalin-induced pain studies, BoNT/A was associated with an inhibition of glutamate release. Significant pain reduction (mean visual analog scale (VAS) of pain) and improvement of sleep quality were reported by this study. Further investigations are necessary to evaluate optimal dosage and underlying mechanisms.

**Electrical nerve stimulation and acupuncture**

Validation of the use of electrical nerve stimulation in the treatment of DPN has been hindered by a lack of adequate clinical trials. A review (Pieber et al., 2010) of the effect of electrotherapy in DPN concluded that 50% of the reviewed studies were poorly designed. TENS (transcutaneous electrical nerve stimulation) may be recommended as nonpharmacologic symptomatic treatment and FREMS (frequency-modulated electromagnetic neural stimulation) reduced pain significantly in one well-designed large study (Bosi et al., 2005). Pieber et al. (2010) could not include articles about PEMF (pulsed electromagnetic fields) in their comparisons due to their different study designs. The effect of electrical stimulation may be based on antiinflammatory effects, release of neurotransmitters such as serotonin and endorphins, an increased production of ATP. Larger, randomized, double-blind and placebo-controlled studies are necessary to evaluate a benefit of electrical stimulation in DPN.

A long-term study of acupuncture in painful symptomatic DPN (Abuaisha et al., 1998) in 46 patients over 10 weeks reported an improvement of symptoms in 77% of patients and a complete remission of pain in 21%. After a follow-up over 18–52 weeks, 67% of patients were able to stop their analgesic medication. There were no significant changes in NDS (Neuropathy Disability Score), VPT, and HbA1c levels. Acupuncture might be an alternative nonpharmacologic treatment for DPN, but further large randomized studies are necessary to prove a positive effect of acupuncture in painful neuropathy.

**Foot care**

Foot ulceration is a leading cause for hospitalization in patients with diabetes and is associated with a high incidence of limb amputation. Distal symmetric polyneuropathy has been reported as the main cause of plantar ulceration. The ability of patients to feel pain, temperature, touch, and pressure is diminished. Furthermore, the impaired proprioception makes it difficult to detect the position of the feet. Deformities occur such as curled toes, prominent metatarsal heads, and limited joint mobility. The most common injury may result from unperceived pressure in bony prominences. Poorly fitting footwear, puncture wounds, and unnoticed pressure leads to necrosis and ulceration. Foot ulcers in diabetic patients have mixed ischemic and neuropathic origin because arterial insufficiency occurs in many diabetic limbs. In addition, diabetic patients are predisposed
for infections and insufficient wound healing. The American Diabetes Association position statement on foot care identifies patients with diabetes for at least 10 years, male sex, poorly controlled glucose, cardiovascular, renal, or retinal complications as high-risk groups for foot ulceration and amputation (American Diabetes Association, 2007). Osteomyelitis frequently occurs in diabetic patients with foot ulceration and might not cause any pain (Newman et al., 1991).

A review of diabetic foot care (O’Loughlin et al., 2010) emphasizes the importance of an aggressive multidisciplinary approach to foot disease. The early control of infection, patient education, appropriate footwear, and artery vascularization can reduce the rate of amputations. Of course, novel agents are also being developed and have been reviewed by O’Loughlin et al. (2010). Hyperbaric oxygen therapy can reduce the need for amputation but studies examining this treatment had small numbers of subjects and were of poor quality. Further investigations are needed. Tissue-engineered biological dressings are supposed to act as skin substitutes for ulcers. Two products (Dermagraft® and Apligraf®) are available in the USA and can improve healing rates in ulcers and prevent development of osteomyelitis (Veeses et al., 2001). They are thought to fill the wounds with extracellular matrix and induce the expression of growth factors. Increasing investigations in stem cell research also provide hope for foot ulcerations. Topical administration of autologous keratinocytes has been showed to initiate wound healing. To date, the most studied agents for wound healing are growth factors. Recombinant platelet-derived growth factor is licensed for the treatment of foot ulcerations in diabetic lower extremities.

Overview of adverse effects, drug interactions, and recommendations for patients with impaired renal and hepatic function

SEROTONIN SYNDROME

Serotonin syndrome is caused by excessive serotonin agonism due to concomitant use of serotoninergic drugs such as TCAs, tramadol, SNRIs, or triptans. Clinical presentation can include autonomic hyperactivity, mental status change, and neuromuscular abnormalities. Early symptoms include agitation, tremor, tachycardia, sweating, diarrhea, and hypertension. Serotonin syndrome can resolve in mild cases and 24 hours after discontinuing serotoninergic drugs.

RISK OF GASTROINTESTINAL BLEEDING

Many patients suffering from neuropathic pain also use NSAIDs to treat musculoskeletal pain; thus, there can be an increased risk of gastrointestinal bleeding in patients taking multiple classes of medications. NSAIDs interfere with coagulation by inhibiting prostaglandin synthesis, and thereby platelet aggregation. The gastrointestinal mucosa itself can be damaged as well by decreased prostaglandin synthesis, as it diminishes the production of protective mucus. Serotonin reuptake inhibitors also influence coagulation by reducing serotonin concentration in platelets. Thus, long-term use of NSAIDs in combination with SSRIs, SNRIs, or TCAs is not recommended. In a population-based study (de Abajo and Garcia-Rodriguez, 2008), the risk of gastrointestinal bleeding was increased in patients who used SSRIs or venlafaxine.

CARDIOVASCULAR CONSIDERATIONS

Cardiovascular side-effects of drugs need to be considered because cardiovascular diseases such as arrhythmia, hypertension, or coronary heart disease are common in adult patients with diabetes mellitus. TCAs have well described cardiac adverse effects (slowed cardiac conduction, orthostatic hypotension, increased heart rate, and antiarrhythmic class IA activity). TCAs with less anticholinergic effect should be used preferentially in the treatment of DPN. TCAs in general are to be avoided in patients with known cardiac conduction disorders or arrhythmia. Duloxetine and venlafaxine can cause a dose-dependent increase of blood pressure. Patients with left ventricular dysfunction should not receive venlafaxine. Opioids have histamine-releasing and anticholinergic characteristics that can lead to hypotension or hypertension, palpitations, and sinus bradycardia. Methadone is able to prolong the QTc interval; the risk of arrhythmia depends on multiple factors such as hypokalemia and structural heart disease.

RENAL IMPAIRMENT

Chronic kidney disease is defined as a glomerular filtration rate (GFR) less than 60 mL/min per 1.73 m². Drug elimination can be impaired and drug dosages should be administered according to the creatinine clearance. In particular, maintenance doses have to be reduced and adapted. Gabapentin and pregabalin are two examples of medications that need to be adjusted based on GFR.

HEPATIC IMPAIRMENT

The pharmacokinetics of most drugs depend on liver function. Liver disease without cirrhosis might cause mild alterations in pharmacokinetics, but cirrhosis is able to cause severe disturbances of drug elimination. Furthermore, plasma protein binding can be impaired from decreased liver protein synthesis. Portosystemic
shunting can inhibit first-pass metabolism. Patients with cirrhosis are often affected by CNS adverse effects due to impaired metabolism of agents dependent upon liver processing (e.g., opioids, carbamazepine, oxcarbazepine, sodium valproate, phenytoin).

**Diabetic autonomic neuropathy**

Autonomic neuropathy is a major complication of diabetes mellitus and associated with high morbidity and mortality. The DCCT demonstrated that intensive treatment can delay autonomic complications in type 1 patients, but it is not known how these findings extrapolate to type 2 diabetic patients. There is evidence that progression of autonomic dysfunction in type 1 patients depends on duration of disease. Associations between cardiac autonomic neuropathy (CAN) and microvascular complications such as diabetic retinopathy, nephropathy, and increased microalbumin excretion rate were found.

A study by Sridhar et al. (2010) demonstrated the benefit of daily exercise (5 minutes warm-up, 30 minutes cycling or treadmill exercise, and 10 minutes cool down) for improvement in increasing heart rate variability (HRV). This trial compared diabetes patients and patients with diabetes and hypertension. Both groups showed a benefit, but the diabetes and hypertension group profited more from exercise. The nonexercised group did not show any improvement in HRV; instead, a continuous decreasing HRV was observed. This study showed that regular exercise training improves the HRV and HbA1c levels in diabetes patients with or without hypertension and lowers high blood pressure. This benefit in improvement of HRV may decrease the risk of a sudden cardiac death in diabetes patients.

Patients with autonomic neuropathy frequently have orthostatic hypotension. The treatment of this condition is difficult and nonpharmacologic approaches should be tried first. This includes avoidance of sudden changes in body posture from supine to standing position, hot baths, medications that induce hypotension such as tricyclic antidepressants or phenothiazines, and straining exercises that increase the intra-abdominal and intrathoracic pressures. Small meals should be taken to avoid postprandial hypotension, supportive stockings can be worn during the day, and exercises to activate the muscle pump of the lower extremities can be performed.

Pharmacologic treatments are available and include the following:

1. **Volume expansion:** the synthetic mineralocorticoid 9-α-fluorodrocortisone (fluorocortisone) causes plasma expansion and increases the sensitivity of blood vessels for circulating catecholamines (Freeman, 2007). Doses should start at 0.05 mg at bedtime and then be titrated to a maximum of 0.2 mg per day. It is necessary to pay attention to supine hypertension, hypokalemia, and hypomagnesemia, which can occur as a result of treatment with fluorocortisone. Other side-effects such as ankle edema, headache, or rarely, congestive heart failure, limit the use of this agent.

2. **Sympathomimetic agents** are able to constrict the blood vessels and reduce the venous capacity. α1 agonists include those with both direct and indirect effects (ephedrine and pseudoephedrine), only direct effects (midodrine and phenylephrine), and only indirect effects (methylphenidate and dextroamfetamine sulfate). Only midodrine, a peripheral α1 adrenoceptor agonist, is approved by the Food and Drug Administration for the treatment of orthostatic hypotension. A single-blind, randomized, placebo-controlled crossover prospective study (Lamarre-Cliche et al., 2008) showed that midodrine and its metabolized product have sympatholytic properties to increase venous return. The dose depends on the individual patient’s sensitivity to this substance and can be titrated between 2.5 and 10 mg three times a day. Side-effects including pruritus, supine hypertension, gastrointestinal complaints, and urinary retention can occur.

3. **Nonselective β-blockers** are limited in their application to orthostatic hypotension treatment because of their ability to cause negative chronotropy and inotropy. Their effect is presumably based on a block of β2 receptors, which induces an α adrenoceptor-mediated vasoconstriction. But further clinical trials for nonselective β-blockers are required to establish a role in the treatment of diabetic autonomic neuropathy.

4. **Clonidine** is a selective α2 adrenoceptor agonist with a sympatholytic central effect and a predominant effect on the postsynaptic α2 adrenoceptors. This latter effect is responsible for clonidine’s ability to increase venous return. But clonidine should not be the first choice for treatment because of its side-effects such as exacerbation of hypotension and somnolence.
5. The acetylcholinesterase inhibitor pyridostigmine inhibits orthostatic hypotension at a dose of 60 mg per day orally. Pyridostigmine is able to increase sympathetic ganglionic neurotransmission by inhibition of acetylcholinesterase. Considering that 20% of patients report anticholinergic side-effects, pyridostigmine should not be considered as a first line treatment.

6. The methylxanthine caffeine is an adenosine receptor antagonist. Administered in a dose between 100 and 250 mg three times a day, caffeine is able to cause some modest pressor effect due to its inhibition of vasodilation.

7. Erythropoietin increases standing blood pressure, although its mechanism is not clear yet. Potentially, the effect of increased blood pressure is with the result of an increased amount of red blood cells and central blood volume. Erythropoietin is administered subcutaneously at doses of 25–75 units per kg.

8. The somatostatin analog octreotide has been used for orthostatic hypotension, postural tachycardia syndrome, and chronic fatigue. Octreotide may suppress the release of vasoactive gastrointestinal peptide and leads to a splanchnic vasoconstrictor effect. But treatment with this drug is often unsatisfactory because its effect lasts just a few hours. An open-label pilot project (Hoeldtke et al., 2006) showed that octreotide has a sustained vasoconstrictor effect in patients with autonomic neuropathy and orthostatic intolerance. But this study also demonstrated that an increased supine blood pressure occurred in three of four patients with autonomic neuropathy. Treatment with octreotide may be reserved for patients with severe hypotension who are refractory to other therapies.

9. Yohimbine is a selective central and peripheral α2 adrenoceptor antagonist that is able to increase systolic blood pressure and the plasma level of norepinephrine compared to placebo (Bharucha et al., 2008). Side-effects include supine hypertension, anxiety, tremor, palpitations, and diarrhea.

Autonomic dysfunction can involve the gastrointestinal system from the esophagus to the colon. The symptoms can range from mild discomfort to severe impairment of daily activities. Gastroesophageal dysfunc- tion can appear as gastroesophageal reflux disease (GERD), delayed gastric emptying, or gastric retention. All these pathologies can cause nausea, vomiting, early satiety, loss of appetite, and epigastric pain (heartburn). Further, delayed gastric emptying can cause problems with the absorption of medications. Colon abnormalities can occur, including constipation, diarrhea, and fecal incontinence. The primary treatment of GI dysfunction is strict glucose control. Eating small frequent meals and avoiding dietary fiber can help to avoid or improve daily problems due to gastrointestinal dysfunction. Also, pharmacologic treatments such as the prokinetic agents metoclopramide and domperidone might relieve the symptoms of gastroparesis. Erythromycin, a macrolide antibiotic, is recommended for short-term use in gastro- parasis as it interacts with motilin receptors (Enweluzo and Aziz, 2013). Diabetic diarrhea is also very common, but other possible causes have to be evaluated and treated first. For example, metformin or arcabose treatment or lactose intolerance should be excluded. Possible bacterial overgrowth, which can be treated with metronida- zole, can be diagnosed by a hydrogen breath test. Loperamide is able to reduce stool frequency but has to be used carefully considering the risk of toxic mega- colon. Clonidine (listed above) can help to improve diarrhea caused by adrenergic overactivity.

Genitourinary autonomic neuropathy affects important and fundamental quality-of-life issues. Erectile dysfunction affects 50% of men over the age of 50 with diabetes. Female sexual dysfunction often manifests as diminished libido. Neurogenic bladder or cystopathy can cause inability to feel bladder fullness or to initiate micturition, which can lead to overflow incontinence, bladder enlargement, and urinary retention. Bethanechol and the α1 adrenergic receptor antagonist doxazosin may reduce urinary retention. The Crede method, an a-bdominal maneuver, can help relieve urinary discomfort in initiating micturition. Self-catheterization may be necessary. The treatment of erectile dysfunction also has to begin with glycemic control. Other causes, such as treatment with β-blockers or antidepressants, have to be evaluated. Phosphodiesterase inhibitors are available and have been considered.

**NEW TREATMENT STRATEGIES**

**Polyol pathway: aldose reductase inhibitors**

Glucose is reduced to sorbitol by the aldose reductase enzyme. Recent data show more oxidative damage is caused by flux through this pathway than by sorbitol accumulation. Diabetic patients with polymorphisms of the 5′ end of the aldose reductase gene have less risk of developing neuropathy. Several aldose reductase inhibitors (ARIs) have been developed and tested.

**Sorbinil**

Sorbinil, the prototype ARI, was used for a period in the 1980s. Several trials demonstrated the efficacy of sorbinil in rodents, but this failed to be reproduced in humans. Some trials (Jaspan et al., 1985) have demonstrated improvement in nerve conduction...
velocities and cardiac autonomic neuropathy. But due to the high incidence of skin rash, sorbinil had to be withdrawn from the market in 1987.

Epalrestat, Ranirestat, and Fidarestat

Epalrestat has been available in Japan for the treatment of diabetic neuropathy and retinopathy since 1992. It is taken orally at a dose of 50 mg three times a day. Several trials had been established to test epalrestat for possible benefit. Small, mostly uncontrolled, studies suggested a daily dose of 150 mg improves motor and sensory nerve conduction velocities, vibration thresholds, and subjective symptoms. Hotta et al. (2001) also conducted an open-label, randomized multicenter trial to evaluate epalrestat at a daily dose of 150 mg. Epalrestat prevented nerve deterioration and alleviated common symptoms such as limb numbness and cramping. Statistical significance was not reached for cardiovascular autonomic nerve function variables. Epalrestat appeared to be well-tolerated, with side-effects including nausea, vomiting, diarrhea, and elevated liver enzymes.

Ranirestat has been tested in a phase II randomized, double-blind, multicenter placebo-controlled efficacy study (Bril and Buchanan, 2004). The question was whether ranirestat is able to penetrate the sural nerve and inhibit the accumulation of sorbitol and fructose in patients with diabetic sensorimotor polyneuropathy. The authors concluded that ranirestat 20 mg daily improves NCV. However, a more recent study (Bril et al., 2009) did not show a significant improvement between ranirestat and placebo for the primary efficacy end point (change in summed sensory NCV). Interestingly, individual and summed motor NCS did demonstrate significant improvement compared to placebo but the explanation for these conflicting results is unclear. In conclusion, more studies are necessary to show the promising benefit of ranirestat.

Fidarestat has been tested at a daily dose of 1 mg in a 1 year placebo-controlled study with type 1 and type 2 diabetes patients (Hotta et al., 2001). In animal models, fidarestat turned out to be one of the most promising ARIs. The study by Hotta et al. (2001) evaluated fidarestat based on electrophysiologic examination of nerve function and assessment of subjective symptoms. Fidarestat showed a significant improvement compared with placebo in F-wave minimum latency and FCV of the median nerve. Subjective symptoms, such as numbness and pain in the upper extremities, hyperesthesia, paresthesias, and numbness in the lower extremities improved significantly in contrast to placebo. These results suggest that fidarestat can delay deterioration of nerve function.

**Hexosamine pathway**

**Benfotiamine**

Thiamine, known as vitamin B1, plays an important role in energy metabolism. Benfotiamine is a synthetic S-acyl derivative of thiamine. Transketolase is a thiamine-dependent enzyme that converts fructose-6-phosphate into pentose-5-phosphate, which results in decreased flux through the hexosamine pathway. The increased flux to the pentose-5-phosphate pathway is associated with increased redox capacity. Transketolase also directs the precursors of advanced glycation end products (AGEs) to the pentose phosphate pathway. This pathway also provides NADPH, and thus contributes to the formation of glutathione. A randomized, double-blind, placebo-controlled clinical trial (Stracke et al., 2008) with benfotiamine in diabetic polyneuropathy demonstrated an improvement of pain. In the US, benfotiamine is currently available as dietary supplement.

**Receptor for advanced glycation end products (RAGE) pathway**

Chronic hyperglycemia accelerates the reaction between glucose and proteins leading to the formation of advanced glycation end products (AGEs). AGEs in turn form irreversible cross-links with many macromolecules such as collagen. In diabetes, AGEs accumulate in tissues at an accelerated rate. Experimental studies have elucidated that binding of AGEs to their specific receptors (RAGE) activates mainly monocytes and endothelial cells, and consequently induces various inflammatory events. Two approaches to targeting this pathway have been considered: decrease the formation of AGEs or block RAGE activation.

Aminoguanidine is a prototypic therapeutic agent for the prevention of formation of AGEs. It reacts rapidly with α,β-dicarbonyl compounds such as methylglyoxal, glyoxal, and 3-deoxyglucosone to prevent the formation of AGEs by blocking carbonyl groups on Amadori products, intermediates in AGE production (Thornalley, 2003). A study (Miyauchi et al., 1996) showed that aminoguanidine had an effect on slowing of motor nerve conduction velocity of the sciatic nerve in streptozocin-induced diabetic rats. However, clinical trials did not show a benefit or had to be discontinued because of flu-like symptoms. Further clinical trials will be necessary to evaluate treatment with aminoguanidine.

Phenylationthiazolium bromide (PTB), an agent of the thiazolium group, is able to cleave AGE cross-links in vitro. This agent was tested in an animal study (Cooper et al., 2000) and showed ability to reduce vascular AGE concentrations in diabetic rats. An analog of

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PTB, alagebrium chloride (ALT-711), demonstrates ability to break glucose-derived cross-links in proteins such as collagen. A study (Guo et al., 2009) reported a decrease of AGE in myocardial tissue of aging rats treated with ALT-711. In vivo trials with patients (Little et al., 2005) reported a benefit of ALT-711 in elderly patients in regression of left ventricular hypertrophy. Another study (Tan et al., 2010) demonstrated the benefit of alagebrium chloride in improvement of creatinine clearance and renal structural parameters in diabetic nephropathy. However, the benefit in DPN is not well evaluated yet and further trials are required to prove the benefit for humans.

A recent study (Matsui et al., 2010) reported that nifedipine, a calcium channel blocker, is able to inhibit inflammatory and fibrogenic gene expression in AGE-exposed fibroblasts. This study showed that AGE is able to evoke inflammatory and fibrogenic reactions in the fibroblast cell line MRC-5 via aldosterone production. This reaction was blocked by the mineralocorticoid receptor antagonistic activity of nifedipine. Nifedipine could become an interesting agent for further trials given its potential to counter the inflammatory and fibrogenic effects of AGE.

Poly (ADP-ribose) polymerase inhibitors

In DPN, elevated glucose levels increase ROS production, and these free radicals induce DNA strand breaks. Excessive DNA damage is a stimulus to overactivate poly (ADP-ribose) polymerase (PARP) and nuclear factor κB alteration (Adaikalakoteswari et al., 2007), which deplete the intracellular NAD⁺. This leads to a slower rate of glycolysis, electron transport, and ATP formation. ADP ribosylation of proteins also causes endothelial dysfunction. PARP inhibitors are being developed and tested for a variety of conditions in which PARP activation may play a pathogenic role. Positive effects of 4-amino 1,8-naphthalimide (4-ANI) against neuropathy have been demonstrated (Sharma et al., 2008) in streptozotocin-induced diabetic rats. Treatment with 4-ANI showed significant improvements in motor nerve conduction velocity, nerve blood flow, allodynia, and hyperalgesia. Nicotinamide is a water-soluble B group vitamin that serves as a precursor of NADP, a free radical scavenger, and a weak PARP inhibitor. Nicotinamide alone and in combination with melatonin showed a benefit in a recent study (Negi et al., 2010) in STZ-diabetic animals. Two different doses (100 mg and 300 mg/kg per day) were used for nicotinamide, but only the higher dose could achieve improvement in nociceptive and functional parameters. The combined therapy with 100 mg/kg nicotinamide and 3 mg/kg melatonin showed a significant improvement of these parameters and a reduction of oxidative stress in the sciatic nerve.

Antioxidants

One approach to treatment is to prevent oxidative stress by antioxidants. α-lipoic acid is a potent antioxidant that has been shown in several studies to improve symptoms and signs in DPN. In a meta-analysis of four placebo-controlled studies with more than 1200 DPN patients (Ziegler et al., 2004), intravenous α-lipoic acid at a dose of 600 mg/day showed an improvement in total symptom score. However, in the ALADIN (Alpha-Lipoic Acid in Diabetic Neuropathy) III study, no differences in symptoms between α-lipoic acid treatment and placebo were found (Ziegler et al., 1999). This agent is available in Germany but not in the USA, although it is available as a food supplement.

α-tocopherol is the most active isoform of vitamin E. A recent study (Pazdro and Burgess, 2010) reported that it can decrease lipid peroxidation, but not the peroxidation of DNA or protein. Small studies have indicated that vitamin E might decrease incidence of certain malignancies, but large studies could not confirm these results.

Resveratrol is a botanical compound extracted from red grapes. A recent study (Jing et al., 2010) tested resveratrol and showed benefit in DM-induced vasculopathy by attenuating RAGE and NF-κB activity in diabetic rats. Another rat study (Kumar and Sharma, 2010) confirmed the NF-κB inhibitory and antiinflammatory activities of resveratrol, which may contribute to neuro-protection in diabetic neuropathy.

Angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists

The ACE inhibitor trandolapril was shown in a placebo-controlled study (Malik et al., 1998) to improve diabetic peripheral neuropathy. In general, ACE inhibitors seem to have some protective effects against microvascular complications and organ damage from diabetes. Another study (Coppey et al., 2006) in diabetic rats reported that treatment with the ACE-inhibitor enalapril or an AT II-receptor antagonist improved nerve function and endoneurial blood flow. In addition, the superoxide level in the aorta had been reduced, but this effect was less in small endoneurial vessels. The diabetic-mediated impairment of CGRP, which causes vasodilation, appears to be attenuated by this therapy. One study (Didangelos et al., 2006) examined the effect of quinapril, an ACE-inhibitor, and losartan, an AT II receptor antagonist, in diabetic patients with diabetic autonomic neuropathy and left ventricular diastolic dysfunction. Monotherapy and combination therapy of these drugs
showed improvement of autonomic neuropathy and cardiac function, the combination of both showing a greater benefit. Larger studies are required to evaluate these results.

MAPK-inhibitors

Mitogen-activated protein kinases play a major role in the intracellular transduction of metabolic and biochemical changes induced by hyperglycemia. Three distinct MAPK have been identified. An inhibitor of p38-α (SD 282) has been tested in a neuropathic model (Sweitzer et al., 2004). Diabetic rats treated with this p38 inhibitor experienced an improvement of mechanical allodynia, and blood glucose levels decreased. Further investigations need to be completed to identify the role of MAPK-inhibitors in treatment of DPN.

SUMMARY

The treatment of diabetic neuropathy requires treatment of the underlying diabetes as well as treatment of neuropathic pain when present. Multiple studies have demonstrated the benefit of enhanced glucose control on the prevention of neuropathy although the effect size is greater in those with type I diabetes (Callaghan et al., 2012). Similarly, many neuropathic pain treatments have been investigated in randomized, controlled clinical trials. Based on the available evidence, Dworkin et al. (2010) recommend TCAs (nortriptyline, desipramine), an SSNRI (duloxetine, venlafaxine), or gabapentin or pregabalin as first line treatment of neuropathic pain (Table 22.2). For patients with localized symptoms, topical lidocaine used alone or in combination with one of the other first line therapies should be considered. For patients with acute neuropathic pain, opioid analgesics or tramadol may be used alone or in combination with one of the first line therapies. If pain relief does not occur sufficiently after an adequate trial, one of the other first line medications can be added. If there is not adequate pain relief at target dosage after an adequate trial, an alternative first line medication should be started. If trials of first line medications alone and in combination fail, second and third line medications can be considered. The European Federation of Neurological Societies (EFNS) published similar guideline recommendations for the pharmacologic treatment of neuropathic pain in 2009. These guidelines list the degree of evidence supporting the use of each medication in the treatment of diabetic neuropathy. Duloxetine, venlafaxine, gabapentin, pregabalin, TCA, oxycodone, and tramadol were classified as level A. Botulinum toxin, dextromethorphan, and levodopa were level B, whereas carbamazepine and phenytoin were considered level C.

### Table 22.2

**Stepwise pharmacological management of neuropathic pain (NP)**

| Step 1 | • Assess pain and establish the diagnosis of NP; if uncertain about diagnosis refer to pain specialist or neurologist |
| Step 2 | • Establish & treat cause of NP; if uncertain about availability of treatments addressing NP etiology, refer to appropriate specialist |
| Step 3 | • Identify relevant co-morbidities (e.g. cardiac, renal, or hepatic disease, depression, gait instability that might be relieved or exacerbated by NP treatment, or that might require dosage adjustment or additional monitoring of therapy |
| Step 4 | • Explain diagnosis and treatment plan to patient and establish realistic expectations |

**Step 2**

- Initiate therapy of disease causing NP, if applicable
- Initiate symptom treatment with one or more of the following:
  - Secondary amine TCA (nortriptyline, desipramine) or an SSNRI (duloxetine, venlafaxine)
  - Calcium channel α2-δ ligand, either pregabalin or gabapentin
  - For localized peripheral NP, topical lidocaine used alone or in combination with one of the other 1st line therapies
  - For patients with acute NP, neuropathic cancer pain or episodic exacerbations of sever pain, and when prompt pain relief during titration of a 1st line medication to an efficacious dosage is required, opioid analgesics or tramadol may be used alone or in combination with one of the 1st line therapies
- Evaluate patient for non-pharmacologic treatments and initiate if appropriate

**Step 3**

- Reassess pain and health-related quality of life frequently
- If substantial pain relief (e.g. average pain reduced to <3/10) and tolerable side effects, continue treatment
- If partial pain relief (e.g. average pain remains >4/10) after an adequate trial, add one of the other 1st line medications
- If no or inadequate trial, switch to alternative 1st line medication
- If no or inadequate pain relief (e.g. <30% reduction) at target dosage after an adequate trial, switch to alternative 1st line medication

**Step 4**

- If trials of 1st line medications alone and in combination fail, consider 2nd and 3rd line medications or referral to a pain specialist or multidisciplinary pain center

(From Dworkin et al., 2007.)

In contrast to the Mayo Clinic’s guidelines, the EFNS classification did not include lidocaine. However, lidocaine patches do have the advantage of fewer side-effects and easy application and should be considered...
for patients with localized symptoms. Overall, the cornerstones of treatment of diabetic neuropathy remain addressing the underlying diabetes and treating neuropathic pain with those medications with the highest levels of evidence.

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Hyperglycemia- and neuropathy-induced changes in mitochondria within sensory nerves


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Abstract

Objective: This study focused on altered mitochondrial dynamics as a potential mechanism for diabetic peripheral neuropathy (DPN). We employed both an in vitro sensory neuron model and an in situ analysis of human intraepidermal nerve fibers (IENFs) from cutaneous biopsies to measure alterations in the size distribution of mitochondria as a result of hyperglycemia and diabetes, respectively.

Methods: Neurite- and nerve-specific mitochondrial signals within cultured rodent sensory neurons and human IENFs were measured by employing a three-dimensional visualization and quantification technique. Skin biopsies from distal thigh (DT) and distal leg (DL) were analyzed from three groups of patients; patients with diabetes and no DPN, patients with diabetes and confirmed DPN, and healthy controls. Results: This analysis demonstrated an increase in mitochondria distributed within the neurites of cultured sensory neurons exposed to hyperglycemic conditions. Similar changes were observed within IENFs of the DT in DPN patients compared to controls. This change was represented by a significant shift in the size frequency distribution of mitochondria toward larger mitochondria volumes within DT nerves of DPN patients. There was a length-dependent difference in mitochondria within IENFs. Distal leg IENFs from control patients had a significant shift toward larger volumes of mitochondrial signal compared to DT IENFs.

Interpretation: The results of this study support the hypothesis that altered mitochondrial dynamics may contribute to DPN pathogenesis. Future studies will examine the potential mechanisms that are responsible for mitochondrial changes within IENFs and its effect on DPN pathogenesis.
Introduction

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes occurring in 60–70% of diabetic patients. Distal symmetric sensorimotor polyneuropathy is the most common complication of type 2 diabetes, resulting in length-dependent injury of the longest projecting axons.1 Possible clinical presentations include asymptomatic neuropathy, painful neuropathy, and painless neuropathy with loss of protective sensation predisposing to foot ulceration and amputation.

Intraepidermal nerve fiber (IENF) density has become an accepted measure of small fiber neuropathies2–6 and specific guidelines for the use of skin biopsy in the diagnosis of small fiber neuropathy have been established.8,9 The loss of IENFs is associated with DPN in human diabetic patients6,10 and animal models of diabetes.14–16 Importantly, there is evidence that therapeutic intervention results in an increase in IENF densities and an improvement in patient assessment of pain in prediabetes associated neuropathy.17 Despite the usefulness of IENF densities as a measure of neuropathy severity, it is necessary to understand the underlying mechanism responsible for the loss or improvement of IENFs.

Mitochondrial dysfunction has been implicated in a number of neurodegenerative diseases including Alzheimer’s disease, amyotrophic lateral sclerosis, Charcot–Marie–Tooth disease, Huntington’s disease, hereditary spastic paraparesis, and optic atrophy.18–22 Notably, a number of studies have demonstrated mitochondrial dysfunction in peripheral neuropathies including chemotherapy- and diabetes-induced neuropathies.23–28 Studies from our laboratory and others have identified hyperglycemia and diabetes-induced changes in mitochondrial dynamics.28–33 In particular, changes in mitochondrial fission were observed in the neurites of sensory neurons.28,30 This study was designed to expand the work on diabetes-induced changes in mitochondrial dynamics. Initial experiments were done in an in vitro sensory neuron model system to examine the effects of hyperglycemia on mitochondria distribution and develop a three-dimensional (3D) analysis technique to measure neurite-specific mitochondria. The novel 3D imaging and analysis technique was then applied to specifically examine mitochondria within IENFs from human cutaneous biopsies in an attempt to translate these in vitro effects to the clinically relevant sensory nerves preferentially lost in DPN.

Materials and Methods

Sensory neuron cultures and image analysis

Timed pregnant rats were purchased from Charles River Laboratories (Wilmington, MA). All rodent care and use was approved and regulated by the Unit for Laboratory Animal Medicine at the University of Michigan. Dissociated dorsal root ganglion (DRG) neurons were isolated from E15 Sprague-Dawley rat embryos following previously published methods.34 Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Neurons were used 1–2 days after plating and were treated with or without additional 20 mmol/L glucose for 6 h to promote a hyperglycemic environment.36,37 Mitochondria were visualized and imaged following previously published methods.30

Fluorescent signals in the images were optimized by deconvolution with Volocity software (PerkinElmer, Waltham, MA) using the iterative restoration feature set at 90% confidence level and a period of 10 cycles. Mitochondrial signals from each image were exported from Volocity as OME TIFF files, imported into Imaris, and then saved as Imaris files (IMS).

The built-in surface creator in Imaris was used to create virtual surfaces around neurites with the “background subtraction” method to account for regional changes in fluorescent intensity within the images. A threshold intensity range was chosen specifically for each image in a way that maximized image signal and minimized noise. A subsequent filtering step allowed for the removal of noise from the data set. A filter ranging from 5 to 40 voxels was used for every image depending on the level of noise in that particular image. All remaining objects were surrounded by a 3D virtual surface that was used to extract data. Values were exported from Imaris and into an Excel spreadsheet for all nerve and mitochondrial surfaces to generate a size distribution of mitochondrial signal.

Patient population recruitment

Three groups of patients were studied: seven patients with diabetes and no DPN (DM), seven patients with diabetes and confirmed DPN, and seven age-matched healthy controls (Control). Study subjects were recruited from a large community-based primary care network at the University of Utah Diabetes Center (Salt Lake City, UT). The definition of definite neuropathy was based on the presence of at least three of the following: symptoms of neuropathy, signs based on an abnormal quantitative neurologic examination, abnormality of nerve conducting studies, quantitative sensory testing, quantitative sudomotor axon reflex testing, and skin biopsy with measurement of intraepidermal nerve fiber density (IENFD). Patients included in the analysis accounted for known differences in IENFDs associated with age and gender.36 Clinical profiles for the patients included in the study are summarized in Table 1. Control subjects were recruited for the study that had no prior history of diabetes or peripheral neuropathy.
and were of similar age to the other groups. All subjects signed an informed consent. The University of Utah Institutional Review Board approved the protocol.

**Human tissue collection**

Each subject underwent a 3-mm skin biopsy from the distal thigh (DT) and distal leg (DL). Samples were subsequently preserved in paraformaldehyde lysine phosphate for 24 h, sectioned at 50 μm, and stored in a cryoprotectant solution (30% glycerin and 30% ethylene glycol in phosphate buffered saline (PBS)).

**Fluorescent immunohistochemistry**

Tissue sections were pretreated with a block solution of 5% goat serum + 0.3% Triton X-100 (Sigma, St. Louis, MO) detergent solution at room temperature for 1 h with gentle shaking. Sections were incubated with primary antibodies to visualize mitochondria (1:100 mouse anti-pyruvate dehydrogenase (PDH), MitoScience, Eugene, OR) and IENFs (1:1000 polyclonal rabbit anti-PGP-9.5, protein gene product 9.5; AbD Serotek, Raleigh, NC) in an incubation solution (1% goat serum + 0.3% Triton X-100) with gentle shaking at room temperature for 1 h then transferred to 4°C with gentle shaking overnight. Sections were washed three times with the incubation solution and allowed to shake for 1 h after each rinse. Fluorescent secondary antibodies were used to visualize mitochondria (1:1000 goat anti-mouse AlexaFluor-594; Invitrogen, Eugene, OR) and IENFs (1:1000 goat anti-rabbit AlexaFluor-488; Invitrogen) with the same incubation process used for the primary antibodies. Sections were washed twice with 1× PBS for 5 min, followed by two extended washes with shaking at room temperature for 30 min each, and finally transferred to freshly filtered 1× PBS. Skin sections were mounted onto glass slides using Prolong Gold with DAPI (Invitrogen) to fluorescently label nuclei.

**Image acquisition and analysis**

Slides were masked as to not influence which regions were chosen for imaging. Images were collected with a Leica TCS SP5 confocal microscope system (Leica Microsystems, Inc., Buffalo Grove, IL, USA) using either a 40 × 1.25 numerical aperture (N.A.) or 63 × 1.40 N.A. (the highest magnification and N.A. available on our Leica SP5 confocal system) oil immersion objective on an inverted Leica DMi6000 microscope. Fluorescent signals were sequentially acquired at each focal plane for nuclei (excitation λ = 405 nm, spectral emission filter λ = 420–480 nm), IENFs (excitation λ = 488 nm, spectral emission filter λ = 505–560 nm), and mitochondria (excitation λ = 543 nm, spectral emission filter λ = 606–670 nm). Z-series were taken through the extent of the tissue with zoom of 1.7×, frame averaging of 2, step size of 0.17 μm, resolution of 512 × 512 pixels, and 12-bit depth. Four Z-series from at least two different tissue sections were taken at 40× from each biopsy site (DT and DL) for every patient. A corresponding 63× image was collected for a subset of the 40× images to directly compare 40× versus 63× as follows: DT: Control n = 2, DM n = 3, and DPN n = 2; and for DL: Control n = 3, DM n = 3, and DPN n = 2. The range and average number of nerves per image were as follows: DT: Control range 4–10, average 5.6, DM range 5–8, average 6.2, and DPN range 4–6, average 4.8; DL: Control range 3–7, average 5.9, DM range 3–10, average 5.9, and DPN range 1–6, average 2.9. Z-series that had poor mitochondria labeling were not included in the final

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**Table 1. Clinical profile of patient population.**

<table>
<thead>
<tr>
<th></th>
<th>Age-matched controls</th>
<th>Diabetic patients (DM)</th>
<th>Diabetic peripheral neuropathy patients (DPN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.8 ± 8.7</td>
<td>53.1 ± 6.3</td>
<td>52.3 ± 9.3</td>
</tr>
<tr>
<td>UENS</td>
<td>–</td>
<td>0.6 ± 1.0</td>
<td>9.7 ± 4.3</td>
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<tr>
<td>MDNS</td>
<td>–</td>
<td>0.6 ± 1.0</td>
<td>5.3 ± 3.4</td>
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<td>MNSI</td>
<td>–</td>
<td>1.3 ± 1.1</td>
<td>7.1 ± 2.0</td>
</tr>
<tr>
<td>HbA1C</td>
<td>–</td>
<td>8.1 ± 1.7</td>
<td>7.2 ± 1.7</td>
</tr>
<tr>
<td>Duration (months)</td>
<td>–</td>
<td>76.9 ± 71.8</td>
<td>69.9 ± 52.4</td>
</tr>
<tr>
<td>Diabetes</td>
<td>–</td>
<td>–</td>
<td>28.7 ± 13.6</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IENFD (fibers per mm)</td>
<td>7.6 ± 2.0</td>
<td>7.0 ± 2.7</td>
<td>3.0 ± 1.7</td>
</tr>
<tr>
<td>Distal thigh</td>
<td>5.8 ± 1.2</td>
<td>3.8 ± 2.0</td>
<td>1.0 ± 0.7</td>
</tr>
</tbody>
</table>

Results are means ± SD. UENS, Utah early neuropathy scale; MDNS, Michigan diabetic neuropathy score; MNSI, Michigan neuropathy screening instrument; HbA1C, glycosylated hemoglobin, hemoglobin A1c; IENFD, intraepidermal nerve fiber density.
analysis. One-two images were removed from four control patients' thighs, two diabetic thighs, one diabetic leg, one neuropathy thigh, and two neuropathy legs.

As with the image acquisition, images were masked as to not influence the analysis. An imbedded selection and cropping tool within Volocity software was used to isolate the epidermis. The lower edge was cropped precisely at the epidermal–dermal border, where a visible change in the appearance of nerve, nuclei, and mitochondria was observed. The upper edge was cropped to exclude the stratum corneum where the rich mitochondrial, nerve, and nuclear stains were no longer present. Image processing and deconvolution was performed using Volocity in the same manner as described above under methods for sensory neuron cultures. These images were exported to Imaris for further analysis.

Imaris classification software made it possible to utilize surfaces created from one fluorescent channel to isolate fluorescent signals in another channel. Thus, the 3D rendering of the nerve signal was used to isolate nervespecific mitochondrial signal within the nerve surface. The nerve surfaces were created first with the “create surface” tool to make a solid surface of the IENFs from the AlexaFluor-488 fluorescent secondary labeling of the PGP-9.5 identified nerves. The “smoothing” tool was not used and absolute intensity was used to set the threshold for the nerve signal since it was significantly brighter than background fluorescence. Threshold was set to values low enough to accurately mark the nerves. Any small, non-nerve surfaces were filtered out based on size or were edited out from the “Edit” tab by selecting and deleting them. Since we were only interested in the mitochondrial staining within the nerves, we eliminated all of the mitochondrial staining outside the boundary of the nerve surfaces with the use of the Mask Properties options in the Edit tab of the Nerve surface. The “mask all” option was used and the mask channel was set for the AlexaFlour-594 fluorescent signal for mitochondrial labeling with the PDH antibody. The “duplicate channel before applying mask” option was selected to preserve the original data, and the mask option was chosen for “Set voxels outside surface to 0.” This removed all mitochondrial signals associated with the keratinocytes within the epithelium.37 The “create surface” tool was then used on the newly created fluorescent channel of the nerve-specific mitochondrial signals that were isolated away from the mitochondrial signal associated with epidermal cells. The “smoothing” tool was not used and the background subtraction option was selected to use local contrast around the mitochondrial signal to render surfaces around the mitochondria. Threshold was set to values low enough to accurately mark mitochondria. Tiny, non-mitochondrial surfaces were filtered out based on size.

Values were exported from Imaris and into an Excel spreadsheet for all nerve and mitochondrial surfaces. Several measurements were collected and calculated for each image: the size distribution of mitochondria in IENFs at the DT and DL regions (placed in bins of increasing size), the size distribution of mitochondria in the keratinocytes of the DT, the number of mitochondria per IENF, and the mitochondrial volume per IENF volume. These values were used to compare between the groups.

Statistical analysis

All values are expressed as means ± SEM. Prism software (version 5.01; GraphPad Software Inc.) was used to determine the overall significance by analysis of variance (ANOVA) and to identify differences between the groups by Tukey’s post-hoc tests. Chi-square analysis was performed using GraphPad Software’s QuickCalcs (www.graphpad.com). Significance was defined as a P < 0.05. Prism was used to plot all graphs.

Results

Visualization and quantification of mitochondria within neurites of cultured sensory neurons

Mitochondria within control DRG neurons and neurons treated with elevated levels of glucose were imaged and processed to create 3D surfaces around the neurite-specific mitochondrial signal to obtain volumetric measurements (Fig. 1A–D). Quantification of mitochondrial volume revealed a significantly greater proportion of mitochondrial signal associated with larger volume ranges after high glucose treatment (Fig. 1E). This 3D analysis technique was used to examine nerve-specific mitochondrial signal in IENFs of human skin biopsies to determine whether the observed hyperglycemic-induced changes in mitochondria within neurites of cultured sensory neurons translated clinically.

Visualization and quantification of mitochondria within human IENFs

Immunofluorescence allowed for simultaneous labeling of multiple signals within the human skin sections that included nerves, mitochondria, and nuclei (Fig. 2A). Signals for nerves and mitochondria were optimized by cropping out regions above and below the epidermis (Fig. 2B). The first step in the analysis was to create 3D surfaces around each nerve (Fig. 2C). The nerve surfaces were then used to crop the nerve-specific fluorescent mitochondrial signal (Fig. 2D). Finally, 3D surfaces were
created around the nerve-specific mitochondrial signal to obtain volumetric measurements (Fig. 2E).

**Mitochondrial distribution in IENFs of the DT**

Patient identities were revealed only after all images were processed for nerve and mitochondrial signals. The data were separated into three groups; age-matched controls (Control), diabetic patients (DM), and patients with DPN. Representative 3D images taken with a 40× objective of DT tissue sections demonstrated a visible change in the size distribution of mitochondrial surfaces in the IENFs among the three groups (Fig. 3A–C). Quantification of this change in distribution revealed a significant shift (Chi-square 19.506, df, 7, *P* = 0.0067) in mitochondrial signal in DPN patients resulting in an increased proportion of mitochondrial signal associated with larger volumes (Fig. 3D). Images were taken with a 63× objective of corresponding 40× images to improve the resolution of the mitochondrial signal. Side-by-side comparisons of 40× versus 63× (Fig. 4A and B) demonstrated a slight improvement in resolution of the signals. Rendered surface volumes of the IENF and mitochondrial signals...
from the 63× objective were all smaller than their corresponding surfaces from 40×. This was due to the improved numerical aperture of the 63× objective (1.4 vs. 1.25). Despite the smaller volumes, the mitochondrial surfaces appeared to be distributed in similar patterns within the IENFs (Fig. 4C and D). The differences in the frequency distribution of the mitochondrial signals taken with the 63× objective were maintained among the three patient groups (Fig. 4E). In particular, significant shifts in mitochondrial signal size distributions were measured

Figure 2. Representative three-dimensional confocal microscopy image of a tissue section from a human epidermal biopsy. (A) Left panel shows nerve (green) and nuclei (Nuc, blue) of keratinocytes within the epidermis. The middle panel is a schematic depicting the corresponding layers of the stratum corneum, epidermis with keratinocytes, and dermis. The right panel includes staining for nerve (green), keratinocyte nuclei (Nuc, blue), and mitochondria (Mt, red). (B) Unprocessed image illustrates the fluorescent labeling of nerve (green) and mitochondria (Mt, red) within the cropped epidermis. White box in (B) represents the magnified region displayed in (D,E). (C) Surface renderings (Nerve Surface, cyan) were created around the nerve signal using the Imaris surface creation tool. (D) Using nerve surfaces, nerve-specific mitochondrial signal (Mt, red) was isolated from the red fluorescent channel. (E) The resulting nerve-specific fluorescent mitochondrial signal was used for further analysis to create surfaces around mitochondria (Mt Surface, magenta). Scale bars = 20 µm.
from both DM (Chi-square 20.89, df, 7, \( P = 0.0019 \)) and DPN patients (Chi-square 48.765, df, 7, \( P < 0.0001 \)), with more mitochondrial signal found within larger volume ranges (Fig. 4E).

**Mitochondrial distribution in keratinocytes of the DT**

To ensure that the change in mitochondrial size distribution was nerve specific, we also quantified mitochondrial volumes from individual keratinocytes. The same surface creation procedure was used to obtain keratinocyte-specific mitochondrial volumes from the DT samples (Fig. 5A). Unlike in the IENFs, no significant changes in mitochondrial size distribution were observed between controls and DM patients, or between controls and DPN patients (Fig. 5B).

**Comparison of mitochondrial distribution in IENFs between DT and DL**

Comparison between DT and DL biopsies was performed in order to determine if there were length-dependent differences in mitochondria within IENFs. Mitochondrial volume within IENFs from DL biopsies was calculated for images acquired with the 40× objective (Fig. 6A, C and...
Figure 4. Corresponding three-dimensional confocal microscopy images taken with either a 40× (A and C) or a 63× (B and D) objective of a distal thigh tissue section from a control human epidermal biopsy. (A and B) Panels show fluorescent signals for nerve (green), nuclei (Nuc, blue), and mitochondria (Mt, red). White box in (A) represents the area taken with the 63× objective in B. (C and D) Rendered mitochondrial signals (Mt Surface, magenta) displayed with fluorescent nerve signals (Nerve, green) were distributed in similar patterns between the images taken with the 40× and 63× objectives. Scale bars for A, B = 20 μm and C, D = 5 μm. (E) Data presented as frequency histograms depicting the proportion of nerve-specific mitochondria binned according to mitochondrial volume (μm³) for the images taken with a 63× objective. When all mitochondria were quantified, nerves from diabetic (DM, gray bars) and diabetic peripheral neuropathy patients (DPN, black bars) exhibited a significant shift toward greater mitochondrial volumes compared to the control group. Values represent means ± SEM.
Objective were also used to Images taken with the 63 objective demonstrated a similar shift toward larger mitochondrial volumes in the DL of diabetic (DM, gray bars) and diabetic peripheral neuropathy (DPN, black bars) patients when compared to controls (white bars). Values represent means ± SEM.

**Figure 5.** Visualization and summary data of mitochondria within epidermal keratinocytes of distal thigh from controls, diabetic patients, and patients with diabetic peripheral neuropathy. (A) Image from a control patient illustrates the fluorescent labeling of mitochondria (Mt, red) and the mitochondria surfaces created within keratinocytes (Mt Surface, magenta). Image also shows nerve-specific mitochondrial surface used in a separate analysis. (B) Data presented as frequency histograms depicting the proportion of keratinocyte-specific mitochondria binned according to appropriate ranges based on mitochondrial volume ($\mu m^3$). There was no significant difference in the volume distribution of keratinocyte mitochondria of diabetic (DM, gray bars) nor diabetic peripheral neuropathy (DPN, black bars) patients when compared to controls (white bars). Values represent means ± SEM scale bar 10 µm.

E) similar to the DT biopsies described above. There was a significant (Chi-square 17.519, df, 7, $P = 0.0143$) change in mitochondrial size distribution between the two biopsy sites in the control group with more mitochondrial signal associated with larger volumes in the DL than in the DT (Fig. 6A). The length-dependent shift in mitochondrial size distribution of the control group was not observed in the DL of DM (Fig. 6C) or DPN patients (Fig. 6E). Images taken with the 63$\times$ objective were also used to compare nerve-specific mitochondrial signals between the DT and DL (Fig. 6B, D and F). A similar shift toward larger mitochondrial volumes in the DL of control group was measured in the 63$\times$ images (Fig. 6B). This shift was also present in the DPN group at 63$\times$ (Fig. 6F).

Standard brightfield IENF density counts confirmed a significant decrease in innervation in the DT ($P < 0.001$) as well as the DL ($P < 0.0001$) of DPN patients (Fig. 7A). There was no significant change in the average number of mitochondria per nerve volume across the three patient groups at either the DT or the DL (Fig. 7B). The percent of mitochondrial surface volume per total nerve surface volume was not significantly different among the three groups, although there was a slight increase in the DM and DPN groups compared to controls in both the DT and DL. Higher percentage of mitochondrial volume per nerve volume was measured in the DL compared to the DT (Fig. 7C).

**Discussion**

Our laboratory and others have focused on alterations in mitochondria as a possible mechanism for neuropathy. A recent study examined the role of mitochondria in small fiber neuropathy, focusing on the mitochondria in subepidermal nerve fibers using a 2D analysis method that relied on the colocalization of nerve and mitochondrial signal for quantification. Our study is the first to 3D visualize and quantify changes in mitochondrial distribution within IENFs in man.

**Visualization and quantification of mitochondria within neurites of cultured sensory neurons and human IENFs**

The combination of fluorescence immunohistochemistry, confocal microscopy, and 3D image analysis allowed for the visualization and measurement of the distribution of mitochondria within neurites and IENFs. The technique was initially developed in an in vitro model system to quantify hyperglycemia-induced changes in the size distribution of mitochondria within neurites of sensory neurons. We then applied this technique to biopsies from diabetic patients and demonstrated that patients with diabetes and DPN have a measurable change in mitochondrial distribution within their IENFs compared to age-matched controls.

**Proportion of mitochondrial signal associated with larger volumes was increased in DT IENFs of patients with DPN**

IENFs in the DT showed a disease-induced change in mitochondrial size distribution. DPN patients had an increased volumetric proportion of mitochondria in their IENFs compared to age-matched control subjects. Interestingly, corresponding higher resolution images (63$\times$, numerical aperture 1.4) of a subset of the data taken with the 40$\times$ objective demonstrated a similar shift toward larger mitochondrial volumes in the DPN group as well as...
the DM group. This suggests that neuropathy could result in increased mitochondria within these nerves. The absence of a similar trend in the keratinocytes from the same samples indicates that this is a nerve-specific phenomenon, and may be associated with neuropathy. A similar increase in mitochondrial size has been reported after 30-day treatment with mitotoxic linezolid, an antibi-otic known to produce sensory peripheral neuropathy.38

Changes in mitochondria size distribution in IENFs of the DT might be due to changes in mitochondrial dynamics such as fission, fusion, or trafficking. Our group and others have shown diabetes and neuropathy-induced changes in the fission/fusion machinery of sensory neurons.28,31,39 It is possible that our observed larger mitochondrial volumes was observed in the distal leg (black hashed bars) of DPN patients compared to distal thigh (black bars) when analyzed from images taken with a 63x objective (F). Values represent means ± SEM. n.s., not significant.

Figure 6. Summary data for mitochondria within intraepidermal nerve fibers of distal thigh compared to distal leg from controls, diabetic patients, and patients with diabetic peripheral neuropathy. Data presented as frequency histograms depicting the proportion of nerve-specific mitochondria binned to mitochondrial volume (μm^3). Analysis of images taken with a 40x objective (A, C and E) were compared to images taken with a 63x objective (B, D, and F). In the control group, distal leg (white hashed bars) samples contained a significantly greater proportion of mitochondria found in larger volume ranges when compared to distal thigh (white solid bars) in both the 40x (A) and 63x images (B). The increased mitochondrial size was not observed in the distal leg (hashed bars) of diabetic patients (DM, gray bars) at either 40x (C) or 63x (D) nor was it shown in the neuropathy (DPN, black hatched bars) patients at 40x (E). A significant shift toward larger mitochondrial volumes was observed in the distal leg (black hashed bars) of DPN patients compared to distal thigh (black bars) when analyzed from images taken with a 63x objective (F). Values represent means ± SEM. n.s., not significant.
an attempt to image at a higher resolution with a 63× objective, the mitochondrial signal was not resolved significantly better than with a 40× objective. Further studies at the electron microscopy level will be necessary to resolve these issues.

Another possibility is that larger mitochondrial signals are the result of a disruption in axonal transport, resulting in aggregation or clustering of mitochondria, or failure of larger, swollen mitochondria to transport compared to smaller mitochondria. Dysfunction in mitochondrial transport contributes to a variety of neurodegenerative diseases. The trafficking and docking of mitochondria are crucial for normal nerve function and our findings and others suggest a potential role of mitochondrial dynamics in the development or progression of peripheral neuropathy.

Figure 7. Summary data for intraepidermal nerve fiber density (IENFD), mitochondrial counts, and volumetric proportion of mitochondria in IENFs from the distal thigh and distal leg. (A) IENFD of diabetic patients (DM, gray bar) decreased slightly in the distal leg and significantly with onset of neuropathy (DPN, black bar) in the distal thigh and distal leg. Bars represent means of IENFD (fibers per mm) for each group. (B) There was no significant change in the number of mitochondria per nerve volume across all groups. Bars represent the mean number of mitochondria per IENF volume for each group. (C) Proportion of mitochondrial volume to nerve volume slightly increased with disease progression in both the distal thigh and distal leg. Bars represent means of the percentage of mitochondrial volume per nerve volume for each group. An overall higher percentage of nerve volume containing mitochondria in the distal leg was observed compared to the distal thigh. Values represent means ± SEM.
Neither diabetes nor DPN significantly altered mitochondria distribution within IENFs of the DL

A well-known characteristic of DPN is the length-dependent loss of sensation. Therefore, mitochondrial signals in IENFs were compared between the DT and DL of all three patient populations. A length-dependent shift toward larger mitochondrial volumes was measured in the DL compared to the DT of the control group. Analysis of the mitochondria from images taken at higher resolution (63× objective) also supported the length-dependent shift in the control group. This length-dependent difference was not observed in the DM and DPN groups in images taken with a 40× objective. However, a shift toward larger mitochondrial volumes was shown in the DL of the DPN group from images taken at the higher resolution. Higher proportions of large mitochondrial volumes in the DL IENFs suggest that mitochondria in these distal nerves are distributed in a similar way to the compromised nerves in the thigh of DPN patients. The shift toward larger mitochondrial volumes distally might support an increased vulnerability of DL IENFs that would make them more susceptible to abnormal mitochondrial functions such as oxidative stress induced by hyperglycemia and diabetes.

DT may provide a better region to monitor the effectiveness of therapeutics designed to improve or prevent DPN

The IENFs from the DT showed a differential distribution of mitochondria that is associated with DPN. An increase in mitochondrial aggregates may be a potential mechanism for increased susceptibility for poor nerve fiber function, potential degeneration, or poor regeneration upon injury. In support of this, DPN subjects showed a slower regeneration of DT IENFs than their control counterparts in response to capsaicin-induced denervation.13 We too have shown that the DT is a better indicator of the beneficial effects of diet and exercise counseling by measuring increased IENF densities after such lifestyle intervention.13,17 The results of our study suggest that the DT would be a better region to assess the effects of therapeutic interventions designed to lessen or reverse the progression of sensory loss associated with DPN. Future studies might benefit using biopsies from the thigh to study changes in mitochondria distribution in IENFs instead of biopsies from the DL.

In summary, we developed an optical technique to specifically image and quantify nerve mitochondria in the epidermis while excluding mitochondria from other cell sources, such as skin keratinocytes. The results of this study support the hypothesis that altered mitochondrial dynamics may contribute to DPN pathogenesis. These findings provide insight into future research designed to elucidate potential therapeutic interventions directed at regulating mitochondrial networks that may ultimately alleviate or prevent DPN.

Acknowledgments

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Conflict of Interest

None declared.

References


BTBR ob/ob mice as a novel diabetic neuropathy model: Neurological characterization and gene expression analyses

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Given the lack of treatments for diabetic neuropathy (DN), a common diabetic complication, accurate disease models are necessary. Characterization of the leptin-deficient BTBR ob/ob mouse, a type 2 diabetes model, demonstrated that the mice develop robust diabetes coincident with severe neuropathic features, including nerve conduction deficits and intraepidermal nerve fiber loss by 9 and 13 weeks of age, respectively, supporting its use as a DN model. To gain insight into DN mechanisms, we performed microarray analysis on sciatic nerve from BTBR ob/ob mice, identifying 1503 and 642 differentially expressed genes associated with diabetes at 5 and 13 weeks, respectively. Further analyses identified overrepresentation of inflammation and immune-related functions in BTBR ob/ob mice, which interestingly were more highly represented at 5 weeks, an observation that may suggest a contributory role in DN onset. To complement the gene expression analysis, we demonstrated that protein levels of select cytokines were significantly upregulated at 13 weeks in BTBR ob/ob mouse sciatic nerve. Furthermore, we compared our array data to that from an established DN model, the C57Blks db/db mouse, which reflected a common dysregulation of inflammatory and immune-related pathways. Together, our data demonstrate that BTBR ob/ob mice develop rapid and robust DN associated with dysregulated inflammation and immune-related processes.

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Introduction

Type 2 diabetes (T2D) has reached epidemic proportions worldwide, affecting over 382 million individuals (International Diabetes Federation, 2013). The associated hyperglycemia, insulin resistance, and/or obesity lead to microvascular complications that impact multiple organs and tissues, including the eyes, kidney, and peripheral nerves. Peripheral diabetic neuropathy (DN), the most common microvascular complication (Centers for Disease Control, Prevention, 2011), is characterized by distal-to-proximal nerve damage that promotes pain and loss of sensation. Sensory deficits combined with poor wound healing can trigger foot ulcer development and non-traumatic lower limb amputations, contributing to the severe morbidity of DN (Malik et al., 2013).

Although the exact etiology remains unclear, the consensus is that DN results from metabolic and physiological imbalances within the peripheral nerve. Persistent hyperglycemia-induced oxidative stress promotes nerve damage (Vincent et al., 2011; Fernyhough et al., 2010), and evidence also suggests that dyslipidemia contributes to aberrant nerve function (Wiggin et al., 2009; Vincent et al., 2009). Numerous biochemical processes, including the formation of advanced glycation end products, increased NADPH oxidase activity, activation of poly-ADP ribose polymerase, and inflammation, are implicated in DN (Vincent et al., 2011; O’Brien et al., 2014a; Wilson and Wright, 2011). Thus, elucidating the precise mechanisms underlying nerve injury is of paramount importance to develop successful therapies.

Various mouse models exist that mimic a diabetes-like phenotype and exhibit key DN features (O’Brien et al., 2014b); however, additional models that accurately recapitulate the complex pathogenesis of T2D are required to gain insight into DN etiology and support translational studies. The leptin-deficient BTBR ob/ob mouse is a robust model of T2D (Clee et al., 2005; Coppari and Bjorbaek, 2012); they are hyperphagic and present with early obesity, insulin resistance, and hyperglycemia at a severity greater than that observed in C57Blks db/db mouse (Clee et al., 2005; Hudkins et al., 2010; Keller et al., 2008). Additionally, these mice develop renal complications closely mimicking human diabetic nephropathy, evidenced by glomerular hypertrophy, capillary basement membrane thickening, and loss of podocytes (Hudkins et al., 2010). The effects on the peripheral nerve in this model, however, have not been characterized.

In the current study, the primary objective was to characterize the neurological phenotype in BTBR ob/ob mice. We confirm that BTBR ob/ob mice present with robust features of T2D and exhibit an early...
and severe neuropathy. We further identified altered genes and pathways in sciatic nerve (SCN) using arrays and bioinformatics analyses, demonstrating that inflammation and immune response factors are overrepresented early in the disease course. These pathways are also altered in SCN of an established DN model, the C57BKS db/db mouse, further supporting the BTBR ob/ob data. Together, our findings suggest that dysregulation of the immune response may play a critical role in DN pathogenesis and support our contention that BTBR ob/ob mice are a valid DN model for mechanistic and therapeutic development research.

Research design and methods

Animals

Male BTBR ob/+ and ob/ob mice (BTBR.Cg-Lepob/WiscJ, Jackson Laboratory, Bar Harbor, ME) were fed a standard diet (5LOD; 13.4% kcal fat; Research Diets, NJ). All procedures were in compliance with protocols established by the Diabetic Complications Consortium (DCC) (Sullivan et al., 2008) and approved by the University of Michigan (U-M) University Committee on Use and Care of Animals (UCUCA). Daily monitoring and maintenance of mice was provided by the U-M Unit for Laboratory Animal Medicine (ULAM).

Metabolic and neuropathic phenotyping

Phenotyping of BTBR ob/+ and ob/ob mice included metabolic and neurological measures to document diabetes onset, duration, and related neurological changes. Body weights and fasting blood glucose (FBG; 4 h fast) were measured weekly. Percent glycosylated hemoglobin (%GHb) was measured by the Chemistry Core at the Michigan Diabetes Research and Training Center (MDRTC). Plasma insulin measurements and fast protein liquid chromatography (FPLC) analysis for cholesterol and triglycerides were performed by the National Mouse Metabolic Phenotyping Center (MMPC; Vanderbilt, TN). Hindpaw thermal latency and nerve conduction velocities (NCVs) were measured according to published protocols (Vincent et al., 2009; Sullivan et al., 2007). At study termination, intraepidermal nerve fiber (JENF) density profiles were determined as previously described (Sullivan et al., 2007).

Affymetrix microarray analyses

Total RNA was isolated from SCN of 5 (n = 8) and 13 (n = 6) week BTBR ob/+ and ob/ob mice using the silica gel-based RNaseasy Mini Kit (QIAGEN, Valencia, CA). RNA integrity and concentration was measured prior to hybridizing to the Affymetrix Mouse Genome 430 2.0 microarray (Santa Clara, CA) by the U-M DNA Sequencing Core as previously described (Pande et al., 2011). Microarray data files were normalized using the BrainArray Custom Chip Definition File version 16 (Dai et al., 2005) and quality was assessed using the affyAnalysisQC.R package (http://arrayanalysis.org/) with BioConductor (Gautier et al., 2004). Intensity-based moderated T-test (IBMT) identified differentially expressed genes (DEGs) using a false discovery rate (FDR) <0.05 cutoff. DEGs were obtained between different genotypes (ob/+ versus ob/ob) for each time point or between age groups (5 weeks versus 13 weeks) for each genotype. For array data validation, DEGs were ranked by fold-change and 8–10 of the top 4% most highly altered DEGs were selected for analysis by real time RT-PCR (RT-qPCR) as previously described (Pande et al., 2011) using the endogenous reference gene YWHAZ. Primers were selected using PrimerBank (Wang et al., 2012) and purchased from Integrated DNA Technologies (Supplementary Table 1).

Microarray data were analyzed using our established in-house microarray data analysis pipeline (Pande et al., 2011; Hur et al., 2011). Over-represented biological functions among the DEGs were identified by Gene Set Enrichment Analysis (GSEA) using Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) (Huang da et al., 2009a,b). Biological functions, represented by Gene Ontology (GO; http://www.geneontology.org) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathways, with a Benjamini–Hochberg (BH) corrected P-value < 0.05 were deemed significant. Heat-maps were generated using the top 10 most over-represented biological functions in each DEG set and clustered based on significance values (log-transformed BH-corrected P-values) to visually represent the overall similarity and differences between the DEGs. DEGs from 5- and 13-week BTBR ob/ob mice were also compared to a previously published data set from 24-week C57BKS db/db mice (Pande et al., 2011) to identify common DEGs associated with DN pathogenesis across models.

Cytokine analyses

To investigate the role of inflammation, cytokine levels were analyzed in SCN from an additional cohort of 5- and 13-week BTBR ob/+ and ob/ob mice. SCN were homogenized and protein lysates were analyzed using MILLIPLEX XMAP magnetic bead technology (Millipore, Billerica, MA) with an MMPI2 (MMP3MAG-79K) and a custom cytokine/chemokine panel (MCYTOMAG-70K) to assess protein levels for 16 pre-selected targets (Supplemental Table 2). Immunoassays used a Bio-Plex200 multiplex array system with Bio-Plex Manager™ 6.0 software (BioRad, Hercules, CA) according to the manufacturer’s instructions.

Statistical analysis

Statistical analyses utilized GraphPad Prism Software, version 6 (GraphPad Software). A two-tailed T-test was performed to compare the BTBR ob/+ mice to ob/ob mice. Values are reported as the mean ± SEM.

Results

Metabolic phenotyping was first performed to delineate the course of diabetes. BTBR ob/ob mice exhibit increased body mass compared to ob/+ controls beginning at 5 weeks (Fig. 1A) and a 1.7-fold and 3.6-fold increase in fasting blood glucose (FBG) at 5 and 13 weeks, respectively (Fig. 1B). Similarly, percent glycosylated hemoglobin (%GHb) was 1.2-fold higher in ob/ob mice at 5 weeks and 2.1-fold higher at 13 weeks, reflecting progressive hyperglycemia (Fig. 1C). Plasma insulin levels were elevated in BTBR ob/ob mice at 5 weeks (10.7 fold) and remained elevated at 13 weeks (13.1 fold) (Fig. 1D), and FPLC lipid fraction analysis indicated that plasma cholesterol and triglyceride levels were also elevated at both time points (Figs. 1E, F). FPLC further revealed a sinistral shift in the HDL fraction of 5-week BTBR ob/ob mice, and a similar peak shift was observed along with an increase in LDL cholesterol at 13-week ob/ob mice, indicating increased uptake of cholesterol at both time points (Supplementary Figs. 1A, B). Significant increases in vLDL triglyceride levels were also present in BTBR ob/ob mice at 5 and 13 weeks (Supplementary Figs. 1C, D). Together, these data confirm that hyperglycemia and dyslipidemia are features of BTBR ob/ob mice.

To characterize neuropathy in BTBR ob/ob mice, thermal hindpaw latency was measured at 9 weeks and electrophysiological NCVs and IENF densities were measured at 9 and 13 weeks. The latency of hindpaw response to a heat stimulus was significantly increased at 9 weeks (Fig. 2A). Both sciatic motor (MNCV) and sural sensory (SNCV) NCVs were significantly slower in BTBR ob/ob mice compared to age-matched ob/+ controls; MNCV was reduced by 29% at 9 weeks and by 40% at 13 weeks, and SNCV was decreased by 16% at 9 weeks and 20% at 13 weeks (Figs. 2B, C). Protein gene product 9.5 (PGP9.5) staining of footpads revealed a 19% reduction in intraepidermal nerve fiber (JENF) density in the hind paw of BTBR ob/ob mice at 13 weeks compared to controls (Figs. 2D, E). Overall, the observed increased...
Fig. 1. Metabolic and lipid profile of BTBR ob/ob mice. A–B. BTBR ob/+ (solid black circles) and ob/ob (open circles) mice were analyzed for body weight (A) and fasting blood glucose (B) at 5 weeks or weekly from 6 until 13 weeks of age. ANOVA confirms significance of the whole curves as well as between each data point. C–F. BTBR ob/+ (solid bars) and ob/ob (open bars) mice underwent terminal glycosylated hemoglobin (%GHb; C), fasting plasma insulin (D), total plasma cholesterol (E), and total triglyceride (F) measurements at 5 and 13 weeks of age. Means ± SEM, n = 7–11 per group. ***P < 0.0001 vs. non-diabetic ob/+ mice. #P < 0.05, ##P < 0.001; ###P < 0.0001 vs. 5 week mice.

Fig. 2. BTBR ob/ob mice display robust neuropathy characterized by electrophysiological and morphological deficits. A. Response time of hindpaw withdrawal latency to thermal stimulus at 9 weeks of age. B–C. Analysis of sural (B; motor NCV) and sciatic (C; sensory NCV) nerve conduction velocity in BTBR ob/+ (black bars) and ob/ob mice (open bars) at 9 and 13 weeks of age. D. Representative images of PGP 9.5-stained nerve fibers in BTBR ob/+ (top) and ob/ob (bottom) mice density at 13 weeks of age. White circles indicate nerve fibers. Bar = 200 μM. E. Quantification of IENF density in 13 week BTBR ob/+ (black bar) and ob/ob (open bar) mice. Means ± SEM, n = 7–11 per group. *P < 0.05; ***P < 0.0001 vs. non-diabetic ob/+ mice. **P < 0.001; ***P < 0.0001 vs. 9 week mice.
hindpaw latency, decreased NCVs, and reduced IENF density confirms that BTBR ob/ob mice develop early and robust neuropathy, as evidenced by changes which are representative of the characteristic nerve fiber damage and distal sensory defects commonly present in human subjects with diabetic neuropathy by 9 weeks of age.

Microarray analyses on SCN isolated from 5- and 13-week mice were then performed to further characterize DN. Using a FDR <0.05, we identified 1184 and 1941 DEGs associated with time in aging BTBR ob/+ and ob/ob mice, respectively (Fig. 3A). Comparisons to determine changes associated with DN also identified 1503 (624 up-regulated and 825 down-regulated) and 642 (331 up-regulated and 301 down-regulated) DEGs in the 5- and 13-week BTBR ob/ob mice compared to age-matched ob/+ controls, respectively (Fig. 4A).

The overlap among these DEG sets is summarized in Supplementary Table 3. RT-qPCR of select DEGs exhibiting the greatest fold-change reflected comparable profiles (Supplementary Fig. 2), validating the microarray findings.

To identify over-represented biological functions in the DEG sets, we performed GSEA and generated a heat map of the top biological terms and pathways (Fig. 4B). Comparison of 5 to 13 week data revealed changes related to aging which were relatively similar for both BTBR ob/ob and ob/+ genotypes; however, BTBR ob/ob mice demonstrated enrichment in membrane trafficking and endocytosis-related terms. When BTBR ob/+ were compared to ob/ob at 5 and 13 weeks to determine changes related to DN, the most significantly enriched hits were...
related to inflammation and the immune response. Notably, the level of regulation as well as the number of associated terms and pathways within these categories was increased at the earlier 5 week time point. We also observed significant enrichment of hits related to chemotaxis at 5 weeks, and MMP12 was the most significantly upregulated gene at both time points (Supplementary Fig. 2). Together, these data suggest that inflammatory mechanisms may play a critical role in early DN development and DN progression.

To complement these analyses and further identify common DEGs and differentially regulated pathways related to DN across diabetes mouse models, we compared our 5 and 13 week data sets to our previously published data set from 24 week C57BKS db/db mice (Pande et al., 2011), an established DN mouse model. Briefly, C57BKS db/db mice display decreased motor and sensory NCVs between 8 and 12 weeks and decreased IENF density at 18 weeks (O’Brien et al., 2014b). Within these three data sets, we identified 189 common DEGs (Fig. 4A) which are listed along with their respective fold-changes in Supplementary Table 4. Interestingly, a number of cytokines and inflammatory mediators are represented across models and ages, and MMP12, the most highly dysregulated gene in BTBR ob/ob mice, is also significantly dysregulated in C57BKS db/db mice. GSEA analysis (Fig. 4B) demonstrated that all three data sets exhibited significant upregulation of immune response-related terms and functions; however, the BTBR ob/ob mice at each age exhibited a larger number of terms and pathways within this category compared to the C57BKS db/db mice, as well as increased levels of dysregulation. We also noted that terms and functions related to lipid metabolism were upregulated only in the C57BKS db/db mice, while those related to neuronal differentiation and outgrowth were downregulated. Overall, these comparisons again support a potential role for inflammation and the immune response in DN.

Finally, to further characterize inflammation within the nerve environment, we examined the levels of select cytokines and chemokines in SCN protein lysates. While no significant differences between BTBR ob/+ and ob/ob mice were observed at 5 weeks, at 13 weeks we saw significant increases in the levels of IL-10, CCL11, CXCL1, and CXCL10, as well as a trending increase in IL-6 in BTBR ob/ob SCN relative to levels in ob/+ SCN (Figs. 5A–E). Similar to the gene expression data demonstrating a higher representation of inflammation and immune

![Fig. 5. Cytokine and MMP protein levels in SCN of BTBR ob/ob mice. Protein levels of IL-6 (A), IL-10 (B), CCL11 (C), CXCL1 (D), CXCL10 (E) and MMP12 (F) in SCN of BTBR ob/+ (black circles) and ob/ob (open circles) mice, measured using MILLIPLEX xMAP magnetic bead technology. *P < 0.05, **P < 0.01, ***P < 0.001 vs. non-diabetic ob/+ mice. #P < 0.05, ##P < 0.01; ###P < 0.001 vs. 5 week mice.](image-url)
response-related terms at the earlier 5 week time point, we also observed decreases in CCL11 and CXCL1 protein levels at 13 weeks compared to at 5 weeks in BTBR ob/ob mice. Finally, MMP12 protein levels were consistently increased at each time point in BTBR ob/ob mice, as seen in the gene expression arrays (Fig. 5F).

Discussion

As the T2D pandemic continues to escalate, there is a critical need for novel animal models that recapitulate the associated diabetic complications in order to develop effective treatment strategies. This is particularly important for DN, a debilitating and common complication with no effective treatments. The current studies demonstrate that BTBR ob/ob mice exhibit numerous features of DN, including insulin hypoaesthesia, delayed NCVs, and reduced IENF density. Furthermore, our gene expression profiling analyses suggest that many inflammatory mediators within the peripheral nerve are dysregulated in diabetes, strengthening the emerging idea that inflammation may contribute to DN pathophysiology in T2D. Thus, we anticipate that BTBR ob/ob mice are a favorable model for disease pathogenesis and therapeutic development studies due to the rapid and robust disease onset and progression.

Despite the surge in diabetes-related studies, the repertoire of available mouse models where DN has been characterized remains limited (O'Brien et al., 2014b; Sullivan et al., 2007). Furthermore, these models are on varying background strains, model both type 1 (T1D) and T2D, and analyses are reported with differing diabetes durations, at different ages, and with different genders; factors which can all impact phenotype. Data indicate that ob/ob mice are susceptible to more robust diabetes on the BTBR background compared to the C57BL6 background (Keller et al., 2008), and we and others have shown that BTBR ob/ob mice are hyperphagic and exhibit early and robust diabetes along with evidence of dyslipidemia as early as 5 weeks of age (Fig. 1, Supplementary Fig. 1; (Clee et al., 2005; Hudkins et al., 2010; Keller et al., 2008)). BTBR ob/ob mice also exhibit a number of microvascular complications associated with diabetes, including nephropathy, cardiomyopathy, and fatty liver disease (Hudkins et al., 2010; O'Brien et al., 2009), suggesting that they may represent an ideal model for diabetic complications research; however, the impact of T2D on the peripheral nervous system in BTBR ob/ob mice was not known prior to the current study.

Our goal was to characterize neuropathy in BTBR ob/ob mice. We demonstrate that BTBR ob/ob mice exhibit increased thermal latency and decreased MNCV and SNCV by 9 weeks and reduced IENF density at 13 weeks (Fig. 2). Reports on established leptin-based models, including the C57BKS db/db and C57BL6 ob/ob mice, exhibit the presence of DN pathology beginning as early as 8 weeks and 11 weeks, respectively, while C57BL6 db/db mice only exhibit significant DN outcomes at 24 weeks when fed a high-fat diet (O'Brien et al., 2014b). Interestingly, our BTBR ob/ob data demonstrate 29% and 40% decreases in MNCV at 9 and 13 weeks, respectively, whereas C57BL6 ob/ob mice exhibit a 22% decrease at 11 weeks (Keller et al., 2008), suggesting that BTBR ob/ob mice develop a more rapid and robust DN phenotype. This is not unexpected given the increased diabetes severity in BTBR ob/ob mice relative to C57BL6 ob/ob mice (Clee et al., 2005; Hudkins et al., 2010; Keller et al., 2008).

While we acknowledge that leptin-based models have limitations and alternative mouse background- and leptin-dependent effects may impact phenotypes (Clee et al., 2005; Coppari and Bjorbaek, 2012; Keller et al., 2008), our results support BTBR ob/ob mice as a valid model for DN research. Because these animals develop other microvascular complications (Hudkins et al., 2010; O'Brien et al., 2009), they also provide a means to study common mechanisms that underlie DN and other diabetic complications. In addition, the leptin deficiency in BTBR ob/ob mice offers an opportunity to examine the reversibility of diabetic complications using exogenous leptin treatment, an approach not possible in models based on leptin receptor mutations (Harris et al., 1998). To examine potential mechanisms underlying DN pathogenesis, we performed gene expression profiling on SCN from 5- and 13-week BTBR ob/ob and ob/+ mice (Fig. 3). Using our in-house bioinformatics pipeline (Pande et al., 2011), we found that aging BTBR ob/ob mice demonstrate dysregulation of pathways related to endocytosis and membrane trafficking between 5 and 13 weeks, and that some of the most highly regulated DEGs are related to the humoral response, suggesting that immune dysregulation within the nerve environment may contribute to DN progression. We observed a 5.02- and 6.33-fold increase in lgfh1 and lgfh2, respectively, in BTBR ob/ob mice at 13 weeks, and previous studies demonstrate that IgGs are elevated in T2D patients and are strongly associated with diabetic nephropathy in T1D patients (Virella et al., 2008). Furthermore, increased IgG deposition is observed in the kidney of NOD mice, a T1D model, likely due to infiltrating B cells (Xiao et al., 2009). Likewise, we observed significant overrepresentation of inflammation and immune response pathways in BTBR ob/ob mice at both 5 and 13 weeks. Interestingly, the level of differential expression and the number of DEGs within the inflammation and immune response categories were significantly increased at the earlier time point, which may suggest that these pathways may underlie DN onset. As we are unable to ascertain at this point whether inflammation plays a causal contribution to DN or if inflammation is a consequence of diabetes itself with no bearing on DN progression, further in-depth experimental analyses are necessary to explore the role of inflammation in the nerve environment.

Inflammation is gaining momentum as a potential driving mechanism in the development and progression of diabetic microvascular complications. Emerging evidence strongly supports a role for inflammation in the progression of diabetic retinopathy and nephropathy (Tang and Kern, 2011; Wada and Makino, 2013), and cross-sectional and prospective studies have demonstrated elevated levels of circulating inflammatory mediators, including cytokines, chemokines, and acute-phase proteins, in the blood of T2D patients (Spranger et al., 2003; Herder et al., 2009). In a rat nerve injury model, infiltration of inflammatory cells and an increase of cytokines are observed in the SCN (Okamoto et al., 2001; Schmid et al., 2013; Camara et al., 2013); however, little is known about the role of inflammation in DN in T1D and T2D models. In BTBR ob/ob mice, we observed significant elevation of IL-10, CCL11, DCCL1, and CXCL10 at 13 weeks, as well as an increasing trend for IL-6. In streptozotocin (STZ)-injected C57BL6 mice, a model of T1D, the pro-inflammatory molecules IL-6, TNF-α, and IL-1β were elevated in SCN (Valsecchi et al., 2011; Kellogg et al., 2007; Bierhaus et al., 2004). In T2D patients, IL-6 is elevated in sural nerve perineurium (Bierhaus et al., 2004), and increased NF-κB activity, a transcription factor responsible for generating a number of immune-related mediators, is also observed in T2D patient sural nerves (Bierhaus et al., 2001). Gene expression profiling studies on T1D and T2D human subjects with progressive DN also implicate inflammation (Hur et al., 2011). Therefore, our current observation of several inflammation pathways in BTBR ob/ob mice strengthens the emerging idea that inflammation plays a role in the pathophysiology of DN.

Of note, the SCN includes a heterogeneous mix of Schwann cells, nerves, fibroblasts, adipocytes, and vascular endothelial cells (Verheijen et al., 2003) as well as resident and infiltrating non-neuronal cells; therefore, it is possible that the observed alterations may be driven by cell types other than the nerves. In addition, it is also possible that circulating immune cells may be recruited into peripheral nerves during DN, similar to observations in diabetic retinopathy and nephropathy (Chow et al., 2007; Omri et al., 2011). Our data demonstrate that CCL2, a chemokine strongly associated with inflammatory cell recruitment to sites of injury (Haberstroh et al., 2002), was among the significantly increased DEGs (fold change of 2.88 at 5 weeks). While we did not detect CCL2 in our SCN protein profiling analysis, elevated CCL2 levels are observed in peripheral blood of T2D patients (Nomura et al., 2000), and increased CCL2 is present in C57BL6 ob/ob mouse plasma (Sartipy and Loskutoff, 2003).
Furthermore, our protein profiling data demonstrates that other classical mediators of chemotaxis, including CCL11, CXCL1, and CXCL10, are upregulated in BTBR ob/ob mouse SCN at 13 weeks (Figs. 5A–D), and chemotaxis terms and pathways are overrepresented at 5 weeks (Figs. 3, 4). Finally, MMP12 was the highest upregulated gene at both 5 and 13 weeks, and increased MMP12 protein was also present in BTBR ob/ob SCN (Supplementary Fig. 2, Fig. 5E). MMPs are involved in extracellular matrix breakdown during normal physiological processes and in disease conditions (Lagente et al., 2009) and are expressed in activated macrophages (Wu et al., 2000) and Schwann cells (Hughes et al., 2002), further indicating that chemotaxis and recruitment of immune cells may play a role in DN pathogenesis. Given that blocking chemotaxis using a CCR2 antagonist in C57BKS db/db mice or knocking out CCL2 in C57BL6 db/db mice attenuates diabetic nephropathy (Chow et al., 2007; Kang et al., 2010), further investigation into therapies targeting chemokines identified in BTBR ob/ob mice or those targeting MMP12 activity may be warranted for DN. In addition, future studies investigating the source of altered immune mediators, whether they are associated with infiltrating cells or produced by resident cells, are warranted to gain additional insight into potential therapeutic targets in DN.

Also of interest are the results and implications stemming from our protein multiplex analyses. Though our protein array data do not exhibit significant differences at 5 weeks of age, this could be explained by the potential variability in the diabetic status of the BTBR ob/ob mice as this time point coincides with hyperglycemia onset. Thus, not all mice may exhibit a robust diabetic phenotype at 5 weeks. In support of this contention, we performed a Pearson correlation on our 5 week MMP12 protein data and found that MMP12 levels correlate with increased levels of %Hb (0.86; $P = 0.0003$). Additional longitudinal measurements of the selected cytokines between 4 and 13 weeks with increased sample sizes may provide less variable outcomes and a clearer picture of any associated alterations in the levels of these mediators throughout the DN disease course.

We also observed a decrease in inflammatory mediators between 5 and 13 weeks for both the diabetic and control mice, a finding that could be attributed to the ongoing nerve developmental processes at the early time point. Chemokines, including CCL5 (Bolin et al., 1998) and CXCL12 (Belmadani et al., 2005), are essential for the development and organization of the hematopoietic/lymphopoietic system and are expressed by different types of cells in the nervous system. Thus, the cytokines that were significantly increased in our 5 week array data may reflect these early processes. While the levels decrease once the nervous system is fully developed in the BTBR ob/+ mice, these levels do not drop to same extent in the diabetic BTBR ob/ob mice. We speculate that hyperglycemia and the associated oxidative stress in the diabetic nerve environment could account for the observed persistence of these inflammatory mediators in BTBR ob/ob mice.

Though the gene expression data demonstrate increased dysregulation of immune related categories at the earlier 5 week time point, our protein data do not exhibit significant differences until 13 weeks. One explanation is that steady-state transcript levels can only partially predict protein levels due to the intricate, heavily regulated processes that span mRNA processing to the post-processed proteome (Vogel and Marcotte, 2012). Of the cytokines measured using the multiplex assay CXCL1 (at 13 weeks) and MMP12 (at 5 and 13 weeks) are differentially expressed in our microarray data set. Measuring the protein products of other dysregulated immune-related genes identified in our GSEA (Supplementary Table 4) could likely provide a more complete reflection of the inflammation and immune response pathways that occur in the BTBR ob/ob mice at 5 weeks. The protein levels of some of these targets will be assessed in subsequent studies.

Comparison of 5- and 13-week BTBR ob/ob DEGs with 24-week C57BKS db/db mouse data identified 189 shared DEGs and a number of common biological functions (Fig. 4A; Supplementary Table 4). Again, MMP12 was significantly upregulated in C57BKS db/db mice, reflecting levels 6.6-fold higher than controls. In addition, changes consistent with demyelination were observed across the 3 datasets. Genes encoding the myelin structural proteins PMP22, MPZ, and ELOVL6 were down-regulated at 5 and 13 weeks and may represent early Schwann cell abnormalities that precede the structural changes of demyelination. Segmental demyelination has been observed in human DN (Malik et al., 2001), but evidence of structural abnormalities is lacking in mouse models, potentially because of the shorter disease course relative to humans. Interestingly, no chemokines were included among the 189 common DEGs, suggesting that these changes may be strain- and age-dependent; however, a number of other inflammation-related DEGs were common, including Il1rn, Il7r, and Thr13. These are reflected in the overrepresented terms related to inflammation, the immune response, and chemotaxis which are shared among models following the functional enrichment analyses.

In summary, a single model that manifests every aspect of human DN does not exist; however, BTBR ob/ob mice exhibit robust DN and provide a great opportunity to elucidate the molecular mechanisms and investigate novel therapies for DN. Our data further suggest that inflammation may underlie DN development and progression, and future studies examining the role of inflammation and the immune response in DN are warranted.

Conflict of interest

No potential conflicts of interest relevant to this article were reported.

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P.D.O.B. conducted animal experiments, performed RT-qPCR validation and immunoassays, and wrote the manuscript. J.H. researched data and edited the manuscript. J.M.H. and C.B. conducted animal experiments. S.A.S. contributed to discussion and reviewed and edited the manuscript. E.L.F. designed and directed the study, contributed to discussion, and reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.10.015.
LETTER

The evaluation of distal symmetric polyneuropathy: utilisation and expenditures by community neurologists

INTRODUCTION

Previous studies show that electrodiagnostic tests and MRIs are frequently ordered in the initial evaluation of neuropathy. However, American Academy of Neurology (AAN) guideline-supported tests, particularly the glucose tolerant test (GTT), are often omitted. Recent evidence suggests that electrodiagnostic studies and MRIs are the primary drivers of expenditures associated with neuropathy testing despite limited data to support their use. However, these results are based on Medicare claims; therefore, it remains unclear if this observation applies to other populations and when using a more rigorous case definition of neuropathy. Furthermore, Medicare claims do not provide detailed clinical information that would allow for investigation of patient-level factors associated with utilisation and expenditures.

Our aim was to determine utilisation and expenditures in the evaluation of a new diagnosis of distal symmetric polyneuropathy (DSP) by community neurologists using a population-based design and a strict case definition. We also sought to determine which patient and physician factors were associated with testing expenditures, electrodiagnostic and MRI utilisation.

METHODS

We attempted to capture all new patients with DSP seen by community neurologists in Nueces County, Texas as previously described. Patients were required to meet the Toronto consensus panel definition of probable neuropathy and have a documented neuropathy diagnosis. From 1 April 2010 to 31 March 2011, we used a validated International Classification of Diseases 9 case capture technique to screen all new patient visits for cases followed by medical record abstraction to confirm that they met our DSP definition. The aetiology at the time of the initial visit to the neurologist was determined by the neurologist’s documented assessment. Utilisation was documented separately for the referring physician and for the neurologist. For neuropathy-related expenditures we used the Medicare physician fee and clinical laboratory schedules. Descriptive statistics were used to describe population demographics and clinical variables, utilisation and expenditures. Multilevel (patients nested within physicians) linear and logistic regression were used to investigate the association between patient-level variables and testing expenditures, electrodiagnostic and MRI utilisation by neurologists. As a measure of the importance of the physician effect on our outcomes, we estimated the percentage of the variance in utilisation attributable to physicians using the intraclass correlation coefficient from a model with only a physician-specific random effect. Our models were also used to calculate the percentage of total variance attributable to patient-level characteristics.

RESULTS

We identified 458 patients meeting our DSP criteria as previously described. Demographics of the population are presented in online supplementary table S1.

Utilisation of the AAN recommended tests, electrodiagnostic testing and MRIs of the neuroaxis are presented in figure 1A (entire population) and B (unknown aetiology prior to diagnostic testing).

The total mean (SD) testing expenditures were US$892 (507) per patient with electrodiagnostic testing (US$553, SD 306) and MRIs (US$203, SD 363) accounting for 62% and 23% of the total testing expenditures, respectively. All other tests resulted in a mean of US$136 (141) in testing expenditures accounting for 15% of the total.

In fully adjusted models, age was the only patient-level variable associated with testing expenditures and MRI utilisation by neurologists (see online supplementary table S2). Physicians accounted for 41.9% of the variation in diagnostic expenditures, 40% in electrodiagnostic utilisation and 13.6% in MRI utilisation. Patient-level factors accounted for 14% of the variance in diagnostic expenditures, 25.7% in electrodiagnostic utilisation and 17.9% in MRI utilisation.

DISCUSSION

Electrodiagnostic testing was performed in over 80% of patients, almost all of which were ordered by neurologists. This finding indicates that neurologists consider electrodiagnostic testing an essential component of the initial evaluation of DSP regardless of the clinical scenario. This practice is in concert with recent AAN quality measures, which state that all patients with DSP should undergo an electrodiagnostic test unless they have an obvious cause for neuropathy and highly suggestive symptoms and signs of neuropathy that cannot be attributable to another condition. However, electrodiagnostic tests accounted for 62% of overall testing expenditures and very little evidence supports or refutes their routine use in patients with DSP. Therefore, future work needs to clarify if all patients with DSP should undergo these tests or if only a subset benefit.

MRIs of the neuroaxis were another large driver of expenditures (23%), being performed in 28% of patients. Unlike electrodiagnostic tests, MRIs have a less intuitive role in the evaluation of DSP. Thus, it remains to be seen what is driving MRI utilisation in DSP populations, and whether the utilisation is appropriate. Possibilities include a high frequency of comorbid conditions that require MRI, concern for a central nervous system localisation causing neuropathic symptoms and signs, and increased visits to physicians leading to more opportunities for testing.

Considering AAN guideline-supported tests, only 7.2% of those with an unclear aetiology prior to diagnostic testing had undergone all recommended tests. The GTT was only ordered in 11.4% of this population. While B12 and serum protein electrophoresis (SPEP) were ordered more frequently, 31% with an unclear aetiology did not receive B12 testing and 49% did not receive SPEP testing. Overall, this data suggests an opportunity to improve guideline concordant DSP testing.

Despite detailed demographic and clinical information, age was the only patient-level factor significantly associated with testing expenditures, electrodiagnostic or MRI utilisation by neurologists. This result implies that the individual patient-level factors assessed in this study are not major drivers of the extent of the diagnostic evaluation of DSP. Conversely, physicians account for a dominant proportion of the variability in testing expenditures and electrodiagnostic testing. Therefore, the physician that a patient chooses is more important than the patient’s specific clinical scenario in determining the extent of the DSP evaluation. These results indicate a need for clinical decision support for DSP testing, particularly for electrodiagnostic tests.

In conclusion, multiple lines of evidence (nationally representative survey, Medicare claims, and now this study) demonstrate that electrodiagnostic tests and MRIs are frequently ordered in the diagnostic evaluation of DSP and account for more than 80% of the
expenditures.1–3 Yet, no prior studies have defined the value of these studies in the evaluation of DSP. Therefore, future studies are needed to determine in which scenarios these tests impact patient management.

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Contributors BCC was involved in study design and statistical analysis, and wrote the manuscript. KAK helped with study design, statistical interpretation, and contributed to the manuscript. MB helped with the statistical analysis plan and interpretation, and contributed to the manuscript. RL performed the screening and medical chart abstraction and participated in the study design. AR assisted in the statistical analysis. PL contributed to the manuscript. LBM, ELF, and LDL were involved in the planning of the project, interpretation of the statistical analysis and contributed to the manuscript. PL, Frank Bonikowski and J Felipe Santos helped coordinate this project at their respective sites.

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Competing interests KAK received speaker honoraria from the American Academy of Neurology and Munson Medical Center, and served as a consultant for the American Academy of Neurology, and The Weinberg Group. KAK and LBM also provide expert medical legal work (modest) that does not involve industry.

Ethics approval University of Michigan IRB.

Provenance and peer review Not commissioned; externally peer reviewed.

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REFERENCES


Longitudinal patient-oriented outcomes in neuropathy
Importance of early detection and falls

ABSTRACT
Objective: To evaluate longitudinal patient-oriented outcomes in peripheral neuropathy over a 14-year time period including time before and after diagnosis.
Methods: The 1996–2007 Health and Retirement Study (HRS)–Medicare Claims linked database identified incident peripheral neuropathy cases (ICD-9 codes) in patients ≥65 years. Using detailed demographic information from the HRS and Medicare claims, a propensity score method identified a matched control group without neuropathy. Patient-oriented outcomes, with an emphasis on self-reported falls, pain, and self-rated health (HRS interview), were determined before and after neuropathy diagnosis. Generalized estimating equations were used to assess differences in longitudinal outcomes between cases and controls.
Results: We identified 953 peripheral neuropathy cases and 953 propensity-matched controls. The mean (SD) age was 77.4 (6.7) years for cases, 76.9 (6.6) years for controls, and 42.1% had diabetes. Differences were detected in falls 3.0 years before neuropathy diagnosis (case vs control; 32% vs 25%, p = 0.008), 5.0 years for pain (36% vs 27%, p = 0.002), and 5.0 years for good to excellent self-rated health (61% vs 74%, p < 0.0001). Over time, the proportion of fallers increased more rapidly in neuropathy cases compared to controls (p = 0.002), but no differences in pain (p = 0.08) or self-rated health (p = 0.9) were observed.
Conclusions: In older persons, differences in falls, pain, and self-rated health can be detected 3–5 years prior to peripheral neuropathy diagnosis, but only falls deteriorates more rapidly over time in neuropathy cases compared to controls. Interventions to improve early peripheral neuropathy detection are needed, and future clinical trials should incorporate falls as a key patient-oriented outcome. Neurology® 2015;85:71–79

GLOSSARY
ADL = activities of daily living; CI = confidence interval; GEE = generalized estimating equations; HRS = Health and Retirement Study; IADL = instrumental activities of daily living; ICD-9 = International Classification of Diseases-9; OR = odds ratio.

Peripheral neuropathy is a highly prevalent disease, particularly in older persons, with more than 15% of those over age 40 years affected.1 While improved glucose control decreases the incidence of neuropathy in type 1 diabetes, the effect is much smaller in type 2 diabetes.2 Similarly, no effective treatments exist for idiopathic peripheral neuropathy (20%–30% of cases).3–5 As new disease-modifying therapeutics are developed for the treatment and prevention of neuropathy, there is a need to better understand the natural history of how peripheral neuropathy affects patient-oriented outcomes over time.

Previous cross-sectional studies have shown that neuropathy is associated with worse quality of life, more pain, and increased number of falls.6–11 However, limited longitudinal data are available to provide stronger epidemiologic support for neuropathy as the prime mediator of these outcomes. Of the longitudinal studies that have been performed, Ahroni and Boyko12 demonstrated that veterans with diabetes who develop neuropathy have a greater decline in quality of life than those who do not develop neuropathy over a 3-year time period. Similarly, DiBonaventura et al.13 reported that a physical quality of life measure in patients with painful
### Table 1  Demographic and clinical variables in the population of patients with peripheral neuropathy compared to propensity score-matched controls

<table>
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<th>Controls (n = 953), n (%) unless otherwise indicated</th>
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<td>63 (6.61)</td>
<td>0.550</td>
</tr>
<tr>
<td>Non-Hispanic white/other</td>
<td>756 (79.33)</td>
<td>769 (80.69)</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic black</td>
<td>122 (12.80)</td>
<td>121 (12.70)</td>
<td></td>
</tr>
<tr>
<td>Education, grade level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-11</td>
<td>312 (32.74)</td>
<td>311 (32.63)</td>
<td>0.775</td>
</tr>
<tr>
<td>12</td>
<td>318 (33.37)</td>
<td>310 (32.53)</td>
<td></td>
</tr>
<tr>
<td>13+</td>
<td>323 (33.89)</td>
<td>332 (34.84)</td>
<td></td>
</tr>
<tr>
<td>Body mass index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>322 (34.18)</td>
<td>295 (31.15)</td>
<td>0.303</td>
</tr>
<tr>
<td>25-29.9</td>
<td>367 (38.96)</td>
<td>398 (42.03)</td>
<td></td>
</tr>
<tr>
<td>30-34.9</td>
<td>181 (19.21)</td>
<td>180 (19.01)</td>
<td></td>
</tr>
<tr>
<td>≥35</td>
<td>72 (7.64)</td>
<td>74 (7.81)</td>
<td></td>
</tr>
<tr>
<td>Diabetes, 6-30 mo pre-index date</td>
<td>401 (42.08)</td>
<td>401 (42.08)</td>
<td>Blocked</td>
</tr>
<tr>
<td>Alcohol, drinks/wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>740 (77.81)</td>
<td>739 (77.71)</td>
<td>0.956</td>
</tr>
<tr>
<td>1-3</td>
<td>86 (9.04)</td>
<td>89 (9.36)</td>
<td></td>
</tr>
<tr>
<td>4-7</td>
<td>68 (7.15)</td>
<td>70 (7.36)</td>
<td></td>
</tr>
<tr>
<td>8-14</td>
<td>40 (4.21)</td>
<td>40 (4.21)</td>
<td></td>
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<tr>
<td>≥15</td>
<td>17 (1.79)</td>
<td>13 (1.37)</td>
<td></td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>85 (8.92)</td>
<td>89 (9.34)</td>
<td>0.722</td>
</tr>
<tr>
<td>Rheumatoid arthritis or osteoarthritis</td>
<td>264 (27.70)</td>
<td>263 (27.60)</td>
<td>0.955</td>
</tr>
<tr>
<td>Cancer</td>
<td>135 (14.17)</td>
<td>129 (13.54)</td>
<td>0.673</td>
</tr>
<tr>
<td>Inpatient Charlson, 2-y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>664 (69.67)</td>
<td>674 (70.72)</td>
<td>0.216</td>
</tr>
<tr>
<td>1</td>
<td>101 (10.60)</td>
<td>104 (10.91)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>84 (8.81)</td>
<td>81 (8.50)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>46 (4.83)</td>
<td>41 (4.30)</td>
<td></td>
</tr>
<tr>
<td>4+</td>
<td>58 (6.09)</td>
<td>53 (5.56)</td>
<td></td>
</tr>
<tr>
<td>Outpatient Charlson, 1-y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>654 (68.63)</td>
<td>656 (68.84)</td>
<td>0.805</td>
</tr>
<tr>
<td>1</td>
<td>167 (17.52)</td>
<td>183 (19.20)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>76 (7.97)</td>
<td>57 (5.98)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>35 (3.67)</td>
<td>39 (4.09)</td>
<td></td>
</tr>
<tr>
<td>4+</td>
<td>21 (2.20)</td>
<td>18 (1.89)</td>
<td></td>
</tr>
<tr>
<td>Auxiliary Charlson, 1-y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>330 (34.63)</td>
<td>335 (35.15)</td>
<td>0.158</td>
</tr>
<tr>
<td>1</td>
<td>241 (25.29)</td>
<td>251 (26.34)</td>
<td></td>
</tr>
</tbody>
</table>

Continued
Table 1 Continued

<table>
<thead>
<tr>
<th>Measure</th>
<th>Cases (n = 953), n (%) unless otherwise indicateda</th>
<th>Controls (n = 953), n (%) unless otherwise indicateda</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>150 (15.74)</td>
<td>162 (17.00)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>115 (12.07)</td>
<td>98 (10.28)</td>
<td></td>
</tr>
<tr>
<td>4+</td>
<td>117 (12.28)</td>
<td>107 (11.23)</td>
<td></td>
</tr>
</tbody>
</table>

aPatients with neuropathy and controls were also balanced on days from Health and Retirement Study interview – 1 to index date, wealth, and presence of other insurance in addition to Medicare.

analysis on falls, self-rated health, and pain to limit the number of comparisons. Other HRS questions were only analyzed at the first HRS interview after the neuropathy diagnosis. Nondichotomous variables were transformed into dichotomous variables by combining adjacent categories based on clinical judgment. For example, for the self-rated health question “Would you say your health is excellent, very good, good, fair, or poor?” we combined those who responded excellent, very good, and good compared with those who responded fair or poor. Four questions pertaining to sleep and 3 questions pertaining to exercise were analyzed. Trouble with activities of daily living (ADL) was defined as some difficulty in 1 of 6 domains (bathing, dressing, eating, bedding, walking, and toileting). Trouble with instrumental ADL (IADL) was defined as some difficulty in 1 of 3 domains (telephone, money, medications). Trouble with mobility was defined as difficulty with 2 or more of the 5 questions focused on walking and climbing stairs. Depression was defined as endorsing 4 or more of the 8 Center for Epidemiologic Studies Depression Scale questions, which is a validated screening instrument.16

Statistical analysis. Matched comparisons between neuropathy cases and controls were conducted using McNemar tests for categorical variables and paired t tests for continuous variables. A generalized estimating equations (GEE) approach was used to analyze differences in falls, pain, and self-rated health (longitudinal binary outcomes) between neuropathy cases and controls. We performed overall as well as stratified analyses by diabetic status. Time since index date (entered as a continuous variable), group (i.e., neuropathy case vs control), and time by group interaction were entered as independent variables. The GEE approach allowed us to investigate the associations between neuropathy status and longitudinal outcomes, while accounting for the correlation among repeated measures in the outcomes. Ignoring the correlation can lead to biased estimates of the standard errors, thereby affecting the inference regarding associations between neuropathy status and outcomes. The interaction term of time by group was used to assess whether the patient-oriented outcome trajectories for neuropathy cases differed from controls over time. For each patient-oriented outcome, we determined the odds ratios (ORs) comparing neuropathy cases to controls at the index date, as well as ORs of change per year for neuropathy cases and for controls. All analyses were performed with SAS 9.3 (Cary, NC).

Standard protocol approvals, registrations, and patient consents. The University of Michigan institutional review board approved this study. All patients participating in the HRS provide oral or implied consent by participating in the interview.

RESULTS A total of 1,039 subjects met our definition of incident peripheral neuropathy. Of this population, 52 died before the HRS interview wave immediately following diagnosis, 19 did not complete an HRS interview within the first 6 years following diagnosis, and 15 had no match within our designated caliper after 3 rounds. Our final cohort consisted of 953 neuropathy cases and 953 controls.

Population demographic and clinical variables are presented in table 1. No statistically significant differences were noted between neuropathy cases and controls. Cases were followed for a mean (SD) 4.7 (2.6) years before and 5.0 (3.1) years after the index date. Controls were followed for 4.7 (2.6) years before and 5.5 (3.2) years after the index date. Table 2 displays the differences in patient-oriented outcomes between neuropathy cases and controls at the first HRS interview after diagnosis.

Evaluating the trajectories of the main patient-oriented outcomes in the entire cohort revealed that neuropathy cases were more likely to fall (OR = 1.41, 95% confidence interval [CI] 1.25–1.58) and to be troubled often by pain (OR = 1.69, 95% CI 1.46–1.94) and less likely to rate their health as good or better (OR = 0.60, 95% CI 0.52–0.70) at the index date (table 3). These results were also identical when looking at the subset of patients with diabetes or the subset without diabetes. However, in the entire cohort, only falls revealed a statistically significant time by group effect (p = 0.002) (figure 1). Neuropathy cases had a 1.11 (95% CI 1.09–1.12) times greater odds of falling each year compared to 1.07 (95% CI 1.05–1.09) times greater odds for controls. The proportion of fallers at the fourth HRS interview before the index date (6.9 years before the index date) was 23% of cases and 29% of controls (figure 1). By the fourth HRS interview after the index date (7.1 years after the index date), the proportion had changed to 56% of cases (33% increase) and 41% of controls (12% increase). While the proportion of cases often troubled by pain increased from 37% to 47% and the proportion of controls remained relatively stable (29%–30%), the time by group interaction effect did not reach statistical significance (p = 0.08). Self-rated health declined slowly in both neuropathy cases and controls over time with no significant difference between the groups. When evaluating for the earliest changes between neuropathy cases and controls, differences were detected in falls at an average of 3.0 years prior to diagnosis (case vs control; 32% vs 25%, p = 0.008), 5.0 years prior to diagnosis for pain (36% vs 27%, p = 0.002), and 5.0 years prior to diagnosis for self-rated health (61% vs 74%, p < 0.0001).

When comparing patients with diabetes and those without diabetes, the patient-oriented outcome trajectories were different (figure 2). Specifically, patients with diabetic neuropathy deteriorated more
slowly on self-rated health compared to diabetic controls ($p < 0.05$), whereas no statistically significant difference was observed when comparing the self-rated health trajectory of patients with nondiabetic neuropathy to nondiabetic controls ($p = 0.1$). In contrast, patients with diabetic and nondiabetic neuropathy fell more frequently over time than controls ($p = 0.02$ and $p = 0.05$, respectively). No statistically significant difference was observed when comparing the self-rated health trajectory of patients with diabetic and nondiabetic neuropathy ($p = 0.05$).

<table>
<thead>
<tr>
<th>HRS question</th>
<th>No.</th>
<th>Case, %</th>
<th>Control, %</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Falls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you fallen down in the last 2 years?</td>
<td>923</td>
<td>46</td>
<td>36</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Percentage of respondents that have injured themselves in a fall enough to need medical treatment</td>
<td>923</td>
<td>17</td>
<td>14</td>
<td>0.0558</td>
</tr>
<tr>
<td>Percentage of respondents that have fallen at least 3 times in the past 2 years</td>
<td>923</td>
<td>17</td>
<td>9</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Have you fractured your hip ever/since previous wave?</td>
<td>926</td>
<td>2</td>
<td>2</td>
<td>0.4927</td>
</tr>
<tr>
<td><strong>Self-rated health</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Would you say your health is excellent, very good, good (vs fair or poor)?</td>
<td>927</td>
<td>49</td>
<td>65</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>Pain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you often troubled with pain?</td>
<td>926</td>
<td>47</td>
<td>32</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>How bad is the pain most of the time? (moderate or severe vs none/mild)</td>
<td>921</td>
<td>36</td>
<td>25</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Does the pain make it difficult for you to do your usual activities such as household chores or work?</td>
<td>921</td>
<td>33</td>
<td>20</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>Sleep</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>How often do you have trouble falling asleep? (most of the time or sometimes vs rarely or never)</td>
<td>617</td>
<td>47</td>
<td>40</td>
<td>0.0077</td>
</tr>
<tr>
<td>How often do you often have trouble with waking up during the night? (most of the time or sometimes vs rarely or never)</td>
<td>617</td>
<td>68</td>
<td>65</td>
<td>0.2484</td>
</tr>
<tr>
<td>How often do you have trouble with waking up too early and not being able to fall asleep again? (most times or sometimes vs rarely or never)</td>
<td>617</td>
<td>44</td>
<td>45</td>
<td>0.8155</td>
</tr>
<tr>
<td>How often do you feel really rested when you wake up in the morning? (most times vs rarely or sometimes)</td>
<td>731</td>
<td>54</td>
<td>66</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>Exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>How often do you take part in sports or activities that are vigorous? (more than once per week vs once/week or less)</td>
<td>525</td>
<td>14</td>
<td>17</td>
<td>0.1227</td>
</tr>
<tr>
<td>How often do you take part in sports or activities that are moderately energetic? (more than once per week vs once/week or less)</td>
<td>524</td>
<td>38</td>
<td>44</td>
<td>0.0825</td>
</tr>
<tr>
<td>How often do you take part in sports or activities that are mildly energetic? (more than once per week vs once/week or less)</td>
<td>526</td>
<td>43</td>
<td>48</td>
<td>0.0909</td>
</tr>
<tr>
<td><strong>ADL, IADL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difficulty with ADL?</td>
<td>930</td>
<td>38</td>
<td>27</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Difficulty with IADL?</td>
<td>930</td>
<td>21</td>
<td>15</td>
<td>0.0012</td>
</tr>
<tr>
<td>Difficulty with mobility?</td>
<td>928</td>
<td>55</td>
<td>41</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>Depression</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Depression</td>
<td>749</td>
<td>23</td>
<td>15</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Since we last talked to you, have you had persistent swelling in your feet or ankles?</td>
<td>495</td>
<td>38</td>
<td>27</td>
<td>0.0007</td>
</tr>
<tr>
<td>Since we last talked to you, have you had persistent dizziness or lightheadedness?</td>
<td>494</td>
<td>21</td>
<td>13</td>
<td>0.0007</td>
</tr>
<tr>
<td>Since we last talked to you, have you had severe fatigue or exhaustion?</td>
<td>491</td>
<td>30</td>
<td>20</td>
<td>0.0002</td>
</tr>
<tr>
<td>Since we last talked to you, have you had back pain or problems?</td>
<td>496</td>
<td>43</td>
<td>37</td>
<td>0.0447</td>
</tr>
<tr>
<td>Have you ever had or has a doctor ever told you that you have arthritis or rheumatism?</td>
<td>927</td>
<td>76</td>
<td>70</td>
<td>0.0042</td>
</tr>
</tbody>
</table>

Abbreviations: ADL = activities of daily living; HRS = Health and Retirement Study; IADL = instrumental activities of daily living.
We found that older adults with neuropathy have more falls and pain and lower self-rated health than diabetic controls. Diabetic neuropathy had a slower deterioration in patient-oriented outcomes within the same study, compared to carefully matched controls without neuropathy. These differences were present 3–5 years prior to a neuropathy diagnosis and persist for several years after diagnosis. However, of these 3 patient-oriented outcomes, the only outcome that worsened more rapidly over time in patients with neuropathy was falls. Surprisingly, patients with diabetic neuropathy had a slower deterioration in self-rated health than diabetic controls.

The observation that neuropathy patients have more falls and pain and lower self-rated health than those without neuropathy is in agreement with several past studies.\(^6-13,17\) Our study builds on these prior findings and provides stronger epidemiologic support for neuropathy as a prime mediator of these outcomes given our ability to track outcome trajectories several years before and after a diagnosis of neuropathy. For all 3 of these patient-oriented outcomes, differences are seen 3–5 years prior to diagnosis. This finding may be partly explained by a delay in diagnosis in this highly prevalent condition, and also highlights the fact that neuropathy often develops slowly over time. Patients typically report neuropathic symptoms to their physician years after their insidious onset. In fact, in a community neurologist setting, patients with a new diagnosis of distal symmetric polyneuropathy had symptoms for a mean of 39 months at their first visit.\(^18\) How long neuropathic symptoms occur prior to seeing a general practitioner is unknown. The implication for future neuropathy clinical trials is that interventions are more likely to be successful if implemented before patients are typically diagnosed with neuropathy. Earlier diagnosis will require studies to evaluate current barriers to a neuropathy diagnosis early in the disease course. Intervening after patients are typically diagnosed may be too late if significant nerve injury has already occurred.

We also demonstrated that falls were the only patient-oriented outcome that increased more rapidly over time between diabetic and nondiabetic patients. This significant difference was seen when comparing pain over time between diabetic and nondiabetic patients with neuropathy and controls (\(p = 0.2\) and \(p = 0.3\)).

**DISCUSSION** Utilizing a nationally representative population, we aimed to evaluate longitudinal trajectories of self-rated health, pain, and falls in those with diabetic and nondiabetic peripheral neuropathy before and after diagnosis over a 14-year follow-up period. Compared to previous longitudinal studies, we were able to investigate our study population for a longer time period, track trajectories prior to a peripheral neuropathy diagnosis, evaluate multiple patient-oriented outcomes within the same study, and compare patients with and without diabetes. We found that older adults with neuropathy have more falls and pain and lower self-rated health compared to carefully matched controls without neuropathy. These differences were present 3–5 years prior to a neuropathy diagnosis and persist for several years after diagnosis. However, of these 3 patient-oriented outcomes, the only outcome that worsened more rapidly over time in patients with neuropathy was falls. Surprisingly, patients with diabetic neuropathy had a slower deterioration in self-rated health than diabetic controls.

The observation that neuropathy patients have more falls and pain and lower self-rated health than those without neuropathy is in agreement with several past studies.\(^6-13,17\) Our study builds on these prior findings and provides stronger epidemiologic support for neuropathy as a prime mediator of these outcomes given our ability to track outcome trajectories several years before and after a diagnosis of neuropathy. For all 3 of these patient-oriented outcomes, differences are seen 3–5 years prior to diagnosis. This finding may be partly explained by a delay in diagnosis in this highly prevalent condition, and also highlights the fact that neuropathy often develops slowly over time. Patients typically report neuropathic symptoms to their physician years after their insidious onset. In fact, in a community neurologist setting, patients with a new diagnosis of distal symmetric polyneuropathy had symptoms for a mean of 39 months at their first visit.\(^18\) How long neuropathic symptoms occur prior to seeing a general practitioner is unknown. The implication for future neuropathy clinical trials is that interventions are more likely to be successful if implemented before patients are typically diagnosed with neuropathy. Earlier diagnosis will require studies to evaluate current barriers to a neuropathy diagnosis early in the disease course. Intervening after patients are typically diagnosed may be too late if significant nerve injury has already occurred.
in cases than controls. While pain and self-rated health were worse in neuropathy cases compared to controls, these outcomes did not worsen more rapidly over time in cases vs controls. The importance of this result to future clinical trials is that falls should be considered as a main outcome measure, especially in an elderly population. When patient-oriented outcomes are assessed at all, most previous trials have focused on pain and/or quality of life. Falls are often ignored as an important patient-oriented outcome, but the quick separation between neuropathy cases and controls over time indicates that falls may be a sensitive and objective neuropathy outcome measure. The magnitude of falls in the neuropathy population, rising from 23% to 56% over the course of this study, also emphasizes the need for clinicians to address falls with their patients. In agreement with this assessment, the American Academy of Neurology recently released quality measures for patients with distal symmetric polyneuropathy with 1 of the 6 measures pertaining to falls. Falls should not only be an important outcome measure of future clinical trials, but also a focus of the clinical care of patients with neuropathy.

Interestingly, self-rated health declined at the same rate in those with neuropathy compared to controls. However, patients with diabetic neuropathy deteriorated more slowly than diabetic controls. One potential explanation for this finding is a possible floor effect. Only 46% of patients with diabetic neuropathy rated their health as good or better 6+ years prior to their neuropathy diagnosis. By comparison, 76% of patients with nondiabetic neuropathy had positive self-rated health. In support of this explanation, previous studies have demonstrated a floor effect when utilizing quality of life measures in stroke patients. Further evidence to support a floor effect is that when we analyzed self-rated health as the proportion that declined by one or more levels compared to their last HRS interview, no difference was observed over time between patients with diabetic neuropathy and diabetic controls. Regardless of the reason, the lack of significant change in self-rated health in patients with diabetic neuropathy either before or after diagnosis makes this a less than ideal patient-oriented outcome for clinical trials.

We found that patients with neuropathy are more likely to have difficulties with sleep and depression, but engagement in exercise is not significantly affected. Previous studies have shown that patients with painful diabetic neuropathy have a higher prevalence of impaired sleep and that the greater the severity of pain, the worse the sleep outcome scores. In agreement with these studies, our results demonstrate that patients with neuropathy are less likely to be well-rested than controls and that the reason for sleep disturbance is likely secondary to problems with falling asleep rather than maintaining sleep. Given that the 2 most likely contributors to sleep disturbance in this population are pain and restless legs syndrome, trouble with sleep initiation would be expected. Similarly, other groups have reported that neuropathy

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Figure 1  Comparison of the patient-oriented outcome trajectories between patients with neuropathy and propensity-matched controls

Patient-oriented outcome trajectories for the entire cohort: (A) falls within the last 2 years, (B) self-rated health, (C) frequent pain. HRS – Health and Retirement Study.
severity is associated with depression severity in both cross sectional and longitudinal studies.\textsuperscript{25,26} We also demonstrate that neuropathy cases are more likely to have depression compared to a propensity-matched control group with a large magnitude of effect (23\% vs 15\%). On the other hand, there were no differences in varying levels of exercise performed comparing neuropathy cases to controls. Our finding is similar to a previous study using the National Health and Nutrition Examination Survey (NHANES) population, which failed to reveal an association between peripheral neuropathy and reduced moderate to
vigorously physical activity as measured by an accelerometer.27 Not only is neuropathy associated with sleep disturbance and depression, but we also found that neuropathy cases were more likely to have difficulties with ADLs, IADLs, and mobility than controls. One previous study revealed an independent association between diabetic neuropathy and the development of mobility limitations, but not new ADL disability.28 A separate group reported an association between women with diabetes and both mobility and ADL limitations that was in part mediated by neuropathy.29 Our results confirm that neuropathy is associated with mobility limitations and provides further support for an association with ADL disability. Furthermore, we report an association with IADL disability, which has not been previously investigated.

Limitations of our study include utilizing ICD-9 codes to identify incident neuropathy cases, which may lead to misclassification bias. Furthermore, patient-oriented outcomes were based on HRS questions and not formally validated questionnaires. While our propensity score–matched controls were well-matched on key demographic and clinical variables, unmeasured residual confounding remains possible. The matching technique made evaluation of obesity-related complications difficult as body mass index and other comorbidities were included in our method. Our propensity score method was maximized to increased length of follow-up; however, this method did not allow us to determine differences in mortality between cases and controls. Although neuropathy status is significantly associated with more falls over time, other important risk factors must be present, as demonstrated by the large proportion of fallers in the control group. Our data are unable to determine how often falls led to the discovery of neuropathy. While falls occurred more rapidly over time in neuropathy cases compared to controls, our study did not have adequate power to determine if fall-related injuries also increased over time. We were unable to determine whether subjects required ambulatory assistance. The generalizability of these results to populations younger than 65 years is unclear.

In older people, peripheral neuropathy significantly impacts patient-oriented outcomes such as falls, pain, and self-rated health. These changes can be observed several years prior to a neuropathy diagnosis and continue for several years after. Falls, an outcome measure that has typically not been used in past clinical trials, was the only patient-oriented outcome that worsened faster over time in neuropathy cases compared to controls. Future studies are needed to explore barriers to earlier diagnosis of neuropathy, and falls should be considered as a main patient-oriented outcome in future intervention trials.

AUTHOR CONTRIBUTIONS
Brian Callaghan was involved in the study design, interpretation of the statistical analysis, and wrote the manuscript. Kevin Kerber, Mousumi Banerjee, Ann Rodgers, Ryan McCammon, Jim Burke, Ken Langa, and Eva Feldman were integrally involved in the study design, interpretation of the data, and critical revisions of the manuscript.

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B. Callaghan receives research support from Impeto Medical Inc. and honoria from the British Medical Journal. He certifies ALS centers for the ALS Association, performs medical consultations for Advance Medical, and consults for a PCORI grant. K. Kerber, K. Langa, M. Banerjee, A. Rodgers, and R. McCammon report no disclosures relevant to the manuscript. J. Burke has received compensation from Astra Zeneca for his role on the adjudication committee of the SOCRADES trial, honoraria from the AAN for contributing to the Continuum series and, consulting fees from Sullivan, Ward, Asher and Paton for reviewing case materials in a medical malpractice defense case. E. Feldman reports no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

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Effects of exenatide on measures of diabetic neuropathy in subjects with type 2 diabetes: results from an 18-month proof-of-concept open-label randomized study

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Abstract

Objective: Experimental studies have reported potential benefit of glucagon-like peptide-1 (GLP-1) receptor agonists in preventing diabetic peripheral neuropathy (DPN). We therefore performed a proof-of-concept pilot study to evaluate the effect of exenatide, a GLP-1 agonist, on measures of DPN and cardiovascular autonomic neuropathy (CAN) in patients with type 2 diabetes (T2D).

Research Design and Methods: Forty-six T2D subjects (age 54 ± 10 years, diabetes duration 8 ± 5 years, HbA1c 8.2 ± 1.3%) with mild to moderate DPN at baseline were randomized to receive either twice daily exenatide (n = 22) or daily insulin glargine (n = 24). The subjects, with similar HbA1c levels, were followed for 18 months. The primary end point was the prevalence of confirmed clinical neuropathy (CCN). Changes in measures of CAN, other measures of small fiber neuropathy such as intra-epidermal nerve fiber density (IENFD), and quality of life were also analyzed.

Results: Glucose control was similar in both groups during the study. There were no statistically significant treatment group differences in the prevalence of CCN, IENFD, measures of CAN, nerve conductions studies, or quality of life indices.

Conclusions: In this pilot study of patients with T2D and mild to moderate DPN, 18 months of exenatide treatment had no significant effect on measures of neuropathy compared with glargine treatment.

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reversed hyperglycemia but only partly improved thermal sensation and epidermal innervation and had no effect on electrophysiological abnormalities (Kan et al., 2012). However, short-term exendin-4 treatment was less effective in T2D mice with neuropathy (Kan et al., 2012). In another study, 4-week exendin-4 treatment in mice with streptozotocin-induced T1D promoted significant neurite outgrowth of DRG neurons and ameliorated the loss of intraepidermal nerve fibers (IENF) (Himeono et al., 2011). It was suggested that these effects were independent of glycemia and possibly mediated via GLP-1 receptor activation and through anti-apoptosis and CAMP signaling pathways (Himeono et al., 2011; Kan et al., 2012), or via stimulating neuronal differentiation in human cells (Luciani et al., 2010). GLP-1 has been also shown to modulate autonomic activity and induce changes in haemodynamic variables. For instance, Griffioen et al. showed that both acute and chronic central administration of exendin-4 increased the resting heart rate and reduced measures of heart rate variability (HRV) in mice, either by altering the inhibition of neurotransmission to cardiac vagal neurons (Griffioen et al., 2011) or up regulation of sympathetic outflow and downstream activation of cardiovascular responses (Yamamoto et al., 2002).

Based on the above experimental evidence, we hypothesized that GLP-1 receptor agonists may have potential beneficial effects on measures of DPN and CAN in humans, something that has not been systematically evaluated. We therefore conducted a pilot, proof-of-concept, randomized, open-label clinical trial to evaluate the effects of exenatide on measures of DPN and CAN in subjects with T2D.

1. Research design and methods

1.1. Study design

This single center, proof-of-concept-pilot, open-label randomized, controlled trial (NCT010855439) was conducted at the University of Michigan between July 2008 and June 2014. The study was reviewed and approved by the University of Michigan Institutional Review Board. All subjects signed a written consent document.

1.2. Study participants

Subjects were eligible to enroll if they were between 18 and 70 years old, had T2D with a hemoglobin A1c (HbA1c) > 7% and fasting blood glucose > 140 mg/dl, had followed a prior stable glucose-lowering regimen that did not include insulin or a GLP-1 receptor agonist, had no known contraindications to treatment with either exenatide or insulin glargine based on FDA prescribing guidelines, and presented with mild-to-moderate DPN as defined by a score of 6 or more on the Michigan Diabetes Neuropathy Scale (MDNS), a validated scale for evaluation of diabetic neuropathy (Feldman et al., 1994) described below in Methods.

Excluded were subjects with a history of kidney, pancreas, or cardiac transplantation, neuropathy independent of diabetes, or any condition other than diabetes associated with neuropathy (e.g. hepatitis C, end stage renal disease, lupus), any lower extremity amputation or severe deformity of lower extremity, Hba1c > 10%, participation in an experimental medication trial within 3 months of starting this study, undergoing therapy for malignant disease other than basal- or squamous cell carcinoma, requiring long-term glucocorticoid therapy, inability or unwillingness to comply with the protocol, and nursing mothers or pregnant women.

1.3. Intervention

Subjects were randomly assigned in a 1:1 ratio to either exenatide (n = 22) or insulin glargine (n = 24) targeting similar levels of glucose control as documented by HbA1c. Exenatide was initiated at a fixed dose of 5 μg twice daily for 4 weeks and then increased to 10 μg twice daily for the remainder of the study. Subjects who did not tolerate the 10 μg dose resumed the reduced 5 μg dose for the duration of the study. Insulin glargine was initiated with 10 units daily and titrated in 2-unit increments to achieve a fasting blood glucose target level of 5.6 mmol/L (100 mg/dL) without recurrent or severe hypoglycemia. The dose of any prior oral agents remained fixed, unless clinical judgment dictated that they should be altered to optimize blood glucose control.

1.4. Assessment of neuropathy

DPN was assessed at baseline and at 12- and 18-month follow-up visits with assessment of symptoms and signs of DPN by a board-certified neurologist as described (Albers et al., 2010), nerve conduction studies of the median (sensory and motor), peroneal motor and sural sensory nerves using a standard protocol, which included replication of baseline limb temperatures at 12 and 18 month assessments (Albers et al., 2010), and quantitative sensory testing for vibration perception (VPT) using the Vibratron II device (Physitemp Instruments, Inc.) as described (Martin et al., 2010). The rate of IENF reinnervation after capsaicin denervation was used as an exploratory measure of small fiber neuropathy and obtained as described (Polydefkis et al., 2004). Briefly, a baseline skin biopsy was obtained from the distal thigh in a subset of consenting T2D subjects (exenatide = 9, glargine = 11). Capsaicin was applied as a 1% topical cream, and the site was covered with an occlusive dressing for 48 hours. Additional skin biopsies were obtained at 48 hours (to confirm denervation) and at 6 and 12 months of treatment (3 months and 9 months post-capsaicin respectively). All skin biopsies were obtained in a standardized fashion by a single examiner, and all IENFD evaluations were analyzed in a blinded manner by Therapath, Inc (New York, NY). In addition, the MDNS was performed at screening to confirm eligibility as described (Feldman et al., 1994). Briefly the MDNS is a 46-point exam that includes testing for vibration, 10-grain monofilament pressure, pin sensation at the great toe, deep tendon reflexes at knee and ankle, and strength. For all evaluations, 1 point was given for reduction on either side, or 2 points when the response was absent, except for pin sensation where 2 points per side were assigned if sharp sensation was absent.

CAN was evaluated at baseline, 12 months, and 18 months with the gold-standard cardiovascular reflex tests (CARTs) (the deep breathing test and the Valsalva maneuver) (Spallone et al., 2011) and measures of HRV obtained during a 5-minute rest and during CARTs using the ANX 3.1 (ANSAR Inc., Philadelphia, PA). Subjects were required to fast for 8 hours and to abstain from tobacco, caffeine, and alcohol prior to testing. Blood glucose was obtained prior to testing, and testing was rescheduled in the presence of hypoglycemia. Testing was performed with the subjects in a supine position, with the head of the bed elevated no more than 30 degrees. Subjects with demonstrable atrial fibrillation (n = 3 glargine) and subjects with a pacemaker (n = 1 glargine) were excluded from the CART analysis.

Neuropathy specific quality of life was evaluated with the Neuropathy Specific Quality of Life Measure (NeuroQOL) (Vileikyte et al., 2003) at baseline, and at 12 and 18 months of follow up. This self-administered, 39-item validated survey that includes: the overall impact of foot problems on quality of life, overall quality of life and 6 other primary domains: 1) painful symptoms and paresthesias; 2) reduced/lost feeling in the feet; 3) diffuse sensory motor symptoms; 4) limitations in daily activities; 5) interpersonal problems; and 6) emotional burden. For the foot problem-specific item, lower scores indicate less negative impact of foot problems on quality of life, and for overall quality of life higher scores indicate worse quality of life. Within each domain, lower scores indicate worse symptoms or greater adverse effect on quality of life (Vileikyte et al., 2003).
1.5. Outcome measures

The primary outcome of the study was the prevalence of confirmed clinical neuropathy (CCN) over 18 months. CCN was defined by a composite score comprised of at least two positive responses among symptoms, sensory signs, or absent or hypoactive reflexes consistent with a distal symmetrical polyneuropathy (Albers et al., 2010), and at least one abnormal nerve conduction study result in two anatomically distinct nerves, e.g. the sural sensory and peroneal motor nerves (defined as an amplitude < 5 μV and a conduction velocity < 40 m/sec for the sural nerve and an amplitude < 2.5 μV and a conduction velocity < 40 m/sec for the peroneal nerve).

Secondary outcomes included: a) individual electrophysiology measures assessed as continuous variables: b) VPT; c) changes in clinical neuropathy (defined as two or more positive findings among symptoms, signs, and reduced or absent deep tendon reflexes); d) changes in IENFD after capsaicin denervation; e) changes in the following measures of CAN: expiration:inspiration (E:I) ratio, Valsalva ratio, resting heart rate, standard deviation of normal RR interval (SDNN), very low frequency power (VLF), low frequency power (LF), and high frequency power (HF) and changes in quality of life measures.

1.6. Power

Seventy people meeting the study inclusion and exclusion criteria by preliminary screening were evaluated for inclusion in the study, and 46 subjects were randomized into the trial (Fig. 1). As a result, there was 90% power to identify a change of one standard deviation (SD) between treatment groups or 80% power to identify a change of 0.87 SDs in the continuous measures using a 2-tailed t-test with a 5% level of significance. With the sample sizes in this study, there was 80% power to identify a difference in the rates of CCN at the end of the study of 40%, i.e., 30% vs. 70%, using a 2-tailed test with a 5% level of significance.

1.7. Statistical analyses

Statistical analyses were done using SAS version 9.2 (SAS Inc, Cary, NC). Differences in continuous and categorical variables between the exenatide and glargine groups at baseline and change from baseline to 18 months were examined by the two-sample t-test (for normally distributed variables) or Wilcoxon rank sum test (for non-normally distributed variables) and chi-square test, respectively. Results were expressed as mean ± standard deviation (SD).

2. Results

At baseline, there were no differences in the mean age of the subjects (51 ± 13 years for exenatide, and 54 ± 9 years for glargine), gender, race, diabetes duration, body mass index (BMI), and glycemic control (P = NS for all) (Table 1). Consistent with the inclusion criteria, all subjects presented with mild-to-moderate neuropathy, as illustrated by similar MDNS scores in both groups at baseline (22 ± 11 and 24 ± 11, P = NS in the exenatide and glargine groups respectively) (Table 1).

The prevalence of CCN was also similar (67% among subjects randomized to exenatide and 75% among subjects randomized to glargine). DPN symptoms were present in 21 (96%) subjects in the exenatide group and 22 (92%) subjects in the glargine group (Table 1). The exenatide and glargine groups also did not differ in any of the electrophysiological measures of median (motor and sensory), sural, and peroneal nerves, or in measures of CAN (Table 2).
group at 12 months (improvement of $4.6 \pm 2.9$ fibers/mm, $P = 0.002$), which was less in the exenatide group (improvement of $2.1 \pm 3.5$ fibers/mm at 12 months, $P = 0.06$). This group difference accounted for a marginally higher regeneration rate with glargine (Fig. 3). There were no differences in baseline characteristics among the participants who agreed to this evaluation and the entire cohort.

Similar to the observations on DPN measures, there was no significant effect of exenatide on any measures of CAN at 12 or 18 months of follow-up compared with glargine (Table 2). In addition, there was a non-significant group difference in resting heart rate, tending to be lower in subjects assigned to exenatide compared with glargine ($70 \pm 74$ bpm respectively, $P = 0.21$).

No group differences were observed over 18 months in either the NeuroQOL scores or overall global quality of life scores. Similar scores between groups were also observed when assessing the differences in the 6 specific domains (painful symptoms and paresthesias, symptoms of reduced/lost feeling in the feet, diffuse sensory motor symptoms, limitations in daily activities, interpersonal problems and emotional burden) (Appendix Table 2).

### 2.2. Adverse events

During the course of the study, 82 adverse events (AEs) were reported; 26 events by 12 exenatide group subjects (1 to 4 events per subject) and 56 events by 20 glargine group subjects (1 to 6 events per subject). Among the events reported by exenatide-treated subjects, 9 events (6 subjects) met serious adverse event criteria, with one of these categorized as possibly related to treatment (hospitalization for severe diverticulitis occurring within three months of starting exenatide) and another considered unlikely related (lipaemia after 12 months of treatment with exenatide, leading to an evaluation that revealed a neuroendocrine tumor of the pancreas, which was surgically removed and determined to be benign). The remaining serious AEs were categorized as unrelated to study treatment or study participation. A summary of all AEs is shown in Table 3.

### 3. Discussion

In this proof of concept, pilot study, treatment with the GLP-1 receptor agonist exenatide did not reduce the prevalence of confirmed DPN over a period of 18 months of treatment, and did not affect electrophysiology or measures of small fiber neuropathy, such as CAN and IENFD, compared to insulin glargine. In addition, exenatide had no effect on symptoms or signs of DPN, or on measure of quality of life. We did not find treatment-group differences with regard to progression (or remission) of neuropathy, as determined by standardized neurologic-focused history and physical examination, nerve conduction measures, CAN measures, or NeuroQOL, in 46 subjects with T2D randomly assigned to 18 months of treatment with exenatide or insulin glargine. An exploratory objective examining change in IENFD in skin before and after capsaicin denervation in a subset of participants also did not reveal treatment group differences, although subjects assigned to glargine experienced more regeneration after capsaicin compared with subjects assigned to exenatide. We believe this is the first study to evaluate the efficacy of exenatide, a GLP-1 receptor agonist, on measures of both small and large fiber neuropathy in subjects with T2D, using very comprehensive assessments.

Emerging experimental evidence discussed above and a small human study suggested potential beneficial effect of GLP-1 receptor agonists or dipeptidyl peptidase-4 (DPP-4) inhibitors on measures of DPN via non-glycemic effects (Barros et al., 2014; Griffioen et al., 2011; Himeno et al., 2011; Jolivalt, Fineman, Deacon, Carr, & Calcutt, 2011; Kan et al., 2012; Luciani et al., 2010; Okawa et al., 2014; Yamamoto et al., 2002). However, our trial, using multiple, sensitive and specific measures for both CAN and PNS, performed under standardized conditions, failed to show any potential beneficial effect.
of a GLP-1 receptor agonist on DPN, CAN or neuropathy-specific quality of life. Although the high prevalence of CCN at baseline may have biased the effects of the intervention, the parametric electrophysiology measures that were used appear very robust and showed expected deterioration in both treatment groups over the short study interval (an expected consequence of diabetes over time), providing expected deterioration in both treatment groups over the short study interval (an expected consequence of diabetes over time), providing

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve conduction and cardiovascular autonomic neuropathy measures at baseline, 12 and 18 months of follow-up.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>12 months</th>
<th>18 months</th>
<th>Difference: Baseline to 18 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median motor amplitude (mV)</td>
<td>Exenatide</td>
<td>9.9 ± 3.6</td>
<td>9.1 ± 3.4</td>
<td>9.2 ± 3.6</td>
</tr>
<tr>
<td>Glargine</td>
<td>8.3 ± 2.9</td>
<td>7.9 ± 3.1</td>
<td>7.7 ± 2.5</td>
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</tr>
<tr>
<td>Median motor CV (m/sec)</td>
<td>Exenatide</td>
<td>49.8 ± 4.6</td>
<td>48.7 ± 4.8</td>
<td>49.4 ± 5.2</td>
</tr>
<tr>
<td>Glargine</td>
<td>50.5 ± 4.4</td>
<td>49.6 ± 4.7</td>
<td>48.5 ± 6.9</td>
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</tr>
<tr>
<td>Median motor F response latency (msec)</td>
<td>Exenatide</td>
<td>30.9 ± 2.7</td>
<td>30.6 ± 2.3</td>
<td>31.6 ± 3.2</td>
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<tr>
<td>Glargine</td>
<td>31.1 ± 2.8</td>
<td>31.1 ± 2.2</td>
<td>31.2 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Median sensory amplitude (μV)</td>
<td>Exenatide</td>
<td>15.5 ± 12.7</td>
<td>15.0 ± 10.9</td>
<td>14.3 ± 10.7</td>
</tr>
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<td>Glargine</td>
<td>13.3 ± 8.8</td>
<td>12.6 ± 8.0</td>
<td>12.6 ± 9.1</td>
<td></td>
</tr>
<tr>
<td>Median sensory CV (m/sec)</td>
<td>Exenatide</td>
<td>45.0 ± 12.5</td>
<td>43.8 ± 13.6</td>
<td>44.9 ± 13.6</td>
</tr>
<tr>
<td>Glargine</td>
<td>41.1 ± 11.2</td>
<td>41.7 ± 10.2</td>
<td>42.5 ± 11.1</td>
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</tr>
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<td>Peroneal motor amplitude (mV)</td>
<td>Exenatide</td>
<td>3.9 ± 1.9</td>
<td>3.7 ± 1.9</td>
<td>3.8 ± 1.8</td>
</tr>
<tr>
<td>Glargine</td>
<td>3.3 ± 2.3</td>
<td>3.7 ± 2.7</td>
<td>3.5 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Peroneal motor CV (m/sec)</td>
<td>Exenatide</td>
<td>40.4 ± 4.6</td>
<td>39.6 ± 5.5</td>
<td>39.9 ± 6.0</td>
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<td>Glargine</td>
<td>38.2 ± 6.2</td>
<td>37.4 ± 5.6</td>
<td>38.4 ± 5.9</td>
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<tr>
<td>Peroneal motor F response latency (msec)</td>
<td>Exenatide</td>
<td>53.0 ± 13.2</td>
<td>58.3 ± 7.6</td>
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<td>Glargine</td>
<td>60.2 ± 7.3</td>
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<td>57.7 ± 8.2</td>
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<td>Sural sensory amplitude (μV)</td>
<td>Exenatide</td>
<td>7.0 ± 7.0</td>
<td>6.2 ± 7.5</td>
<td>5.8 ± 6.4</td>
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<td>Glargine</td>
<td>5.0 ± 4.8</td>
<td>5.6 ± 5.6</td>
<td>4.52 ± 4.4</td>
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<tr>
<td>Sural sensory CV (m/sec)</td>
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<td>41.0 ± 6.7</td>
<td>41.7 ± 9.2</td>
<td>39.2 ± 5.3</td>
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<td>40.9 ± 7.1</td>
<td>41.7 ± 9.2</td>
<td>38.8 ± 5.6</td>
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<td>HR, beat/min</td>
<td>Exenatide</td>
<td>50 ± 18.2</td>
<td>40 ± 13.6</td>
<td>38 ± 9.4</td>
</tr>
<tr>
<td>Glargine</td>
<td>50 ± 18.2</td>
<td>40 ± 13.6</td>
<td>38 ± 9.4</td>
<td></td>
</tr>
<tr>
<td>SDNN, msec</td>
<td>Exenatide</td>
<td>43 ± 25</td>
<td>43 ± 32</td>
<td>40 ± 26</td>
</tr>
<tr>
<td>Glargine</td>
<td>50 ± 24</td>
<td>30 ± 13</td>
<td>38 ± 24</td>
<td></td>
</tr>
<tr>
<td>RMSSD, msec</td>
<td>Exenatide</td>
<td>28 ± 20</td>
<td>34 ± 35</td>
<td>32 ± 36</td>
</tr>
<tr>
<td>Glargine</td>
<td>31 ± 24</td>
<td>15 ± 6</td>
<td>27 ± 21</td>
<td></td>
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<tr>
<td>LF/HF ratio</td>
<td>Exenatide</td>
<td>4.5 ± 3.8</td>
<td>4.8 ± 4.6</td>
<td>4.9 ± 5.7</td>
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<td>Glargine</td>
<td>11.1 ± 17.2</td>
<td>6.7 ± 6.7</td>
<td>5.0 ± 3.6</td>
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<td>Valsalva ratio</td>
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<td>1.4 ± 0.5</td>
<td>1.4 ± 0.3</td>
<td>1.3 ± 0.2</td>
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<td>Glargine</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>1.4 ± 0.4</td>
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<tr>
<td>E/I ratio</td>
<td>Exenatide</td>
<td>1.2 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>Glargine</td>
<td>1.2 ± 0.3</td>
<td>1.1 ± 0.03</td>
<td>1.1 ± 0.04</td>
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</tr>
</tbody>
</table>

Data are presented as mean ± SD.

HR: heart rate, SDNN: standard deviation of normal RR interval, RMSSD: root mean square difference of successive RR interval, LF: low frequency power, HF: high frequency power, E/I ratio: expiration inspiration ratio.

The effects on heart rate observed in this study contrast, however, with those observed in the majority of human trials that evaluated the glucose-lowering effects of GLP-1 receptor analogs involving diabetic and/or obese subjects which all reported persistent increase in heart rate, usually associated with significant decreases in systolic blood pressure, which seems to occur before weight loss (Bharucha et al., 2008; Cefalu et al., 2014; Drucker, Sherman, Bergenstal, & Buse, 2011). Lastly, we did not find any significant differences in any of the NeuroQOL scores in the glargine or exenatide groups. This is somewhat in contrast with other studies that reported some benefit with insulin glargine on more general quality of life measures or treatment satisfaction (Brod, Cobden, Lammert, Bushnell, & Raskin, 2007; Cheng et al., 2015; Lam, 2003) whereas the NeuroQOL specifically captures neuropathy-related quality of life measures.

The treatment of diabetic neuropathy continues to be challenging, with important consequences on patients' morbidity and quality of life.
This investigation were specified to ensure uniformity between treatment groups.

Weaknesses of our study are the small sample size, the relatively short duration of the intervention when placed in perspective with the natural history of DPN and CAN (Dyck et al., 2011), and the high prevalence of CCN at baseline, in spite of the relatively short duration of diabetes. However, higher than expected rates for diabetes complications, including neuropathy, were reported by others even in newly diagnosed patients with T2D (Ratzmann, Raschke, Gander, & Schimke, 1991) or in patients with impaired glucose tolerance (Smith & Singleton, 2008) and metabolic syndrome (Smith & Singleton, 2006). These complications are likely associated with several years of exposure to hyperglycemia prior to a formal diagnosis of diabetes, and the presence of multiple co-morbidities and risk factors including hypertension, hyperlipidemia and obesity among most T2D patient (Callaghan & Feldman, 2013). Given the high prevalence of CCN at baseline, only treatments that promoted improvement, not prevention, could be identified, which further highlights the relevance of the secondary measures that were included in this study design (such as regeneration shown with skin biopsy). Despite the relatively short duration of diabetes, both the groups had a similar prevalence and severity of diabetic neuropathy at baseline which could potentially be explained by the neuropathology stage that might have developed in the pre-diabetic stage and explain the functional changes observed.

In conclusion, in this pilot, proof-of-concept study, exenatide was not superior to insulin glargine in preventing progression of DPN or CAN in patients with T2D at similar levels of glucose control. This is possibly associated with the complexity of the mechanisms involved in the pathogenesis of diabetic neuropathies which could require an integrated approach targeting multiple mechanisms for successful outcomes over a relatively short duration of follow-up. Nevertheless, this study provides some important lessons regarding selection of subjects, study design, and difficulties in translating experimental observations to human trials which may be applied to the design of future neuropathy trials.

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Carolina, 1 0

Cardiovascular 2 7
CABG, 2 vessel 0 1
Atrial fibrillation 0 1
Bilateral ankle cellulitis 0 1
Chest pain 0 1
CHF exacerbation 1 1
NSTEMI 0 1
Pedal edema 1 0
Atrial fibrillation 0 1
Dental 2 0
Bleeding post dental work 1 0
Dental infection 1 0
Endocrine/Metabolic 5 1
Severe hypoglycemia 0 1
Neuroendocrine tumor 1 0
Serum creatinine increase 1 0
Hypothyroid 1 0
Thyrotoxicosis 1 0
Gastrointestinal 6 4
Cholelithiasis 1 0
Diarrhea 1 0
Diverticulitis 1 1
Epi gastric pain nausea 0 1
Lipemia 1 0
Incurred ventral hernia 0 1
Intracatable N/V with fever 0 1
Nausea 2 0
Vomiting 1 0
Persistent nausea 1 0
Hematologic 0 1
Thalassemia B minor 0 1
Infection skin or soft tissue 2 7
Tie Infection 1 1
Infection, skin 0 1
LE cellulitis 0 2
Left athlete's foot 0 1
Left toe cellulitis 0 2
Plantar ulcer and cellulitis 0 1
Mood/Psychological 0 2
Depression 0 2
Musculoskeletal 1 5
Back pain 1 0
Ganglion cyst right knee 0 1
Knee pain 0 1
Plantar fasciitis 0 2
Right knee pain 0 1
Neurological 0 6
CTS 0 1
Dizziness 0 1
Headache after fall 0 1
Left arm numbness 0 1
Lightheadedness 0 1
Neuropathic pain, worsened 0 1
Pregnancy 1 0
Renal 1 0
Nephrolithiasis 1 0
Respiratory 3 10
Asthma exacerbation 0 1
Colds 1 0
Hospitalization – bronchitis 0 2
Idiopathic pulmonary fibrosis 0 1
Pneumonia 0 1
Pulmonary embolism 1 0
Sinusitis 0 1
Status asthmaticus 0 1
strep throat/ear infection 0 1
Upper respiratory tract infection 1 2

Table 3 (continued)

Skin/Dermatologic 1 0
Squamous cell carcinoma 1 0
Surgical procedure 0 6
Ingrown toenail extraction — bilaterally 0 1
Left toe amputation 0 1
Basal cell carcinoma 0 1
Nasal septal repair 0 1
Gastric bypass 0 1
Scheduled surgery 0 1

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Appendix A. Supplementary data

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References


The Metabolic Syndrome and Microvascular Complications in a Murine Model of Type 2 Diabetes

To define the components of the metabolic syndrome that contribute to diabetic polyneuropathy (DPN) in type 2 diabetes mellitus (T2DM), we treated the BKS \(db/db\) mouse, an established murine model of T2DM and the metabolic syndrome, with the thiazolidinedione class drug pioglitazone. Pioglitazone treatment of BKS \(db/db\) mice produced a significant weight gain, restored glycemic control, and normalized measures of serum oxidative stress and triglycerides but had no effect on LDLs or total cholesterol. Moreover, although pioglitazone treatment normalized renal function, it had no effect on measures of large myelinated nerve fibers, specifically sural or sciatic nerve conduction velocities, but significantly improved measures of small unmyelinated nerve fiber architecture and function. Analyses of gene expression arrays of large myelinated sciatic nerves from pioglitazone-treated animals revealed an unexpected increase in genes related to adipogenesis, adipokine signaling, and lipoprotein signaling, which likely contributed to the blunted therapeutic response. Similar analyses of dorsal root ganglion neurons revealed a salutary effect of pioglitazone on pathways related to defense and cytokine production. These data suggest differential susceptibility of small and large nerve fibers to specific metabolic impairments associated with T2DM and provide the basis for discussion of new treatment paradigms for individuals with T2DM and DPN.

Nearly 387 million people have diabetes worldwide, and the epidemic continues to rise at an alarming rate (1). Type 2 diabetes mellitus (T2DM) accounts for 95% of diagnosed diabetes (2), and its complications, including heart disease and stroke, result in significant morbidity and mortality, representing the first and fourth most common causes of death, respectively, in the U.S. (3). The best predictor of T2DM macrovascular complications is the preceding presence of microvascular complications, particularly diabetic polyneuropathy (DPN) and diabetic nephropathy (DN). Although the exact etiology of DPN and DN remain a source of intensive investigation, it is generally believed that hyperglycemia underlies both complications and that glycemic control is the cornerstone treatment for DPN and DN, preventing ulcers, lower-limb amputations, and renal failure (4,5).

We completed a Cochrane review of all available evidence on the role of glycemic control in DPN and discovered that glucose control positively affects DPN in patients with type 1 diabetes mellitus (T1DM) but has little beneficial effect on DPN in patients with T2DM (6), thus supporting the emerging concept that DPN in T2DM is due to the metabolic syndrome and not hyperglycemia alone. Contrasting with DPN, glucose control ameliorates renal injury in T2DM rodents (7), suggesting that glucotoxicity is more important in the pathogenesis of DN in T2DM and complications-specific pathological mechanisms. The metabolic syndrome is present when a patient has at least three of the following five metabolic features: central obesity, hypertension, hyperglycemia, hypertriglyceridemia, and low levels of HDL cholesterol. Although nearly all individuals with T2DM have the metabolic syndrome (8), the combination of features underlying the onset and progression of DPN in T2DM remains unknown. This knowledge is critical if we are to make meaningful inroads into treatment of this common and disabling disorder.
To gain insight into which components of the metabolic syndrome contribute to DPN in T2DM, we turned to the BKS db/db mouse, an established T2DM murine model. The leptin receptor mutation in BKS db/db mice produces robust T2DM and metabolic syndrome features that parallel the human disorder, including hyperglycemia, hyperinsulinemia, hypertriglyceridemia, insulin resistance, and obesity (9,10). At 8 weeks of age, these mice develop painful alloynia, a common early sign of human DPN, and as in man, the disease progresses to frank nerve fiber loss with concomitant sensory loss and abnormal electrophysiology by 16 weeks of age (11). The animals also develop DN, with the expected pathologic glomerular hypertrophy, capillary basement membrane thickening, and podocyte loss as well as decreased renal function as quantitated by lower albumin-to-creatinine ratios (ACRs) (12,13).

In the current study, we treated BKS db/db mice with the thiazolidinedione (TZD) pioglitazone. Pioglitazone stimulates the nuclear receptor peroxisome proliferator–activated receptor (PPAR)-γ and to a lesser degree PPAR-α. When activated by pioglitazone, these genes regulate the expression of insulin-sensitive genes that improve glycemia, decrease triglyceride levels, and increase HDL cholesterol in patients with T2DM. In the current study, pioglitazone treatment of BKS db/db mice for 11 weeks restored glycemic control, normalized measures of serum oxidative stress and triglycerides, and caused significant weight gain with no effect on LDL or total cholesterol. This improved metabolic control normalized renal function but had no effect on nerve conduction velocities (NCVs), measurements of large myelinated fiber function. In contrast, measures of small unmyelinated nerve fiber architecture and function reflected by intraepidermal nerve fiber density (IENFD) and thermal latency testing were significantly improved. Analyses of gene expression arrays of large myelinated sciatic nerves (SCNs) and dorsal root ganglia (DRGs) identified differential pathway regulation by both T2DM and pioglitazone treatment. These results suggest that small and large nerve fibers are differentially impaired by components of the metabolic syndrome.

RESEARCH DESIGN AND METHODS

Animals

Male BKS db/+ and db/db mice (BKS.Cg-m +/+ Leprdb/J, stock number 000642; The Jackson Laboratory, Bar Harbor, ME) were fed a standard diet (5LOD, 13.4% kcal fat; Research Diets, New Brunswick, NJ) with or without 15 mg/kg pioglitazone (112.5 mg pioglitazone/kg chow for a final dose of 15 mg/kg to the mouse) starting at 5 weeks of age and maintained through 16 weeks of age, totaling 11 weeks of pioglitazone treatment. Animals were maintained in a pathogen-free environment and cared for by the University of Michigan (U-M) Unit for Laboratory Animal Medicine. All protocols were approved by the U-M University Committee on Use and Care of Animals and followed the Diabetes Complications Consortium guidelines (www.diacom.org/shared/protocols.aspx).

Metabolic Phenotyping

Body weights were measured weekly, as was fasting blood glucose (FBG) using an AlphaTRAK glucometer (Abbott Laboratories, Abbott Park, IL). Glycosylated hemoglobin (HbA1c) level was measured by the Chemistry Core at the Michigan Diabetes Research and Training Center. The National Mouse Metabolic Phenotyping Center (Vanderbilt University, Nashville, TN) completed plasma insulin measurements and fast-protein liquid chromatography (FPLC) analysis for cholesterol and triglycerides. Plasma hydroxyoctadecadienoic acids (HODEs) were quantified by reverse-phase C-18 high-performance liquid chromatography (JASCO, Essex, U.K.) using a Beckman ODS Ultrasphere C18 Column (Beckman Coulter, Inc., Fullerton, CA) to analyze triphenylphosphine-reduced lipid extracts after base hydrolysis. Analyses of the dansylated derivatives of tyrosine and O,O’-dityrosine were also completed by reverse-phase high-performance liquid chromatography as previously described (14) and quantified using authentic O,O’-dityrosine and tyrosine standard curves.

DPN and DN Phenotyping

At 16 weeks of age, all animals were phenotyped for DPN and DN according to the Diabetes Complications Consortium guidelines (15,16). Briefly, large nerve fiber involvement was assessed through NCV measurements, and small nerve fiber involvement was assessed through IENFD and thermal latency measurements as previously described (10,17). Periodic acid Schiff (PAS) staining was performed on 3-μm-thick fixed kidneys to determine mesangial area as previously described (18,19). Urinary albumin levels, ACRs, glomerular area, and glomerular PAS-positive area were measured using published protocols (18,19).

Affymetrix Microarray Analyses

Total RNA was isolated from SCNs and DRGs from db/+ (n = 6), db/db (n = 6), db/+ with pioglitazone (db/+ PIO) (n = 6), and db/db with pioglitazone (db/db PIO) (n = 6) mice using the RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA integrity was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples meeting RNA quality criteria were analyzed by microarray as previously described (20). Briefly, total RNA (75 ng) was amplified and biotin labeled using the Ovation Biotin-RNA Amplification System (NuGEN Technologies Inc., San Carlos, CA) per the manufacturer’s protocol. Amplification and hybridization were performed by the U-M Comprehensive Cancer Center Affymetrix and Microarray Core Facility using the Affymetrix GeneChip Mouse Genome 430 2.0 Array.

Microarray Data Analysis and Validation

Microarray data were analyzed using our locally established microarray analysis pipelines (20,21). Briefly, microarray files were Robust Multi-array Average normalized using the BrainArray Custom Chip Definition File version 15 (22). Quality was assessed using the affyAnalysisQC R package (http://arrayanalysis.org) with Bioconductor (www.bioconductor.org). Differentially expressed genes
(DEGs) were determined using the intensity-based moderated T-statistic (IBMT) test (23) with a false discovery rate (FDR) cutoff < 0.05. DEGs were obtained by pairwise comparisons either between genotypes (db/+ vs. db/db) or between treatment groups (untreated vs. pioglitazone treatment) for each genotype. The DEG sets were then compared against each other to identify overlapping and unique gene expression changes. Analyses focused on the db/+ versus db/db and db/db versus db/db PIO DEG sets to identify genes that were affected by T2DM and/or pioglitazone treatment.

To identify and compare the overrepresented biological functions among DEG sets, Gene Set Enrichment Analysis was performed using a locally implemented version of the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov) (24). Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were selected as the functional terms, and those with a Benjamini-Hochberg corrected P < 0.05 were selected as significantly overrepresented biological functions in each DEG set. A heat map was generated using the top 10 overrepresented biological functions in each DEG set, with clustering based on significance values (log-transformed Benjamini-Hochberg corrected P values), to visually represent overall similarity and differences between DEG sets.

For technical array data validation, four DEGs (Ucp1, Acca1b, Cidea, Ppargc1a) from DRGs among those with the highest fold change were evaluated by real-time RT-PCR (RT-qPCR) as previously described (20). Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein (Ywhaz) was used as the endogenous reference gene. Primers were selected using PrimerBank (http://pga.mgh.harvard.edu/primerbank) and purchased from Integrated DNA Technologies (Coralville, IA) (Supplementary Table 1).

Statistical Analysis
Statistically significant differences in phenotypic measurements between groups were determined using Prism 6 software (GraphPad Software, La Jolla, CA) and the two-tailed t test. Values are reported as the mean ± SEM.

RESULTS
Diabetes, Oxidative Stress, and Dyslipidemia
BKS db/db mice were significantly heavier than db/+ controls, and pioglitazone treatment increased db/+ and db/db body weights by 19% and 83%, respectively, making db/db PIO animals morbidly obese (Fig. 1A and Supplementary Fig. 1). FBG and %GHb (mmol/mol) were significantly elevated in db/db compared with db/+ animals, whereas pioglitazone treatment normalized both parameters to db/+ control levels (Fig. 1B and C; see Supplementary Fig. 2 for GHb [mmol/mol]). Plasma insulin, however, was elevated in both untreated and pioglitazone-treated db/db animals (Fig. 1D). Conversely, plasma HODE and nitrotyrosine levels were significantly increased in db/db animals and normalized to control levels by pioglitazone treatment (Fig. 1E and F).

Lipid analyses revealed a 5- and 12-fold elevation in total plasma triglycerides and VLDL triglycerides, respectively, in db/db relative to db/+ mice; both parameters were normalized by pioglitazone treatment (Fig. 2A and B). In contrast, there was no statistically significant difference in total plasma cholesterol across experimental groups, regardless of treatment (Fig. 2C); however, FPLC analysis of pooled plasma samples revealed an apparent increase in the LDL fraction of pioglitazone-treated db/db mice compared with untreated db/db mice (Fig. 2D).

DPN Phenotyping
Sural and sciatic NCVs, measures of large fiber function, were decreased in db/db relative to db/+ mice, with no treatment effect on either measurement (Fig. 3A and B). Hindpaw withdrawal latency, a behavioral assessment of thermal sensation that represents small fiber function, was significantly increased in db/db compared with db/+ animals and was normalized by pioglitazone treatment (Fig. 3C). IENFD, a measure of small fiber architecture, was significantly decreased in db/db compared with db/+ animals and was significantly improved by pioglitazone treatment (Fig. 3D and E).

DN Phenotyping
Quantitation of renal function by measuring 24-h urinary albumin secretion and ACR revealed a 300- and 1,000-fold increase, respectively, in db/db mice (Supplementary Fig. 3A and B), and pioglitazone treatment normalized both functional measurements to control db/+ levels. In parallel, mesangial matrix accumulation, as measured by the mesangial index, was significantly increased in db/db animals and normalized with pioglitazone treatment (Supplementary Fig. 3C). Morphological changes in glomeruli were also observed in the db/db mice and were rescued by pioglitazone treatment (Supplementary Fig. 4A). Likewise, glomerular area and glomerular PAS-positive area (Supplementary Fig. 4B and C) were significantly increased in db/db animals and normalized with pioglitazone treatment.

DRG and SCN Transcriptomic Profiling
To better understand the differential effect of pioglitazone treatment on small and large nerve fibers, we performed microarray analyses on DRGs and SCNs from the four experimental groups. We identified DEGs, and RT-qPCR of selected DEGs in DRGs reflected comparable profiles to microarray data (Supplementary Table 2), thus validating the microarray findings.

DRGs
In DRGs, 2,082 genes showed significant differential expression between the db/+ and db/db groups (FDR < 0.05), whereas pioglitazone treatment significantly changed the expression of 1,811 and 24 genes in the db/db and db/+ animals, respectively (Fig. 4A). The most highly enriched biological functions among these four DEG sets are shown in Fig. 4B, and the overlap among these sets is summarized in Supplementary Table 3. Further analysis of the db/+ versus db/db and db/db versus db/db PIO DEG sets
to examine the effects of pioglitazone treatment on DPNs (Fig. 4A) also demonstrated that of 1,811 genes affected by pioglitazone, 1,356 were not affected by T2DM (Fig. 4C). In addition, of the 2,082 DEGs regulated by diabetes, 455 genes (22%) were also significantly regulated by pioglitazone treatment, whereas the remaining 1,627 genes were not changed by pioglitazone treatment (Fig. 4C).

As illustrated in Fig. 4D, expression levels of 75% (339 of 455) of the common DEGs were reversed by pioglitazone treatment (e.g., if diabetes increased expression, pioglitazone treatment decreased expression or vice versa). For the remaining 116 genes, pioglitazone treatment exacerbated gene expression in the same direction (Fig. 4D).

We then identified the overrepresented biological functions of the four DEG subsets by functional enrichment analysis (Fig. 4E). Focusing on those enriched among the 455 DEGs regulated by diabetes and altered by pioglitazone treatment, we determined that pioglitazone treatment exacerbated DEGs belonging to several functional enrichment groups, spanning fat cell differentiation, adipocytokine signaling, and HDL binding, with genes including adiponectin (Adipoq), leptin (Lep), Cd36 antigen (Cd36), and GPI-anchored HDL-binding protein 1 (Gpihbp1) (Supplementary Table 5). DEGs regulated by T2DM and reversed by pioglitazone treatment were highly enriched in functions related to defense response and regulation of cytokine production (Fig. 4E).

SCNs
In SCNs, 1,066 genes showed significant differential expression between the db/+ and db/db groups, whereas pioglitazone treatment significantly changed the expression of
4,537 and 1,182 genes in db/db and db/+ animals, respectively (Fig. 5A). The most highly enriched biological functions among the four DEG sets are shown in Fig. 5B, with the overlap among these sets summarized in Supplementary Table 8. A siN DRG s, further analysis of the db/+ versus db/db and db/db versus db/db PIO DEG sets to examine the effect of pioglitazone treatment demonstrated that of the 1,066 DEGs between db/+ and db/db, 484 genes (45%) were also identified as DEGs between db/db and db/db PIO, whereas the remaining 582 genes were not affected by pioglitazone (Fig. 5C). Furthermore, 67% (323 of 484) of the common DEGs were reversed by pioglitazone treatment, whereas the expression of 161 DEGs was exacerbated by pioglitazone treatment (Fig. 5D). The 20 most upregulated and 20 most downregulated genes in these four SCN DEG sets are listed in Supplementary Tables 9–12.

Functional enrichment analysis identified the over-represented biological functions of the four DEG sets, including the 323 reversed genes, 161 exacerbated genes, 582 genes unchanged by treatment, and 4,053 genes

Figure 2—Effects of pioglitazone on plasma triglyceride and cholesterol profiles. After 11 weeks of pioglitazone treatment, total fasting triglycerides (A), plasma triglyceride profiles (B), total fasting cholesterol (C), and plasma cholesterol profiles (D) were assessed. Fasting plasma samples were pooled for each lipid profiling group and fractionated by FPLC. Total triglyceride and total cholesterol profiles were measured in each fraction. In all panels, n = 6. *P < 0.05, **P < 0.01.
Dysregulated only by pioglitazone treatment (Fig. 5E). Specifically, pioglitazone treatment exacerbated DEGs belonging to several functional enrichment groups, spanning protein-lipid complex, endoplasmic reticulum, and response to nutrient levels, with genes including apolipoprotein C-IV (Apoc4), apolipoprotein C-II (Apoc2), and Gpihbp1 (Supplementary Table 11). Alternatively, DEGs regulated by T2DM and reversed by pioglitazone treatment were highly enriched in functions related to collagen, lipid biosynthesis, insulin signaling, neurofilament, and polyol pathway (Fig. 5E).

**DISCUSSION**

Large-scale clinical trials confirm that glucose control alone has little impact on DPN in individuals with T2DM (6), and recent clinical studies suggest that DPN in T2DM is more likely secondary to a constellation of metabolic imbalances that define the metabolic syndrome (25). In contrast, glucotoxicity appears to be more important in the pathogenesis of DN, regardless of diabetes type (7). Thus, the goal of the current study was to confirm that DN is positively affected by glycemic control in T2DM and to define the components of the metabolic syndrome responsible for DPN in a murine model of T2DM. This identification provides not only a first step in identifying modifiable risk factors but also a window into understanding the pathogenesis of DPN.

We chose to study the well-characterized db/db mouse, which by 6 weeks of age is hyperglycemic with hyperphagia and evidence of dyslipidemia. We (9,10) and others (1,26) have also reported that these mice exhibit microvascular complications associated with diabetes, including DPN, manifested as early-onset small (11) and later-onset large fiber neuropathy (9,10) as well as DN (27), retinopathy, and cardiomyopathy. Moreover, with age, these mice become obese with T2DM and severe dyslipidemia (28). In the current study, we treated db/db mice with pioglitazone, a commonly used TZD in man, with the goal of controlling certain, but not all, aspects of the metabolic syndrome. We show that 11 weeks of pioglitazone treatment, given from 5 to 16 weeks of age, results in morbid obesity in diabetic animals, but despite this significant weight gain, pioglitazone treatment normalized glycemic control (FBG and %GHb) and circulating triglycerides, but not insulin or cholesterol levels, to those of nondiabetic animals. These data agree with other studies...
demonstrating that weight gain is a significant adverse effect of various TZDs, including pioglitazone, rosiglitazone, and other PPAR agonists (29,30). Examination of DN in db/db mice following pioglitazone treatment revealed that renal function and architecture are normalized with treatment. These results agree with data from multiple laboratories showing that pioglitazone ameliorates renal injury in T2DM rodents (reviewed in Ko et al. [7]). Similarly, we also reported that the TZD rosiglitazone ameliorates murine DN in T1DM, reduces renal and plasma markers of oxidative injury, and reverses urinary metabolite abnormalities (18).

In contrast to the beneficial effects of pioglitazone on DN, pioglitazone did not completely normalize DPN. We found that only small fiber neuropathy, assessed through IENFD and thermal latency measurements, was normalized with pioglitazone treatment in db/db animals, with no beneficial effects on large fiber function (NCVs). These results are especially interesting in light of the fact that pioglitazone normalized serum %GHb, total triglycerides, and VLDL triglycerides but had no effect on plasma cholesterol levels while promoting gross obesity. Collectively, these data suggest that small fiber neuropathy may be linked to systemic glycemic control and triglycerides, whereas large fiber neuropathy may be linked to systemic cholesterol and gross obesity. Others have reported beneficial effects of TZDs on DPN in T1DM mouse models, but results vary in T2DM rodent models. Yamagishi et al. (31) reported that pioglitazone is beneficial for DPN in streptozotocin-induced T1DM Wistar rats, improving both sciatic and sural NCVs. Troglitazone also improves tibial motor NCVs in streptozotocin-induced T1DM rats.
as well as morphometric measures of myelinated nerve fiber area and axon/myelin ratios (32). Alternatively, other groups reported results in T2DM rodents similar to our own, where TZD treatment normalizes glycemia but has little effect on measures of large fiber function (33,34). Similarly, both groups also reported that TZD treatment has no effect on elevated serum cholesterol levels and promotes animal obesity (33,34). The current data showing that TZD treatment restores small fiber function as measured by thermal latencies and IENFD have also been reported in neuropathic rodent models (35,36). Thus, in the absence of a beneficial effect on large fiber function, it appears that there would be no role for TZD treatments for DPN in man; however, TZD murine treatment paradigms have informed us of both the disease mechanisms and the differential susceptibility of fiber types to metabolic derangements.

As such, transcriptomic profiling of DRGs and SCNs in the current study to assess alterations associated with pioglitazone treatment offers important insight into the mechanisms underlying DPN in T2DM. Although the efficacy of pioglitazone treatment was limited to small fiber measures of DPN, pioglitazone reversed the diabetes-induced changes in 323 genes in SCNs, including genes related to collagen, lipid biosynthesis, insulin signaling, neurofilament, and the polyol pathway, suggesting the...
importance of SCN structure and energy homeostasis in small fiber neuropathy. Additionally, the 339 genes reversed by pioglitazone treatment in diabetic DRGs implicate local immune dysregulation in small fiber neuropathy. Previously, we reported that the PPAR signaling pathway is dysregulated in the SCNs of 24-week-old db/db mice with advanced DPN (20). Indeed, PPAR-γ itself is upregulated in db/db SCNs at 24 weeks (20), suggesting that PPAR-γ agonism through pioglitazone treatment in the current study may stimulate an already upregulated pathway in the db/db nerve, an observation that may contribute to the lack of effect of pioglitazone on large fiber DPN.

Alternatively, pathways exacerbated by pioglitazone treatment in SCNs and DRGs include protein-lipid complex and HDL binding, with the top upregulated genes being Gpihbp1 in both DRGs and SCNs, Apoc4 and Apoc2 in SCNs, and Cdh36 in DRGs. Of note, HDL is involved in reverse cholesterol transport and has antioxidant and anti-inflammatory properties (37). Furthermore, class C apolipoproteins are expressed on the surface of HDLs in the fasting state (38), and mice in the current study were fasted. CD36 is a class B scavenger receptor that binds a number of ligands, including HDLs (39) and oxidized LDLs (40). Dysfunctional HDL signaling is linked to neuron and glial reactive oxygen species production and apoptosis (41). Moreover, pioglitazone treatment upregulated the oxidized LDL (lectin-like) receptor 1 gene (Olr1/LOX1) in db/db SCNs; we have previously reported oxLDL-mediated DRG neuron injury (17). Collectively, these data suggest a role for dysfunctional lipoprotein signaling and cholesterol in large fiber neuropathy in T2DM.

In addition to the exacerbating effect on diabetic changes in gene expression, pioglitazone treatment also affected >5,000 genes in db/db mice that were not affected by diabetes itself (Figs. 4E and 5E). These genes regulated only by pioglitazone in db/db mice were highly associated with mitochondrion in DRGs and with electron transport chain, generation of precursor metabolites and energy, and death in SCNs. Pioglitazone also increased expression of adipogenin (Adig), resistin (Retn), cell death–inducing DNA fragmentation factor α (DFFA) subunit-like effector A (Cidea), cell death–inducing DFFA-like effector C (Cidea), perilipin 5 (Plin5), and leptin, together suggesting local adipogenesis, with lipid accumulation and adipokine signaling (42). The epineurium contains resident adipocytes (43) that secrete paracrine adipokines (e.g., leptin) to modulate peripheral nerve activity (44). Furthermore, rosiglitazone stimulation of the PPAR-γ pathway upregulates adipocyte Cidea expression and increases lipid deposition (42). We propose that with these findings taken together, pioglitazone treatment enhances epineurial adipocyte lipid storage, likely affecting local lipid and protein trafficking to modulate peripheral nerve function.

The current murine data support local dysregulation of cholesterol-lipoprotein signaling and adipogenesis, with local lipid accumulation exacerbated by pioglitazone treatment. We contend that locally secreted factors from modified epineurial adipocytes negatively affect peripheral nerve function, contributing to the maintained large fiber dysfunction observed with pioglitazone treatment. We propose that the treatment effect on systemic glycemia and hypertriglyceridemia, superimposed on T2DM-mediated local mitochondrial dysfunction, is not sufficient to prevent large fiber nerve damage.

These results together with the accumulating published literature on rodent models of T2DM and DPN parallel observations in several large human clinical trials (reviewed in Callaghan et al. [6]). Collectively, these clinical studies strongly support the concept that hyperglycemia is not the singular metabolic derangement underlying DPN in T2DM in man, similar to the current results in mouse, and that abnormalities in other components of the metabolic syndrome contribute to nervous system damage. Indeed, multiple studies have reported that the incidence of DPN increases with increasing number of abnormal metabolic syndrome components (reviewed in Callaghan and Feldman [45]).

Of note, the current murine data suggest that both elevated cholesterol and obesity may be particularly instrumental in inciting nervous system damage. We contend that dyslipidemia and visceral adiposity in man form a network with insulin resistance, hypertension, and hyperglycemia to injure the peripheral nervous system, particularly myelinated large fibers. These murine data support that this network of metabolic impairments activates detrimental feed-forward cycles of local and systemic oxidative stress and dysregulated energy homeostasis with local mitochondrial dysfunction and inflammation, thus resulting in neural injury and DPN. In further support of this idea are the clinical studies showing that dyslipidemia is strongly correlated with DPN (46) and that lowering cholesterol, not glycemia, is significantly associated with decreasing lower-extremity amputations among patients with diabetes (47).

In summary, we report that glycemic control alone is not sufficient to ameliorate injury to large myelinated fibers in murine models of T2DM and DPN, likely because of the persistence of hypercholesterolemia as well as local neural dysfunctional lipoprotein signaling. These findings along with similar results in several large clinical trials in patients with T2DM and DPN collectively suggest that treatment of the metabolic syndrome as a whole and not just hyperglycemia is required to effectively target DPN in T2DM.

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Gender-specific differences in diabetic neuropathy in BTBR ob/ob mice

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Gene expression

A B S T R A C T

Aims: To identify a female mouse model of diabetic peripheral neuropathy (DPN), we characterized DPN in female BTBR ob/ob mice and compared their phenotype to non-diabetic and gender-matched controls. We also identified dysregulated genes and pathways in sciatic nerve (SCN) and dorsal root ganglia (DRG) of female BTBR ob/ob mice to determine potential DPN mechanisms.

Methods: Terminal neuropathy phenotyping consisted of examining latency to heat stimuli, sciatic motor and sural sensory nerve conduction velocities (NCV), and intraepidermal nerve fiber (IENF) density. For gene expression profiling, DRG and SCN were dissected, RNA was isolated and processed using microarray technology and differentially expressed genes were identified.

Results: Similar motor and sensory NCV deficits were observed in male and female BTBR ob/ob mice at study termination; however, IENF density was greater in female ob/ob mice than their male counterparts. Male and female ob/ob mice exhibited similar weight gain, hyperglycemia, and hyperinsulinemia compared to non-diabetic controls, although triglycerides were elevated more so in males than in females. Transcriptional profiling of nerve tissue from female mice identified dysregulation of pathways related to inflammation.

Conclusions: Similar to males, female BTBR ob/ob mice display robust DPN, and pathways related to inflammation are dysregulated in peripheral nerve.

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1. Introduction

The diabetes epidemic is a major medical concern of the 21st century, affecting over 387 million people globally (International Diabetes Federation, 2013). The majority of cases (>90%) have type 2 diabetes (T2D) and display insulin resistance that is typically acquired from poor lifestyle choices combined with genetic susceptibility. T2D is associated with increased morbidity, and the debilitating nature of this disease stems from an array of macrovascular and microvascular complications. Diabetic peripheral neuropathy (DPN) is one such complication that presents in approximately 50% of diabetic patients (Edwards, Vincent, Cheng, & Feldman, 2008) and is a leading cause of diabetes-related hospital admissions and non-traumatic foot amputations in the USA (Centers for Disease Control & Prevention, 2014).

Current therapeutic options for DPN rely on controlling and treating T2D; however, significant gender dimorphisms in the responsiveness of patients to anti-diabetic drugs have been reported (Donnelly, Doney, Hattersley, Morris, & Pearson, 2006; Kim et al., 2005; Osterbrand, Fahlen, Oden, & Eliasson, 2007). These observations highlight the importance of elucidating gender-specific differences in diabetes disease manifestation; a decree which has been iterated by the National Institutes of Health (Clayton & Collins, 2014). To date, clinical studies investigating the role of gender in relation to obesity and diabetes are limited (Gale & Gillespie, 2001) but demonstrate that although T2D does not discriminate between gender, typically affecting males and females alike, young males are at a higher risk of developing insulin resistance (Geer & Shen, 2009; Macotela, Boucher, Tran, & Kahn, 2009) and T2D (Ding, Song, Malik, & Liu, 2006; Wild, Roglic, Green, Sicree, & King, 2004). Clearly, with differences in prevalence and drug-responses between males and females, treatments tailored to gender-type are needed. Moreover, pre-clinical research utilizing both male and female models of diabetes is required, as the varying responses of gender to anti-diabetic drugs is a likely consequence of pre-clinical studies primarily utilizing male mouse models to avoid the distinct differences in female physiology and metabolism. Although female
mouse models of diabetes are readily available, reports on the gender-specific differences observed in diabetic complications are either limited, or in the case of DPN, absent. Thus, characterization of DPN in female mice is important to establish the differences in physiological profiles between the sexes, including time of onset, degree of severity, and response to disease modifying agents.

In the current study, effects of gender were investigated in female and male BTBR ob/ob mice, with an emphasis on identifying differences in DPN severity in the context of comprehensive diabetes phenotyping. Both male and female BTBR ob/ob mice present with a condition similar to T2D (Clee, Nadler, & Attie, 2005), and we recently confirmed that male BTBR ob/ob mice display a robust neuropathic phenotype as early as 9 weeks (O’Brien et al., 2014). Previous examination of diabetes phenotypes in male and female BTBR ob/ob mice have revealed marked deficits in metabolic homeostasis between gender, with more severe metabolic perturbations in males that include increased hyperglycemia, hypertriglyceridemia, insulin resistance, and dyslipidemia (Clee et al., 2005; Hudkins et al., 2010). Thus, as these components of the metabolic syndrome are known to be involved in DPN pathogenesis, we hypothesized that females would display a milder neuropathic phenotype, similar to observations seen in the human population (Aaberg, Burch, Hud, & Zacharias, 2008). As this was the first instance of DPN characterization in a female model, we also performed gene expression profiling on dorsal root ganglia (DRG) and sciatic nerve (SCN) of female mice to identify differentially expressed genes (DEGs) that contribute to DPN in female mice and may provide insight into underlying disease mechanisms.

2. Materials and methods

2.1. Animals

Male and female BTBR ob/+ and ob/ob mice (n = 4; BTBR.Cg-Lepob/ob; Wisc, Jackson Laboratory, Bar Harbor, ME) were fed a standard diet (SLOD: 13.4% kcal fat; Research Diets, NJ). All procedures complied with protocols established by the Diabetic Complications Consortium (DCC) (Sullivan, Lentz, Roberts, & Feldman, 2008) and approved by the University of Michigan (U-M) University Committee on Use and Care of Animals (UCUCA). Daily monitoring and maintenance of mice was provided by the U-M Unit for Laboratory Animal Medicine (ULAM).

2.2. Metabolic and neuropathic phenotyping

Male and female BTBR ob/+ and ob/ob mouse phenotyping included both metabolic and neurological measures at ~24 wks. Terminal body weights and fasting blood glucose (FBG; 4 hr fast) were measured. Percent glycosylated hemoglobin (%GHB) was measured by the Chemistry Core at the Michigan Diabetes Research and Training Center (MDRTC), while plasma insulin, cholesterol and triglyceride measurements were performed by the National Mouse Metabolic Phenotyping Center (MMPC; Vanderbilt, TN and University of Washington, WA). Nerve conduction velocities (NCVs) were measured according to published protocols (Sullivan et al., 2007; Vincent et al., 2009), and at study termination, intraepidermal nerve fiber (IENF) density profiles were determined as previously described (Sullivan et al., 2007).

2.3. Affymetrix microarray

RNA isolated from DRG and SCN of five female BTBR ob/ob and BTBR ob/+ mice was used for microarray hybridization. Total RNA (75 ng) from each sample was amplified and biotin-labeled using the Ovation™ Biotin-RNA Amplification and Labeling System (NuGEN Technologies Inc., San Carlos, CA) according to the manufacturer’s protocol. Amplification and hybridization was performed at the University of Michigan DNA Sequencing Core’s Affymetrix and Microarray Core Group (Ann Arbor, MI) using the Affymetrix GeneChip Mouse Genome 430 2.0 Array. To validate microarray data, DEGs were ranked by fold-change (Tables 1 and 2) and several of the most highly altered DEGs were analyzed by real time RT-PCR (RT-qPCR) using Ywhaz as the endogenous reference gene as previously described (O’Brien, Hur, et al., 2014). The genes chosen for validation along with fold-change compared to controls are provided (Supplemental Table 1). Primers were designed in house, optimized, and purchased from Integrated DNA Technologies (Supplemental Table 2).

2.4. Data and microarray analyses

Body weight, blood glucose and %GHB levels, plasma cholesterol and triglyceride levels, thermal latency measures, IENF density, and NCVs of BTBR ob/+ and ob/ob mice for each gender were compared using two-tailed T-test in GraphPad Prism version 6 for Windows (San Diego, California).

Microarray data were analyzed using our established in-house microarray data analysis pipeline (Hur et al., 2011; O’Brien, Hur, et al., 2014; Pande et al., 2011). Briefly, Affymetrix raw data files (CEL files) were processed using a local copy of GenePattern, a bioinformatics platform from the Broad Institute (Reich et al., 2006). The samples were Robust Multi-array Average (RMA) normalized using the BrainArray Custom Chip Definition File (CDF) ENSEMBL version 16 (Dai et al., 2005). The raw and processed microarray data have been deposited into the NCBI Gene Expression Omnibus data repository (http://www.ncbi.nlm.nih.gov/geo, accession # GSE70852). Intensity-Based Moderated T-test (IBMT) (Sartor et al., 2006) identified DEGs using a false discovery rate (FDR) < 5% cutoff. DEGs were obtained between control (ob/+) and diabetic mice (ob/ob) in DRG and SCN. Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) (Huang da, Sherman, & Lempicki, 2009a, 2009b) identified significantly enriched biological functions among the DEGs in terms of Gene Ontology (GO; http://www.geneontology.org/) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathways. Heatmaps were generated using the top 10 most enriched biological functions in each DEG set based on significance values (log-transformed Benjamini-Hochberg (BH)-corrected P-values) to visually represent the overall similarity and differences between the DEG sets. DEGs from our previously published 5- and 13-week BTBR ob/ob mice were included in the comparison identify commonly dysregulated pathways between male and female DPN models.

3. Results

3.1. Female and male BTBR ob/ob mice develop robust DPN

We recently reported that male BTBR ob/ob mice rapidly develop considerable peripheral nerve deficits after diabetes onset (O’Brien, Hur, et al., 2014). Thus, our initial goal for this study was to assess phenotypic differences associated with gender using this robust mouse model of diabetes with established DPN. Male mice display signs of extreme morbidity sooner than females; therefore, male and female mice were sacrificed at 22 wk and 26 wk, respectively. At terminal stages, electrophysiological testing and quantification of IENF densities confirmed the presence of neuropathy in both obese male and female mice compared to gender-matched non-diabetic controls (Fig. 1). NCVs of the sciatic nerve (motor; MNCV) and sural nerve (sensory; SNCV) were both significantly decreased (Fig. 1A, B), with obese male mice demonstrating a ~1.7-fold decrease in MNCV and obese females displaying a ~1.5-fold decrease compared to their respective controls. Similar SNCV deficits were also observed; males
exhibited a ~1.3-fold decrease while females exhibited a ~1.4-fold decrease relative to lean control mice. Morphological analysis was performed to assess IENF innervation (Supplementary Fig. 1) and quantification of IENF densities revealed significantly lower IENF levels in diabetic mice compared to the respective non-diabetic controls (Fig. 1C). Further assessment indicated that IENF loss was greater in obese males (~2.7-fold decrease) than females (~1.4-fold decrease). Additional behavioral testing of nerve function confirmed a loss of sensation in female ob/ob mice, as evidenced by decreased sensitivity to thermal stimuli in tail flick and hind-paw testing (Supplementary Fig. 2A, B). Combined, these physiological and anatomical DPN measures confirm a peripheral neuropathic phenotype in both male and female ob/ob mice that is similar to that seen in T2D patients, although female BTBR ob/ob mice display a less severe loss of IENFs despite being 4 wk older than males.

### Table 1

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### Table 2

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Fig. 1. Female BTBR ob/ob mice display robust neuropathy characterized by electrophysiological and morphological deficits. Analysis of sciatic (A; Motor NCV) and sural (B; Sensory NCV) nerve conduction velocity in male and female BTBR ob/+ and ob/ob mice ~24 weeks of age. C. Quantification of IENF density in male and female BTBR ob/+ and ob/ob mice. Male BTBR mice are represented by blue filled circles while female mice are shown in pink filled circles. Means ± SEM, n = 4–5 per group. *P < 0.05; ***P < 0.0001 vs. gender-matched, non-diabetic ob/+ mice.

3.2. Female and male BTBR ob/ob mice share a similar, robust diabetic phenotype

Both male and female BTBR ob/ob mice exhibit the key physical and metabolic features of obesity and T2D (Fig. 2). At study termination, both male and female BTBR ob/ob mice were severely obese (68.1 ± 4.04 and 61.8 ± 3.56 g, respectively), exhibiting a
males and 1.65-fold higher in female obese mice relative to controls, non-diabetic ob/+ controls, respectively (Fig. 2A). Likewise, %GHb was 1.9-fold higher in female BTBR ob/ob mice further confirmed severe hyperinsulinemia compared to control mice (female ob/+ = 1.32 ± 0.13 S.E.M.; male ob/+ mice = 1.28 ± 0.16 S.E.M.), with levels exceeding the threshold of detection (maximum threshold = 10 ng/ml).

3.3. Gene set enrichment analysis indicates inflammation as a prominent dysregulated pathway in the peripheral nerve of female BTBR ob/ob mice

To complement our neuropathy phenotyping, microarray analysis was performed on RNA isolated from SCN and DRG to identify dysregulated genes in female BTBR ob/ob mice. Using a 5% FDR cutoff, 584 and 1,105 DEGs were identified in SCN and DRG, respectively. The 10 most up-regulated and down-regulated DEGs identified in DRG and SCN tissues are listed in Tables 1 & 2, respectively. Subsequently, gene set enrichment analysis identified numerous biological functions dysregulated in female mice with established DPN. Overrepresented pathways in female BTBR ob/ob mice included functions related to inflammation and the immune response (Fig. 3), similar to our findings in 13 wk male BTBR ob/ob mice (O’Brien, Hur, et al., 2014). There were 131 common DEGs between the DRG and SCN sets, 124 of which show concordant changes in gene expression. The common DEGs are listed in Supplementary Table 3, while Supplementary Fig. 3 illustrates the most over-represented (enriched) biological functions among these DEGs. We found numerous significantly enriched terms among the concordant DEGs, including complement activation, humoral immune response, and inflammatory response (Supplementary Fig. 3), suggesting that inflammatory processes occur within both tissues.

4. Discussion

The rising interest in gender-based differences in diabetes (Clayton & Collins, 2014) has highlighted the need for further study into the effects of sex on T2D pathogenesis and the development of diabetic complications. We report here the first instance of a female T2D mouse model presenting with a neuropathic phenotype. Similar

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to male BTBR ob/ob mice, female BTBR ob/ob mice exhibit robust peripheral neuropathy, including decreased IENF density, impaired motor and sensory NCVs, and thermal hypalgesia. Moreover, application of our established microarray and gene set enrichment analysis approach (Hur et al., 2015; Hur et al., 2011; O’Brien, Hur, et al., 2014; Pande et al., 2011) to DRG and SCN of female BTBR ob/ob mice identified genes and biological functions related to inflammation and the immune response. These functions are similar to our previously reported findings in male BTBR ob/ob mice (O’Brien, Hur, et al., 2014), providing further evidence that these pathways are implicated in DPN pathogenesis.

We are the first to report that female BTBR ob/ob mice display significant deficits in nerve function as well as decreased IENF, thus indicating a similar neuropathic phenotype to that observed in T2D patients. With the general consensus that male BTBR ob/ob mice are at greater risk for development of hyperglycemia, insulin resistance, and related metabolic abnormalities (Clee et al., 2005; Hudkins et al., 2010) whereas females display mild protection (Gale & Gillespie, 2001), we anticipated a more robust DPN phenotype in males compared to females. However, upon examination of terminal electrophysiological measures, we observed no noticeable differences in MNCV and SNCV between genders at study termination (Fig. 1, A,B), suggesting that electrophysiological functions are similarly impaired across gender. It is unknown, however, whether metabolic imbalances between genders at disease onset may impact nerve function earlier than 26 wk. Thus, further assessment of sex-dimorphisms in nerve function is warranted, as differences may be identified during early stages of DPN development. Future studies will include side-by-side NCV assessments earlier in the disease course to determine if gender-related differences are present. Although no discernable nerve electrophysiology differences were observed, assessment of IENF density, considered the most accurate measure of small fiber neuropathy (Pittenger et al., 2004), revealed that male BTBR ob/ob mice exhibited greater IENF loss than females (Fig. 1C). These findings suggest that the greater small fiber loss in male BTBR ob/ob mice may possibly be a result of more robust increased metabolic perturbations (described below). In support of this idea, small fiber neuropathy in humans is associated with obesity and dyslipidemia, as seen in the male BTBR ob/ob mice, more than hyperglycemia (Smith & Singleton, 2013).

Our terminal metabolic phenotyping involved measuring weight, %GHb, plasma triglycerides, and fasting plasma insulin (Fig. 2), hallmarks typically increased in diabetes and obesity. BTBR ob/ob mice were profoundly heavier than their controls (Fig. 2A) and had hyperglycemia and poor glycemic control (Fig. 2B), hypertriglycerideremia (Fig. 2C), and hyperinsulinemia. %GHb, a marker of glucotoxicity and a hallmark of diabetes, was only mildly more elevated in male than in female BTBR ob/ob mice at study termination when comparing fold-change in relation to non-diabetic controls (Fig. 2B). This mild increase in plasma glucotoxicity was expected due to the increased FBG typically found in males and is consistent with previous reports in this model (Askari et al., 2014; Clee et al., 2005).

As dyslipidemia has recently been identified as a contributory factor in DPN progression in both humans and mice (Vincent, Hinder, Pop-Busui, & Feldman, 2009), plasma triglycerides were measured in male and female BTBR ob/ob mice. Although male and female BTBR ob/ob mice similarly display an abnormal lipid profile compared to controls (Fig. 2), male mice exhibited a greater degree of hypertriglycerideremia which agrees with previous reports (Hudkins et al., 2010). As the increase in triglycerides correlates with decreased IENF, which was more profound than the difference in %GHb between genders, our findings are in line with the hypothesis that lipids, and not just glycaemia, may contribute to DPN progression. A recent Cochrane review investigating blood glucose control in the prevention and treatment of DPN in man found that targeting hyperglycaemia has little effect on neuropathy outcomes in T2D (Callaghan, Little, Feldman, & Hughes, 2012). The observed sex-based dimorphism in our study was anticipated due to greater metabolic imbalances seen in male mice that, as a consequence, would promote DPN and greater IENF loss. Indeed, studies have reported that elevated plasma triglycerides significantly correlate with both decreased sural nerve fiber density in T2D patients (Wiggin et al., 2009) and a loss of small unmyelinated nerve fibers (Smith & Singleton, 2013). Although the morphology of sural nerves was not measured, our findings support that decreased IENFs in ob/ob mice may similarly be the result of lipotoxicity and elevated triglycerides (Fig. 2C), and that lipid lowering therapies may therefore halt DPN progression. Indeed evidence suggests that such therapies may alter the progression of diabetic complications, including diabetic neuropathy (Vincent, Hinder, et al., 2009). Results from the FIELD trial demonstrates that fibrates are effective in treating diabetic nephropathy and retinopathy (Forsblom et al., 2010; Keech et al., 2007), while a report from the Fremantle Diabetes Study suggests that fibrates and statins may protect against the development of sensory neuropathy in patients with type 2 diabetes (Davis, Yeap, Davis, & Bruce, 2008). Furthermore, we have recently published data from C57BKS ob/ob mice treated with pioglitazone, a PPAR-γ agonist, in which decreased plasma triglycerides are associated reduced small fiber neuropathy, as determined by IENF density and latency to thermal response (Hur et al., 2015). We contend that insulin resistance in peripheral nerves is another potential contributor to DPN (Grote et al., 2013). Our results demonstrate that both male and female mice have profound hyperinsulinemia, as fasting plasma insulin exceeded the threshold for detection (10 ng/ml). A comprehensive assessment of insulin resistance in peripheral nerves was beyond the scope of this study; however, as men are at greater risk of developing systemic insulin resistance (Gale & Gillespie, 2001; Geer & Shen, 2009), confirmed in the BTBR ob/ob mouse model (Clee et al., 2005), this sex dimorphism would suggest a similar situation in peripheral nerves where insulin resistance is more pronounced in males, thus promoting greater nerve dysfunction. Furthermore, the increased risk of developing insulin resistance in males may explain the higher fold-change of fasting plasma triglycerides when compared to females. As a consequence of systemic insulin resistance, impaired insulin action on adipocytes results in decreased uptake of circulating triglycerides (Guilherme, Virbasius, Puri, & Czech, 2008), an important feature in maintaining lipid homeostasis. Interestingly, female BTBR ob/ob mice retain adipose tissue insulin sensitivity (Clee et al., 2005). Thus, our findings demonstrating higher levels of circulating triglycerides in male mice may be due to increased adipose tissue insulin resistance, thus contributing even further to nerve dysfunction that is brought upon by peripheral nerve insulin resistance.

We previously performed gene expression profiling on male BTBR ob/ob mice which resulted in the identification of numerous biological functions dysregulated in the peripheral nerve of diabetic mice compared to controls (O’Brien, Hur, et al., 2014); therefore, we performed an identical analysis on SCN and DRG from 26 wk old female BTBR mice. We identified numerous DEGs with large fold changes in female BTBR ob/ob mice compared to controls (Tables 1 and 2). Among the DEGs identified in DRG tissue, several encode proteins implicated in inflammatory signaling pathways, including Crh (corticotropin releasing hormone), which promotes macrophage foam cell formation (Cho, Kang, & Park, 2015), and Sf3a3 (stefin A3), a cysteine protease inhibitor upregulated in lipopolysaccharide-stimulated glial cells (Hosoi et al., 2005) that has a role in protecting cells from inappropriate proteolysis. Saa3 (serum amyloid A3) is an acute phase protein that was also highly overexpressed and is associated with diabetic complications (Hamano et al., 2004). In addition, SAA3 is also a mediator in diabetic kidney disease (Anderberg et al., 2015), and was previously identified as overexpressed in male BTBR ob/ob mouse SCN (O’Brien, Hur, et al., 2014).

Using the publically available DNMKB database (a repository of our completed gene expression profiling in nerves of diabetic animals;
http://jdrf.neurology.med.umich.edu/DNMKB/) to compare the 26 wk old female DEG set with the 13 wk male DEG set, a post-hoc comparison identified 161 commonly dysregulated DEGs between the two genders (Supplementary Table 3). Similar to our findings in female BTBR ob/ob SCN, those with the greatest fold change for both genders included several immunoglobulin family members (Ighg1, Ighg2c and Ighg2b), Mmp12 (matrix metalloproteinase 12), and Ucp1. In addition, S100a8, S1009, Pon1 (paraoxonase 1), and Pmp2 were also similarly dysregulated in both genders. While our current analyses examined gene expression changes in females later in the disease course than our male mouse analyses, we similarly identified dysregulation of biological functions related to inflammation in both the DRG and SCN of female BTBR ob/ob mice, suggesting a common mechanism of nerve injury in both genders (Fig. 3). Although several branches of inflammatory response are likely to be involved in the peripheral nerve environment, collectively, our microarray data suggest an increase in peripheral nerve antigens in BTBR ob/ob mice. As antibodies to neural myelin antigens have been identified in demyelinating diseases (Allen et al., 2005), an increase of IgG immunoglobulins in SCN may similarly due to an increase in neural autoantigens. For example, neuropeptide Y (Npy), a widely expressed protein in the peripheral nervous system that is increased in response to nerve injury (Ji, Zhang, Wiesenfeld-Hallin, & Hokfelt, 1994) is a DEG in our female BTBR ob/ob mice and is a known autoantigen in T1D and T2D patients (Skrarstrand, Dahlin, Lernmark, & Vaziri-Sani, 2013). Interestingly, Mmp12 and S100a8 are involved in inflammation, tissue remodeling, and injury. MMP12 is an extracellular matrix protein involved in collagen degradation and tissue destruction that is produced by Schwann cells (Hughes, Wells, Perry, Brown, & Miller, 2002). S100 proteins are calcium binding proteins expressed in neural tissues, increased in patients with T2D (Krisp et al., 2013), that stimulate a local inflammatory response through binding to RAGE receptors causing a release in proinflammatory cytokines.

One notable downregulated shared DEG identified was Pon1 (similarly decreased in BKS db/db mice Pande et al., 2011). PON1 is an anti-atherosclerotic component of high-density lipoprotein (HDL) and plays a role in the prevention of lipid peroxidation. The PON1 gene is activated by PPARγ, whose expression itself has been found to be decreased in the sural nerve of patients with progressive DPN (Hur et al., 2011). Pomp2 was another common DEG decreased in male and female BTBR ob/ob SCN. Pmp2 is one of the most abundant myelin proteins in the peripheral nervous system, with 15% of myelin comprising of Pmp2, and it is predominantly expressed in myelinated Schwann cells where it has a role as a lipid binding protein and is thought to mediate lipid transport. PMP2-deficient mice exhibit decreased NCVs (Zenker et al., 2014), so decreased expression of Pmp2 in our mouse models of DPN is not surprising, as they too display decreased NCV.

We acknowledge that this current study has some caves. First, while there are multiple concerns with leptin-deficient models in diabetes research, these models remain favorable to high fat diet (HFD)-fed models for generating a robust diabetes with a predictable and extensively characterized neuropathic phenotype (O’Brien, Sakowski, & Feldman, 2014). However, due to the rapid onset of a severe neuropathic phenotype in BTBR ob/ob mice (O’Brien, Hur, et al., 2014), this model may not be suitable for understanding the early subtle changes that occur in the peripheral nerve as a result of metabolic imbalances. In addition, our non-diabetic control animals consisted only of heterozygote BTBR ob/+ mice. As these mice are known to exhibit subtle metabolic differences to wild-type mice (Hudkins et al., 2010) that may also manifest in mild neurological differences, the inclusion of wild-type BTBR mice in future in vivo studies is warranted. Second, for reasons related to study design and execution, side-by-side comparisons between gender at exact ages could not be performed. The primary reason for this is that male BTBR ob/ob mice exhibited signs of ill health at 22 weeks requiring study termination, while female mice remained healthy until the prescribed 26 week termination. Lastly, as this was a preliminary investigation, only a small cohort of animals was phenotyped. Despite these caveats, these analyses support the use of female BTBR ob/ob mice as a novel model for evaluating the effects of gender on DPN mechanisms and treatments.

With the global incidence of T2D on the rise and an increase in the aging population predicted, the number of men and women with diabetes and diabetic complications is set to increase. This inevitable crisis highlights the need for further investigation into how gender influences the development of diabetic complications. Though marginal, there is a higher prevalence of T2D in young men than women (Ding et al., 2006; Wild et al., 2004), likely attributed to the fact that males are more susceptible to insulin resistance than females (van Gennugten et al., 2006). Studies have also demonstrated that (i) the insulin analogue glargine causes a significantly greater decrease in HbA1c in males than females (Osterbrand et al., 2007) and that (ii) males respond better to sulfonylureas than females (Donnelly et al., 2006), while (iii) females respond more favorably to rosiglitazone than males (Kim et al., 2005). Our understanding of sex dimorphisms in diabetes is compounded by underlying physiological differences which are numerous and include differences in glucose control and energy homeostasis (Basu et al., 2006), insulin disposal and clearance (Jensen, Nielsen, Gupta, Basu, & Rizza, 2012), regional fat disposition (Geer & Shen, 2009; Macotela et al., 2009), and sex steroid hormones (Shi & Clegg, 2009). For instance, high levels of estrogen confer protection against diabetes development in women (Le May et al., 2006; Margolis et al., 2004; Shi & Clegg, 2009; Tiano & Mauvais-Jarvis, 2012), and the decreased estrogen production along with increased longevity in post-menopausal women promotes a greater incidence of T2D in this population compared to males, which is also due to increased longevity in this sex (Gale & Gillespie, 2001). Evidence has shown that the incidence of diabetes in females is similar to males prior to puberty or after onset of menopause, suggesting that protection is conferred by female hormone, estrogen. Indeed, estrogen prevents β cell failure in most rodent models of diabetes, demonstrating protection through various pathways (Tiano & Mauvais-Jarvis, 2012). Similar to T2D incidence, DPN appears to be more prevalent in men than women. The impact of gender on peripheral nerve function is evidenced by nerve conduction studies and quantitative sensory testing that demonstrate earlier development of DPN in male patients with either type 1 diabetes (T1D) and T2D relative to their female counterparts (Aaberg et al., 2008). Further studies are needed to verify whether this DPN-predominance persists in men with T2D, as males with T1D are more susceptible to DPN (Gale & Gillespie, 2001).

Our findings demonstrate that female BTBR ob/ob mice exhibit a robust DPN phenotype. Although terminal measures of body weight, hyperglycemia, and hyperinsulinemia were relatively similar at the study conclusion irrespective of gender, male mice exhibited a greater degree of dyslipidemia. The hypertriglyceridemia in male BTBR ob/ob mice highlights the presence of gender-specific differences in this T2D mouse model, and based on recent studies, identifies a feature which may explain why male BTBR ob/ob mice exhibit a greater decrease in iENP densities suggesting a more robust small fiber neuropathy in males compared to females. Although we have provided preliminary data into how sex dimorphisms in diabetes may influence DPN progression, further investigation is required to identify the biological components that confer male susceptibility/female resistance so that tailored gender-specific therapeutic strategies can be devised and implemented.

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N.J.R. P.D.O.B., and J.M.H. conducted animal experiments, researched data, and wrote the manuscript. S.A.S reviewed and edited the manuscript. J.H. researched data and reviewed and edited the manuscript. E.L.F. designed and directed the study, contributed to discussion, and reviewed the manuscript.

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The authors are deeply saddened by the passing of our dear friend and colleague Lisa L. McLean. Lisa died on July 5, 2015 from a sudden illness. Her selfless dedication facilitated many studies in the field of diabetic complications. She will be deeply missed by everyone who knew her.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jddiaco.2015.09.018.

References


The Importance of Rare Subtypes in Diagnosis and Treatment of Peripheral Neuropathy
A Review

Brian C. Callaghan, MD, MS; Raymond S. Price, MD; Kevin S. Chen, MD; Eva L. Feldman, MD, PhD

**IMPORTANCE** Peripheral neuropathy is a prevalent condition that usually warrants a thorough history and examination but has limited diagnostic evaluation. However, rare localizations of peripheral neuropathy often require more extensive diagnostic testing and different treatments.

**OBJECTIVE** To describe rare localizations of peripheral neuropathy, including the appropriate diagnostic evaluation and available treatments.

**EVIDENCE REVIEW** References were identified from PubMed searches conducted on May 29, 2015, with an emphasis on systematic reviews and randomized clinical trials. Articles were also identified through the use of the authors' own files. Search terms included common rare neuropathy localizations and their causes, as well as epidemiology, pathophysiology, diagnosis, and treatment.

**FINDINGS** Diffuse, nonlength-dependent neuropathies, multiple mononeuropathies, polyradiculopathies, plexopathies, and radiculoplexus neuropathies are rare peripheral neuropathy localizations that often require extensive diagnostic testing. Atypical neuropathy features, such as acute/subacute onset, asymmetry, and/or motor predominant signs, are frequently present. The most common diffuse, nonlength-dependent neuropathies are Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, multifocal motor neuropathy, and amyotrophic lateral sclerosis. Effective disease-modifying therapies exist for many diffuse, nonlength-dependent neuropathies including Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, multifocal motor neuropathy, and some paraprotein-associated demyelinating neuropathies. Vasculitic neuropathy (multiple mononeuropathy) also has efficacious treatment options, but definitive evidence of a treatment effect for IgM anti-MAG neuropathy and diabetic amyotrophy (radiculoplexus neuropathy) is lacking.

**CONCLUSIONS AND RELEVANCE** Recognition of rare localizations of peripheral neuropathy is essential given the implications for diagnostic testing and treatment. Electrodiagnostic studies are an important early step in the diagnostic evaluation and provide information on the localization and pathophysiology of nerve injury.

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Peripheral neuropathy includes all conditions resulting in injury to the peripheral nervous system and is best categorized by the localization of the nerve injury. Distal symmetric polyneuropathy, mononeuropathy, and lumbar/cervical radiculopathy are the most common peripheral neuropathies and have been detailed in a separate review. Rare localizations of peripheral neuropathy include diffuse, nonlength-dependent neuropathies, multiple mononeuropathies, polyradiculopathies, plexopathies, and radiculoplexus neuropathies; these neuropathies are particularly important to recognize because they require different diagnostic evaluations and potentially different treatments.

Methods

References were identified from PubMed searches conducted on May 29, 2015, with an emphasis on systematic reviews and randomized clinical trials. Articles were also identified through the use of the authors’ own files. Search terms used included Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP), multifocal motor neuropathy (MMN), amyotrophic lateral sclerosis (ALS), vasculitic neuropathy, sensory neuropathy, motor neuropathy, polyradiculopathy, plexopathy and radiculoplexus, epidemiology, pathophysiology, diagnosis, and treatment.

Rare Subtypes of Peripheral Neuropathy

Diffuse, nonlength-dependent neuropathies present with numbness, tingling, pain, and/or weakness in the arms and legs. Unlike distal symmetric polyneuropathy, symptoms can be present in the upper extremities prior to spreading to the level of the knees and can involve proximal extremities early in the disease course. Atypical neuropathy features are commonly observed, including nonlength-dependent distribution (by definition), acute/subacute onset, asymmetry, and/or motor predominant signs. Symptoms and examination features can involve sensory and motor nerves (most common) or can be confined to sensory (sensory neuropathy) or motor nerves (motor neuropathy). Sensory neuropathies typically present with a sensory ataxia, leading to gait imbalance and frequent falls. Proprioception is often quite impaired, such that when patients close their eyes, they move their extremities without their knowledge. Sensory deficits can be asymmetric and pseudoathetosis may be observed. Patients may experience weakness, but when they visually attend to their muscles, they are able to generate normal force on confrontation testing. A painful form where patients demonstrate mechanical hyperalgesia also exists. Motor neuropathies present with painless weakness, fasciculations, and muscle atrophy without numbness, paresthesias, or other sensory symptoms/signs.

Multiple mononeuropathies present with symptoms in the distribution of multiple nerves. For example, patients may present with numbness, paresthesias, and/or pain in the distribution of the right sural, right tibial, left sural, and left peroneal nerves, with sparing of the left tibial and right peroneal nerves. Pain is a common presenting symptom. Atypical neuropathy features are frequent including asymmetry, acute/subacute onset, motor predominant signs, and non-length-dependent pattern.

Polyradiculopathies present with symptoms and signs that are in a dermatomal and/or myotomal pattern, although this can be difficult to discern if many nerve roots are involved. Radicular pain is a common presenting symptom and is often accompanied by neck and/or back pain. Plexopathies present with symptoms and signs in the distribution of the brachial or lumbosacral plexus. Clues to this localization include symptoms/signs that involve multiple nerves that pass through the same trunk or cord of the plexus but do not share the same nerve root origin. Radiculoplexus neuropathies present with abnormalities in the distribution of multiple nerve roots and the brachial or lumbosacral plexus.

Causes of Rare Subtypes

The differential diagnosis of diffuse, nonlength-dependent neuropathies depends on the underlying pathophysiology and the types of nerves involved (sensory, motor, or both). Common causes are summarized in Table 1.

In those with demyelinating neuropathy affecting sensory and motor nerves, the most common conditions are GBS and CIDP. Guillain-Barré syndrome typically presents with maximal symptoms before 4 weeks from onset, whereas CIDP symptoms usually nadir after more than 8 weeks. In both conditions, electromyogram and nerve conduction studies (EMG/NCS) often reveal acquired demyelinating features (conduction block and/or temporal dispersion) along with other demyelinating features (prolonged latencies, decreased conduction velocities, and prolonged F responses). A lumbar puncture frequently shows elevated protein levels with a normal white blood cell count. Mimics include toxic exposures, diphtheria, and CIDP variants, such as polynuropathy, organomegalgy, endocrinopathy, monoclonal gammapathy, and skin changes (POEMS) syndrome; multifocal acquired demyelinating sensory and motor neuropathy (MADSAM); and IgM anti-MAG neuropathy. Demyelinating motor neuropathies include MMN, whereas demyelinating sensory neuropathies include rare sensory-only GBS and CIDP variants. Diffuse, nonlength-dependent neuropathies that are axonal and involve sensory and motor nerves include toxic exposures, acute sensory motor axonal neuropathy (or axonal GBS), and acute intermittent porphyria. Causes of axonal sensory neuropathies include paraneoplastic syndromes (anti-Hu, SJögren syndrome, chemotherapy (cisplatin), and vitamin B toxicity, and some remain idiopathic. Slowly progressive forms may be seen in those with hereditary sensory and autonomic neuropathy (autonomic symptoms) and Friedrich ataxia (cerebellar and upper motor neuron signs and dorsal column dysfunction). Axonal motor neuropathies include primary muscular atrophy, ALS (upper motor neuron and bulb signs), postpolio syndrome, human immunodeficiency virus, human T-lymphotrophic virus (thoracic myelopathy), West Nile virus, enterovirus D68, MMN without conduction block, radiation injury, and monomelic amyotrophy. Slowly progressive forms may be seen in those with hereditary motor neuropathy, spinal muscular atrophy (including Kennedy disease), and complicated hereditary spastic paraplegia.

The most common cause of multiple mononeuropathies is vasculitic neuropathy (mononeuritis multiplex). Systemic and nonsystemic forms exist, emphasizing the need to evaluate for other systemic features. Systemic vasculitic neuropathy often occurs in the setting of longstanding rheumatologic conditions, such as microscopic polyangiitis, Wegener granulomatosis, polyarteritis nodosa, Churg-Strauss syndrome, cryoglobulinemia, SJögren syndrome, rheumatoid arthritis, and systemic lupus erythematosus. Patients starting with a nonsystemic vasculitic neuropathy rarely evolve sys-
temic manifestations, with most spread restricted to the skin.5 Multiple mononeuropathies can also be seen with neoplasms (including leukemia, lymphoma, lipomas, and neurofibromas), hereditary neuropathy with liability to pressure palsies (HNPNPs), sarcoidosis, amyloidosis, MMN, and MADSAM.

Polyradiculopathy can be caused by compressive lesions such as disc herniation and spondylosis, neoplasms, or osteomyelitis.6 Infectious (cytomegalovirus, varicella-zoster virus, Lyme disease, and tuberculosis), inflammatory (sarcoid), and neoplastic (infiltrative) causes are also seen, as well as radiation-induced injury. The etiology of plexopathy is most commonly trauma/stretch (postpartum and iatrogenic), compression (neoplastic, hemorrhage, and iliac/subclavian aneurysm), radiation, ischemic (aortoiliac occlusive disease), infectious (varicella-zoster virus), inflammatory (brachial plexitis and vasculitis), or inherited (HNPP and hereditary neuralgic amyotrophy).

Radiculopexies neuropathies are most commonly seen in patients with diabetes mellitus. Lumbar involvement (diabetic amyotrophy) is the most common localization and is often seen after weight loss.7 Patients often have severe subacute pain, asymmetric involvement of one leg more than the other, and proximal predominance that improves over the course of several months but often leaves substantial morbidity.8 Cervical involvement is becoming increasingly recognized and patients without diabetes mellitus can also develop this subtype of neuropathy.9 Nerve biopsy often shows signs of a microvasculitis.10 Lumbar and cervical radiculoplexus neuropathies can also be seen in patients after surgery (postsurgical inflammatory neuropathy).11 The nerve damage can occur remotely from the surgical site and can develop within 24 hours to a few weeks postsurgery.

Epidemiology

The most common diffuse, nonlength-dependent neuropathies are GBS, CIDP, MMN, and ALS. The adjusted incidence rate of GBS in the United States was estimated to be between 1.7 and 1.8 per 100,000 person-years from 2000-2004.12 This is similar to the reported average incidence in Canada and Western Europe, described in the eTable in the Supplement. Electrophysiologically and pathologically, GBS can demonstrate either demyelinating or axonal features. In the United States and Western Europe, most patients have acute inflammatory demyelinating polyradiculoneuropathy, as demonstrated by a multicenter study of electrophysiologic features in 369 patients with GBS in whom 69% were demyelinating, only 3% axonal, and 23% equivocal.13 In contrast, in northern China, 65% of patients with GBS have the motor predominant axonal form (acute motor axonal neuropathy) and only 24% have the demyelinating form.14 The in-hospital mortality rate in the United State was 2.58% (128 of 4954) from 2000-2004.12 Beyond the typical ascending paralysis of classic GBS, there are multiple rare variants of GBS, including the Miller-Fisher syndrome, cranial polyneuritis, and pure sensory neuropathy with liability to pressure palsies (HNPNPs), CIDP, chronic inflammatory demyelinating polyneuropathy, orthomyloneuropathy, monoclonal IgG protein, cold agglutinins, disialosyl antibodies; CANVAS, cerebellar ataxia neuropathy vestibular areflexia syndrome; CIDP, chronic inflammatory demyelinating polyneuropathy; CMV, cytomegalovirus; CRMP-5, collapsin response mediator protein 5; DADS, distal-acquired demyelinating syndrome; DMS, distal motor sensory; HNPP, hereditary neuropathy with liability to pressure palsies; HSN, hereditary sensory autonomic neuropathy; HSP, hereditary spastic paraplegia; HSV, herpes simplex virus; HTLV, human T-lymphotropic virus; MADSAM, multifocal acquired demyelinating sensory and motor neuropathy; MGUS, monoclonal gammopathy of unclear significance; MMN, multifocal motor neuropathy; POEMS, progressive systemic sclerosis; POEMS syndrome; CIDP variants: POEMS syndrome, IgM anti-MAG neuropathy, Waldenstrom acroglobulinemia, and MGUS; diphtheria; and toxic exposures (hexane, arsenic, and amiodarone).

Demyelinating sensory

Sensory CIDP or AIDP; and DADS (IgM anti-MAG neuropathy)

Demyelinating motor

MMN

Axonal sensory motor

Toxic exposures; ASAN; and AIP

Axonal sensory

Paraneoplastic (Hu, CRMP-5, and amphiphysin)7; SJögren syndrome; chemotherapy (platinum based, bortezomib); vitamin B12 toxicity; idiopathic; HIV, HTLV; autoimmune hepatitis; celiac disease; HASAN, Friedrich ataxia; CANVAS; SANDO; and CANOMAD

Multiple mononeuropathies

Systemic vasculitic neuropathy: microscopic polyangiitis, Wegener granulomatosis, polyarteritis nodosa, Churg-Strauss syndrome, cryoglobulinemia, SJögren syndrome, rheumatoid arthritis, and SLE; nonsystemic vasculitic neuropathy; neoplasms (malignant and benign); HNPP; sarcoidosis; amyloidosis; MMN; and MADSAM

Polyradiculopathy

Compressive: disc herniation/spondylosis, osteomyelitis, and neoplasm; noncompressive: infection (CMV, VZV, Lyme, and tuberculosis), inflammatory (sarcoidosis), neoplastic (leukemia and lymphoma), and radiation

Plexopathy

Compressive: neoplasm and hemorrhage; noncompressive: infection (VZV, HSV, CMV, and Lyme), inflammatory (sarcoidosis), neoplastic (leukemia, lymphoma), and radiation

Radiculoplexus neuropathy

Diabetic lumbar (diabetic amyotrophy); diabetic cervical; postsurgical inflammatory; nondiabetic lumbar or cervical; infection (VZV, HSV, CMV, and Lyme); inflammatory (sarcoidosis); neoplastic (leukemia and lymphoma); and radiation

Table 1. Common Causes of Rare Subtypes of Peripheral Neuropathy

<table>
<thead>
<tr>
<th>Localization</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse, nonlength-dependent neuropathy; demyelinating sensory motor</td>
<td>AIDP; CIDP; CIDP variants: POEMS syndrome, IgM anti-MAG neuropathy, Waldenstrom acroglobulinemia, and MGUS; diphtheria; and toxic exposures (hexane, arsenic, and amiodarone)</td>
</tr>
<tr>
<td>Demyelinating sensory</td>
<td>Sensory CIDP or AIDP; and DADS (IgM anti-MAG neuropathy)</td>
</tr>
<tr>
<td>Demyelinating motor</td>
<td>MMN</td>
</tr>
<tr>
<td>Axonal sensory motor</td>
<td>Toxic exposures; ASAN; and AIP</td>
</tr>
<tr>
<td>Axonal sensory</td>
<td>Paraneoplastic (Hu, CRMP-5, and amphiphysin); SJögren syndrome; chemotherapy (platinum based, bortezomib); vitamin B12 toxicity; idiopathic; HIV, HTLV; autoimmune hepatitis; celiac disease; HASAN, Friedrich ataxia; CANVAS; SANDO; and CANOMAD</td>
</tr>
<tr>
<td>Multiple mononeuropathies</td>
<td>Systemic vasculitic neuropathy: microscopic polyangiitis, Wegener granulomatosis, polyarteritis nodosa, Churg-Strauss syndrome, cryoglobulinemia, SJögren syndrome, rheumatoid arthritis, and SLE; nonsystemic vasculitic neuropathy; neoplasms (malignant and benign); HNPP; sarcoidosis; amyloidosis; MMN; and MADSAM</td>
</tr>
<tr>
<td>Polyradiculopathy</td>
<td>Compressive: disc herniation/spondylosis, osteomyelitis, and neoplasm; noncompressive: infection (CMV, VZV, Lyme, and tuberculosis), inflammatory (sarcoidosis), neoplastic (leukemia and lymphoma), and radiation</td>
</tr>
<tr>
<td>Plexopathy</td>
<td>Compressive: neoplasm and hemorrhage; noncompressive: infection (VZV, HSV, CMV, and Lyme), inflammatory (sarcoidosis), neoplastic (leukemia, lymphoma), and radiation</td>
</tr>
<tr>
<td>Radiculoplexus neuropathy</td>
<td>Diabetic lumbar (diabetic amyotrophy); diabetic cervical; postsurgical inflammatory; nondiabetic lumbar or cervical; infection (VZV, HSV, CMV, and Lyme); inflammatory (sarcoidosis); neoplastic (leukemia and lymphoma); and radiation</td>
</tr>
</tbody>
</table>

Abbreviations: AIDP, acute inflammatory demyelinating polyneuropathy; AIP, acute intermittent porphyria; ALS, amyotrophic lateral sclerosis; ASAN, acute sensory motor axonal neuropathy; CANOMAD, chronic axonic neuropathy, orthomyloneuropathy, monoclonal IgG protein, cold agglutinins, disialosyl antibodies; CANVAS, cerebellar ataxia neuropathy vestibular areflexia syndrome; CIDP, chronic inflammatory demyelinating polyneuropathy; CMV, cytomegalovirus; CRMP-5, collapsin response mediator protein 5; DADS, distal-acquired demyelinating syndrome; HIV, human immunodeficiency virus; HNPN, hereditary motor neuropathy; HNPP, hereditary neuropathy with liability to pressure palsies; HASAN, hereditary sensory autonomic neuropathy; HSP, hereditary spastic paraplegia; HSV, herpes simplex virus; HTLV, human T-lymphotropic virus; MADSAM, multifocal acquired demyelinating sensory and motor neuropathy; MGUS, monoclonal gammopathy of unclear significance; MMN, multifocal motor neuropathy; POEMS, progressive systemic sclerosis; POEMS syndrome; CIDP variants: POEMS syndrome, IgM anti-MAG neuropathy, Waldenstrom acroglobulinemia, and MGUS; diphtheria; and toxic exposures (hexane, arsenic, and amiodarone); lymphoma, and radiation

The prevalence of CIDP has been reported to vary from 1.2 to 8.9 patients per 100,000 (eTable in the Supplement). The reasons for this wide range include differences in the electrodiagnostic criteria used, inclusion or exclusion of CIDP variants, and the environment and genetic background of the populations studied. Multifo-
Pathophysiology

The catalog of pathophysiologic processes that can adversely affect peripheral nerves is quite extensive. Immunologic, metabolic, genetic, infectious, toxic, and even traumatic processes can damage the peripheral nerves at multiple levels via many molecular pathways. To achieve some degree of organization, it can be worthwhile to understand peripheral nerve pathophysiology in the context of the microscopic anatomy and the sites where disease processes occur (Figure 1).

The origin of the peripheral nerve lies in the neuronal cell bodies located in the dorsal root ganglion for sensory nerves and in the ventral horn of the spinal cord for motor nerves. Naturally, any pathologic process affecting the cell body will result in downstream degeneration of the cell’s axon. Primary motor neuron diseases, such as ALS or spinal muscular atrophy, demonstrate axonal pathology peripherally when central neurons degenerate. Similarly, metabolic conditions, such as diabetes mellitus, metabolic syndrome, nutritional deficiencies, or chronic renal failure, affect dorsal root ganglion cell bodies by mechanisms involving insulin resistance, oxidative stress, and apoptosis.

Pathologic damage may also be considered to take place directly at the axon, independent of the cell body. For example, processes that affect the cytoskeletal components of axons may give rise to neuropathy. Thus, chemotherapeutics, particularly those that affect microtubules, have a propensity to produce neuropathic adverse effects by disrupting axonal structure. Other toxic exposures may disrupt metabolic homeostasis, myelin composition, and mitochondrial function, producing demyelinating and axonopathic disease to varying degrees.

Schwann cells and the myelin sheath are often selectively targeted in immune-mediated processes such as GBS, CIDP, paraproteinemias, and their variants. It is theorized that a phenomenon of molecular mimicry occurs in these diseases, wherein glycoprotein epitopes found in myelin bear structural similarity to those found in other infectious agents (e.g., 

Campylobacter jejuni, cytomegalovirus, and Epstein-Barr virus). Immune recognition of these pathogens then spreads to include normal epitopes on the myelin sheath. Pathologic studies reveal both humoral and cellular immune activation and lymphocytic infiltration with patchy demyelination and remyelination (the classic onion bulb formation). A number of GBS variants that are more prevalent in Asia and Central/South America also damage axons along with myelin and are commonly associated with Campylobacter jejuni infection. Anti-MAG-negative IgM gammopathies, as well as less common gammopathies, can also present with a mixed demyelinating/axonopathic picture.
Atypical neuropathy features include nonlength-dependent distribution, acute/subacute onset, asymmetry, and/or motor predominant signs. Electrodiagnostic testing is the first step in further categorizing the peripheral neuropathy subtype and determines further diagnostic evaluation. Abs indicates antibodies; AMA, antimitochondrial antibody; ANA, antinuclear antibody; ANCA, antineutrophil cytoplasmic antibody; ASMA, antismooth muscle; BJS, Bence-Jones proteins; CBC, complete blood count; COMP, comprehensive metabolic panel; CRP, C-reactive protein; CXL, chest x-ray; EMG, electromyogram; ESR, erythrocyte sedimentation rate; FLC, serum free light chain; GM, GM1 ganglioside antibody; HIV, human immunodeficiency virus; HTLV, human T-lymphotrophic virus; IF, immunofixation; MRI, magnetic resonance imaging; NCS, nerve conduction study; RF, rheumatoid factor; SPEP, serum protein electrophoresis; SSA, Sjögren syndrome antigen A; UA, urinalysis; UPEP, urine protein electrophoresis; VEGF, vascular endothelial growth factor; WNV, West Nile virus.

Hereditary neuropathies can also affect both axons and/or their myelin sheaths. The most common type, hereditary motor sensory neuropathy (or Charcot-Marie-Tooth disease), is classified into many clinical subtypes. Type 1 encompasses demyelinating processes and results from mutations in proteins integral to myelin formation. Type 2 chiefly results in axonal pathologies and involves mutations that affect cellular structure or metabolism. A characteristic of inherited neuropathies is that the entire length of the nerve is affected more or less uniformly given the genetic underpinnings of the disease. Many other genetic syndromes also produce varying disruption of Schwann cell, axon, and/or neuronal function.

Both the Schwann cells and axons of the peripheral nerve depend on delicate vasa nervorum for perfusion and metabolic support. Many metabolic and inflammatory processes, while directly affecting peripheral nerves and neuronal cell bodies, can also result in damage to nerve vasculature and indirectly produce ischemic damage, particularly to axons. Primary vasculitides and other rheumatologic disorders (eg, systemic lupus erythematosus, Sjögren syndrome, and nongranulomatous vasculitis of the peripheral nerves) may compromise vascular supply to nerves. Thus, ischemic changes represent a common neuropathologic mechanism by which peripheral nerves may be damaged.

The interstitial spaces between nerve fibers or between fascicles can represent the principal site of pathology. Infiltrative disorders, such as sarcoidosis or amyloidosis, demonstrate accumulation of material in the endoneurium, perineurium, and epineurium. In lepromatous leprosy, proliferation of the Mycobacterium leprae organism in the interstitial spaces may also contribute to axonal damage. These conditions may involve pathologic mechanisms of ischemia or even damage by direct compression of axons.

As is obvious from the overview presented here, pathologies of the peripheral nervous system can be quite diverse. Often, results from nerve biopsy are a helpful contribution to the overall corpus of evidence when diagnosing atypical neuropathies.

Diagnostic Evaluation
The evaluation of a diffuse, nonlength-dependent neuropathy, summarized in Figure 2, begins with a comprehensive history and examination. Particular attention should be paid to identifying atypical neuropathy features such as acute/subacute onset, asymmetry, and/or motor predominant signs. The medical history is also important to identify conditions known to be associated with atypical neuropathies. The next step is to perform an EMG/NCS to further define the pattern of nerve injury and underlying pathophysiology. Electromyogram/NCS can determine whether the patient has a sensory motor neuropathy, sensory neuronopathy, or motor neuropathy. Furthermore, occasionally, polyradiculopathies and multiple mononeuropathies can be hard to distinguish clinically from diffuse, nonlength-dependent neuropathies, and EMG/NCS can identify these patterns. Importantly, EMG/NCS can also identify whether the underlying pathophysiology is axonal or demyelinating, which has important implications for the differential diagnosis. For demy-
eliminating neuropathies, a lumbar puncture is often helpful to evaluate for elevated protein level and/or white blood cell count.

Patients with a sensory neuronopathy typically require evaluation with Sjögren antibodies, antinuclear antibodies, erythrocyte sedimentation rate and C-reactive protein, paraneoplastic panel, and vitamin B₁₂ levels. Additional testing to consider includes dsDNA, celiac testing, and antismooth muscle and antimitochondrial antibodies. History should focus on identifying previous chemotherapy (platinum based, bortezomib) or rheumatologic symptoms (dry eyes/mouth). For those with a chronic sensory neuronopathy, a focus on family history, including questions regarding autonomic symptoms, is important. If a motor neuronopathy is identified on EMG/NCS, then patients need evaluation for upper motor neuron signs because ALS is a common cause of this localization. The EMG/NCS should thoroughly investigate for conduction block, which is the hallmark of MMN, although conduction block is not always identified in this condition. Physicians should inquire about previous poliomyelitis, previous fever, and family history.

The evaluation of multiple mononeuropathies includes detailed questioning regarding rheumatologic symptoms/signs and symptoms of systemic vasculitis. The diagnostic evaluation should begin with an EMG/NCS to confirm the pattern of nerve involvement and axonal pathophysiology, as well as evaluate for other potential mimics such as MMN (motor nerve conduction block), MADSAM (motor and sensory nerve conduction block), and HNPP (diffuse distal slowing of motor and sensory latencies with slowing across common sites of entrapment), among other possibilities. A detailed rheumatologic workup, including antinuclear antibodies, antineutrophil cytoplasmic antibodies, rheumatoid factor, Sjögren antibodies, dsDNA, and cryoglobulins should be performed, as well as human immunodeficiency virus and hepatitis panel testing. A sural or radial nerve biopsy is needed if concern for vasculitis neuropathy is high. A comprehensive metabolic panel, complete blood count, urinalysis, and chest x-ray are needed to evaluate for systemic manifestations of vasculitis. A detailed family history is important, especially in those with painless multiple mononeuropathies that occur with compression, as is often seen in HNPP.

Those suspected of polyradiculopathy, plexopathy, or radiculoplexus neuropathies also need a comprehensive history and examination to evaluate for systemic conditions such as infectious, inflammatory, and neoplastic causes, as well as previous radiation exposure. Electromyogram/NCS is an invaluable tool to confirm the pattern of injury and distinguish between these 3 entities. Magnetic resonance imaging of the nerve roots and/or plexus is often needed to evaluate for a compressive vs noncompressive etiology, as well as to evaluate for infiltrative conditions. A lumbar puncture is particularly helpful in the evaluation of a polyradiculopathy or radiculoplexus neuropathy.

**Disease-Modifying Therapy**

The importance of identifying these rare subtypes of peripheral neuropathy, such as GBS, CIDP, POEMS syndrome, MMN, and vasculitic neuropathy, is that their course can be modified with particular treatment regimens. The common effective treatments include corticosteroids, intravenous immunoglobulin (IVIGs), plasma exchange, and other immunosuppressive medications. Precise categorization of these rare subtypes is essential because each condition responds to different treatment regimens.

**Guillain-Barré Syndrome**

Many studies have investigated the effects of steroids, IVIGs, and plasma exchange on the rate and degree of recovery in GBS. Each therapy has been the topic of a Cochrane systematic review. The conclusion of the Cochrane systematic review on corticosteroids in 587 patients was that they do not significantly hasten recovery from GBS or affect the long-term outcome. A detailed description of the larger randomized clinical studies that led to this conclusion and other treatment recommendations in inflammatory demyelinating polyneuropathies is provided in Table 2. In contrast to corticosteroids, treatment of patients with GBS with plasma exchange has been shown to increase the rate of recovery and the likelihood of full recovery (5 trials with 404 participants; risk ratio, 1.24; 95% CI, 1.07-1.45) and decrease the likelihood of residual severe weakness compared with supportive care (6 trials with 649 patients; risk ratio, 0.65; 95% CI, 0.44-0.96). Additionally, there have been 2 large studies evaluating the most efficacious number of plasma exchanges in GBS. Compared with 2 sessions, 4 sessions of plasma exchange were shown to reduce the median time to walk with assistance (24 days vs 20 days, respectively; P = .04) and the median time to hospital discharge (26 days vs 21 days, respectively; P = .04), as well as increase the proportion of patients with full recovery of strength at 1 year (64% vs 48%; P = .006). In 161 patients, there was no clear difference between 4 and 6 plasma exchanges. Because plasma exchange was established as an effective treatment of GBS, studies of IVIG in GBS were compared with plasma exchange and not placebo. Intravenous immunoglobulin appears to have similar improvement in disability at 1 month as plasma exchange.

**Chronic Inflammatory Demyelinating Polyneuropathy**

Unlike GBS, patients with CIDP can be treated with corticosteroids, IVIG, or plasma exchange. In CIDP, the largest studies demonstrating efficacy of treatment over placebo were with IVIG. There have been a total of 269 patients in 5 blinded randomized clinical trials of IVIG vs placebo, with the largest being the ICE Study in 2008 with 117 patients. When pooling this data in a Cochrane systematic meta-analysis, IVIG reduced disability in patients with CIDP after 1 month of treatment compared with placebo, with a number needed to treat for an additional beneficial outcome of 3.03 (95% CI, 2.33-4.35). There are 2 small trials of about 30 patients each comparing plasma exchange to sham exchange in patients with CIDP. Both studies demonstrated that plasma exchange provided significant short-term improvement in disability over sham exchange at 4 weeks. A single small trial of 32 patients compared IVIG and plasma exchange in patients with CIDP and found no significant difference in the neuropathy disability score at 6 weeks. Similarly, there have been 2 studies comparing either IVIG and oral prednisolone or IVIG and monthly intravenous methylprednisolone that did not show a statistically significant difference in disability improvement at 6 weeks. More patients discontinued methylprednisolone than IVIG at 6 months for perceived lack of efficacy. Of note, to our knowledge, there have not been any blinded randomized clinical studies demonstrating the efficacy of corticosteroids over placebo in CIDP. Alternative immunosuppressive medications for the treatment of CIDP currently lack high-quality randomized clinical trials demonstrating efficacy. Furthermore, the comparative effectiveness of different treatment regimens for the long-term treatment of CIDP is unclear.
Multifocal Motor Neuropathy

The first-line treatment for MMN is IVIG. A Cochrane systematic review in 2005 found 4 randomized clinical trials with a total of 34 patients that demonstrated improved strength in 78% of patients treated with IVIG compared with only 4% of patients treated with placebo.\(^2\) Since then, Hahn et al.\(^4\) published the largest randomized clinical trial of IVIG in MMN with 44 patients and showed that strength deteriorated in 35.7% of patients taking placebo compared with 11.9% taking IVIG (P = .02), and that disability increased in 31% of patients taking placebo compared with 7.1% taking IVIG (P = .01). To our knowledge, there have been no randomized clinical trials of corticosteroids in MMN. Corticosteroids are not used based on case series and reports. In 1991, Feldman et al.\(^5\) described 13 patients who were treated with oral prednisone regimens of 30 to 100 mg for 6 weeks to 3 years without clinical improvement or reduction of GM1 antibody titer. Of note, 4 of these patients were also treated with plasma exchange without improvement. Four patients reported by Donaghy et al.\(^6\) showed marked motor deterioration within 4 weeks of starting treatment with prednisolone, 60 mg daily. Similarly, in 2 patients described by Van den Berg et al.\(^7\) treated with dexamethasone every 28 days for 6 months, there was marked deterioration of strength. Moreover, to our knowledge, there have been no randomized clinical trials of plasma exchange in MMN and case series and reports have not suggested efficacy.\(^8\) Little data exist to inform long-term treatment of MMN or when refractory to IVIG.

Vasculitic Neuropathy

Nonviral-associated systemic vasculitis is typically treated with corticosteroids and cyclophosphamide to induce remission.\(^9\) Rituximab is an alternative therapy, with evidence supporting that it is noninferior to cyclophosphamide in antineutrophil cytoplasmic antibody-associated vasculitis.\(^6\) Maintenance therapy with azathioprine, methotrexate, or rituximab is often given for 24 months after remission has been achieved.\(^8\) Data in a population with antineutrophil cytoplasmic antibody-associated vasculitis revealed that maintenance with rituximab was superior to azathioprine.\(^7\) Randomized clinical trials in other forms of systemic vasculitis and in patients presenting with vasculitic neuropathy and/or reporting on neuropathy outcomes have not been performed. Similarly, a 2007 Cochrane review revealed no randomized clinical trials have been performed on which to base immunosuppressive treatment for non-systemic vasculitic neuropathy.\(^5\) In the largest observational study in this population (48 patients followed up for at least 6 months), 61% of patients treated with corticosteroids monotherapy had a long-term benefit, while 69% of patients treated with cyclophosphamide had a long-term benefit.

Table 2. Large Randomized Clinical Trials for Inflammatory Demyelinating Polyneuropathies

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of Patients</th>
<th>Treatment</th>
<th>Control</th>
<th>Duration</th>
<th>Disability Improvement at 1 mo(^a)</th>
<th>Common Adverse Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCCTR Group,(^3) 1993 (GBS steroid)</td>
<td>242</td>
<td>IV methylprednisolone, 500 mg</td>
<td>Saline placebo</td>
<td>48 wk</td>
<td>Total of 0.8 vs 0.73 (P = .06)</td>
<td>Hypertension in placebo</td>
</tr>
<tr>
<td>Van Koningsveld et al,(^4) 2004</td>
<td>225</td>
<td>IV methylprednisolone, 500 mg, and IVIG, 0.4 g/kg, for 5 d</td>
<td>IVIG, 0.4 g/kg, for 5 d</td>
<td>1 y</td>
<td>≥1 point in 68% vs 56% (P = .06)</td>
<td>Hypertension and UTI in placebo, elevated blood glucose in treatment group</td>
</tr>
<tr>
<td>GBSS Group,(^5) 1985</td>
<td>245</td>
<td>3-5 PE in 7-14 d</td>
<td>Supportive care</td>
<td>6 mo</td>
<td>≥1 point in 59% vs 39% (P = .01)</td>
<td>No significant difference</td>
</tr>
<tr>
<td>FCGPEGBS,(^6) 1987</td>
<td>220</td>
<td>4 PE in 8 d</td>
<td>Supportive care</td>
<td>6 mo</td>
<td>≥1 point in 61.5% vs 37% (P &lt; .001)</td>
<td>Sepsis in treatment group, blood pressure instability and pneumonia in control group</td>
</tr>
<tr>
<td>PSGBS Study Group,(^7) 1997</td>
<td>383</td>
<td>IVIG, 0.4 g/kg, for 5 d</td>
<td>5-6 PE in 8-13 d or PE followed by IVIG</td>
<td>48 wk</td>
<td>IVIG = 0.8; PE = 0.9; and PE = 1.1 followed by IVIG (no statistically significant difference between any of these 3 groups)</td>
<td>No significant difference</td>
</tr>
</tbody>
</table>

CIDP

Hughes et al,\(^8\) 2008 (ICE Trial) | 117 | IVIG, 2 g/kg, over 2-4 d and then 1 g/kg over 1-2 d every 3 wk | Albumin 24 wk | 5-6 PE in 8-13 d or PE followed by IVIG | ≥1 point in 54% vs 21% (P < .001)\(^b\) | Headache and fever were more common in IVIG group |

**Paraprotein-Associated Demyelinating Neuropathy**

The IgG- and IgA-associated demyelinating neuropathies usually respond to the same immunotherapies as CIDP. In contrast, IgM anti-MAG neuropathy has not been shown to respond to immunosuppressive therapy. In a blinded randomized clinical crossover trial of IVIG vs placebo, there was no significant difference in the strength or functional outcomes in the 9 patients studied.\(^9\) Similarly, in 2 randomized clinical trials of rituximab vs placebo of 26 patients each, there was no significant difference in the primary outcome of sensory improvement.\(^9\) The POEMS syndrome causes a demyelinating neuropathy in the context of osteosclerotic bone lesions. Radiation of these lesions is one potential therapy for localized disease. For patients with clonal plasma cells on bone marrow biopsy, high-dose melphalan followed by autologous stem cell transplantation results in a 75% 5-year progression-free survival.\(^10\)

Abbreviations: CIDP, chronic inflammatory demyelinating polyneuropathy; DCCTR, Diabetes Control and Complications Trial Research; FCGPEGBS, French Cooperative Group on Plasma Exchange in Guillain-Barré Syndrome; GBS, Guillain-Barré Syndrome; GBSS, Guillain-Barré Syndrome Study; IV, intravenous; IVIG, intravenous immunoglobulin; PE, plasma exchange; PSGBS, Plasma Exchange/Sandoglobulin Guillain-Barré Syndrome; UTI, urinary tract infection.

**Values represent points on a 7-point disability scale.**

**Disability improvement on the Inflammatory Neuropathy Cause and Treatment Scale.**
term response, whereas 95% of patients treated with corticoste-
roids in combination with another immunosuppressive agent, usu-
cally cyclophosphamide, had a long-term response. Further high-
quality treatment trials are needed to determine the appropriate ini-
tial and long-term therapeutic strategy in patients with systemic and nonlymphatic vasculitic neuropathy.

Diabetic Amyotrophy

To our knowledge, only 1 randomized trial has been performed in pa-
patients with diabetic lumbosacral radiculoplexus neuropathy. A total of 75 patients were randomized to intravenous methylpredni-
solone vs placebo. The study did not show a statistically signific-
ant difference in the primary end point, but neuropathic pain symptoms were reduced. Overall, this study provides evidence that intrave-
nous methylprednisolone is effective at reducing neurologic deficits.

Conclusions

The presence of key warning signs, such as a non-length-
dependent distribution, acute/subacute onset, asymmetry, and/or motor predominant signs, increases the likelihood of a rare local-
ization of peripheral neuropathy. The identification of these rare pe-
ripheral neuropathy localizations has important diagnostic testing and treatment implications. Electrodiagnostic studies are an impor-
tant early piece of the diagnostic evaluation by providing informa-
tion on the localization and pathophysiology of nerve injury.

REFERENCE


Progress in inflammatory neuropathy —the legacy of Dr Jack Griffin

Eva L. Feldman, Richard A. C. Hughes and Hugh J. Willison

Abstract | The past quarter of a century has brought incredible advances in our understanding of inflammatory neuropathies, and the insights into Guillain–Barré syndrome (GBS) began in the 1990s with the seminal work of Dr Jack Griffin and his colleagues. In this essay, we provide a tribute to Jack, and review the recent progress in a field that he termed his personal favourite. In particular, we discuss the new developments in our understanding and diagnosis of inflammatory neuropathies, the recent emergence of the node of Ranvier and the paranode as sites of intensive investigation, and the mechanistic evidence that is providing a platform for therapeutic development studies.

Introduction

John W. ‘Jack’ Griffin, MD (1942–2011) served as the Editor-in-Chief of the fledgling journal Nature Clinical Practice Neurology from its launch in 2005 (Figure 1, Box 1). Like all things that Jack began, the journal, rebranded as Nature Reviews Neurology since 2009, is a highly successful enterprise. This essay is both a tribute to Jack and a review of the recent developments in a field that he termed his personal favourite: inflammatory neuropathies. Those of us who had the privilege of knowing Jack can say with certainty that he would be unabashedly enthusiastic about the new developments in our understanding and diagnosis of inflammatory neuropathies, and the recent emergence of the node of Ranvier and paranode as sites of intensive investigation.

Inflammatory neuropathies comprise a family of PNS disorders characterized by primary insults to myelin, to the node and/or paranode, to the axon, or to a combination of these structures, resulting in distinct clinical and pathological presentations. The two main classes of inflammatory neuropathies are Guillain–Barré syndrome (GBS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP). The past quarter of a century has brought incredible advances in our understanding of the inflammatory neuropathies, especially GBS. These insights began in the 1990s with the classification and characterization of the GBS variants acute motor axonal neuropathy (AMAN)1 and acute inflammatory demyelinating polyradiculoneuropathy (AIDP)2—findings that were facilitated by collaborative studies assessing a clinical cohort of patients affected by seasonal GBS epidemics in Northern China. Since that time, many of the pathological features of AMAN have been recapitulated in animal models,3–6 which have provided essential mechanistic evidence as well as a platform for subsequent therapeutic development studies.

This remarkable progress would not have been possible without the seminal work of Jack Griffin and his colleagues.1,2,7–13 In particular, his initial examination of autopsy tissue from the Chinese cohort defined the pathological features of AMAN, which included damage focused in motor nerve roots, with macrophage infiltration into axons at nodes of Ranvier, and axonal Wallerian degeneration. These observations supported a proposed immunopathological disease course that involved a Campylobacter-induced antibody response targeted at a ganglioside on the axolemma of motor nerves at the nodes of Ranvier, which triggered complement binding, membrane attack complex formation, and subsequent ion channel dysfunction and impaired nerve conduction. Over time, macrophage recruitment occurred, with invasion of these cells into the perinodal space of affected nerves. Building on Griffin’s earlier work, which identified anti-glycolipid antibodies in GBS,13 these studies also recognized the presence of anti-GD1a antibodies in addition to anti-GM1 antibodies in AMAN.12 Additional research by Griffin and colleagues identified distal axonal degeneration and regeneration, which is one of several possible mechanisms that drive rapid recovery in certain cases.11

Guillain–Barré syndrome

The clinical syndromes

The pathological studies led by Jack Griffin redefined the field of inflammatory neuropathy, and clearly showed different underlying mechanisms in AIDP and AMAN.2,8,10 These discoveries, in turn, presented neurologists with a challenge to distinguish the disease subtypes clinically. Early attempts to make neurophysiological distinctions identified most patients with GBS in North America and Europe as having AIDP with prolonged distal motor latencies, slowed motor nerve conduction velocities with motor nerve conduction block, temporal dispersion, and prolonged F-wave latencies. In these regions, only about 5% of GBS patients seemed to have a primary axonal disease consisting of absent or reduced compound muscle action potentials with relatively preserved motor nerve conduction velocities, compared with 38–67% in northern China, Bangladesh, Japan, and Central America.14–17 Griffin’s work clearly established that preceding diarrhoea and Campylobacter jejuni infection, as well as IgG antibodies to gangliosides, are more common in AMAN than in AIDP, although other studies suggest that there are no discriminating biomarkers for these conditions.18

A third GBS subtype, described initially by Feasby et al.19 and later by Griffin et al.,7 is acute motor and sensory axonal neuropathy (AMSAN). Despite similar antibody profiles, the clinical course of AMSAN is generally more severe than that of AMAN, with slower recovery and a poorer prognosis.20 AMAN involves the ventral roots and

Competing interests

The authors declare no competing interests.
motor nerves, and Griffin discovered that the pathology in AMSAN also extends to dorsal roots and sensory nerves. A related form of acute neuropathy is Miller Fisher Syndrome (MFS), which was initially described nearly 60 years ago by Charles Miller Fisher. In contrast to AIDP, AMAN and AMSAN, this self-limiting illness has few motor nerve electrophysiological abnormalities, but presents with a classic triad of ophthalmoplegia, ataxia and areflexia and, in its pure form, no limb muscle weakness. Table 1 summarizes the key clinical parameters of the three main GBS subtypes and MFS. Table 2 complements Table 1 by presenting the defining pathological studies completed by Griffin and colleagues that served as the foundation for the current classification of GBS subtypes.

More recently, serial neurophysiological studies have shown that the traditional criteria outlined above for AIDP (demyelination), and AMAN and AMSAN (axonal loss) could be leading to underdiagnosis of AMAN, and that the proportion of AMAN cases in European GBS cohorts is higher than originally thought. The difficulty in distinguishing between these conditions arises in part from the occurrence of reversible partial conduction block in motor nerves—most commonly held to be a feature of demyelinating neuropathies—in AMAN (Table 1). This phenomenon is attributable to the engagement of antibodies to gangliosides such as GM1, and possibly other antigens, at the nodes, paranodes and/or juxtaparanodes. Rajabally et al. suggested refinements to the criteria for distinguishing AIDP from AMAN and AMSAN, which might improve their accuracy when used in a single study at an early stage, but these new criteria need to be validated by comparing the results of single studies with those from serial studies in larger numbers of patients.

**Clinical trials and outcome measures**

Early distinction between AMAN and AIDP is potentially important, because the divergent underlying pathologies of these diseases, as discovered by Jack Griffin and colleagues, must reflect fundamentally different disease mechanisms, thereby necessitating distinct treatments. To date, however, treatment trials have not stratified patients according to their neurophysiological subtype, that is, demyelinating versus axonal neuropathy. In view of the effectiveness of corticosteroids in CIDP, it was surprising that trials of corticosteroids showed no evidence of benefit in GBS as a whole.

We can speculate, however, that an adverse effect of corticosteroids in AMAN—not inconceivable in view of their often negative effect in multifocal motor neuropathy—might have masked a beneficial effect in AIDP. Comparative trials over a decade ago showed that intravenous immunoglobulin and plasma exchange produced comparable benefits in patients with GBS, but more evidence is required to determine the relative efficacies of the two treatments in the individual subtypes.

In the past 10 years, no substantial new trials of treatment for GBS have been conducted, although a trial of complement inhibition, predicted to be effective from Jack Griffin’s original pathological observations and supported by animal modelling studies, is ongoing.


Box 1 | John W. ‘Jack’ Griffin, MD (1942–2011)

Jack Griffin was appointed as the Editor-in-Chief of *Nature Clinical Practice Neurology* in 2005 and continued to serve on the Advisory Board when the journal was relaunched as *Nature Reviews Neurology* in 2009. Jack spent his career as a clinician neuroscientist at the Johns Hopkins University School of Medicine in Baltimore, MD, USA, where he served as Chair of the Department of Neurology between 1999 and 2006. In 2007, he founded the Brain Sciences Institute at Johns Hopkins and served as Director until the month before he died.

Jack published over 300 scientific articles, edited textbooks on neuropathy, and served as the President of the Peripheral Nerve Society, the Society for Experimental Neuropathology, and the American Neurological Association. His contributions to neuroscience and neurology were widely acknowledged, and he was elected into the Institute of Medicine (now known as the National Academy of Medicine) in 2004, and received the Johns Hopkins Heritage Award in 2007.

Posthumously, a supplement to the *Journal of the Peripheral Nervous System* (July 2012) was dedicated to him. The articles in this supplement were written by the many grateful neurologists who had the opportunity to work with Jack, including two authors of this essay (E.L.F. and H.J.W.).
need for mechanical ventilation.\textsuperscript{31} These predictive models, which are publicly available on the International GBS Outcome Study (IGOS) website,\textsuperscript{32} are easy to apply, clinically on the International GBS Outcome Study dictive models, which are publicly available need for mechanical ventilation.\textsuperscript{31} These pre- populations being tested.\textsuperscript{35,36} To overcome long-term disability or impairments in the ing the full range of both short-term and and familiarity, they had serious defects, running’.\textsuperscript{37} Their new scale fulfilled the bio- Disability Scale (R-ODS) with items ranging constructed a 24-item Rasch-built Overall disability scales.39 Future trials will benefit from embracing this scale, initi- metric requirements of a linearly weighted scale, and showed more responsiveness to change in patients with GBS or CIDP than did a scale similar to the ODSS,\textsuperscript{38} or motor or sensory impairment scales.\textsuperscript{39} Future trials will benefit from embracing this scale, initially alongside but ultimately replacing the traditional measures.

The nodal complex

Jack Griffin’s early observations two decades ago on the pathology of the node of Ranvier in GBS (Table 2), and the important role of glycolipid antibodies, provided the backdrop for today’s exciting advances in our understand- ing of inflammatory neuropathies. The appreciation that peripheral nerve myelin is organized into discrete subdomains with unique molecular signatures expressed on their surface has opened up a new era for investigation of potential immune targets in inflammatory demyelinating neuro- pathies.\textsuperscript{40,41} Historically, searches for clinically relevant myelin antigens focused on the compact myelin proteins, most notably, peripheral myelin protein 2, 22 and 0 (P2, PMP22 and P0, respectively).\textsuperscript{42} As these proteins can induce an active immunization model of AIDP and CIDP, known as experimental autoimmune neuritis (EAN), they were considered to be prime antigen targets in human disease; however, the human correlative studies have in general yielded disappointing results, despite decades of searching. Equally, and in contrast to the very clear glycolipid antibody biomarker and pathogenesis data in AMAN (as described by Griffin and colleagues\textsuperscript{43,44}) and in MFS,\textsuperscript{45,46} the search for clinically relevant glycolipid anti- gens underlying AIDP and CIDP has been disappointing, despite a wealth of ancillary information pointing us in their direction.

In an attempt to overcome this impasse, attention is currently being focused on axoglial junction adhesion molecules within the nodal complex (Figure 2). Prominent autoimmune targets identified from the plethora of surface-expressed molecules at these sites include neurofascin 155 and 186, gliomedin, contactin-1, and Caspr1.\textsuperscript{45} Autoantibodies against these molecules are identified in only a small proportion of cases, but there is compelling evidence that, when present, they are likely to be pathophysiologically relevant and under-lie disease onset and progression. A prime example is the IgG antibody to contactin-1, which maps to a predominantly subacute-onset, distal, sensory-dominant CIDP with tremor.\textsuperscript{46,47} This condition is resistant to conventional treatment, owing to the IgG4 subclass of the antibodies, but responds well to rituximab therapy\textsuperscript{48}—a finding analogous

### Table 1 | Clinical presentation of the common GBS subtypes

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Demographics</th>
<th>Presentation</th>
<th>Electrophysiology</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute inflammatory demyelinating polyneuropathy (AIDP)</td>
<td>Most common in the USA and Europe, with a prevalence of two cases per 100,000 individuals</td>
<td>Ascending motor weakness with sensory loss; respiratory failure and dysautonomia (25% and 15% of cases, respectively)</td>
<td>Demyelinating neuropathy with prolonged distal latencies and F waves, slowed conduction velocities, temporal dispersion and conduction block</td>
<td>Active area of investigation, but no antibodies yet clearly identified</td>
</tr>
<tr>
<td>Acute motor axonal neuropathy (AMAN)</td>
<td>Most common in Asia, and Central and South America</td>
<td>Ascending motor weakness with no sensory loss; respiratory failure (&gt;50% of cases) and little dysautonomia</td>
<td>Axonal neuropathy with absent or low motor nerve action potentials; normal sensory nerve action potentials</td>
<td>GM1a, GD1a, GM1b, and GalNAc-GD1a</td>
</tr>
<tr>
<td>Acute motor and sensory axonal neuropathy (AMSAN)</td>
<td>Most common in Asia, and Central and South America</td>
<td>As with AMAN, but with sensory involvement</td>
<td>As with AMAN, but with low or absent sensory nerve action potentials</td>
<td>GM1 and GD1a</td>
</tr>
<tr>
<td>Miller Fisher Syndrome (MFS)</td>
<td>Represents 10–25% of GBS cases depending on geographical region</td>
<td>Ophthalmoplegia, ataxia and areflexia, but no muscle weakness</td>
<td>Usually normal except for slowed F waves</td>
<td>GQ1b and GT1a</td>
</tr>
</tbody>
</table>

Abbreviation: GBS, Guillain–Barré syndrome.

### Table 2 | Histological studies by Griffin and colleagues

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cases studied</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>McKhann et al. (1993)\textsuperscript{1}</td>
<td>10 autopsies out of 90 axonal GBS cases in China</td>
<td>Wallerian degeneration of motor nerve fibres, with no inflammation</td>
</tr>
<tr>
<td>Griffin et al. (1995)\textsuperscript{6}</td>
<td>Nine autopsies out of 12 cases of axonal GBS in China, three cases of AMAN; three cases of AMSAN</td>
<td>Wallerian degeneration of motor and sensory nerve fibres, with minor inflammation</td>
</tr>
<tr>
<td>Griffin et al. (1996)\textsuperscript{5}</td>
<td>Seven autopsies—six from the 1995 report and one new case</td>
<td>Ultrastructural analyses revealing the nodes of Ranvier and intermodal axolemma as initial sites of immunopathology</td>
</tr>
<tr>
<td>Griffin et al. (1996)\textsuperscript{5}</td>
<td>Four autopsies—three from the 1995 report and one new case</td>
<td>Ultrastructural analyses confirming Wallerian degeneration in motor and sensory axons</td>
</tr>
</tbody>
</table>

The studies described in this table were instrumental to our current understanding of the different GBS subtypes. Abbreviations: AMAN, acute motor axonal neuropathy; AMSAN, acute motor and sensory axonal neuropathy; GBS, Guillain–Barré syndrome.
to the situation with anti-MuSK antibodies in myasthenia gravis.49

This close phenotype–serotype association in node-protein-antibody-positive CIDP is reminiscent of the earlier historical developments that emerged with anti-ganglioside antibodies, starting with the stratification of IgM paraproteinaemic neuropathy on the basis of anti-myelin-associated glycoprotein and anti-disialosyl antibodies, and proceeding to the categorization of AMAN and MFS variants through anti-ganglioside antibody profiling.50 As a result of such studies, it seems inevitable that CIDP will eventually be segregated into multitudinous clinical phenotypes through immune biomarker discovery, in much the same way that Charcot–Marie–Tooth disorders became subcategorized through identification of novel genes.31

The missing antigens in AIDP

AIDP biomarker discovery still remains shrouded in immunological mist. Nodal complex proteins have not, to date, been shown to be prominent targets, although they have not been exhaustively explored. A report that identified moesin—a component of the ezrin–radixin–moesin (ERM) complex family of ubiquitous cytoskeletal proteins, which in Schwann cells are localized to microvilli abutting the nodal axolemma—as the key target in cytomegalovirus-associated AIDP remains tantalizing yet unconfirmed52 and, indeed, has been challenged.53 The clinical evolution of AIDP, its postinfectious nature and spontaneous immune resolution, response to therapy, and close relationship to AMAN and MFS all suggest that an acute-phase, short-lived myelinotoxic antibody should be identifiable, yet such antibodies, and the nature of their antigens (for example, proteins, glycolipids, or a combination of the two), still evade discovery. Development of new methods and assay platforms involving cell-surface-expressed recombinant proteins, as have been successfully applied to CNS autoimmune disorders, will be an essential step towards addressing this knowledge gap.54

One area, pioneered by Kaida and Kusunoki,55 that possibly warrants further investigation was originally highlighted 10 years ago in the first issue of *Nature Clinical Practice Neurology*.56 These still somewhat theoretical studies indicate that multiple glycolipid and lipid antigens are able to coalesce in the plasma membrane to form new antigens that are not visible in their component parts.57 Recent evidence indicates that these paired antigens are important targets in some cases of AMAN and MFS, in terms of both enhancing and inhibiting antibody binding.58 Determination of the molecular topography underlying this phenomenon is ripe for rapid advances in this multidisciplinary field.59 The extent to which heteromeric and multimeric antigens that accumulate in myelin and other specialized Schwann cell membranes, including those within the nodal complex, are targets for AIDP-associated antibodies requires detailed exploration in future studies.60 So far, no conclusive evidence is available, and methodological improvement in anti-glycolipid assays is an essential step towards further progress in this complex field.61

Concluding remarks

The next decade will surely bring significant advances in our understanding of AIDP, and new therapeutics for all subtypes of GBS. As Jack Griffin always said, “there is nothing better than being a friend of the Schwann cell.” The future will undoubtedly bear out this assertion.


Diabetes and Alzheimer’s Disease
Insulin resistance as a key link for the increased risk of cognitive impairment in the metabolic syndrome

Bhumsoo Kim and Eva L Feldman

Metabolic syndrome (MetS) is a cluster of cardiovascular risk factors that includes obesity, diabetes, and dyslipidemia. Accumulating evidence implies that MetS contributes to the development and progression of Alzheimer’s disease (AD); however, the factors connecting this association have not been determined. Insulin resistance (IR) is at the core of MetS and likely represent the key link between MetS and AD. In the central nervous system, insulin plays key roles in learning and memory, and AD patients exhibit impaired insulin signaling that is similar to that observed in MetS. As we face an alarming increase in obesity and T2D in all age groups, understanding the relationship between MetS and AD is vital for the identification of potential therapeutic targets. Recently, several diabetes therapies that enhance insulin signaling are being tested for a potential therapeutic benefit in AD and dementia. In this review, we will discuss MetS as a risk factor for AD, focusing on IR and the recent progress and future directions of insulin-based therapies.

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Metabolic syndrome (MetS) is a major contributor for the development of diabetes.1 A subject is diagnosed with MetS when he/she has central obesity plus any two of four additional factors, which include elevated triglycerides, reduced high density lipoprotein (HDL) cholesterol, hypertension, or abnormal fasting plasma glucose. The National Health and Nutrition Examination Survey study found that MetS affects 34% of adults in the US.2 The incidence increases with both higher body mass index (BMI) and advancing age. Insulin resistance (IR) is a state of decreased responsiveness of target tissues to normal circulating levels of insulin and is a major feature of type 2 diabetes (T2D), glucose intolerance, obesity, dyslipidemia and hypertension; that is, MetS.3 Both genetic and environmental factors such as a lack of exercise, obesity, smoking, stress, and aging affect the development of IR.4

Alzheimer’s disease (AD) is a slow progressing terminal neurodegenerative disease that can that can remain asymptomatic for several decades.5 It is the most common form of dementia, accounting for over 70% of all cases. Aging is the most definitive risk factor for AD, with the incidence doubling every five years in the population over 65 years old; 50% of people over 85 years old are affected by various degrees of AD. Currently, 5.2 million Americans have AD, and this number is expected to rise to 16 million by 2050 without a breakthrough in the treatment and prevention of the disease (http://www.alz.org/downloads/Facts_Figures_2014.pdf). AD is estimated to cost American society $214 billion in direct medical expenses; a cost expected to rise to $1.2 trillion (in today’s dollars) in 2050. At the cellular level, the most prominent neuropathological features of AD are the appearance of senile plaques composed of amyloid β (Aβ) peptides and neurofibrillary tangles (NFTs) derived from the aggregation of the microtubule-associated protein, tau.6 Clinically, AD is characterized by the loss of memory and other cognitive functions necessary to perform complex daily activities.5

Multiple studies demonstrate a strong connection between MetS and the increased risk of AD.7-9 Accumulating evidence also suggests that AD is closely related to dysfunction of both insulin signaling and glucose metabolism in the brain, prompting some investigators to refer AD as type 3 diabetes or an insulin-resistant brain state.10,11 In this manuscript, we will review recent findings connecting MetS and AD, focusing on IR as the major link between the two diseases.

METABOLIC SYNDROME AND INSULIN SIGNALING

Insulin plays a critical role in glucose homeostasis by regulating the balance between glucose production by the liver and glucose uptake by muscle and adipose tissues. In adipocytes and myocytes, insulin regulates glucose transport by controlling the translocation of the glucose transporter, (Glut)4.12 IR has a rather loose definition, but generally refers to the fact that tissues do not respond sufficiently to physiological insulin
concentrations. T2D patients usually have hyperinsulinemia, and poor insulin sensitivity is a common characteristic of obesity and hyperlipidaemia. In general, IRS serine/threonine phosphorylation inhibits insulin signaling by antagonizing tyrosine phosphorylation. Once activated, the InsR recruits and phosphorylates intracellular substrates, including IRS family proteins and Shc. Phosphorylated IRS residues on IRS and Shc then recruit downstream signaling molecules containing Src homology 2 (SH2) domains, such as the p85 subunit of phosphatidylinositol 3 kinase (PI3K), which activates Akt-mediated signaling, and growth factor receptor-binding protein 2 (Grb2), which leads to the activation of mitogen-activated protein kinase (MAPK) signaling pathway.

In addition to over 20 tyrosine residues, IRS proteins contain more than 50 potential serine/threonine phosphorylation sites. In general, IRS serine/threonine phosphorylation inhibits insulin signaling by antagonizing tyrosine phosphorylation. Increased serine/threonine phosphorylation of IRS-1 accelerates its dissociation from the InsR and downstream signaling molecules, induces mislocalization, and accelerates its degradation by the ubiquitin-proteasome pathway. Multiple IRS serine kinases are activated during IR, resulting in increased IRS serine phosphorylation and impaired insulin signaling.

Increased IRS serine phosphorylation in IR states, including obesity and T2D, are reported in both animal and human studies. Akt (Akt1, Akt2, and Akt3), also known as PKB (PKBα, PKBβ, and PKBγ), is a serine/threonine kinase activated by PI3K downstream of growth factors and various cellular stimuli. Many molecules involved in Akt signaling are the key therapeutic targets for the treatment of human diseases, including T2D and cancer. Akt mediates the bulk of insulin’s action, including glycogen, lipid, and protein synthesis, cell survival, and the anti-inflammatory response. Alterations in Akt activity are one of the key characteristics of IR. Akt2 activation is closely correlated to Glut4 translocation via insulin-activated PI3K signals in adipocytes, and T2D patients have reduced Akt activation in adipocytes and skeletal muscle. Akt2 knockout mice have impaired insulin action in liver and skeletal muscle and develop diabetes with hyperglycemia, hyperinsulinemia, glucose intolerance, and impaired muscle glucose uptake.

In contrast to the decrease in PI3K-Akt activity, the MAPK pathway is relatively unaffected by IR. MAPK pathway activation by insulin signaling is responsible for gene expression, cell growth, and mitogenesis. MAPK acts as an IRS serine kinase in certain conditions; therefore, inappropriate activation of MAPK may worsen IR by increasing serine phosphorylation of IRS and interfering with insulin signaling.

**INSULIN SIGNALING IN THE BRAIN**

Insulin, a peptide secreted by pancreatic beta cells, enters the central nervous system by crossing the blood–brain barrier in a regulated and saturable fashion, although de novo synthesis of insulin in the brain is still under debate. InsRs are widely expressed in the brain, including in the olfactory bulb, cerebral cortex, hippocampus, hypothalamus, and amygdala. InsRs are more concentrated in neurons relative to glial cells and are especially high in post-synaptic densities.

Brain insulin signaling plays critical roles in the regulation of food intake, body weight, reproduction, and learning and memory. Intranasal insulin administration improves working memory in both human and animal studies, and intrahippocampal delivery of insulin improves hippocampal-dependent spatial working memory. In addition, InsR mRNA and protein levels are increased in the hippocampus CA1 region in association with short-term memory formation after a spatial memory task, suggesting that neuronal insulin sensitivity could be enhanced during learning.

Disruption of insulin signaling, however, makes neurons more vulnerable to metabolic stress, thus accelerating neuronal dysfunction. Defective insulin signaling is associated with decreased cognitive ability and the development of dementia, including AD. Poor cognitive performance in diabetes and AD are associated with a decrease in InsR expression and cerebrospinal fluid (CSF) insulin levels. A recent study demonstrated decreases in the phosphorylation of similar insulin signaling molecules in both AD and T2D patient brains, and this decrease was more severe in the brains of the patients with both AD and T2D. Decreased insulin signaling, including altered kinase activity and IRS expression, in AD gets worse with disease progression, and increased basal IRS-1 phosphorylation, a key signature of IR, is evident in the AD brain. Interestingly, the brain regions with the highest densities of InsR, such as the hippocampus and temporal lobe, are also the major targets of neurodegeneration in AD. Therefore, impaired insulin signaling caused by IR can have a profound effect on cognitive decline and the development of AD.

**METS AND AD**

Multiple studies report that patients with MetS have an increased risk of developing AD compared to age- and gender-matched controls, and accumulating evidence suggests that AD is closely related to dysfunction of both insulin signaling and glucose metabolism in the brain, prompting some investigators to refer AD as type 3 diabetes, or an insulin resistant brain state. The incidence of both T2D and dementia rise in later life, increasing the prevalence of the comorbidity of these age-related diseases. Indeed, T2D predicts cognitive decline in older adults and is related not only to vascular dementia (VD), but also to AD. One of the pioneering epidemiological studies about the connection between T2D and dementia is the Rotterdam study, which demonstrates that T2D almost doubles the risk of dementia AD; interestingly, people who were insulin-treated (therefore, with more severe diabetes) at baseline were at the highest risk. Another study on a Japanese-Hawaiian cohort further showed a 1.5-, 1.8-, and 2.3-fold increased risk of total dementia, AD, and VD, respectively, for people with diabetes. The Religious Order Study demonstrated a 65% increased risk of AD among...
T2D patients. Among the diabetes-related factors, higher levels of 2-hour post-load plasma glucose, fasting insulin, and homeostasis model assessment-estimated IR (HOMA-IR) were associated with increased risk for senile plaques after adjustment for age, sex, systolic blood pressure, total cholesterol, BMI, habitual smoking, regular exercise, and cerebrovascular disease. A detailed analysis of 14 high quality longitudinal studies from MEDLINE and EMBASE searches further demonstrates that individuals with T2D have a greater than two-fold increased risk of developing AD compared to individuals without T2D, adjusted for age, sex, education, and vascular risk factors (including a history of stroke, hypertension, and heart disease). The duration of diabetes is also a risk factor for increased cognitive decline, and this may be related to the length of exposure to high levels of insulin combined with the severity of disease. The converse is also true, as patients with AD are also more likely to develop diabetes. The Mayo Clinic AD Patient Registry reveals that 80% of AD patients have either T2D or an impaired fasting glucose level.

Animal studies also demonstrate the connection between diabetes and AD. We have reported that db/db mice, a model of T2D, exhibit age-dependent increases in tau phosphorylation and cleavage. Inducing type 1 diabetes (T1D) and IR by streptozotocin (STZ) treatment or by feeding with a high fat diet (HFD) in AD animal models exacerbates both amyloid and tau accumulation. High-fat and high carbohydrate diet feeding of AD rats significantly increased hyperphosphorylated tau and total tau mRNA compared to rats with T2D or AD alone, and there was also a significant difference in spatial memory deficits between AD and AD+T2D groups.

Obesity is a pathologic state defined by an excessive accumulation and maintenance of adipose tissue. BMI is a simple index of weight-for-height that is commonly used to classify overweight and obese adults (kg m$^{-2}$). Worldwide, obesity has nearly doubled between 1980–2008, with ~35% and ~11% of adults currently overweight or obese, respectively (http://www.who.int/mediacentre/factsheets/fs311/en/). In the United States, the epidemic is even worse; 35% of adults and 15% of children were obese in 2010 and 80% of obese individuals are insulin resistant. Obesity has been associated with several processes related to the acceleration of aging, including the excessive production of free radicals, oxidation, and inflammation. Visceral adipose tissue is a metabolically active endocrine organ, and dysfunction in this organ is responsible for increased plasma free fatty acids (FFAs). The inappropriate accumulation of lipids in muscle and liver due to abnormal fatty acid metabolism is one of the main features of IR. Visceral fat is also infiltrated with inflammatory cells and secretes proinflammatory cytokines, such as interleukin 6 and tumor necrosis factor-α, which are implicated in the development of IR. Furthermore, these proinflammatory cytokines produced by adipocytes can cross the blood-brain barrier (BBB) and induce neuroinflammation and subsequent neurodegeneration. Increased inflammation induces accelerated Aβ deposition and/or decreased clearance and facilitates the polymerization of tau. FFAs also increase tau phosphorylation through astroglia-mediated oxidative stress.

Midlife obesity (measured by BMI) consistently demonstrates a strong and independent association with late-onset dementia and AD. In a 30 year longitudinal study involving 6583 members of Kaiser Permanente of Northern California, central obesity (sagittal abdominal diameter) alone doubled the risk of dementia after adjusting for age, sex, race, education, marital status, diabetes, hypertension, hyperlipidemia, stroke, heart disease, and medical utilization. Studies examining later onset obesity, however, have generated mixed results. In the Cardiovascular Health Study, which involved people 65 years or older, underweight individuals (BMI < 20) had an increased risk of dementia (hazard ratio [HR] = 1.62), whereas obese individuals (BMI > 30) exhibited a reduced risk (HR = 0.63) relative to normal BMI controls. Another study found a decreased risk of dementia with higher BMI in subjects over 76 years old, and BMI and dementia further exhibited a U-shaped correlation in individuals younger than 76 years.

Similar results have been observed in animal studies. Rats fed with diets high in saturated or unsaturated fat for 3 months display impairments in learning and memory tasks and mice fed with a HFD have impaired spatial working memory, as assessed by a T-maze. Furthermore, brains of HFD-fed mice exhibit dysfunctional Akt signaling and increased IRS serine phosphorylation, a marker of IR, and mice fed with a high fat/high cholesterol diet display increased APP C-terminal fragment accumulation and increased tau phosphorylation. These results suggest that obesity-induced peripheral IR alters central insulin signaling and leads to AD-like cognitive impairment.

Dyslipidemia is an important component of IR. Because insulin plays a critical role in lipid metabolism by stimulating lipogenesis and suppressing lipolysis, perturbations in insulin signaling lead to accelerated lipolysis and increased production of FFAs. Approximately 30% of total body cholesterol is contained in the brain; therefore, slight changes in lipid metabolism may have profound effects on cognitive function. Cholesterol is the key component of the plasma membrane, and the processing of APP into Aβ occurs in the plasma membrane. The interaction between cholesterol and APP in the plasma membrane is critical for Aβ production and clearance. In Tg2576 AD mice, a high-fat/high-cholesterol diet significantly increased the production of Aβ, and cholesterol lowering drugs reduced this brain amyloid load by more than two-fold. Lowering cholesterol levels in vitro also stimulates non-amyloidogenic processing of APP, thereby reducing Aβ production.

A meta-analysis of 18 prospective studies ranging from 3–29 years revealed a consistent association between high midlife total cholesterol and an increased risk of AD and dementia. Interestingly, no evidence was found to support an association between total cholesterol later in life and AD. Cohorts of the Seven Countries Study by a Finnish group concluded that high serum total cholesterol is an independent risk factor for AD.
while another Finnish study further demonstrated that high midlife cholesterol levels are positively connected with an increased risk of AD later in life.73 Further, a retrospective cohort study of members of the Kaiser Permanente Medical Care Program of Northern California showed that midlife cardiovascular risk factors including smoking, hypertension, high cholesterol, and diabetes are all positively connected with increased dementia in later life, with diabetes and high cholesterol being the strongest risk factors.74 Despite these findings, however, the connection between cholesterol and dementia is still complex and inconclusive, as some studies show no correlation, and others even demonstrate a protective role for cholesterol.75,76

IR AND TAU
Abnormal phosphorylation of tau has been implicated as a mechanism of AD pathophysiology since the mid-1980s.77 Tau is commonly regulated by post-translational modifications, including phosphorylation, glycation, glycosylation, sumoylation, O-GlcNAcylation, and cleavage.78 In AD, tau is abnormally phosphorylated at its over 80 serine/threonine residues, which leads to the aggregation of tau filaments, appearing as NFTs, in cell bodies and proximal dendrites. Several kinases, including glycogen synthase kinase-3 beta (GSK3β), cyclin-dependent kinase 5, MAPK, and microtubule affinity-regulating kinases, and phosphatases, such as protein phosphatase 2A, are responsible for tau phosphorylation.78 These kinases and phosphatases are the targets of insulin regulation.78–80 As in peripheral tissues, IR mostly affects PI3K-Akt signaling in the brain, and chronic hyperinsulinemia prevents insulin-stimulated Akt phosphorylation in cortical neuron cultures.81 Increased basal Akt phosphorylation is also present in the cortex of T2D db/db mice, and ex vivo insulin stimulation could not increase cortical Akt phosphorylation as it did in non-diabetic control mouse (db+).81 GSK3β is one of the key signaling molecules downstream of Akt82 and is a major tau kinase. Impaired insulin signaling results in aberrant GSK3β activation and increased tau phosphorylation and accumulation.83 Therefore, the precise regulation of Akt signaling is critical for both amyloid and tau neuropathology in AD.

Impaired glucose metabolism due to IR can affect tau pathology via the dysregulation of O-GlcNAcylation. Similar to phosphorylation, O-GlcNAcylation is a dynamic post-translational modification involving the attachment of N-acetyl-D-glucosamine (GlcNAc) moieties to the hydroxyl group of serine and threonine residues.83 O-GlcNAcylation is affected by nutrients in circulation, especially glucose;84 therefore, the accumulation of excess energy associated with obesity and IR can result in the dysregulation of O-GlcNAcylation. Furthermore, in some cases, O-GlcNAcylation may occur at or near the residues that can also be phosphorylated.85 Tau has at least 12 O-GlcNAcylation sites which are mostly inversely correlated with phosphorylation status.83 Recent reports demonstrate that reduced brain glucose metabolism and O-GlcNAcylation leads to increased tau phosphorylation in both in vivo and in vitro models.86 Conversely, increased O-GlcNAcylation prevents pathological tau accumulation.87 Thus, the failure of proper insulin signaling can promote the accumulation of neurofibrillary tau, disrupt neuronal cytoskeletal networks and axonal transport, and lead to a loss of synaptic connections and progressive neurodegeneration.

Abnormal phosphorylation of IRS-1 is also a pathological feature of AD. A recent study examining 157 human brain autopsies demonstrated that IRS-1 serine phosphorylation is increased in multiple sites in AD, and in other tauopathies such as Pick’s disease, corticobasal degeneration and progressive supranuclear palsy.88 Furthermore, increased IRS-1 serine phosphorylation was frequently co-expressed with pathologic tau in neurons and dystrophic neurites.88 Similar results were observed in transgenic PS19 tau mouse brains, where abnormally increased IRS-1 serine phosphorylation co-localized with tangle-bearing neurons.89 Conversely, inducing IR by feeding mice a HFD increased tau phosphorylation and impaired insulin signaling,62,64,90 and high-fat-feeding of animal models of AD exacerbated the pathologies. After 23 wk on HFD, APPswe/PS1dE9 mice displayed severe hyperinsulinemia along with increased tau phosphorylation, Aβ levels, and amyloid burden.91 HFD-feeding from an early age in THY-Tau22 mice also potentiated spatial learning deficits and significantly increased tau phosphorylation,92 and direct disruption of insulin signaling by inducing T1D using STZ93 or IRS-2 knockout94 increased tau pathology. In addition, STZ injections in pR5 tau transgenic mice greatly increased insoluble hyperphosphorylated tau and the later deposition of NFTs, features not observed in control pR5 mice.47 Together, these results suggest that IR accelerates the onset and increases the severity of AD, especially in situations with a predisposition to developing tau pathology.

IR AND AB
Brain insulin signaling plays a critical role in the regulation of food intake, body weight, reproduction, and learning and memory,23 and defective insulin signaling is associated with decreased cognitive ability and the development of dementia and AD.27 AD is characterized by improper expression and accumulation of APP and the accumulation of insoluble neurotoxic Aβ into subsequent senile plaques. Studies show that insulin signaling regulates multiple steps of the amyloid cascade and affects Aβ aggregation in the brain. Insulin increases the transcription of anti-amyloidogenic proteins, such as the insulin-degrading enzyme (IDE) and α-secretase, and stimulates Aβ clearance.95 While GSK3β is the major tau kinase, GSK3α increases Aβ production by stimulating γ-secretase activity.96 Constitutively active Akt inhibits APP trafficking and Aβ secretion through feedback inhibition of IRS and PI3-K.97 APP is modified by O-GlcNAc in a region that may affect its degradation, and a recent report demonstrated that O-GlcNAcylation of APP encourages non-amyloidogenic α-secretase processing, thus decreasing Aβ secretion.98

Conversely, Aβ can affect insulin signaling by competing with or reducing the affinity of insulin binding to its own
receptor or by regulating intracellular signaling. Soluble Aβ binds to the InsR in hippocampal neurons to inhibit receptor autophosphorylation and subsequent activation of PI3K/Akt, and Aβ derived diffusible ligands (ADLs) induce the abnormal expression of InsR and interrupt insulin signaling, thereby potentially contributing to the development of central IR. In addition, Aβ inhibits insulin signaling by c-Jun N-terminal kinase (JNK)-mediated increases in IRS serine phosphorylation. Intracellularly, Aβ prevents the interaction of pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1) with Akt to inhibit Akt activation. Therefore, a feed-forward interaction between impaired insulin signaling and increased Aβ production exacerbates AD pathology in the presence of IR.

In a recent cross-sectional study of the Wisconsin Registry for Alzheimer’s Prevention that included late middle-aged adults, higher IR levels were connected with increased amyloid deposition, as measured by Pittsburgh compound B uptake. Inducing IR in rats using fructose-containing water also enhanced Aβ production by increasing the expression of β-secretase, stimulating γ-secretase activity, and decreasing IDE levels. In cultured primary cortical neurons, insulin reduced Aβ buildup by enhancing the conversion of oligomers to monomers and also prevented Aβ oligomer-induced synaptic toxicity at the level of both synapse composition and structure. In contrast, inhibiting InsR activity by transfecting a kinase-dead mutant receptor or a tyrosine kinase inhibitor increased Aβ oligomer aggregation; similar results were obtained through either InsR knockdown or PI3K pathway inhibition. Thus, dysfunctional insulin signaling due to IR accelerates amyloid pathology, both in human and animal models.

In summary, peripheral MetS induces central IR in the brain. The resulting impaired insulin signaling, which mainly impacts the PI3K/Akt pathway, then increases APP processing/ Aβ levels and tau phosphorylation. Finally, increased Aβ further disrupts insulin signaling to exacerbate AD pathology and cognitive decline (Figure 1).

INSULIN SIGNALING AS A THERAPEUTIC TARGET OF AD

Given the close correlation between brain IR and cognitive impairment, therapeutic approaches using anti-diabetic drugs to improve insulin levels or signaling have been tested to treat AD. In rats, intracerebroventricular (icv) administration of insulin improved spatial learning and memory and reversed AD. In rats, intracerebroventricular (icv) administration of to improve insulin levels or signaling have been tested to treat impairment, therapeutic approaches using anti-diabetic drugs Given the close correlation between brain IR and cognitive AD

Figure 1 MetS and AD Aβ/tau pathology may act in a feed-forward mechanism to accelerate AD pathology in the presence of IR.
facilitated Aβ clearance, reduced amyloid plaques, decreased tau phosphorylation, and improved cognitive function in AD mouse models,\textsuperscript{120,121} and pioglitazone prevented IR and Aβ overproduction that are associated with fructose-drinking in rats.\textsuperscript{104} Some studies, however, show inconsistent results depending on gender and/or genotype of the selected animal models.\textsuperscript{122,123}

Likewise, there are mixed results from human clinical trials with TZDs. An early study demonstrated that mild AD or amnestic MCI patients who received rosiglitazone for 6 months exhibited better delayed recall and selective attention,\textsuperscript{124} and rosiglitazone protected cognitive decline in older individuals with both T2D and MCI.\textsuperscript{125} Similar to intranasal insulin treatment, the effect of rosiglitazone was effective only on the individuals with ApoE-ε4-negative genotypes.\textsuperscript{126} More recent follow-up studies show disappointing results with rosiglitazone. A multicenter trial proved no effect of rosiglitazone on brain atrophy or cognitive function in AD patients,\textsuperscript{127} and two large phase III clinical trials also demonstrated no evidence of clinically significant efficacy in cognition or global function, regardless of ApoE-ε4 genotype.\textsuperscript{128,129} Pioglitazone has produced similarly mixed results. Two prospective randomized open-labelled studies demonstrated that pioglitazone induced cognitive and functional improvements and stabilization of the disease in the individuals with mild AD and MCI with T2D.\textsuperscript{130,131} However, another study evaluating the safety of pioglitazone as the primary outcome exhibited no significant treatment effect on cognition after 6 months of treatment in non-diabetic AD patients.\textsuperscript{132}

Incretins, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1), are a group of gastrointestinal hormones secreted by intestinal epithelial cells in response to food intake that can affect whole body glucose utilization.\textsuperscript{133} GLP-1 receptors are widely expressed throughout the body, including in the pancreas, intestines, heart, and lungs, and in both the central and the peripheral nervous system.\textsuperscript{134} The activation of GLP-1 receptors leads to the facilitation of glucose utilization and antiapoptotic effects in various organs,\textsuperscript{135,136} and two long-lasting GLP-1 analogues, exenatide (Byetta) and liraglutide (Victoza), are approved for the treatment of T2D. More importantly, GLP-1 can readily cross the BBB and enhance insulin signaling in the brain.\textsuperscript{137,138} Therefore, GLP-1 analogues are an attractive therapeutic approach to improve IR in AD and in T2D because they can activate pathways common to insulin signaling and facilitate brain synaptic plasticity, cognition, and cell survival.\textsuperscript{139,140}

Exendin-4 prevented glucose-induced tau hyperphosphorylation or Aβ-mediated toxicity in cultured neurons,\textsuperscript{141,142} and subcutaneous injection of liraglutide for 30 days reduced icv-STZ-induced tau hyperphosphorylation and significantly improved learning and memory in mice.\textsuperscript{143} Two months of liraglutide injections in APP/PS1 mice reduced plaque load, APP and Aβ oligomer levels, and overall inflammation and increased IDE levels.\textsuperscript{144} Furthermore, long-term potentiation was significantly enhanced and liraglutide also improved cognitive function, as measured by novel object recognition and Morris water maze tests. Liraglutide not only has a protective effect at an early stage of AD (7 mo),\textsuperscript{145} but it is also able to reverse AD-related changes in older (14 mo) APP/PS1 mice.\textsuperscript{144} Moreover, liraglutide reduced tau phosphorylation and restored Akt and GSK3β phosphorylation in a HFD-induced model of T2D in rats.\textsuperscript{146} Other GLP-1 analogues demonstrated similar beneficial effects on AD pathologies and cognitive function in AD mouse models.\textsuperscript{147–149}

Although the first small clinical trial of liraglutide in AD patients did not lead to any improvement in cognition or changes in Aβ deposition, as measured by Pittsburgh compound B PET,\textsuperscript{150} a recent clinical trial of exenatide in Parkinson’s disease patients demonstrated clinically relevant improvements in motor and cognitive measures.\textsuperscript{151} Currently two additional clinical trials are in progress with exenatide (http://clinicaltrials.gov/ct2/show/NCT01255163?term) and liraglutide (http://clinicaltrials.gov/ct2/show/NCT01843075?term) in MCI and early AD patients, and completion is estimated in 2016 and 2017, respectively.

CONCLUSION

Recent evidence supports the contention that AD may be a slow-progressing brain metabolic disease, and numerous studies demonstrate an intricate connection between MetS and AD. Individuals with MetS features, such as T2D and obesity, have a higher risk of developing AD, while AD patients often develop hyperglycemia and IR. IR due to impaired insulin signaling is a common characteristic of both MetS and AD, and likely represents the key link between the two diseases. Insulin signaling regulates Aβ and tau, and Aβ has negative effects on insulin signaling; therefore, dysfunctional insulin signaling can enhance Aβ and tau pathology, and increased Aβ production can further exacerbate IR. Thus, several diabetes treatments that enhance insulin signaling are being tested for therapeutic potential in AD and dementia, and even though the results from the TZD clinical trials were disappointing, intranasal insulin and GLP-1 analogues are still being actively pursued as a potential treatments for AD and have exhibited some promising results. Intranasal insulin, however, is only effective in early AD and MCI patients, and individuals with the ApoE-ε4 allele do not respond well. In addition, exenatide and liraglutide are still in the early stages of therapeutic development, and large clinical trials are currently in progress.

Effective AD and MCI treatment demands the development of specific biomarkers to support the diagnosis of these conditions as early as possible. Currently, total tau, hyperphosphorylated tau, and Aβ40/Aβ42 ratios in the CSF exhibit over 80% specificity as biomarkers of AD,\textsuperscript{152,153} Continued research to discover the precise mechanism of how IR contributes to the onset and progression of AD, is also imperative for the development of improved therapeutic interventions. Given the current obesity epidemic among all ages and increased life expectancy, there is a critical need to understand the underlying causes of cognitive impairment due to IR, which may be the key link for the increased incidence of AD in individuals with metabolic disorders.
Insulin resistance and cognitive impairment

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The Role of Oxidized Cholesterol in Diabetes-Induced Lysosomal Dysfunction in the Brain

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Abstract Abnormalities in lysosomal function have been reported in diabetes, aging, and age-related degenerative diseases. These lysosomal abnormalities are an early manifestation of neurodegenerative diseases and often precede the onset of clinical symptoms such as learning and memory deficits; however, the mechanism underlying lysosomal dysfunction is not known. In the current study, we investigated the mechanism underlying lysosomal dysfunction in the cortex and hippocampi, key structures involved in learning and memory, of a type 2 diabetes (T2D) mouse model, the leptin receptor deficient db/db mouse. We demonstrate for the first time that diabetes leads to destabilization of lysosomes as well as alterations in the protein expression, activity, and/or trafficking of two lysosomal enzymes, hexosaminidase A and cathepsin D, in the hippocampus of db/db mice. Pioglitazone, a thiazolidinedione (TZD) commonly used in the treatment of diabetes due to its ability to improve insulin sensitivity and reverse hyperglycemia, was ineffective in reversing the diabetes-induced changes on lysosomal enzymes. Our previous work revealed that pioglitazone does not reverse hypercholesterolemia; thus, we investigated whether cholesterol plays a role in diabetes-induced lysosomal changes. In vitro, cholesterol promoted the destabilization of lysosomes, suggesting that lysosomal-related changes associated with diabetes are due to elevated levels of cholesterol. Since lysosome dysfunction precedes neurodegeneration, cognitive deficits, and Alzheimer’s disease neuropathology, our results may provide a potential mechanism that links diabetes with complications of the central nervous system.

Keywords Type 2 diabetes · Brain · Lysosome · Cathepsin D · Cholesterol · Central nervous system

Introduction

Diabetes mellitus, which currently affects 25.8 million Americans, is a complex metabolic disorder characterized by hyperglycemia. Type 2 diabetes (T2D) accounts for approximately 90–95 % of all diabetes cases and is associated with obesity and hyperinsulinemia. Various complications are associated with diabetes, including retinopathy, nephropathy, neuropathy, and cardiovascular disease [1], and the impact of diabetes on the central nervous system is gaining attention. It is also believed by some that diabetes accelerates brain aging [2, 3]. Aging and age-related diseases may involve abnormalities in the endosomal-lysosomal system, which is an early manifestation of neurodegeneration [4]. Furthermore, lysosomal dysfunction contributes to the accumulation of protein aggregates, a common occurrence in neurodegenerative disorders [5, 6].

Lysosomes are involved in numerous functions, including cell death, exocytosis, endocytosis/phagocytosis, and autophagy. Many of these functions are dependent upon the action of acid hydrolase enzymes within the lysosome that can degrade lipids, carbohydrates, proteins, nucleic acids, or cellular debris. Hexosaminidase A is a lysosomal enzyme that converts GM2 ganglioside to G3M by removing an N-acetyl-
normal accumulation of protein aggregates [14]. In addition, cathepsin D is one of the major lysosomal proteases that contribute to the conversion of proinsulin to insulin in Langerhans cells and rat hepatocytes [9], and it is also involved in the degradation of insulin [10, 11].

Thiazolidinediones (TZDs), a class of drugs known to improve insulin sensitivity, are commonly used for the treatment of diabetes. TZDs are ligands for peroxisome proliferator-activated receptors (PPAR-γ) and improve insulin sensitivity by lowering serum glucose and insulin levels, increasing peripheral glucose uptake, and decreasing triglyceride levels [12, 13]. In fact, studies have demonstrated the beneficial effects of TZDs in neuropegenerative diseases associated with the abnormal accumulation of protein aggregates [14–17]. One such TZD is pioglitazone (Actos), which improves hyperglycemia, reduces hyperinsulinemia, and ameliorates hypertriglyceridemia in a variety of animal models of obesity and insulin resistance [18–23]. Pioglitazone does not improve cholesterol levels in mice (personal communication, [24]).

Hypercholesterolemia is present in 70 % of adults diagnosed with diabetes [25]. In T2D, serum cholesterol is elevated secondary to altered cholesterol synthesis and absorption [26, 27]. When in excess, cholesterol is oxidized by enzymatic or reactive oxygen species (ROS)-mediated pathways. Oxidized cholesterol is increased in T2D [28, 29] and disrupt cellular membranes [30], especially lysosomal membranes [31]. Disrupted lysosomes are incapable of effectively removing ROS-damaged macromolecules [32]. This leads to a feedback cycle of damage, where ROS promote the oxidation of cholesterol, resulting in lysosomal injury.

Alterations in lysosomal function in diabetes have been documented in the liver, kidney, heart, saliva, whole brain, and plasma [33–36]. Our previous work in the hippocampi of a well-characterized mouse model of T2D, the db/db mouse, revealed differential expression in genes related to the lysosome [37]. Previous studies have reported alterations in the central nervous system including cognitive impairment and evidence of neurodegeneration in the db/db mouse [38–44]. Therefore, in this study, we characterized lysosomal function in the cortex and hippocampus of the db/db mouse. To determine a potential mechanism underlying lysosomal dysfunction in diabetes, we treated the db/db mice with pioglitazone to ameliorate diabetes and performed subsequent in vitro studies to confirm.

Materials and Methods

Animals

Control db+ and T2D db/db mice (BKS.Cg-m +/+ Leprdb/J, JAX mice stock no. 000642) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were fed a standard rodent chow from Lab Diet (#5053) ad libitum. For pioglitazone studies, db+ and db/db mice were fed a standard diet (5LOD; Research Diets, New Brunswick, NJ) supplemented with or without 112.5 mg of pioglitazone per kg of chow for a final dosage of 15 mg/kg to the mouse beginning at 5 weeks of age. To document the persistence of diabetes, fasting blood glucose levels were measured every 4 weeks by analyzing one drop of tail blood after a 6-h fast using a standard Glucometer (One Touch Ultra, Milpitas, CA). Mice were euthanized at either ~8 or ~20 weeks of age.

Tissue Preparation

The mice were euthanized according to our published protocols with an overdose of sodium pentobarbital, and the tissue was processed as follows per our previously published protocols [45]. For western immunoblotting analyses and enzyme activity assays, the hippocampus and cortex from the dissected brains were prepared as previously described by homogenizing the tissues in tissue protein extraction reagent (Pierce, Rockford, IL) containing a protease inhibitor cocktail (Calbiochem, San Diego, CA). For immunohistochemistry (IHC), mice were perfused with 30 ml of 2 % paraformaldehyde-lysine-periodate, the whole brains were removed and immersed in the same fixative overnight, and the brains were then cryoprotected in PBS (0.1 M, pH 7.2) with 30 % sucrose prior to embedding in OCT compound (Sakura Finetek, Torrance, CA). The brains were then sectioned (20 μm) using a CM1850 cryostat (Leica Microsystems Inc., Bannockburn, IL), mounted onto SuperFrost glass slides (Fisher Scientific, Pittsburgh, PA), and stored at −20 °C until use. For cell fractionation and flow cytometry, the mice were perfused with 15 ml PBS, and the cortex and hippocampus were removed and immediately processed as described below.

Western Immunoblotting

Western immunoblotting was performed as previously described [45, 46]. Briefly, the tissue lysates were either separated by SDS-PAGE and transferred to a nitrocellulose membrane or used to determine enzyme activity as described below. Tris-buffered saline with Tween-20 supplemented with 5 % milk was used to block the membrane and to dilute the antibodies. Polyclonal antibodies against cathepsin D (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), β-hexosaminidase (ProteinTech Group, Inc., Chicago, IL), and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), as well as appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.), were used for western immunoblotting. The signal was visualized using LumiGLO-enhanced chemiluminescence reagent (Cell Signaling Technology, Danvers, MA). Images were captured using
the Chemidoc XRS system and analyzed by Quantity One software (Bio-Rad Laboratory, Hercules, CA).

### Enzyme Assays

The activity of β-hexosaminidase A and cathepsin D was measured in 4–5 μg of protein cell lysate from the cortex and hippocampus at 8 and 20 weeks of age (*n*≥5 for each group) in a 96-well plate. To measure β-hexosaminidase A activity, 3.2 mM 4-methylumbelliferyl-6-sulfo-N-acetyl-β-D-glucosaminide (MUGS) potassium salt (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the cell lysate and incubated for 1 h at 37 °C. Next, 2-amino-2-methyl-1-propanol (0.1 M) was added, and fluorescence was read immediately with a 355-nm excitation filter and 460-nm emission filter using a Fluoroskan Ascent II plate reader (LabSystems, Helsinki). Cathepsin D activity was assessed using the cathepsin D activity assay kit (BioVision, Mountain View, CA) according to the manufacturer’s instructions. Fluorescence was read with a 320-nm excitation filter and 460-nm emission filter.

### Cell Fractionation

Dissected cortex and hippocampus were homogenized in homogenization medium (HM: 0.32 M sucrose, 1 mM Na₂EDTA, 10 mM HEPES; pH 7.0) and centrifuged at 800 g for 10 min at 4 °C. The supernatant was kept on ice, and the pellet was resuspended in HM and centrifuged again. The supernatants from both centrifugation steps were combined and centrifuged at 20,000 g for 15 min at 4 °C. The supernatant was centrifuged at 300,000 g for 2 h at 4 °C to obtain the cytosol, and the pellet was resuspended in HM and layered over a 27 % Percoll solution (Sigma) diluted with Percoll diluent (2.5 M sucrose, 10 mM Na₂EDTA, 100 mM HEPES; pH 7.0). The sample was centrifuged for 95 min at 20,000 g. Lysosomes were collected from the layer near the bottom of the gradient and centrifuged for 50 min at 100,000 g.

### Measurement of Intralysosomal pH

The measurement of intralysosomal pH was performed using flow cytometry with minced cortex and hippocampus (separately) that were trypsinized at 37 °C for 6 min. The tissue was triturated with Leibovitz (L15) media, filtered (70 μm), and centrifuged at 155 g for 5 min at 4 °C. The pellet was resuspended in PBS. A standard curve was generated in LysoSensor Yellow/Blue DND-160 (2 mM; Invitrogen Molecular Probes) dye-loaded cell suspension using a series of phosphate-citrate buffers containing various mixtures of 300 mM KH₂PO₄ and 300 mM citric acid ranging in pH 2–6, supplemented with the inophores nigericin and monensin (Sigma Aldrich; 10 μmol/L) to facilitate the equilibration of intralysosomal pH with the buffer. The cell suspension was allowed to equilibrate for 10 min. The standard curve was generated by exciting at 355 nm and plotting the emission fluorescence ratio (550/21 nm) of DND-160-loaded cells as a function of the actual pH, which was assessed on a standard pH meter. The intralysosomal pH of the lysosomes in the cortex or hippocampus from db+ and db/db mice was calculated by extrapolation from the standard curve.

### Immunohistochemistry

Brain sections were heated on a 55 °C slide warmer for 10 min, hydrated in PBS for 5 min, and permeabilized with PBS containing 0.3 % Triton X-100 and 3 % milk. Sections were incubated in primary antibodies diluted in PBS containing 0.3 % Triton X-100 and 1 % BSA in a humidified chamber overnight at 22 °C. A polyclonal antibody against lysosomal associated membrane protein-1 (LAMP-1; Abcam, Cambridge, MA) was used for IHC. After rinsing with PBS, sections were incubated with the appropriate secondary antibody conjugated with AlexaFluor 594 (Molecular Probes, Eugene, OR) for 1 h at room temperature. Following three rinses with PBS, the sections were incubated for 3 h at room temperature in the dark with 10 μg/ml filipin complex (Sigma, St. Louis, MO). After rinsing with PBS, coverslips were mounted with Prolong anti-fade mounting medium (Molecular Probes, Eugene, OR). Images were captured using a Spot-RT camera (Diagnostic Instruments Inc., Sterling Heights, MI) attached to a Nikon Microphot-FXA microscope.

### Primary Cortical Neuron Experiments

Primary cortical neurons (CN) were prepared as previously described [47]. Briefly, the cortex from E13 B6C3F1/J mice were dissected, dissociated with trypsin, and plated on poly-L-lysine (PLL)-coated tissue culture plates or coverslips. CN were maintained in neurobasal media (Invitrogen, Grand Island, NY) containing 5 mM glucose, supplemented with 1× B27 (without antioxidant; Invitrogen), antibiotics (penicillin, streptomycin, and neomycin; Sigma), 2.5 μg/ml albumin, 10 μg/ml apo-transferrin, 0.1 μg/ml biotin, 15 μg/ml D-galactose, 7 ng/ml progesterone, 16 μg/ml putrescine, 4 ng/ml selemium, 3 ng/ml β-estradiol, 4 ng/ml hydrocortisone, 3 μg/ml catalase, and 2.5 μg/ml superoxide dismutase. CN were cultured for 6 days prior to use, with an addition of fresh media on day 3. CN treatment media (neurobasal media without B27 and antioxidants) was used to carry out the experiments indicated below.

In vitro experiments involved treating primary CN with 25 mM of glucose [47] with or without 5 μM oxidized cholesterol (27-hydroxycholesterol; Medical Isotopes, Inc., Pelham, NH; Prasanthi et al. 2009) for 72 h. Lysosomal destabilization was measured using acridine orange (AO), a
lysosomotropic weak base, and metachromatic fluorochrome. Briefly, 5 μg/ml AO in neurobasal medium was incubated with CN on coverslips for 15 min at 37 °C. After the cells were washed and treated as outlined above, microscopic assessment of lysosomes was performed using an Olympus IC-71 inverted confocal microscope and FluoView v4.3 software. Quantitative analysis was performed after staining and treatment of CN in a 96-well clear bottom/black walled plate using a Fluoroskan Ascent FL instrument. When excited by a blue light (450 nm), AO fluoresces red (612-nm wavelength emission) at high lysosomal concentrations and green (520-nm wavelength emission) at low nuclear and cytosolic concentrations. Rupture of AO-loaded lysosomes shows an increase in cytoplasmic diffuse green fluorescence and a decrease in granular red fluorescence [48, 49].

Statistical Analyses

Data analyses were performed using Prism v6 (GraphPad Software, Inc.). Assumptions about Gaussian distribution of data were made using the D’Agostino and Pearson omnibus normality test. Data not corresponding to a Gaussian distribution were analyzed using an appropriate mathematical transformation, log (x). At least 10 mice per group at 8 and 20 weeks of age were used for metabolic studies. For all other measures, at least 5 mice per group were used. t test was used in experiments where T2D was compared to the nondiabetic control. For all experiments, *p<0.05, **p<0.01, ***p<0.001, and #p<0.0001 and bar graphs illustrate the mean±standard error of the mean (SEM).

Results

Cortical and Hippocampal Changes in Hexosaminidase A Protein Expression and Activity in T2D

We evaluated the lysosomal enzyme β-hexosaminidase A to examine lysosomal function in T2D. While hexosaminidase A protein expression did not change in the cortex, it was significantly increased in the hippocampus by 33 % at 8 weeks and 28 % at 20 weeks in T2D compared to the nondiabetic control (Fig. 1a, b). Likewise, the activity of hexosaminidase A did not change in the cortex; however, it decreased by 10 % in the hippocampus of T2D mice at 8 weeks of age and increased by 8 % in the hippocampus of T2D mice at 20 weeks of age compared to the nondiabetic control (Fig. 1c, d).

Cortical and Hippocampal Changes in Cathepsin D Protein Expression and Activity in T2D

To further examine lysosomal function in T2D, the protein expression and activity of the major lysosomal protease, cathepsin D, were also evaluated. The protein expression of cathepsin D in T2D mice significantly increased by 55 % at 8 weeks and 118 % at 20 weeks in the cortex, and by 46 % at 8 weeks and 64 % at 20 weeks in the hippocampus, compared to nondiabetic control (Fig. 2a, b). This increase in protein expression in the cortex at 20 weeks of age was associated with a 21 % decrease in the activity of cathepsin D in T2D compared to the nondiabetic control, whereas the activity of cathepsin D did not change in the hippocampus (Fig. 2c, d).

The processing of cathepsin D in the Golgi, endosomes, and lysosomes correspond to the 3 major forms—immature, intermediate, and mature, respectively. Alterations in the activity of cathepsin D are associated with the mature form of the protein. Since the protein expression of cathepsin D is significantly increased in the cortex of T2D but the activity is decreased, we assessed the 3 major forms of cathepsin D in the cortex to evaluate whether or not T2D alters the trafficking pattern of cathepsin D. We observed a significant increase in both the intermediate and mature forms of cathepsin D in T2D compared to the nondiabetic control (Fig. 2e, f).

Lysosomal Membrane Integrity, But Not pH, Is Compromised in T2D

The activity of cathepsin D was expected to parallel the direction of the protein expression of the mature form of cathepsin D; however, we observed a decrease in the activity with an increase in the protein expression of the mature form of cathepsin D in the T2D db/db mouse cortex. This may be due to either a compromised lysosomal membrane with subsequent leakage of the mature form of cathepsin D into the cytosol, or an alteration in intralysosomal pH in the db/db mouse cortex that renders mature cathepsin D inactive. Thus, western immunoblotting following cell fractionation was used to assess the integrity of lysosomal membranes in T2D. The protein expression of both lysosomal enzymes hexosaminidase A and cathepsin D in the cytosol was increased by at least 100 % in the db/db mouse hippocampus/cortex compared to the nondiabetic control (Fig. 3a, c), whereas the protein expression levels of the lysosomal enzymes were not significantly different in the lysosomal fraction of db/db mice compared with the nondiabetic control (Fig. 3b, d). Flow cytometry was then utilized to assess the potential alterations in the intralysosomal pH in T2D cortex and hippocampus. The average intralysosomal pH in the cortex of the db+ and db/db mice was 4.75±0.87 and 4.71±1.1, respectively (data not shown). Similarly, the average intralysosomal pH in the hippocampus of the db+ and db/db mice was 4.75±0.39 and 4.68±0.39, respectively (data not shown).
Elevated Levels of Glucose Can Cause Destabilization of the Lysosomal Membrane

To determine a potential mechanism underlying the lysosomal destabilization and leakage associated with T2D, AO staining was used on live primary CN cultures in the presence or absence of 25 mM glucose treatment. An increase in the green cytosolic fluorescence and/or a decrease in red punctate lysosomes are indicative of a loss of membrane integrity. Microscopy of live cells revealed that 25 mM of glucose treatment for 72 h led to the loss of red lysosomal staining (Fig. 4).

Fig. 1 Protein expression and activity of the lysosomal enzyme hexosaminidase A in the brain of T2D mice. a, b Protein expression and c, d activity of hexosaminidase A in the cortex (a, c) and hippocampus (b, d) of db+ and db/db mice at 8 and 20 weeks of age. Actin was used as the loading control (*p<0.05 and **p<0.01 compared with db+; n≥6). Activity is expressed in relative fluorescence units (RFU)

Fig. 2 Protein expression and activity of the major lysosomal protease cathepsin D in the brain of T2D mice. a, b Protein expression and c, d activity of cathepsin D in the cortex (a, c) and hippocampus (b, d) of db+ and db/db mice at 8 and 20 weeks of age. e Representative immunoblot and f densitometric analysis of the protein expression and subcellular distribution of cathepsin D in the cortex. Actin was used as the loading control (*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 compared with db+; n≥6). Activity is expressed in relative fluorescence units (RFU)
Pioglitazone Improves the Hyperglycemic Phenotype But Not Alterations in Lysosomal Membrane Integrity Associated with T2D

Pioglitazone treatment worsens the obesity phenotype of db/db mice and also significantly increases the weight of db+ mice; however, it reverses the elevated levels of blood glucose, glycosylated hemoglobin, and triglycerides in db/db mice back to levels similar to those in control db+ mice (personal communication, [24]). Thus, to determine if pioglitazone can effectively reverse the leakage of lysosomal enzymes into the cytosol, the protein expression levels of hexosaminidase A and cathepsin D were evaluated following pioglitazone treatment in db+ and db/db mice. While pioglitazone did not alter the total protein expression levels of hexosaminidase A or cathepsin D (data not shown), pioglitazone treatment did elevate the levels of cathepsin D in the cytosol following cell fractionation (Fig. 5).

Elevated Levels of Cholesterol Within Lysosomes in T2D

Fast protein liquid chromatography analyses indicate that pioglitazone treatment does not reverse the levels of cholesterol and promotes an increase in the low density lipoprotein (LDL) cholesterol fraction in db/db mice compared to nondiabetic controls [24]. Thus, to evaluate cholesterol load in the lysosomes of db/db mice, filipin staining was used. We observed an increase in the colocalization of filipin and the lysosomal membrane protein LAMP-1 in the cortex and hippocampus of db/db mice compared with db+ control mice (Fig. 6).

Cholesterol Can Cause Destabilization of the Lysosomal Membrane

To investigate cholesterol as a potential mediator underlying the destabilization and leakage of lysosomes associated with T2D, AO staining was used in live CN treated with and without oxidized cholesterol. Quantitative analysis of the AO staining of live CN revealed that oxidized cholesterol treatment for 72 h led to an increase in green fluorescence and a decrease in the red lysosomal staining (Fig. 7).

Discussion

In the current study, we investigated the mechanisms under-lying alterations in lysosomal function induced by diabetes. Hyperglycemia is a key player in many complications associated with diabetes. Thus, according to the American Diabetes Association, tight glycemic control with either drugs or diet...
and exercise is the most effective method for preventing diabetic complications. We show for the first time that cholesterol, and not hyperglycemia, may mediate the changes observed in lysosomal function during diabetes in the hippocampus/cortex. Abnormalities in the lysosomal system are early manifestations of neurodegeneration \[4\]; thus, altered cholesterol metabolism may play a role in diabetes-induced lysosomal changes and neurodegeneration.

The expression of both hexosaminidase A and cathepsin D is upregulated in neurodegenerative diseases \[50–53\]. Hexosaminidase A removes N-acetyl-glucosamine residue from GM2 ganglioside, converting it to GM3. Obesity leads to a dramatic increase in the protein levels of GM2 in adipose tissue in a mouse model of T2D \[54\]. Hence, the increase in protein expression observed in the hippocampus of the db/db mouse may be due to obesity. Consistent with our observed increase in hexosaminidase A activity in the db/db mouse hippocampus, T2D patients have increased activity of hexosaminidase A in plasma and serum \[36\]. Thus, increased activity of hexosaminidase A in plasma and peripheral blood mononuclear cells may have diagnostic value for the detection of the early stages of dementia in Alzheimer’s disease (AD) patients with and without T2D \[36\].

Cathepsin D is the major lysosomal protease in neurons, contributing to nearly 90 % of protease degradation in the brain \[55\]. The increase in protein expression we observed in both the cortex and hippocampus is consistent with previous studies in T2D patients reporting that the expression of cathepsin D is increased in serum and leucocytes \[56, 9\]. On the other hand, the decrease in the activity of cathepsin D we observed is unique to our studies in T2D and may be indicative of abnormalities in the trafficking of cathepsin D to lysosomes. The trafficking of cathepsin D was assessed by evaluating the 3 different isoforms—immature, intermediate, and mature—which correspond to trafficking from the Golgi, endosomes, and lysosomes, respectively \[57\]. The acidic environment of lysosomes provides a platform for proteolytic cleavage of the intermediate form of cathepsin D to the mature form \[57\]. Thus, any alterations in the pH of lysosomes may impact the levels of the mature form of the enzyme. Our data indicate that the pH within lysosomes is not altered during T2D. Alternatively, the elevated levels of cathepsin D in the cytosolic fraction in db/db mice suggests that the lysosomal membrane may be compromised. This would explain the elevated protein levels of cathepsin D and the decrease in the activity due to lack of acidic pH in the cytosol. The
mechanism underlying this damage to the membrane of lysosomes is not known; however, oxidative stress is associated with leakage of cathepsin D into the cytosol from the lysosome in both in vivo and in vitro models [58, 59].

Hyperglycemia is the key player leading to oxidative stress during T2D. To investigate hyperglycemia as the mechanism underlying lysosomal membrane damage, we exposed CN to hyperglycemic conditions, demonstrating that hyperglycemia causes destabilization of the lysosomal membrane. A previous study demonstrated that hyperglycemia inhibited lysosomal function in macrophages and may contribute to diabetes-associated atherosclerosis [60]. Thus, reversing hyperglycemia may have a profound impact on lysosomal membrane stability.

Pioglitazone is known to improve hyperglycemia, reduce hyperinsulinemia, and ameliorate hypertriglyceridemia in a variety of animal models of obesity and insulin resistance [22, 21, 20, 19, 18]; however, pioglitazone did not reverse the alterations in lysosomal enzymes and in fact lead to a further increase in the levels of cathepsin D in the cytosol. We previously demonstrated that pioglitazone does not improve hypercholesterolemia in db/db mice [24]. In addition, in patients with T2D, serum cholesterol is elevated secondary to altered cholesterol synthesis and absorption [27, 26]. When in excess, cholesterol is oxidized by enzymatic or reactive oxygen species-mediated pathways to generate cholesterol oxides. Cholesterol oxide derivatives, known as oxysterols, are common components of oxidized LDLs, are increased in T2D [29, 28], and disrupt lysosomal membranes [31]. We demonstrate that oxysterols are capable of disrupting lysosomal membranes in primary CN. Thus, it is possible that pioglitazone did not improve the T2D-induced effects on lysosomes due to its inability to reverse hypercholesterolemia in db/db mice.

In summary, it is likely that multiple mechanisms contribute to the alterations in lysosomal enzymes during diabetes. We demonstrate that improving hyperglycemia, insulin resistance, and hypertriglyceridemia alone is not sufficient to reverse diabetes-induced changes in lysosomal membrane stability, although our studies further demonstrate that hypercholesterolemia plays a role in lysosomal membrane destabilization. Future studies will focus on cholesterol as a potential therapeutic target to reverse or prevent the impact of T2D on lysosomal enzymes.

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Conflict of Interest The authors declare that they have no conflict of interest.

Compliance with Ethical Standards All protocols and procedures were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA; approval number PRO00003694), and were approved by the University of Michigan Committee on the Use of Laboratory Animals. The University’s Animal Welfare Assurance Number on file with NIH Office of Laboratory Animal Welfare (OLAW) is A3114-01, and the University is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Intl.).

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References

Insulin Resistance Prevents AMPK-induced Tau Dephosphorylation through Akt-mediated Increase in AMPK$^{\text{Ser-485}}$ Phosphorylation*

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Background: Insulin resistance is a risk factor for Alzheimer disease.
Results: AMPK$^{\text{Ser-485}}$ is responsible for AMPK-mediated Tau phosphorylation.
Conclusion: Abnormal phosphorylation of AMPK$^{\text{Ser-485}}$ may be the link for the increased Alzheimer disease risk in metabolic syndrome
Significance: With the rapid increase in both metabolic syndrome and Alzheimer disease, it is crucial to understand the underlying mechanism linking two diseases

Metabolic syndrome (MetS) is a cluster of cardiovascular risk factors including obesity, diabetes, and dyslipidemia, and insulin resistance (IR) is the central feature of MetS. Recent studies suggest that MetS is a risk factor for Alzheimer disease (AD). AMP-activated kinase (AMPK) is an evolutionarily conserved fuel-sensing enzyme and a key player in regulating energy metabolism. In this report, we examined the role of IR on the regulation of AMPK phosphorylation and AMPK-mediated Tau phosphorylation. We found that AMPK$^{\text{Ser-485}}$, but not AMPK$^{\text{Thr-172}}$, phosphorylation is increased in the cortex of db/db and high fat diet-fed obese mice, two mouse models of IR. In vitro, treatment of human cortical stem cell line (HK-5320) and primary mouse embryonic cortical neurons with the AMPK activator, 5-aminoimidazole-4-carboxamide 1-$\beta$-d-ribofuranoside (AICAR), induced AMPK phosphorylation at both Thr-172 and Ser-485. AMPK activation also triggered Tau dephosphorylation. When IR was mimicked in vitro by chronically treating the cells with insulin, AICAR specifically induced AMPK$^{\text{Ser-485}}$, but not AMPK$^{\text{Thr-172}}$, hyperphosphorylation whereas AICAR-induced Tau dephosphorylation was inhibited. IR also resulted in the overactivation of Akt by AICAR treatment; however, preventing Akt overactivation during IR prevented AMPK$^{\text{Ser-485}}$ hyperphosphorylation and restored AMPK-mediated Tau dephosphorylation. Transfection of AMPK$^{\text{S485A}}$ mutant caused similar results. Therefore, our results suggest the following mechanism for the adverse effect of IR on AD pathology: IR → chronic overactivation of Akt → AMPK$^{\text{Ser-485}}$ hyperphosphorylation → inhibition of AMPK-mediated Tau dephosphorylation. Together, our results show for the first time a possible contribution of IR-induced AMPK$^{\text{Ser-485}}$ phosphorylation to the increased risk of AD in obesity and diabetes.

Alzheimer disease (AD) is a progressive neurodegenerative disease characterized by loss of memory and other cognitive functions necessary to perform complex daily activities (1). The most prominent neuropathological features of AD are the appearance of senile plaques composed of amyloid $\beta$ peptides and neurofibrillary tangles derived from the aggregation of the microtubule-associated protein Tau (2, 3). AD is the most common form of dementia, accounting for over 70% of all cases, and it currently affects 5.4 million Americans. The incidence is expected to reach over 13.8 million by 2050, and it is estimated that the cost for caring for people with AD will dramatically increase from $203 billion for 2013 to $1.2 trillion by 2050.

Insulin resistance (IR) is defined as a state of reduced responsiveness of target tissue(s) to normal circulating levels of insulin. It is the central feature of metabolic syndrome (MetS), a constellation of disorders related to an increased risk of cardiovascular disease, and it is the major contributor for the development of diabetes (4). Multiple studies report that patients with MetS have an increased risk of developing AD compared with age- and gender-matched controls (5). In addition, accumulating evidence suggests that AD is closely related to dysfunction of both insulin signaling and glucose metabolism in the brain, prompting some investigators to refer to AD as type 3 diabetes or an insulin-resistant brain state (6, 7). Epidemiological studies also demonstrate an association between obesity caused by dietary fat intake and an increased risk for AD (8), and multiple studies report that patients with diabetes have a 50–75% increased risk of developing AD compared with age- and gender-matched control groups (9–13). In parallel, a study of the Mayo Clinic AD Patient Registry revealed that 80% of AD patients have either type 2 diabetes or impaired fasting glucose (14).

There are many theories to explain the connection between AD and diabetes (and MetS in general), including impaired
Role of AMPKSer-485 in Tau Phosphorylation in IR

Experimental Procedures

Antibodies and Chemicals—Polyclonal antibodies against phosphorylated Tau (Ser(P)-199/202, Ser(P)-262, Ser(P)-396, and Thr(P)-231) were purchased from Life Technologies Inc. Tau1 (recognizing dephosphorylated Tau), Tau5 (for total Tau), and anti-GAPDH antibodies were from Millipore (Billerica, MA). LY294002 and U0126 were also from Millipore. Ser(P)-485-AMPK and actin antibodies were from Abcam (Beverly, MA). The antibodies for Thr(P)-172-AMPK, and AMPK were purchased from Cell Signaling (Beverly, MA).

TABLE 1

<table>
<thead>
<tr>
<th>Diet composition</th>
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AICAR was purchased from Sigma-Aldrich. All other chemicals were purchased from either Sigma-Aldrich or Fisher Scientific (Fair Lawn, NJ).

Mouse Models and Brain Preparation—BKS-db/db and db+ (BKS.Cg-m+/-Leprdb/J, JAX mice stock #000642) mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL6 (B6) mice, a commonly used model to study diet-induced obesity (20, 21, 22, 23), were also from Jackson Laboratory. Obesity was induced in B6 mice by placing them on a high fat diet (HFD) consisting of 54% kcal from fat (D05090701; Research Diets Inc., New Brunswick, NJ) at 4 weeks of age until 24 weeks of age, whereas control mice were fed a standard diet consisting of 10% kcal from fat (D12450B; Research Diets Inc.) (Table 1). Fasting blood glucose levels were measured every 4 weeks using a standard glucometer (OneTouch; LifeScan Inc., Milpitas, CA). All mice were housed in a pathogen-free environment and cared for following the University of Michigan Committee on the Care and Use of Animals guidelines.

Mice were euthanized per our published protocols with an overdose of sodium pentobarbital (33, 34). For Western immunoblotting analyses, brains were cut in half, the cortex and hippocampus were separated, and then tissues were snap frozen in liquid nitrogen and stored at −80 °C until use. At least six animals were analyzed for each treatment, and three or four representative Western blotting results are shown on each figure.

Cell Culture and Treatments—HK-532 human cortical stem cells were provided by Neuralstem, Inc. (Rockville, MD). Cells were incubated in differentiation medium (NSDM) for 7–10 days, and the medium was changed to NSDM without insulin 24 h before treatment.

Primary cortical neurons were prepared from embryonic day 15 embryos of Sprague-Dawley rats. Cells were maintained in feed medium (neurobasal media; Invitrogen) supplemented with B27 without antioxidant (Invitrogen) and other supple-

In AD, the role of AMPK is controversial, with the reports suggesting both beneficial and detrimental effects on the progression of AD (20). It is reported that AMPK activation reduces amyloid β production in rat cortical neurons (24) and that leptin and resveratrol reduce amyloid β levels and Tau phosphorylation through AMPK activation (25–27). In contrast, AMPK can directly phosphorylate Tau, and amyloid β-induced increases in Tau phosphorylation require AMPK-activated Ca2+/calmodulin-dependent protein kinase kinase β (28). Furthermore, increased AMPK activation is observed in tangle- and pretangle-bearing neurons in AD brain (29). This activation of AMPK requires phosphorylation at threonine 172 (AMPKThr-172), a residue in its α-subunit, by upstream kinases such as liver kinase B1 (LKB1) or Ca2+/calmodulin-dependent protein kinase kinase β (21, 30). AMPK is also phosphorylated at serine 485 (AMPKSer-485) by Akt, which reduces Thr-172 phosphorylation and inhibits AMPK activation (31, 32); however, the direct contribution of Ser-485 on AMPK activation has not been extensively studied.

In this study, we report a novel role of AMPKSer-485 on Tau phosphorylation. We demonstrate that AMPKThr-172, but not AMPKThr-172- phosphorylation is increased in two animal models of diabetes and obesity. Our results also indicate that activation of AMPK by the AMPK activator AICAR decreases Tau phosphorylation and that this change is specifically correlated with changes in AMPKSer-485 phosphorylation. Finally, we demonstrate that Akt hyperactivation caused by IR may be responsible for the increased AMPKSer-485 phosphorylation and subsequent inhibition of AICAR-mediated Tau dephosphorylation. These results show for the first time how AMPKSer-485 phosphorylation may contribute to the increased risk of AD in obesity and diabetes.

In AD, the direct contribution of Ser-485 on AMPK activation increased risk of AD in obesity and diabetes. Finally, we demonstrate that AMPKSer-485, but not AMPKThr-172, phosphorylation is increased in two animal models of diabetes and obesity. Our results also indicate that activation of AMPK by the AMPK activator AICAR decreases Tau phosphorylation and that this change is specifically correlated with changes in AMPKSer-485 phosphorylation. Finally, we demonstrate that Akt hyperactivation caused by IR may be responsible for the increased AMPKSer-485 phosphorylation and subsequent inhibition of AICAR-mediated Tau dephosphorylation. These results show for the first time how AMPKSer-485 phosphorylation may contribute to the increased risk of AD in obesity and diabetes.
Role of AMPK<sub>Ser-485</sub> in Tau Phosphorylation in IR

Results

db/db mice demonstrates an elevation of plasma insulin at ~2 weeks and of blood sugar at 4–6 weeks. By 8 weeks of age, hyperinsulinemia and hyperglycemia were stable phenotypes of the db/db mice (36). At 24 weeks, db/db mice display the full blown diabetes phenotypes with the increased body weight and blood glucose and glycated hemoglobin (Fig. 1A). However, as the diabetic phenotype continues to deteriorate the mice prone to a spontaneous death, so we harvested the mice at 24 weeks. Mice fed with high fat diet usually display signs of prediabetes starting around 16 weeks of age (37, 38). Their blood glucose level and body weight continue to increase up to a year (39). We observed that HFD mice display increased body weight (Fig. 1A) and impaired glucose tolerance test (Fig. 1B) but no significant changes in blood glucose and glycated hemoglobin levels indicating that they are prediabetic.

AMPK is an evolutionarily conserved serine/threonine kinase that plays a pivotal role in maintaining cellular homeostasis (22); therefore, we first examined the changes in AMPK phosphorylation in the brain of two IR mouse models; diabetic mice (db/db) or obese mice (HFD-fed). AMPK phosphorylation at Thr-172 in the cortex of db/db mice was not significantly different from that in control db+ mice (Fig. 2A). Similarly, mice fed a HFD for 24 weeks did not affect AMPK<sub>Thr-172</sub> phosphorylation in the cortex (Fig. 2B). In contrast, however, AMPK<sub>Ser-485</sub> was significantly increased in both db/db and obese (1.7-fold) mouse cortices compared with control animals (Fig. 2, A and B). In addition, Tau phosphorylation was increased in HFD mouse cortex compared with controls (Fig. 2C). Increased Ser/Thr phosphorylation of IRS proteins is one of the key features of IR (40). We observed consistently increased IRS phosphorylation (Fig. 2D). Basal phosphorylation of Akt was also increased in HFD mouse cortex; the increase in ERK phosphorylation was less significant compared with control animals. We previously reported similar changes in Tau phosphorylation as well as insulin signaling components in db/db mouse brains (36). These results imply that MetS factors, such as diabetes and obesity, may underlie the observed changes in AMPK and Tau phosphorylation.

To examine the role of AMPK on Tau phosphorylation in more detail, we used in vitro cell culture models utilizing HK-532 cortical stem cells and primary rat cortical neurons. Treatment of HK-532 cells with the AMPK activator, AICAR, resulted in time- and concentration-dependent increases in AMPK phosphorylation at both Thr-172 and Ser-485 (Fig. 3A). AMPK phosphorylation was maintained at least 6 h after AICAR treatment. Because of serine and threonine phosphorylation, total AMPK immunoblotting resulted in a diffuse pattern. Acetyl-CoA carboxylase phosphorylation by AMPK at Ser-79, a surrogate marker for AMPK activation (41), was also increased following AICAR treatment in a manner that corresponded to the observed AMPK phosphorylation (Fig. 3A). In parallel, AMPK activation following treatment of HK-532 cells with AICAR for 2 h reduced Tau phosphorylation at Ser-199/202, Thr-231, and Ser-396 (Fig. 3B). Tau1 immunoreactivity is also increased after AICAR treatment (Fig. 3B), consistent with the prefer-
ential reaction of the Tau1 antibody with Tau when it is dephosphorylated at serines 195, 198, 199, and 202 (42). Similar changes in AMPK and Tau phosphorylation were observed in primary cortical neurons by AICAR treatment (Fig. 3C).

IR is at the core of MetS (4); therefore, we next tested the effect of IR on AMPK-mediated Tau dephosphorylation utilizing HK-532 cortical stem cells and primary rat cortical neurons. We previously reported that chronic insulin treatment of dorsal root ganglia and primary cortical neurons results in a blunted

FIGURE 1. Metabolic phenotyping of db/db and HFD-fed mice. A, body weight, blood glucose, and glycated hemoglobin (GHb) of 24-week-old db/db (left panels) mice and mice fed with HFD for 20 weeks (right panels). B, HFD mice display impaired glucose tolerance test (GTT). At least six mice were used for each group. *, p < 0.05 by t test.
insulin response, suggesting that these models are an excellent means to study neuronal IR in vitro (43, 44). To examine the effect of IR on AMPK phosphorylation, we treated HK-532 cells without or with 50 nM insulin overnight and then with 1 mM AICAR for 0, 2, or 6 h. The increase in AMPKThr-172 phosphorylation by AICAR was not significantly different with or without insulin pretreatment (Fig. 4, A and B, upper panel); however, interestingly, AICAR-induced AMPKSer-485 phosphorylation was significantly higher after insulin pretreatment (Fig. 4, A and B, lower panel). As expected, AICAR treatment resulted in Tau dephosphorylation, and induction of IR by chronic insulin treatment prevented AICAR-induced Tau dephosphorylation (Fig. 4, C and D). We also observed a similar inhibitory effect of IR on AICAR-induced Tau dephosphorylation in embryonic cortical neurons (Fig. 4E). Together, these results suggest a connection between increased AMPKSer-485 phosphorylation and Tau phosphorylation, which is abolished in the setting of IR.

To examine the possible mechanism behind IR-mediated changes in AMPK and Tau phosphorylation, we next looked at the contribution of insulin signaling pathway components. To first confirm that the cells are insulin-responsive, we treated HK-532 cells with insulin and observed an increase in Akt phosphorylation, as well as a slight increase in ERK phosphorylation (Fig. 5A). It is reported that Akt is the kinase responsible for AMPKSer-485 phosphorylation and the subsequent inhibition of
AMPK activity (32), and it is also reported that AMPK can activate PI3-K/Akt pathway (45, 46). Here, we demonstrate that AICAR treatment of HK-532 cells also increases Akt phosphorylation, whereas insulin pretreatment significantly increased AICAR-mediated Akt phosphorylation (Fig. 5B). AICAR treatment did not increase ERK phosphorylation with or without insulin pretreatment (Fig. 5B). We previously reported that prevention of Akt hyperactivation restored insulin responsiveness and reversed IR in DRG and cortical neurons (43, 44). Similarly, treatment of HK-532 cells with insulin along with LY294002 to inhibit the hyperactivation of Akt prevented the overphosphorylation of AMPKSer-485 by AICAR (Fig. 5, C and D, panel b). As expected, AMPKThr-172 phosphorylation was not affected by insulin or LY294002 treatment (Fig. 5, C and D, panel a). LY294002 treatment also restored AICAR-mediated dephosphorylation of Tau (Fig. 5, C and D, panel c). Treatment with the MAPK inhibitor, U0126, had no effect on AMPKSer-485 or Tau phosphorylation (Fig. 5C). These results strongly suggest that hyperactivation of Akt increases AMPKSer-485 phosphorylation and ultimately leads to the inhibition of AMPK-mediated dephosphorylation of Tau.

Finally, to confirm the specific contribution of AMPKSer-485 phosphorylation, we transfected HK-532 cells with lentiviral vectors expressing nonphosphorylatable mutants of AMPKSer-485, AMPK S485A. Transfection of AMPK S485A decreased basal as well as AICAR-induced AMPKSer-485 phosphorylation and slightly increased AMPKThr-172 phosphorylation (Fig. 6A). AMPK S485A transfection suppressed AMPKSer-485 hyperphosphorylation after insulin pretreatment (Fig. 6B). Tau phosphorylation levels correlated well with the reduced AMPKSer-485 phosphorylation, and more importantly, AMPK S485A transfection restored the ability of AICAR to reduce Tau phosphorylation after insulin pretreatment (Fig. 6C). Stable transfection of S485A, but not S485D, mutant also reversed the inhibitor effect of IR on AICAR-induced Tau phosphorylation (Fig. 6, D and E). These results emphasize the direct and important role of Ser-485 in AMPK-mediated Tau phosphorylation.

**Discussion**

It is now well established that MetS, including obesity and diabetes, is a risk factor for AD (5, 6). IR is at the core of MetS, and studies suggest that IR has an important role for the development/acceleration of AD and further emphasize the need for precise regulation of energy expenditure (6, 7). There is significant gap in our knowledge, however, about the molecular link between IR and increased risk of AD. Considering the important role of AMPK in energy metabolism, we can naturally speculate that it is the crucial link between IR and AD. In the current study, we report that the activation of AMPK by AICAR resulted in Tau dephosphorylation and that chronic insulin treatment prevented AICAR-induced Tau dephosphorylation. Furthermore, we demonstrated for the first time that chronic insulin treatment specifically increased AICAR-induced AMPKSer-485 phosphorylation, consistent with what is observed in the brains of the diabetes and obesity mouse models, and preventing hyperactivation of Akt during chronic insulin...
treatment prevented the overphosphorylation of Ser-485 and subsequently restored the ability of AMPK to reduce Tau phosphorylation. Together, these results emphasize the important role of AMPKSer-485 in AMPK regulation of Tau phosphorylation, especially in IR.

Given that most of the studies of AMPK are focused on peripheral tissues, there is very limited knowledge about the regulation AMPK in brain regions other than the hypothalamus (47, 48). Our results from mouse brains demonstrate that MetS induces a specific increase in AMPKSer-485 phosphorylation in the cortices of both db/db and obese mice. We also observed increased Akt phosphorylation in these brains caused by IR (44), suggesting that increased Akt may contribute to the increased AMPKSer-485 phosphorylation (31, 32). In a very recent report, Arnold et al. (49) demonstrated for the first time that AMPK phosphorylation is increased in the brain of HFD-fed mice; however, their results only report AMPKThr-172 phosphorylation, which showed no changes in our study. This discrepancy may arise from apparent differences in the feeding scheme of the HFD between our study and theirs regarding the fat content (54% versus 60%), duration of feeding (24 weeks versus 17 days), and when the diet was initiated (4 weeks versus 8 weeks). Similarly, in another report, intracerebroventricular injection of streptozotocin decreased AMPK phosphorylation and increased Tau phosphorylation, both of which were reversed by AICAR administration (50). Because there are very few studies about AMPK in the brain, more research is required to obtain consistent results.

The exact contribution of AMPK to AD pathology is still controversial, with the results supporting both beneficial and detrimental effects (20, 24–29). Our results demonstrate a causal relationship between increased AMPKSer-485 phosphorylation and Tau phosphorylation in db/db and obese mouse brains. Our current study does not clearly describe whether Tau phosphorylation is regulated directly by AMPK, especially AMPKSer-485 phosphorylation, or through other kinases. However, the close correlation between AMPK and Tau phosphorylation observed in vivo along with our in vitro results strongly suggest the important contribution of AMPKSer-485 on Tau phosphorylation. Most of the studies about the connection between AMPK and AD pathology focus only on AMPKThr-172 phosphorylation. It is possible that the status of AMPKSer-485 phosphorylation (which was not examined in previous reports) might have resulted in the contradictory findings.

The important role of AMPKSer-485 phosphorylation on Tau phosphorylation is supported by our in vitro results. In agreement with published reports (26, 27), we show that activation of AMPK by AICAR reduced Tau phosphorylation, whereas induction of IR correlates with a specific increase in AMPKSer-485 phosphorylation and prevents AICAR-induced Tau dephosphorylation. However, our results demonstrating that increased AMPKSer-485 phosphorylation during IR did not affect AMPKThr-172 phosphorylation conflict with previous reports demonstrating that increases in AMPKSer-485 phosphorylation are generally accompanied by decreased AMPKThr-172 phosphorylation (31, 32, 51). This may possibly be due to the relatively small increase in AMPKSer-485 phosphorylation observed during IR, or it could be that AMPKSer-485-mediated regulation of Tau phosphorylation is independent of AMPKThr-172 phosphorylation. Thus, the precise role of AMPK on Tau phosphorylation is still controversial and likely depends on the context of the experiments.

AMPKThr-172 is phosphorylated by LKB1 or Ca2+/calmodulin-dependent protein kinase kinase β (21, 30), whereas AMPKSer-485 is phosphorylated by Akt (31, 32). Our in vitro data further demonstrate that AMPK reciprocally increases the phosphorylation and activation of Akt. Interestingly, AMPK activation during IR significantly increased Akt phosphorylation, correlating well with the specific increase of AMPKSer-485 phosphorylation. Although some studies suggest cooperative interactions between AMPK and Akt activation (45, 46), we believe, at least in our system, that AICAR-mediated Akt activation serves as a negative feedback regulator that limits AMPK activity. Indeed, we observe that AMPKThr-172 phosphorylation returns to basal levels after long term (>8 h) AICAR treatment (data not shown). Thus, IR induces overactivation of Akt and a subsequent increase in AMPKSer-485 phosphorylation, which results in the disruption of AMPK signaling.

In the current study, we further demonstrate that Akt hyperphosphorylation by AMPK in IR results in inhibition of AMPK-mediated Tau dephosphorylation and that AMPKSer-485 phosphorylation has a direct role in Tau phosphorylation using two Ser-485 phosphorylation-resistant AMPK mutant constructs. There are reports for both increased (28, 29) and decreased (25–27, 50) Tau phosphorylation by AMPK activation; however, none of the studies examined the role of AMPKSer-485 phosphorylation, and very few examined the connection between IR and AMPK in the brain (49, 50). Of note, insulin antagonizes AMPK activation by increased phosphorylation of AMPKSer-485 (31, 52); therefore, we believe that IR overactivates Akt, resulting in increased AMPKSer-485 phosphorylation and subsequent inhibition of Tau dephosphorylation.

Our results are summarized in the model (Fig. 7). In normal conditions, AMPK regulates Tau phosphorylation through balanced phosphorylation of Thr-172 and Ser-485. AMPK-mediated Akt phosphorylation may act as a negative feedback mechanism for a precise regulation of AMPK activity. In the MetS status, IR induces impaired insulin/Akt signaling, with supporting data for not only reduced Akt activity.
Role of AMPK<sub>Ser-485</sub> in Tau Phosphorylation in IR

A

Insulin (h)

0 1 4 24

pAkt
Akt
pERK
ERK
actin

B

Ins AICAR

- - + +

pAkt
Akt
pERK
ERK
actin

D

\[
\text{Relative density (Akt/actin)}
\]

Control AICAR

no Insulin Insulin

Inhibitors

1 h

Insulin (20 μM)
Inhibitors

(50 nM)
O/N

AICAR

(1 mM)
2 h

Harvest

Ins

- - + +

inhibitors

- - + +

AICAR

- - + +

pAPMK (T172)
pAMPK (S485)
pTau (S262)
pTau (S199/202)
actin

Relative density

pAMPK (T172)

1.00 1.57 0.98 1.89 1.64 2.77

pAMPK (S485)

1.00 1.84 1.32 3.69 1.86 3.48

pTau (pS199/202)

2.35 1.66 3.07 2.94 2.96 2.43

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vation but also instances of chronic overactivation of Akt (43, 44). Overactivation of Akt in IR subsequently leads to the hyperphosphorylation of AMPKα SER-485. These changes result in the elevated phosphorylation of Tau. It is not clear from our study whether AMPKα SER-485 affects Tau phosphorylation through inhibitory action on AMPKα THR-172 or direct independent action on Tau. Our results suggest for the first time a possible contribution of AMPKα SER-485 phosphorylation toward the increased risk of AD in IR associated with diabetes and obesity. Furthermore, this study supports a new

FIGURE 5. Hyperactivation of Akt by insulin pretreatment is responsible for the increased AMPKα SER-485 phosphorylation in HK-532 cells. A, HK-532 cells were treated with 20 nM insulin and Akt, and ERK phosphorylation was examined. B, HK-532 cells were incubated overnight without or with 50 nM insulin (Ins) and then treated with 1 mM AICAR for 2 h. Insulin pretreatment significantly increased AICAR induced Akt phosphorylation. *, p < 0.05; #, p < 0.01 by t test. C, the cells were incubated with insulin along with 20 μM LY294002 (LY) or U0126 (U) overnight. The cells were washed and treated with 1 mM AICAR for 2 h. D, inhibiting Akt overactivation during insulin pretreatment by LY294002 prevented AMPKα SER-485 hyperphosphorylation (b) and restored AICAR-induced Tau dephosphorylation. (c) but did not affect AMPKα THR-172 phosphorylation (a). *, p < 0.05 compared with insulin + LY294002 + AICAR; #, p < 0.05 compared with insulin or insulin + AICAR; †, p < 0.05 compared with control.

FIGURE 6. Preventing AMPKα SER-485 hyperphosphorylation by mutating Ser-485 restored AICAR-induced Tau dephosphorylation by insulin pretreatment. A, HK-532 cell were infected with a lentiviral vector expressing S485A AMPK mutants or control for 5 days. S485A mutant transfection reduced both basal and AICAR-induced Ser-485 phosphorylation by AICAR. The cells were treated without or with 50 nM insulin over night and then treated with 1 mM AICAR for 2 h. B and C, cell lysates were examined for AMPK and Tau phosphorylation. D, HK-532 cells were transfected with S485A or S485D AMPK mutant, and the stable clones were selected by puromycin. AICAR-induced Tau dephosphorylation was examined after without or with 50 nM overnight insulin pretreatment. E, densitometry of the result of stable AMPK mutant transfection. *, p < 0.05; #, p < 0.005.
Role of AMPK<sub>Ser-485</sub> in Tau Phosphorylation in IR

**References**


