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Identification of Epigenetically Altered Genes in Sporadic Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a terminal disease involving the progressive degeneration of motor neurons within the motor cortex, brainstem and spinal cord. Most cases are sporadic (sALS) with unknown causes suggesting that the etiology of sALS may not be limited to the genotype of patients, but may be influenced by exposure to environmental factors. Alterations in epigenetic modifications are likely to play a role in disease onset and progression in ALS, as aberrant epigenetic patterns may be acquired throughout life. The aim of this study was to identify epigenetic marks associated with sALS. We hypothesize that epigenetic modifications may alter the expression of pathogenesis-related genes leading to the onset and progression of sALS. Using ELISA assays, we observed alterations in global methylation (5 mC) and hydroxymethylation (5 HmC) in postmortem sALS spinal cord but not in whole blood. Loci-specific differentially methylated and expressed genes in sALS spinal cord were identified by genome-wide 5mC and expression profiling using high-throughput microarrays. Concordant direction, hyper- or hypo-5mC with parallel changes in gene expression (under- or over-expression), was observed in 112 genes highly associated with biological functions related to immune and inflammation response. Furthermore, literature-based analysis identified potential associations among the epigenes. Integration of methylomics and transcriptomics data successfully revealed methylation changes in sALS spinal cord. This study represents an initial identification of epigenetic regulatory mechanisms in sALS which may improve our understanding of sALS pathogenesis for the identification of biomarkers and new therapeutic targets.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and terminal neurodegenerative disease characterized by the selective degeneration of motor neurons within the motor cortex, brainstem and spinal cord [1]. In the United States, approximately 14 cases of ALS are diagnosed each day and 30,000 people are living with the disease. The average time from disease onset to death is 3 years and no treatment that substantially improves the clinical course of the disease is currently available [1].

Proposed pathogenic mechanisms of ALS include oxidative stress, glutamate excitotoxicity, impaired axonal transport, neurotrophic deprivation, neuroinflammation, apoptosis, altered protein turnover, and mitochondrial dysfunction [1,2]. Moreover, influences from astrocytes and microglia in the motor neuron microenvironment contribute to pathogenesis [3]. In the last 20 years, a search for genetic factors has identified several genes associated with familial ALS (fALS) and a few with sporadic ALS (sALS) [4–6]. Because fALS only accounts for 5–10% of all cases of ALS, the causes leading to the vast majority of ALS (sALS) are poorly understood [1].

Environmental exposure to toxins, excessive physical activity, dietary factors, and changes in immunity increase the risk of developing ALS [7]. These factors may drive epigenetic changes, which are well suited to explain disease onset and progression in sALS, as they may be acquired throughout life. Epigenetic modifications, including covalent modifications of DNA and histones as well as RNA editing, dynamically regulate gene expression without altering the genetic code [8,9]. These modifications are important in chromosome integrity, cellular differentiation, development, and aging [8,10]. Two such modifications, 5-methylcytosine (5 mC) and 5-hydroxymethylcytosine (5 HmC) are associated with repression or activation of gene expression, respectively, in response to environmental and developmental factors linked to age-related diseases [11].


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† These authors contributed equally to this work.
5mC at CpG (cytosine nucleotide separated by a phosphate from a guanine nucleotide) sites is a reversible mechanism facilitated by DNA (cytosine-5)-methyltransferases (DNMTs). Conversely, the Fe(II) and α-ketoglutarate (α-KG)-dependent ten-eleven translocation (TET) family of proteins catalyze oxidation and decarboxylation reactions of 5mC leading to 5-hydroxymethylcytosine (5 HmC), 5-formylcytosine (5 fC) and 5-carboxylcytosine (5 caC) [12,13]. 5 HmC may be an intermediate for passive (during DNA replication) and active demethylation and/or serve as a docking site for proteins with high affinity for 5 HmC, thereby dissociating interactions between the transcriptional repression machinery and 5 mC [14].

In addition to the identification of alterations in global 5 HmC associated with sALS, this study represents one of the first methylation assessments of sALS by integrating methylome and transcriptome profiles of postmortem frozen human spinal cord samples. We identified differentially methylated sALS spinal cord genes exhibiting concordant mRNA expression overrepresented in functional categories implicated in sALS. These data support a role for epigenetic regulation in sALS and it may provide a better understanding of disease pathogenesis and facilitate the discovery of new therapeutic targets.

Results

A workflow of our data analysis is provided in Fig. 1.

Global 5mC is Increased in sALS Spinal Cord

Chestnut et al. recently reported an increase in DNMTs and 5mC immunoreactivity in ALS brain and spinal cord, suggesting that a global increase in 5mC is associated with the pathogenesis of ALS [15]. We assessed global 5mC of genomic DNA extracted from postmortem human spinal cord samples (sALS, n = 11; matching controls, n = 11; Tables 1, S1) using a colorimetric ELISA approach. We observed a modest but significant 1.4-fold increase in global 5mC in sALS (3.58±0.10) compared to controls (2.56±0.10) (p = 0.0006, Fig. 2), confirming previous observations [15].

Genome-wide 5mC Profiling in sALS Spinal Cord

To identify loci-specific differentially methylated genes (DMGs) in sALS, postmortem spinal cord tissue (Tables 1, S1) was subjected to an unbiased genome-wide methylation screen using the Infinium Human Methylation27 DNA BeadChip (HM27K) array. We identified 4,261 significant differentially methylated autosomal CpG sites, representing 3,574 genes. Functional enrichment analysis identified biological categories of extracellular region (p = 3.5E-30), defense response (p = 2.3E-18), cytokine activity (p = 2.7E-16), immune response (p = 6.9E-15), JAK-STAT signaling pathway (p = 2.2E-7), steroid hormone biosynthesis (p = 3.6E-7), and drug metabolism (p = 3.1E-5) were over-represented in spinal cord DMGs (Fig. 3). This suggests a role for epigenetic regulation in molecular mechanisms known to drive sALS pathogenesis [16–18]. Furthermore, the identified DMGs included previously reported epigenetically regulated genes such as runt-related transcription factor 3 (RUNX3), TNF-related apoptosis-inducing ligand (TRAIL/TNFSF10), H19, neutrin 1 (MRV1) and signal transducer and activator of transcription 5A (STAT5A) [19–23], validating our approach. The ALS-dependent differential methylation of three genes encoding the chemokines CCL1-3, like MARVEL transmembrane domain-containing proteins 2 and 3 (CMTM2 and CMTM3) and the chemokine C-X-C motif ligand 12 (CXCL12), as well as the two transcription factors, STAT5A and the CCAAT/enhancer binding protein beta (C/EBPB) was assessed by pyrosequencing (Fig. S1). These results indicate parallel direction of differential methylation between the two assays.

Genome-wide Expression Profiling in sALS Spinal Cord

An increase in promoter 5mC (hyper-methylation) is associated with gene silencing, while a decrease (hypo-methylation) reflects up-regulation of gene expression [8]. To identify functionally relevant epigenes, we performed genome-wide gene expression profiling of total RNA from sALS (n = 12) and control (n = 10) spinal cord (Tables 1, S1). We found 1,102 DEGs in sALS enriched with immune response-related biological categories such as defense response (p = 2.3E-20), inflammatory response (p = 1.3E-18), T-cell activation (p = 3.0E-9), leukocyte activation (p = 1.6E-9), cytokine binding (p = 1.7E-7) as well as carbohydrate binding (p = 7.2E-6), transmembrane receptor protein tyrosine kinase signaling (p = 2.6E-5), cell death (p = 1.3E-5), and JAK-STAT signaling (p = 3.6E-2) (Fig. 3). Several of these biological categories overlap with the 5mC profile, suggesting methylation may play a role in the regulation of these DEGs.

Comparison between DMGs and DEGs in sALS Spinal Cord

DMGs and DEGs were analyzed for their direction of change to select genes demonstrating concordant and potentially true regulation of gene expression by methylation. Between 3,574 DMGs and 1,182 DEGs, 251 genes were common (Table S2), of which approximately 70% were hyper-methylated as observed with global 5mC. Among these common genes, 112 epigenes...
demonstrated concordant direction of methylation and expression changes (hypo-methylation/up-regulation (51 genes; Table 2) or hyper-methylation/down-regulation (61 genes; Table 3) (Fig. 4). These concordant epigenes were enriched in biological categories including immune response (p = 7.1E-5), plasma membrane part (p = 9.6E-5), defense response (p = 0.03), and neuron adhesion (p = 0.05) (Fig. 3).

Gene co-citation network analysis revealed potential associations between some of the concordant genes identified in our screens. Among the 112 concordant epigenes, 53 genes were co-cited at least once in PubMed abstracts at the sentence or abstract-level. Within the co-citation network, the epigenes were classified into representative biological functions such as immune response, antigen presentation, tumor/suppressor related, and extracellular matrix repair (Fig. 5). In this network, the transcription factors C/EBPB and STAT5A were highly connected, suggesting a role in sALS pathogenesis. Moreover, some of these connections were directly or indirectly associated with concordant genes previously related to neuronal development such as muscle segment homeobox (msx) 2 (MSX2), megalencephalic leukoencephalopathy with subcortical cysts 1 (MLC1), RUNX3, catenin delta-2 (CTNND2), receptor tyrosine kinase (AXL), neuronatin (NNAT), and NRN1 [24–30] (Fig. 5).

SciMiner identified 4,128 genes from ALS-related publications (as of 7/23/2012), which were compared to our 112 concordant epigenes. Fourteen genes were identified in two or more ALS-related publications with frequencies that were significantly different from those in over 20 million abstracts in PubMed (p < 0.05). Fifty-one genes demonstrated 2-fold altered expression, including the chitinase 3-like protein 2 (CHI3L2), the triggering receptor expressed on myeloid cells-2 (TREM2), cathepsin Z (CTSZ), the lumican precursor protein (LUM), H19, and TRAIL/TNFSF10 (Tables 2, 3). Thus, bioinformatics evaluation of the concordant epigenes identified by integrating methylomic and transcriptomic analyses detected both novel and previously known ALS-related genes.

Experimental Confirmation using Real-time Polymerase Chain Reaction (RT-PCR)

Expression of 14 concordant epigenes selected either from the ALS-related literature or from the expression array data was confirmed by RT-PCR (Figs. 6 and S2, Table 4). Of the genes previously related to the ALS literature, NRN1, FMO1, and the lumican precursor protein (LUM) were under-expressed, while the lysosomal protease CTSZ was over-expressed in sALS. No significant difference between sALS and control subjects was observed for FES-upstream region (FURIN). Novel sALS-associ-
ated epigenes such as STAT5A, TREM2, the high-affinity IgE receptor (FCER1G), CHI3L2, and the proton-couple divalent metal ion transporter solute carrier family 11 member 1A (SLC11A1) were over-expressed in sALS. SLC11A1 presented the highest increase by 12.7-fold. Down-regulation was validated for gap junction β-2 (GJB2)/Connexin-26 as well as imprinted genes such as H19, NNAT, and the paternally expressed 10 (PEG10). In summary, the RT-PCR expression data indicate high concordance with the microarray expression data, validating our results.

Global 5 HmC Increases in sALS Spinal Cord

5 HmC, an alternate epigenetic modification of DNA, is increased in brain compared to other human tissues, and alterations in global 5 HmC are associated with age-related neurodegenerative disorders, suggesting an important role of 5 HmC in neuronal the function [31–34]. We measured global 5 HmC for sALS and control spinal cord samples previously analyzed for global 5mC. We observed an approximately 3.0-fold increase in global 5 HmC in sALS (0.31 ± 0.02) compared to controls (0.11 ± 0.03) \( p < 0.0001 \) (Fig. 7). This is the first report of

<table>
<thead>
<tr>
<th>ID</th>
<th>Terms</th>
<th>DMG</th>
<th>Concordant Epigene</th>
<th>DEG</th>
</tr>
</thead>
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<tr>
<td>GO:0005576</td>
<td>extracellular region</td>
<td>3,574</td>
<td>112</td>
<td>1,182</td>
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<td>GO:0006952</td>
<td>defense response</td>
<td>17.64</td>
<td>1.46</td>
<td>19.64</td>
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<tr>
<td>GO:0005125</td>
<td>cytokine activity</td>
<td>15.56</td>
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<td></td>
</tr>
<tr>
<td>GO:0006955</td>
<td>immune response</td>
<td>14.16</td>
<td>4.15</td>
<td>21.84</td>
</tr>
<tr>
<td>hsa04600</td>
<td>Cytokine-cytokine receptor interaction</td>
<td>13.67</td>
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<td>2.60</td>
</tr>
<tr>
<td>GO:005887</td>
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<td>11.01</td>
<td>3.50</td>
<td>13.42</td>
</tr>
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<td></td>
<td>1.32</td>
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<tr>
<td>GO:0044559</td>
<td>plasma membrane part</td>
<td>8.60</td>
<td>4.02</td>
<td>13.40</td>
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<td>hsa00780</td>
<td>Metabolism of xenobiotics by cytochrome P450</td>
<td>8.51</td>
<td></td>
<td></td>
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<td>GO:0030246</td>
<td>carbohydrate binding</td>
<td>7.80</td>
<td>3.33</td>
<td>5.14</td>
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<tr>
<td>GO:0019825</td>
<td>oxygen binding</td>
<td>7.04</td>
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<tr>
<td>GO:0045087</td>
<td>innate immune response</td>
<td>6.81</td>
<td>8.84</td>
<td></td>
</tr>
<tr>
<td>GO:0031640</td>
<td>killing of cells of another organism</td>
<td>6.76</td>
<td></td>
<td></td>
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<tr>
<td>hsa00982</td>
<td>Drug metabolism</td>
<td>6.76</td>
<td></td>
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<tr>
<td>hsa04630</td>
<td>Jak-STAT signaling pathway</td>
<td>6.66</td>
<td>1.44</td>
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<tr>
<td>GO:0005786</td>
<td>digestion</td>
<td>6.61</td>
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<tr>
<td>hsa05320</td>
<td>Autoimmune thyroid disease</td>
<td>6.61</td>
<td>1.76</td>
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<tr>
<td>GO:009611</td>
<td>response to wounding</td>
<td>5.86</td>
<td>14.84</td>
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<td>GO:000954</td>
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<td>GO:0025684</td>
<td>positive regulation of immune system process</td>
<td>5.30</td>
<td>1.23</td>
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<td>GO:001775</td>
<td>cell activation</td>
<td>4.24</td>
<td>1.00</td>
<td>9.97</td>
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<td>GO:0050865</td>
<td>regulation of cell activation</td>
<td>3.73</td>
<td>9.76</td>
<td></td>
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<tr>
<td>GO:002694</td>
<td>regulation of leukocyte activation</td>
<td>3.51</td>
<td>9.20</td>
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<tr>
<td>GO:0001817</td>
<td>regulation of cytokine production</td>
<td>3.33</td>
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<td>3.07</td>
<td>9.74</td>
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<td>GO:0051249</td>
<td>regulation of lymphocyte activation</td>
<td>3.06</td>
<td>9.32</td>
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<td>GO:0050778</td>
<td>positive regulation of immune response</td>
<td>2.22</td>
<td>1.22</td>
<td>13.02</td>
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<td>GO:0042110</td>
<td>T cell activation</td>
<td>2.09</td>
<td>1.37</td>
<td>8.52</td>
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<td>GO:0045321</td>
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<td>1.99</td>
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<tr>
<td>GO:0002253</td>
<td>activation of immune response</td>
<td>1.86</td>
<td>10.18</td>
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<tr>
<td>GO:002757</td>
<td>immune response-activating signal transduction</td>
<td>1.86</td>
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<td>GO:0031224</td>
<td>intrinsic to membrane</td>
<td>3.50</td>
<td>6.64</td>
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<tr>
<td>GO:0019882</td>
<td>antigen processing and presentation</td>
<td>1.84</td>
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<td>GO:007169</td>
<td>transmembrane receptor protein tyrosine kinase signaling pathway</td>
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<td>GO:007167</td>
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<tr>
<td>GO:0001779</td>
<td>natural killer cell differentiation</td>
<td>1.61</td>
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<tr>
<td>GO:004537</td>
<td>mucosal-associated lymphoid tissue development</td>
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<td>GO:0048541</td>
<td>Peyer's patch development</td>
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<td>GO:0030670</td>
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<td>GO:0096002</td>
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<tr>
<td>GO:0007158</td>
<td>neuron adhesion</td>
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Figure 3. Biological functions of DMGs, DEGs, and concordant epigenes. Overrepresented biological functions were identified using DAVID. The top 20 biological functions ordered by p-value were collected from each gene set, and redundant terms were combined. The values in the table correspond to -log10 (DAVID p-value), ranging from 0 (white) to 30 (bright red). The values are not normalized across different gene sets with variable numbers of genes. The order of biological terms was based on the log-transformed p-values of DMGs and DEGs.

doi:10.1371/journal.pone.0052672.g003
### Table 2. Hypo-methylated and up-regulated concordant epigenes.

<table>
<thead>
<tr>
<th>GeneID</th>
<th>Description</th>
<th>Symbol</th>
<th>ΔS mc (%)</th>
<th>DS</th>
<th>FC</th>
<th>SM</th>
<th>ALSoD &amp; OS</th>
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<tr>
<td>58475</td>
<td>membrane-spanning 4-domains, subfamily A, member 7</td>
<td>M54A7</td>
<td>6</td>
<td>−190</td>
<td>4.6</td>
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<td>1439</td>
<td>colony stimulating factor 2 receptor, beta, low-affinity</td>
<td>CSF2RB</td>
<td>6.6</td>
<td>−35.4</td>
<td>3.8</td>
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<td>9056</td>
<td>solute carrier family 7 (amino acid transporter light chain, y+L system), member 7</td>
<td>SLC7A7</td>
<td>6.6</td>
<td>−18.6</td>
<td>3.5</td>
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<td>822</td>
<td>capping protein (actin filament), gelsolin-like</td>
<td>CAPG</td>
<td>5.8</td>
<td>−13.9</td>
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<td>−34.4</td>
<td>3.1</td>
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<td>triggering receptor expressed on myeloid cells 2</td>
<td>TREM2</td>
<td>7.1</td>
<td>−26.0</td>
<td>2.9</td>
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<td>CD1d molecule</td>
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<td>glycoprotein (transmembrane) nmb</td>
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<td>4046</td>
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aberrant levels of global 5 HmC associated with the pathogenesis of sALS.

Global 5mC and 5 HmC in sALS Whole Blood

High correlation of epigenetic marks in spinal cord and blood may be useful for diagnostic and therapeutic application in ALS. We investigated whether global 5mC and 5 HmC would be altered in sALS whole blood similarly to spinal cord. Whole blood genomic DNA from a different cohort (Tables 5, S1) was subjected to global 5mC and global 5 HmC by ELISA. The levels of percent global 5mC and 5 HmC in whole blood were 10 fold lower compared to spinal cord, in agreement with recent reports [33,35]. Contrary to spinal cord, no differential percent global 5mC or global 5 HmC were observed in whole blood. Although several genes have been implicated in the pathogenesis of ALS, the causes leading to most cases remain unknown. Environmental factors may be associated with the onset and development of sALS by altering epigenetic regulation [7,8]. The aim of this study was to identify sALS-associated epigenetic marks resulting in aberrant gene expression. Abnormal 5mC patterns of repetitive elements such as Alu and LINE1, as well as altered function of methylation regulators such as the DNMTs, lead to changes in global 5mC or 5 HmC associated with neurodegeneration [15,36]. We demonstrated increased global methylation in sALS spinal cord, perhaps due to an increase in DNMT activity [31]. Furthermore, we report for the first time an increase in global 5 HmC in sALS spinal cord. Increased 5mC and 5 HmC may be associated with spinal muscular atrophy, a neurodegenerative disease with similar pathogenesis as ALS, and it provides oligodendrocyte protection, which in turn favors neuronal damage surpasses the capacity of the nervous system to respond to immunosuppressive and anti-inflammatory therapies at later stages of ALS, implicating a need for biomarkers identifying early immune-related changes in sALS.

Co-citation network and literature mining approaches identified connections among novel and previously implicated ALS-related epigenes and pathways [51,52]. The transcription factors STAT5A and C/EBPB are highly connected in our co-citation network and their interplay promote activation of various genes including interleukin-6 (IL-6) [53]. Moreover, recent reports implicate C/EBPB and STAT5A in ALS pathogenesis and neurodegeneration. For instance, expression of C/EBPB in ALS microglia from spinal cord suggests an important role of C/EBPB in the regulation of neurotoxic genes in the ALS neuronal microenvironment [45,54]. Furthermore, changes in STAT5A expression may reflect an altered inflammatory response contributing to the pathogenesis of ALS. Over-expression of STAT5A reduces neuronal degeneration associated with spinal muscular atrophy, a neurodegenerative disease with similar pathogenesis as ALS, and it provides oligodendrocyte protection, which in turn favors neuronal environment preservation [55–57]. Whether positive regulation of STAT5A in sALS is due to an anti-apoptotic response to compensate for the degeneration of the nervous system, or its over-expression is responsible, in part, for the pathogenesis of the disease remains to be determined. Interestingly, we observed potential transcription factor binding sites (TFBSs) for STAT5A and C/EBPB in 40% and 48% of the promoters of our identified DEGs, respectively; the binding sites for STAT5A and C/EBPB are 1.2 (p = 4.1E-12) and 1.3 (p = 3.8E-13) times more frequent in the DEGs than in the vertebrate promoters, respectively. Our observations suggest epigenetic mechanisms, in part, drive the expression of central regulators of downstream targets in sALS.

Our study identified ALS-dependent methylation dysregulation of several genes previously implicated in neuronal development, differentiation, and proliferation, including Slit-Robo Rho

### Table 2. Cont.

<table>
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<tr>
<th>GeneID</th>
<th>Description</th>
<th>Symbol</th>
<th>ΔS mc (%)</th>
<th>DS</th>
<th>FC</th>
<th>SM</th>
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Genes are ordered by expression fold change (FC). Percent methylation change (ΔS mc (%)) is calculated by $\frac{\beta_{\text{ALS}} - \beta_{\text{control}}}{\beta_{\text{control}}}$. Differential methylation (a transformation of the p-value; $p < 0.05$, $p = 0.01$, $p = 0.001$ are equivalent to DiffScore = 13, 20, 30, respectively). Fold change (FC) in gene expression $\Delta V$ indicates that the corresponding gene was identified as an ALS-associated gene by SciMiner (SM), the ALSoD database, and other high-throughput microarray studies (OS) on human ALS spinal cord samples, respectively. See Table S2 more details.

doi:10.1371/journal.pone.0052672.t002
Table 3. Hyper-methylated and down-regulated concordant epigenes.

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<td>6.7</td>
<td>49.5</td>
<td>-1.8</td>
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<tr>
<td>168537</td>
<td>GTPase, IMAP family member 7</td>
<td>GMAP7</td>
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<tr>
<td>4199</td>
<td>malic enzyme 1, NADP(+)-dependent, cytosolic</td>
<td>ME1</td>
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<td>-1.7</td>
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<tr>
<td>113932</td>
<td>transmembrane protein 139</td>
<td>TMEM139</td>
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<td>26.9</td>
<td>-1.7</td>
<td></td>
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<tr>
<td>6932</td>
<td>transcription factor 7 (T-cell specific, HMG-box)</td>
<td>TCF7</td>
<td>5.3</td>
<td>87.4</td>
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<tr>
<td>79827</td>
<td>CXADR-like membrane protein</td>
<td>ASAM</td>
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<td>-1.6</td>
<td></td>
<td></td>
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<tr>
<td>152330</td>
<td>contactin 4</td>
<td>CNTN4</td>
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<td>48.8</td>
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<tr>
<td>55228</td>
<td>PNMA-like 1</td>
<td>PNML1</td>
<td>8.8</td>
<td>30.6</td>
<td>-1.6</td>
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<td></td>
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<tr>
<td>150330</td>
<td>ENTH domain containing 1</td>
<td>ENTHD1</td>
<td>4.8</td>
<td>81.9</td>
<td>-1.5</td>
<td></td>
<td></td>
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<tr>
<td>27237</td>
<td>Rho guanine nucleotide exchange factor (GEF) 16</td>
<td>ARHGEF16</td>
<td>7.5</td>
<td>101.6</td>
<td>-1.5</td>
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<tr>
<td>57415</td>
<td>chromosome 3 open reading frame 14</td>
<td>C3orf14</td>
<td>7.9</td>
<td>78.2</td>
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<tr>
<td>84909</td>
<td>chromosome 9 open reading frame 3</td>
<td>C9orf3</td>
<td>0.1</td>
<td>40.4</td>
<td>-1.5</td>
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<tr>
<td>2326</td>
<td>flavin containing monooxygenase 1</td>
<td>FMO1</td>
<td>9.4</td>
<td>61.1</td>
<td>-1.5</td>
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<tr>
<td>5172</td>
<td>solute carrier family 26, member 4</td>
<td>SLC26A4</td>
<td>9.4</td>
<td>23.5</td>
<td>-1.5</td>
<td>✓</td>
<td></td>
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<tr>
<td>9467</td>
<td>SH3-domain binding protein 5 (BTK-associated)</td>
<td>SH3BP5</td>
<td>7</td>
<td>50.6</td>
<td>-1.5</td>
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<td></td>
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<tr>
<td>166</td>
<td>amino-terminal enhancer of split</td>
<td>AES</td>
<td>5.8</td>
<td>42.7</td>
<td>-1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3768</td>
<td>potassium inwardly-rectifying channel, subfamily J, member 12</td>
<td>KCNJ12</td>
<td>14.5</td>
<td>343.9</td>
<td>-1.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Another sALS-related epigene, CTSX, warrants further investigation since its aberrant expression is associated with neurodegeneration by promoting neurotoxin elimination in the damaged cellular environment [62]. Expression of CTSX as well as two other members of the cathepsin family, cathepsins B and D, increases in the cellular environment [62]. Expression of CTSX in the regulation of neuronal homeostasis. Nevertheless, more studies need to be done to address the role of the promoter region of SOD1, PCP4, and flavin containing monoxygenase 1 (FMO1), and five of our concordant genes presented opposite direction of expression when compared to known ALS-dependent differentially expressed gene. Our data indicate that epigenetic mechanisms are potential regulators of these key genes in ALS.

Based on the large number of genes identified in the methylated (3,574 genes) and expression (1,182 genes) arrays, relatively few sALS-associated genes presented concordant direction between methylation and gene expression. The low occurrence of a small subset of genes potentially regulated by CpG modification in such a way that hyper-methylation promotes gene silencing and hypo-methylation promotes gene expression has been previously documented [73]. 5mC within promoter regions is associated with repression of gene expression by interfering with transcription factor binding or by providing a binding site for transcriptional repressors [10]. Interestingly, over half (53%) of the 251 common DMGs/DEGs presented same direction of 5mC and gene expression when compared to known ALS-dependent differential-expression studies (OS) on human ALS spinal cord samples, respectively. See Table S2 more details.

Table 3. Cont.

<table>
<thead>
<tr>
<th>GeneID</th>
<th>Description</th>
<th>Symbol</th>
<th>DSMc (%</th>
<th>DS</th>
<th>FC</th>
<th>SM</th>
<th>ALSoD &amp; OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>22927</td>
<td>hyaluronan binding protein 4</td>
<td>HABP4</td>
<td>12.2</td>
<td>45.1</td>
<td>-1.4</td>
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</tr>
<tr>
<td>8357</td>
<td>breast carcinoma amplified sequence 1</td>
<td>BCAS1</td>
<td>8.7</td>
<td>59.2</td>
<td>-1.4</td>
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<td></td>
</tr>
<tr>
<td>8817</td>
<td>fibroblast growth factor 18</td>
<td>FGF18</td>
<td>7.8</td>
<td>15.8</td>
<td>-1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5213</td>
<td>phosphofructokinase, muscle</td>
<td>PFKM</td>
<td>5.9</td>
<td>29.3</td>
<td>-1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>85453</td>
<td>TSPY-like 5</td>
<td>TSPYLS</td>
<td>11.7</td>
<td>32</td>
<td>-1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4233</td>
<td>met proto-oncogene (hepatocyte growth factor receptor)</td>
<td>MET</td>
<td>6</td>
<td>22.9</td>
<td>-1.4</td>
<td></td>
<td></td>
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<tr>
<td>135250</td>
<td>retinoic acid early transcript 1E</td>
<td>RAET1E</td>
<td>9.6</td>
<td>89.7</td>
<td>-1.3</td>
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<td></td>
</tr>
<tr>
<td>2260</td>
<td>fibroblast growth factor receptor 1</td>
<td>FGFFR1</td>
<td>5.1</td>
<td>18.7</td>
<td>-1.3</td>
<td></td>
<td></td>
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<tr>
<td>90135</td>
<td>BTB (POZ) domain containing 6</td>
<td>BTBD6</td>
<td>9.3</td>
<td>23.5</td>
<td>-1.3</td>
<td></td>
<td></td>
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<tr>
<td>744</td>
<td>metallophosphoesterase domain containing 2</td>
<td>MPPED2</td>
<td>7.3</td>
<td>41.8</td>
<td>-1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3749</td>
<td>potassium voltage-gated channel, Shaw-related subfamily, member 4</td>
<td>KCNC4</td>
<td>3.2</td>
<td>28.2</td>
<td>-1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>91977</td>
<td>myozenin 3</td>
<td>MYOZ3</td>
<td>5</td>
<td>15.3</td>
<td>-1.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genes are ordered by expression fold change (FC). Percent methylation change (DSmc (%) is calculated by |BetaALS – Betacontrol|/Betacontrol. DiffScore (DF), Differential methylation (a transformation of the p-value; p = 0.05, p = 0.01, p = 0.001) are equivalent to DiffScore = ±13, ±20, ±30, respectively. Fold change (FC) in gene expression ‘+’ indicates that the corresponding gene was identified as an ALS-associated gene by SciMiner (SM), the ALSoD database, and other high-throughput microarray studies (OS) on human ALS spinal cord samples, respectively. See Table S2 more details.

doi:10.1371/journal.pone.0052672.g004

![Figure 4. Overlapping of DMGs and DEGs.](image-url)
Literature-derived information for potential associations among the concordant epigenes was obtained using Genomatix Pathway System (GePS). Among the 112 concordant epigenes, 53 genes were co-cited at least once in PubMed abstracts at sentence-level (solid lines) or abstract-level (dashed lines). These epigenes were grouped by their representative biological role: immune response (dark pink), antigen presentation (light pink), tumor/suppressor related (green), extracellular matrix repair (yellow), and others (gray). Neuronal development-related genes are enclosed by a square. The epigenes with more than 5 connections to other genes are enlarged. A diamond-shape represents a transcription factor.

doi:10.1371/journal.pone.0052672.g005

Table 4. Confirmation of microarray differential expression in spinal cord using RT-PCR.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Control</th>
<th>sALS</th>
<th>~p-value</th>
<th>Fold-Change</th>
<th>Microarray Fold-Change</th>
<th>Confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTSZ</td>
<td>1.10±0.07</td>
<td>2.06±0.23</td>
<td>0.001</td>
<td>1.9</td>
<td>2.2</td>
<td>Yes</td>
</tr>
<tr>
<td>STAT5A</td>
<td>1.10±0.12</td>
<td>1.58±0.20</td>
<td>0.05</td>
<td>1.4</td>
<td>1.6</td>
<td>Yes</td>
</tr>
<tr>
<td>TREM2</td>
<td>1.20±0.17</td>
<td>3.18±0.72</td>
<td>0.05</td>
<td>2.6</td>
<td>2.9</td>
<td>Yes</td>
</tr>
<tr>
<td>Fcer1g</td>
<td>1.10±0.11</td>
<td>2.30±0.19</td>
<td>0.001</td>
<td>2.1</td>
<td>2.4</td>
<td>Yes</td>
</tr>
<tr>
<td>Chi3l2</td>
<td>1.32±0.29</td>
<td>3.13±0.48</td>
<td>0.01</td>
<td>1.6</td>
<td>2.1</td>
<td>Yes</td>
</tr>
<tr>
<td>SLC11A1</td>
<td>1.34±0.23</td>
<td>17.00±3.38</td>
<td>0.001</td>
<td>12.7</td>
<td>3.1</td>
<td>Yes</td>
</tr>
<tr>
<td>NRN1</td>
<td>1.19±0.18</td>
<td>0.49±0.05</td>
<td>0.001</td>
<td>−2.4</td>
<td>−2.5</td>
<td>Yes</td>
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<tr>
<td>NNAT</td>
<td>1.14±0.20</td>
<td>0.52±0.06</td>
<td>0.05</td>
<td>−2.2</td>
<td>−2.2</td>
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<tr>
<td>FMO1</td>
<td>1.41±0.40</td>
<td>0.38±0.07</td>
<td>0.001</td>
<td>−3.7</td>
<td>−1.5</td>
<td>Yes</td>
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<tr>
<td>GJB2</td>
<td>1.38±0.27</td>
<td>0.35±0.07</td>
<td>0.001</td>
<td>−3.9</td>
<td>−4.7</td>
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<tr>
<td>FURIN</td>
<td>1.11±0.16</td>
<td>0.83±0.13</td>
<td>0.17</td>
<td>−1.3</td>
<td>1.6</td>
<td>No</td>
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<tr>
<td>H19</td>
<td>1.28±0.15</td>
<td>0.68±0.20</td>
<td>0.05</td>
<td>−1.9</td>
<td>−2.3</td>
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<tr>
<td>Peg10</td>
<td>1.08±0.08</td>
<td>0.79±0.07</td>
<td>0.01</td>
<td>−1.4</td>
<td>−1.8</td>
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<tr>
<td>LUM</td>
<td>1.40±0.29</td>
<td>0.32±0.13</td>
<td>0.01</td>
<td>−4.4</td>
<td>−4.1</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Yes* indicates the differential expression of the corresponding gene is statistically significant and demonstrated the same direction of change as in the microarray data.

doi:10.1371/journal.pone.0052672.t004
expression. In some cases, 5mC positively regulates gene transcription by promoting transcription factor binding at promoter regions [74] or, more commonly, by modifying intragenic CpG sites facilitating transcription efficiency, histone conformation, and regulating levels of sense and antisense mRNA [75]. Furthermore, 5 HmC, a highly enriched modification in brain, correlates with increased gene expression [10]. HM27K does not differentiate between 5mC and 5 HmC; therefore, some of the common epigenes presenting same direction of methylation and expression may be regulated by 5 HmC.

Although the high incidence of same direction sALS concordant epigenes parallels the high levels of global 5 HmC in spinal cord, loci specific 5 HmC modifications associated with sALS remain to be identified. Gene expression of non-common DEG (non-DMGs) could be determined, in part, by 5mC-dependent regulation of transcription factors. In addition to STAT5 and C/EBPB, we identified several transcription factors as concordant genes such as the transcription factor 7 (TCF7), RUNX3, IKAROS family zinc finger 1 (IKZF1), MSX2, and hypoxia inducible factor 3, alpha subunit (HIF3A). Furthermore, regulation of gene expression is a dynamic and complex mechanism and the interplay of several epigenetic pathways has been reported to modulate adult neurogenesis [76]. Therefore, alterations to epigenetic networks in conjunction with genetic predisposition may result in the development of sALS.

The prospect of identifying sALS epigenetic biomarkers in blood is exciting as it provides a minimally invasive alternative for sALS diagnostic and prognostic assessments. Although we did not detect significant global 5mC and 5 HmC differences in blood and inflammation-related epigenetic biomarkers may reflect systemic inflammatory changes rather than neuronal changes, further investigation of individual loci may provide potential epigenetic biomarkers for sALS.

There were several limitations to our study. First, a relatively small number of samples were analyzed and loci-specific 5 HmC analysis is still needed. Nevertheless, this is an initial step towards...
identifying epigenetic mechanisms altering key pathways leading to sALS, which will be validated in larger cohorts. Second, sALS postmortem tissue reflects the terminal disease stage rather than the pathogenic mechanisms leading to disease onset and progression. As sALS-affected motor neurons deteriorate at the terminal stage and heterogeneous tissue consisting of both gray and white matter was analyzed, our results may represent epigenetic regulation of the neuronal microenvironment, including microglia activation and the scarce neurons surviving the degenerative process [54,72]. This may explain, in part the discrepancy in the direction of expression of common and concordant genes reported here with other sALS genome-wide expression profiles, as well as the heavily represented inflammation-related genes, in our concordant epigenes, which are not differentially expressed specifically in sALS motor neurons or ventral horns [68]. Finally, more studies are needed to concretely identify whether or not the genes identified in this study are involved in ALS pathogenesis.

Advances in identifying epigenetic regulators in disease states have led to new therapeutic approaches. Interestingly, demethylating agents have been extensively studied to reverse aberrant epigenetic changes associated with cancer [77] and more recently, histone deacetylase inhibitors have shown to have neuroprotective properties in animal models of neurodegenerative diseases [78]. These observations suggest reversible epigenetic modifications carry the potential for therapeutic treatment in sALS. We contend that environmental life exposures result in failure to maintain epigenetic homeostasis in the nervous system microenvironment leading to global and loci specific aberrant regulation of gene expression in sALS-affected tissue. Ascertainment of the role of epigenetic regulation may provide a better understanding of the pathogenesis of sALS and new therapeutic targets.

Methods

Subjects and Tissue

Frozen human spinal cord samples from 12 Caucasian sALS subjects and 11 age and gender-matched neurologically-normal controls were obtained from the National Center for Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD (Table 1). Whole blood was collected in EDTA tubes from a different cohort of 11 Caucasian sALS and 11 age-(56 years) and gender-matched neurologically-normal control subjects at the University of Michigan ALS Consortium (Table 4). Table S1 summarizes the samples used for each assay.

Ethics Statement

The participants donating blood reviewed and signed a written informed consent under a protocol reviewed and approved by the

Table 5. Characteristics of sALS and control subjects used for global 5mC and 5 HmC in whole blood.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>sALS group</th>
<th>Control group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>11</td>
<td>12</td>
<td>–</td>
</tr>
<tr>
<td>Age (years) [a]</td>
<td>60 (48–68)</td>
<td>59 (48–70)</td>
<td>NS</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>7</td>
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</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Onset</td>
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<td></td>
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</tr>
<tr>
<td>Bulbar</td>
<td>3</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Limb</td>
<td>8</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Disease duration (months) [a,b]</td>
<td>45 (16–64)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Health condition of controls [c]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety</td>
<td>0</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Asthma</td>
<td>0</td>
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<td>–</td>
</tr>
<tr>
<td>Cancer</td>
<td>0</td>
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<tr>
<td>Healthy</td>
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<td>5</td>
<td>–</td>
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<tr>
<td>Hearing loss</td>
<td>0</td>
<td>1</td>
<td>–</td>
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<tr>
<td>Hypercholesterolemia</td>
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<td>–</td>
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<tr>
<td>Hypothyroidism</td>
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<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

[a] median (range);
[b] mean ± standard deviation;
[c] some patients presented more than one condition; NS, not significant; ‘–’, value not available.

doi:10.1371/journal.pone.0052672.t005
Methylation Profiling and Identification of DMGs

For high-throughput methylation profiling, 200 ng of bisulfite-converted DNA was whole-genome amplified (WGA), enzymatically fragmented, purified, and hybridized to the Infinium Human Methylation27 DNA BeadChip array (HM27K; Illumina, Inc., San Diego, CA) following the manufacturer’s instructions at the University of Michigan Sequencing Core. The HM27K quantitatively determines DNA methylation for 27,578 CpG sites spanning 14,495 genes. DMGs were identified using Illumina’s GenomeStudio software [79]. Single-base resolution corresponding to DNA methylation levels for each locus was reported and the methylation level is given by a beta (β) value describing the percentage of the degree of methylation ranging from 0 (no methylation) to 1 (complete methylation). Any methylation value with a detection P-value >0.05 was excluded. Differential methylation of the selected CpG target regions of autosomal chromosomes between sALS and control groups were tested using Illumina Custom algorithm with multiple testing corrections applied. DiffScore, GenomeStudio’s statistical significance score for differential methylation, of >13 for hyper-methylation or <13 for hypo-methylation, equivalent to False Discovery Rate (FDR) <5%, were used.

Global 5mC and 5 HmC

Differences in genomic DNA global methylation (Global 5mC) and hydroxymethylation (Global 5 HmC) from sALS and control spinal cord or whole blood were determined in duplicate using the colorimetric enzyme-linked immuno-sorbent assay (ELISA) MethylFlash (Methylated or Hydroxymethylated) DNA Quantification Kits according to the manufacturer’s directions. Total RNA was extracted from the same tissue for methylation profiling (Table S1) according to the manufacturer’s instructions (Qiagen, Valencia, CA). Automated genomic DNA extraction from whole blood was performed at the Michigan Institute for Clinical & Health Research (MICHR) at the University of Michigan using Agilent FlexStar (Agilent Technologies, Palo Alto, CA). Nucleotide concentration was assessed using a Nanodrop 2000 (Thermo Scientific) and RNA integrity was determined by microfluid electrophoresis with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Global 5mC and 5 HmC were used for hypo-methylation, equivalent to False Discovery Rate (FDR) <5%, were used.

Genome-wide Expression Profiling

Microarray gene expression analysis was performed as previously described in our published protocols [80]. Briefly, RNA samples with an RNA integrity number (RIN) >6.4 were used for further microarray and real-time PCR analysis. Total RNA (75 ng) 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 instructions. Amplification and hybridization was performed at the University of Michigan DNA Sequencing Core Affymetrix and Microarray Core Group (Ann Arbor, MI) using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array measuring over 47,000 transcripts representing over 20,000 human genes.

Affymetrix CEL files were analyzed using a local version of the GenePattern genomic analysis platform from the Broad Institute [81]. Samples were Robust Multi-array Average (RMA) normalized using the BrainArray Custom CDF HGU133Plus2_Hs_EN-TREZG version 14 [82]. Microarray quality was assessed as previously published [80]. Briefly, probe-level modeling (PLM) and quality metrics provided by the BioConductor affy package were used to identify low-quality arrays [83–85]. Outlier arrays,
skewed away from other arrays, identified by Principal Component Analysis (PCA) were excluded from further analyses. Intensity-Based Moderated T-statistic (IBMT) [86] was employed to identify DMGs with a 10% FDR cut-off between sALS and control samples.

**Identification of Differentially Expressed Genes (DEGs)**

Concordant epigenes are those exhibiting significant differential methylation (hyper- or hypo-methylation) and a parallel change of gene expression (under- or over-expression, respectively) between sALS and control. Differentially methylated (DMGs)/expressed (DEGs) genes were subjected to bioinformatics analyses.

**Bioinformatics Analysis of Concordant Epigenes**

**Functional enrichment analysis.** Database for Annotation, Visualization and Integrated Discovery (DAVID); [http://david.abcc.ncifcrf.gov/](http://david.abcc.ncifcrf.gov/) [87,88] was used to identify enriched molecular biological functions and ALS-relevant pathways of concordant epigenes. A Benjamini-Hochberg corrected P-value of 0.05 was used as the cut-off for statistically significant over-representation.

**Literature mining analysis.** A literature mining approach was used to obtain a comprehensive list of potential ALS-associated targets (genes/proteins). SciMiner, a web-based literature mining tool [89,90], retrieves, processes documents, and identifies potential ALS-associated targets from the ALS-related literature, defined by a PubMed-style query of “Amyotrophic Lateral Sclerosis”. The concordant epigenes were compared against the literature-derived ALS-associated targets that were observed in at least 2 or more papers and whose frequency (in terms of the number of papers) was significantly different from the background. Fisher’s exact test \( (p \text{-value} < 0.05) \) was used to determine whether each gene’s frequency was significantly different from the complete collection of abstracts of over 20 million papers in PubMed. The concordant genes identified by the high-throughput arrays were compared with these literature-derived ALS-related genes to identify which known disease-relevant genes are most highly methylated/expressed and, consequently, likely involved in disease pathogenesis. The resulting genes were designated as literature-derived ALS-associated epigenes.

**Transcriptional network analysis.** To elucidate the functional relationships among the concordant epigenes, we generated transcriptional networks using Genomatix Pathway Systems (GePS; Genomatix Software GmbH, Munich, Germany) with a sentence-level co-citation filter. Two genes co-cited at the sentence level in the literature are linked, resulting in a co-citation network. Additionally, transcriptional regulatory information of predicted transcription factor binding sites (TFBS) in promoter regions of genes could be further incorporated. The network allows the visualization of concordant epigenes, their potential associations, and transcriptional regulation with each other. Therefore, it helps genes could be further incorporated. The network allows the identification of key genes that are highly connected to genes, and which play potentially important roles in the pathogenesis of sALS. Potential TFBSs of two highly connected genes in the network, \( STAT3A \) and \( C/EBPB \), were searched among the promoters of the concordant epigenes using MatInspector (Genomatix) [91].

**Pyrosequencing**

To validate the HM27K arrays, we assessed gene-specific methylation of three selected cytokine genes based on the fact that immune response is associated with the pathogenesis of sALS [92] and two transcription factors. Amplicons of the promoter regions of the genes coding for the Cklf-like MARVEL transmembrane domain-containing proteins 2 and 3 (CMTM2 and CMTM3), the chemokine (C-X-C motif) ligand 12 (CXCL12), signal transducer and activator of transcription 5A (STAT5A), and CCAAT/enhancer binding protein \( \beta (C/EBPB) \) were generated using bisulfite-converted genomic DNA as previously described [93]. PCR conditions: 95°C for 15 min, 50 cycles [95°C for 30 s, 40–50°C for 30 s, 72°C for 20 s], 72°C for 10 min. Ten ml of the amplicon was Streptavidin Sepharose (Amersham Bioscience, Uppsala, Sweden) were purified, denatured with 0.2 M NaOH, and pyrosequenced using 0.5 mM of sequencing primer in a PSQ96 HS System (Qiagen) following the manufacturer’s protocol. Percent methylation of the region analyzed containing the identified Illumina methylation site or individual sites are presented as mean \( \pm \) SEM with a two-sample equal variance \( t \)-test using GraphPad Prism 5.

**RT-PCR**

cDNA was generated by reverse transcription from total RNA isolated for microarray analysis using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). RT-PCR was performed in triplicate using sequence-specific primers (Table S4) with SYBR Green PCR reagents (Bio-Rad, Hercules, CA). The PCR amplification profile was as follows: 95°C for 5 min, [denaturation at 95°C for 30 s, annealing at 55–60°C for 60 s, and extension at 72°C for 30 s] x40 cycles, and a final phase of 72°C for 5 min. The fluorescence threshold \( C_T \) value, representing mRNA expressed in sALS samples, was calculated by the iCycler iQ system software. mRNA levels were normalized to an endogenous reference \( \Delta C_T \) and then relative to the control group \( \Delta A C_T \). Levels of PCR products are demonstrated as mean \( \pm \) SEM and a two-sample equal variance \( t \)-test was performed using GraphPad Prism 5 to confirm that mRNA levels were significantly different between sALS and control.

**Supporting Information**

Figure S1 Validation of HM27K arrays using pyrosequencing. Amplicons to the promoter regions identified by HM27K of cytokines CXCL12, CMTM3, CMTM2, C/EBPB, and \( STAT3A \) were generated using bisulfite-converted genomic DNA from human postmortem spinal cord and used as templates for pyrosequencing [sALS \( n = 11 \); Ctrl \( n = 11 \)]. Results are presented as mean of percent methylation of all CpG sites within the area tested on each gene (A) or as percent methylation of individual sites for \( STAT3A \); site 2 was identified with the HM27K (B). Results are presented as mean \( \pm \) SEM and a two-sample equal variance \( t \)-test was used. \( * p < 0.05, ** p < 0.01 \) compared to control group (Ctrl). (EPS)

Figure S2 RT-PCR confirmation of concordant epigenes in spinal cord. Total RNA was extracted from postmortem human spinal cord tissue used for the methylation analysis from sALS subjects (\( n = 8–11 \)) and controls (\( n = 8–11 \)) and subjected to RT-PCR. Results were normalized to housekeeping genes [TATA-box Binding Protein \( (TBP) \) for \( CTS2, FCER1G, TREM2, NRM1 \) and \( Nvx1 \); ribosomal 18S subunit for \( CHE3L2, H19, PEG10, \) and \( LUM \)], and are presented as fold-changes calculated by the \( 2^{-\Delta A C_T} \) method. \( * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 \). (EPS)

Table S1 Samples used for methylation and expression analyses. (DOC)
human oligonucleotide sequences of primers used for real-time RT-PCR.

(DOC)

Acknowledgments

We are grateful to the participants in this study and their families, as well as to Stacey Sakowski, PhD, Lisa McLean, J. Simon Lunn, PhD, and Yu Hong for critical revisions, suggestions, and assistance in the submission of the manuscript. We thank Crystal PcaLt for technical assistance and Aaron C. Goldstrohm, PhD for allowing us to use his automated Maxwell 16 System Instrument.

Author Contributions

Conceived and designed the experiments: CFR JH DEB CDMR RY ELF. Performed the experiments: CFR DEB CDMR ELF. Analyzed the data: JH CFR DEB ELF CDMR RY. Contributed reagents/materials/analysis tools: ALS BCC DMR ELF. Wrote the paper: CFR JH DEB CDMR ELF.

References


An Imbalance Between Excitatory and Inhibitory Neurotransmitters in Amyotrophic Lateral Sclerosis Revealed by Use of 3-T Proton Magnetic Resonance Spectroscopy

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IMPORTANCE A lack of neuroinhibitory function may result in unopposed excitotoxic neuronal damage in amyotrophic lateral sclerosis (ALS).

OBJECTIVE To determine whether there are reductions in γ-aminobutyric acid (GABA) levels and elevations in glutamate-glutamine (Glx) levels in selected brain regions of patients with ALS by use of proton magnetic resonance spectroscopy.

DESIGN Case-control study using short echo time and GABA-edited proton magnetic resonance spectroscopy at 3 T with regions of interest in the left motor cortex, left subcortical white matter, and pons; data analyzed using logistic regression, t tests, and Pearson correlations; and post hoc analyses performed to investigate differences between riluzole-naive and riluzole-treated patients with ALS.

SETTING Tertiary referral center.

PARTICIPANTS Twenty-nine patients with ALS and 30 age- and sex-matched healthy controls.

EXPOSURE Fifteen patients were taking 50 mg of riluzole twice a day as part of their routine clinical care for ALS.

MAIN OUTCOMES AND MEASURES Levels of GABA, Glx, choline (a marker of cell membrane turnover), creatine (a marker of energy metabolism), myo-inositol (a marker of glial cells), and N-acetylaspartate (a marker of neuronal integrity).

RESULTS Patients with ALS had significantly lower levels of GABA in the motor cortex than did healthy controls ($P < .01$). Patients with ALS also had significantly lower levels of N-acetylaspartate in the motor cortex ($P < .01$), subcortical white matter ($P < .05$), and pons ($P < .01$) and higher levels of myo-inositol in the motor cortex ($P < .001$) and subcortical white matter ($P < .01$) than did healthy controls. Riluzole-naive patients with ALS had higher levels of Glx than did riluzole-treated patients with ALS ($P < .05$ for pons and motor cortex) and healthy controls ($P < .05$ for pons and motor cortex). Riluzole-naive patients with ALS had higher levels of creatine in the motor cortex ($P < .001$ for both comparisons) and subcortical white matter ($P \leq .05$ for both comparisons) than did riluzole-treated patients with ALS and healthy controls. Riluzole-naive patients with ALS had higher levels of N-acetylaspartate in the motor cortex than did riluzole-treated patients with ALS ($P < .01$).

CONCLUSIONS AND RELEVANCE There are reduced levels of GABA in the motor cortex of patients with ALS. There are elevated levels of Glx in riluzole-naive patients with ALS compared with riluzole-treated patients with ALS and healthy controls. These results point to an imbalance between excitatory and inhibitory neurotransmitters as being important in the pathogenesis of ALS and an antiglutamatergic basis for the effects of riluzole, although additional research efforts are needed.

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Amyotrophic lateral sclerosis (ALS) is a progressive degenerative motor neuron disease involving the motor cortex, corticospinal tract, brainstem, and spinal anterior horn neurons. The disease is uniformly fatal, although the clinical presentation and course are heterogeneous, with median survival times between 2 and 4 years. Patients present most commonly with combined upper motor neuron (UMN) and lower motor neuron (LMN) features, although, earlier in the disease course, only UMN or LMN signs may be present. Although electromyography confirms LMN signs in ALS, UMN signs are solely assessed on clinical grounds that can delay diagnosis. This is due in part to the fact that UMN signs in ALS can be masked by LMN weakness and secondary hypertreflexia rather than hyperreflexia. Riluzole, the only medication for ALS approved by the US Food and Drug Administration, has limited efficacy, extending average life expectancy by only 3 to 6 months. Riluzole is postulated to modulate excitatory neurotransmission, although the exact in vivo pharmacologic actions are not well understood.

To further the understanding of the disease process and perhaps to diagnose the disease at an earlier stage, advanced magnetic resonance imaging techniques have been applied to the study of ALS. Proton magnetic resonance spectroscopy (1H-MRS) is one such imaging technique that has been used in a number of studies to investigate ALS. The techniques of 1H-MRS require the placement of a voxel or region of interest to specify a volume of brain tissue in which to measure brain metabolites. Conventional in vivo 1H-MRS at 3 T can quantify various brain metabolites, including N-acetylaspartate (NAA; a marker of neuronal integrity), choline (Cho; a marker of cell membrane turnover), creatine (Cr; a marker of energy metabolism), myo-inositol (mI; a marker of glial cells), and glutamate-glutamine (Glx). γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter, is difficult to quantify using conventional 1H-MRS but can be measured using spectral-editing techniques. There is increasing evidence that reduced inhibitory function may play an important role in the pathogenesis of ALS. Given our limited prior knowledge of in vivo GABA changes in ALS, direct interrogation of GABA may lead to a new understanding of this complex disease and may provide opportunities for the development of new disease-modifying treatments.

The most common finding reported in 1H-MRS studies of ALS is reduced NAA in the motor cortex, which is generally interpreted as neuronal loss. Although riluzole is thought to modulate excitatory neurotransmission, only 2 published 1H-MRS studies have explored the effect of riluzole on brain metabolites and measured only Cho, Cr, and NAA. We recently published the first 1H-MRS study reporting a decrease in GABA motor cortex levels in a small ALS cohort, which suggested reductions in inhibitory neurotransmission. However, our prior study did not consider the excitatory contribution of Glx. The aim of the present study was to study both inhibitory neurotransmission, as measured by GABA, and excitatory neurotransmission, as measured by Glx. Our hypothesis is that there is an imbalance between excitatory and inhibitory neurotransmitters in ALS. The GABA and Glx levels were measured in the left motor cortex and left subcortical white matter, and Glx levels were measured in the pons as well. A secondary aim was to determine differences between brain metabolite profiles of riluzole-treated vs riluzole treatment-naive patients with ALS.

### Methods

Twenty-nine patients with ALS were recruited from our institution's ALS clinic. Thirty age- and sex-matched healthy controls were also recruited. The patients with ALS met the El Escorial Criteria for probable (n = 15), probable laboratory-supported (n = 10), or definite (n = 4) ALS. Participants were excluded if they had a history of central nervous system infection, head injury, or cerebrovascular disease; were active substance abusers; or had a contraindication for magnetic resonance imaging. All participants were right-handed. Our institutional review board approved all study protocols, and informed consent was obtained from all participants. The UMN scores were graded by combining the Ashworth Spasticity Scale (range, 0-8) with the presence of pathological reflexes (range, 0-24). A scale for measuring the pseudobulbar affect (range, 0-1), with a total scale ranging from 0 to 33 (a higher score reflecting higher disease burden). Participant characteristics are presented in **Table 1**.

### Table 1. Participant Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy Controls (n = 30)</th>
<th>Patients With ALS (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59 (10) [29-79]</td>
<td>60 (10) [32-78]</td>
</tr>
<tr>
<td>Male to female ratio</td>
<td>20:10</td>
<td>17:12</td>
</tr>
<tr>
<td>Type or location of onset</td>
<td>NA</td>
<td>Bulbar (7 patients), limb (22 patients)</td>
</tr>
<tr>
<td>Disease duration, mo</td>
<td>NA</td>
<td>29 (15) [4-64]</td>
</tr>
<tr>
<td>UMN score</td>
<td>NA</td>
<td>16 (7) [1-27]</td>
</tr>
<tr>
<td>ALSFRS-R score</td>
<td>NA</td>
<td>34 (8) [18-47]</td>
</tr>
</tbody>
</table>

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised Amyotrophic Lateral Sclerosis Functional Rating Scale; NA, not applicable; UMN, upper motor neuron.
Conventional PRESS Data Acquisition

The PRESS spectra (with a repetition time [TR] of 2000 milliseconds and an echo time [TE] of 35 milliseconds) were acquired using VAPOR (variable power and optimized relaxation delays) water suppression: 32 averages were performed for the motor cortex and subcortical white matter voxels, and 96 averages were performed for the pons voxel. Conventional PRESS data were analyzed using LCModel version 6.1-4A.21 Metabolite concentrations from LCModel were only used for statistical analysis if the Cramér-Rao lower bounds were less than 20% for the motor cortex and subcortical white matter voxels and less than 25% for the pons voxel. Cerebral spinal fluid correction was performed for each voxel using magnetization-prepared rapid acquisition gradient echo images and Statistical Parametric Mapping version 5 (Wellcome Trust Centre for Neuroimaging).

MEGA-PRESS Data Acquisition

The MEGA-PRESS experiment for editing GABA was performed with the following parameters: $TE_1 = 15$ milliseconds and $TE_2 = 53$ milliseconds; $TR = 1.8$ seconds; 256 averages; and frequency-selective editing pulses (14 milliseconds) applied at 1.9 ppm (on) and 7.46 ppm (off). Slice-selective refocusing was performed using amplitude-modulated pulse “GTST1203” (length, 7 milliseconds; bandwidth, 1.2 kHz); MEGA-PRESS was analyzed using in-house postprocessing software in Matlab 2012a (Mathworks) with Gaussian curve fitting to the GABA and inverted N-acetylaspartate (NAA) peaks. The GABA levels were expressed relative to the NAA signal in the edited spectra.22 The GABA:NAA ratio was then multiplied by the NAA concentration determined from LCModel analysis of a short-TE PRESS spectrum of the same voxel to provide an estimate of GABA concentration. Note that the metabolite concentration is in international units (IU) because various factors are not corrected, including editing efficiency and relaxation times.

Statistical Analyses

Logistic regression analyses were performed between disease status and individual metabolites using scanner type.
Achiva or Ingenia) as a covariate to determine if there were significant differences between the Philips Achieva 3T and Ingenia 3T systems. Two-tailed independent-sample t tests were performed to determine differences in brain metabolites between patients with ALS and healthy controls. Pearson correlations were performed for associations between brain metabolites and clinical status (using UMN scores, disease duration, the revised ALS Functional Rating Scale [ALSFRS-R], and the rate of disease progression [defined as \((48 - \text{ALSFRS-R score at evaluation}/\text{disease duration from symptom onset to evaluation})\]). A subset analysis was also performed to compare riluzole-treated vs riluzole-naive patients with ALS. Stata version 11 (StataCorp) was used for statistical analysis. The significance threshold was set a priori at \(P = .05\).

**Results**

For the conventional PRESS spectra, 2 patients with ALS had an inadequate signal to noise ratio from the pons voxels and were excluded, and the pons Glx value from 1 patient with ALS was excluded owing to a high Cramér-Rao bound. For the MEGA-PRESS spectra, the GABA spectra in the motor cortex of 2 patients with ALS and 1 healthy control and in the subcortical white matter of 1 patient with ALS had an inadequate signal to noise ratio. Four of the patients with ALS were unable to complete the entire imaging protocol and did not undergo \(^1\)H-MRS of the subcortical white matter. The logistic regression analysis showed no significant effects of scanner type \((z > 0.05)\) for all of the metabolites, which indicates that the data from the cohorts scanned on the different machines are comparable.

**MEGA-PRESS Results**

As shown in Figure 2, patients with ALS demonstrated significantly lower levels of GABA in the motor cortex than did healthy controls \((P = .002)\). There were no significant differences in GABA levels in the left subcortical white matter between patients with ALS and healthy controls \((P = .30)\). There was a significant correlation between GABA level in the motor cortex and disease duration \((r = -0.39; P = .05)\). The patients with ALS who had the 5 lowest GABA levels had a disease duration (33 months) that was almost double that of the patients with ALS who had the 5 highest GABA levels (18 months), although the ALSFRS-R and UMN scores were close (ie, the ALSFRS-R score was 5 points lower, and the UMN score was 2 points higher, for the lowest GABA levels).

**Figure 2. Decreased \(\gamma\)-Aminobutyric Acid (GABA) Levels in the Motor Cortex of Patients With Amyotrophic Lateral Sclerosis (ALS)**

The diamonds represent GABA levels in the left motor cortex (A) and subcortical white matter located caudal to the motor cortex (B) for individual healthy controls (open diamonds) and patients with ALS (filled diamonds). The horizontal bars indicate the mean. Patients with ALS have reduced levels of GABA in the left motor cortex compared with healthy controls. There is no difference in GABA levels in the left subcortical white matter between patients with ALS and healthy controls.
Table 2. Conventional Point-Resolved Spectroscopy Results

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Motor Cortex</th>
<th>Subcortical White Matter</th>
<th>Pons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Controls</td>
<td>Patients With ALS</td>
<td>Healthy Controls</td>
</tr>
<tr>
<td>Cho</td>
<td>1.15 (0.18)</td>
<td>1.23 (0.14)</td>
<td>1.34 (0.21)</td>
</tr>
<tr>
<td>Cr</td>
<td>4.93 (0.33)</td>
<td>5.09 (0.59)</td>
<td>4.21 (0.35)</td>
</tr>
<tr>
<td>Glx</td>
<td>5.62 (1.00)</td>
<td>5.88 (1.37)</td>
<td>4.34 (1.13)</td>
</tr>
<tr>
<td>mI</td>
<td>3.18 (0.46)</td>
<td>3.79 (0.74)</td>
<td>3.10 (0.40)</td>
</tr>
<tr>
<td>NAA</td>
<td>8.20 (0.48)</td>
<td>7.72 (0.81)</td>
<td>7.73 (0.57)</td>
</tr>
</tbody>
</table>

Abbreviations: ALS, amyotrophic lateral sclerosis; Cho, choline; Cr, creatine; Glx, glutamine and glutamate; mI, myo-inositol; NAA, N-acetylaspartate.

Conventional PRESS Results

Results are summarized in Table 2. The levels of NAA in the motor cortex (P < .008), subcortical white matter (P < .02), and pons (P < .003) were significantly lower in patients with ALS than in healthy controls. The levels of mI in the motor cortex (P < .001) and subcortical white matter (P < .002) were significantly higher in patients with ALS than in healthy controls. There were significant correlations between NAA level in the motor cortex and ALSFRS-R score (r = 0.39; P < .05), between mI level in the subcortical white matter and disease duration (r = 0.43; P < .05), and between Glx level in the pons and UMN score (r = −0.63; P < .001). There was a significant correlation between NAA and GABA levels (r = 0.57; P < .002). There were no significant correlations between GABA and Glx levels within the same voxel location or between voxel locations.

Riluzole Treatment Subanalyses

Subgroup characteristics are presented in Table 3. As seen in Figure 3, there were significantly higher levels of Cr, Glx, and NAA in the motor cortex of riluzole-naive patients with ALS compared with the riluzole-treated patients with ALS. There were also significantly higher levels of Glx in the pons (P < .01) and higher levels of Cr in the subcortical white matter (P < .05) of the riluzole-naive patients with ALS compared with the riluzole-treated patients with ALS. There were no significant differences in the levels of GABA or other metabolites between the 2 subgroups. The levels of Glx in the motor cortex (P < .03) and the pons (P < .04) were significantly higher for riluzole-naive patients with ALS (mean [SD] levels of 6.49 [1.52] IU and 8.28 [2.52] IU, respectively) than for healthy controls (mean [SD] levels of 5.62 [1.00] IU and 6.83 [1.77] IU, respectively). The levels of Cr in the motor cortex (P < .001) and the subcortical white matter (P < .03) were significantly higher for riluzole-naive patients with ALS (mean [SD] levels of 5.46 [0.58] IU and 4.47 [0.30] IU, respectively) than for healthy controls (mean [SD] levels of 4.93 [0.33] IU and 4.21 [0.35] IU, respectively).

Discussion

Our study demonstrates reductions of GABA levels in the motor cortex of patients with ALS compared with healthy controls. In addition, it was found that Glx levels were elevated in riluzole-naive patients with ALS compared with riluzole-treated patients with ALS and healthy controls. The inclusion of an additional 19 patients with ALS augments the significance of our prior study that found lower levels of GABA in the motor cortex of 10 patients with ALS. To our knowledge, this is the first report of in vivo measurements of both GABA and Glx in patients with ALS, thereby investigating both the GABAergic (inhibitory) and the glutamatergic (excitatory) neurotransmitter systems in the same patients.

The cause of ALS remains elusive, although excitotoxic neuronal injury is thought to play an important role, which is primarily mediated through glutamate toxicity. There is increasing evidence that an “interneuronopathy” may be a central player in the pathophysiology of ALS. Interneuronopathy is the hypothesis that inhibitory or GABAergic dysfunction results in relatively unopposed excitotoxic neuronal damage. Neuroimaging, animal, histochemical, genetic, and clinical studies support the role of an interneuronopathy in ALS. An ALS functional magnetic resonance imaging study demonstrated increased functional connectivity in ALS, suggesting a loss of inhibitory neuronal tone. An animal model of ALS demonstrated that cortical excitability is explained by reduced GABAergic inhibition. In addition, human histochemical and positron emission tomography studies have implicated GABA receptor alterations in the motor cortex of patients with ALS.

A significant barrier to further supporting the interneuronal hypothesis has been the challenge of directly measuring in vivo GABA concentrations. γ-Aminobutyric acid has chemical shift overlap with other brain metabolites such as Cr, Glx, and NAA, which are present in brain tissue at higher concentrations than GABA. The 1H-MRS editing techniques allow for improved differentiation of metabolites and quantification of GABA by editing out the overlapping Cr peak at 3.0 ppm. The current result of decreased levels of GABA in the motor cortex provides further in vivo evidence of reduced inhibitory function in the pathophysiology of ALS and suggests the possibility of the development of new ALS disease-modifying treatments aimed at increasing inhibitory neuronal tone.

Higher levels of Glx were also found in the motor cortex and pons of riluzole-naive patients compared with riluzole-treated patients and healthy controls. Glutamate and glutamine are difficult to resolve independently at field strengths.

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of 3 T or lower, and are usually reported as a combined measure (ie, Glx); however, in a normal brain, glutamate is the larger contributor to this peak by about a 4:1 ratio.\textsuperscript{30} Owing to concerns of a potential relationship between NAA and Glx, we confirmed that there was not a significant correlation between the NAA levels and the Glx levels for the overall group or the treatment subgroups. Riluzole is thought to act on both the glutamatergic and GABAergic systems.\textsuperscript{31,32} Riluzole has been shown to decrease glutamatergic neurotransmission by acting as an antagonist of presynaptic N-methyl-D-aspartate and \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptors, as well as by increasing glutamate transporter uptake.\textsuperscript{33-35} Riluzole has been shown to increase GABA levels in cell cultures, although the levels required for GABA modulation are thought to be higher than those required for glutamatergic inhibition, which may explain the lack of effect of riluzole on the GABA levels seen in the present study.\textsuperscript{36,37} Reduced motor cortex excitability has been demonstrated after riluzole administration in healthy subjects, as well as partial normalization of increased cortical excitability in patients with ALS, which is thought to be mediated through glutamatergic rather than GABAergic interactions.\textsuperscript{38,39}

A moderate negative correlation between GABA levels and disease duration was found. The patients with ALS who had the lowest GABA levels had longer disease durations than did the patients with ALS who had the highest GABA levels. It may be that patients with ALS who have a more rapid course have a reflexive increase in inhibitory tone relative to those with a more prolonged disease course. There may be a release of GABA from rapidly denervated interneurons resulting in higher relative levels in patients with ALS who had a short disease duration. Interestingly, both Filippini et al\textsuperscript{40} and Iwata et al\textsuperscript{41} found in their cross-sectional studies that fractional anisotropy, a measurement of white matter integrity, was paradoxically higher in patients with ALS who had the longest disease duration. Fractional anisotropy is a different advanced neuroimaging metric, but, taken together, these findings suggest that long-term survivors have different pathophysiologic processes. Although there was no significant direct correlation between GABA and Glx levels, it is possible that there may be a relative GABA threshold for each respective patient, allowing for the excitotoxic effects of glutamate rather than a linear relationship between GABA and glutamate. It is interesting to note that there was a significant correlation between NAA and

Table 3. Riluzole Subgroup Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD) [Range]</th>
<th>Riluzole-Naive Patients With ALS (n = 14)</th>
<th>Riluzole-Treated Patients With ALS (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male to female ratio</td>
<td>8:6</td>
<td>9:6</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>60 (13) [32-78]</td>
<td>59 (7) [47-72]</td>
<td></td>
</tr>
<tr>
<td>Disease duration, mo</td>
<td>25 (16) [4-64]</td>
<td>31 (13) [12-60]</td>
<td></td>
</tr>
<tr>
<td>ALSFRS-R score</td>
<td>38 (6) [26-47]</td>
<td>30 (9) [18-25]</td>
<td></td>
</tr>
<tr>
<td>UMN score</td>
<td>15 (7) [1-24]</td>
<td>17 (7) [3-27]</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised Amyotrophic Lateral Sclerosis Functional Rating Scale; UMN, upper motor neuron.

* \(P \leq 0.05\) (no significant differences in other characteristics between subgroups).

Figure 3. Metabolite Levels in the Left Motor Cortex for Riluzole-Naive Patients With Amyotrophic Lateral Sclerosis (ALS) and Riluzole-Treated Patients With ALS

A

B

C

The diamonds represent the respective brain metabolites of creatine (Cr [A]), glutamate and glutamine (Glx [B]), and N-acetylaspartate (NAA [C]). Riluzole-naive patients with ALS have elevated levels of Cr, Glx, and NAA in the left motor cortex compared with riluzole-treated patients with ALS.
GABA levels, which suggests an association between neuronal integrity and neuronal inhibitory tone. A longitudinal imaging trial is required to address these issues.

The riluzole-treated patients with ALS had lower levels of Cr than both the riluzole-naive patients with ALS and the healthy controls. It has been proposed that an energy-depleted state may be an inciting factor in ALS\(^9\) and that riluzole may reduce neuronal cellular energy demand.\(^{42}\) A potential confounding factor to these findings is that the riluzole-treated patients with ALS had lower levels of NAA than those with riluzole-naive patients with ALS. There was a moderate correlation between NAA and Cr levels in the motor cortex (\(r = 0.49; P = .01\)) for the 29 patients with ALS. Two prior studies\(^{43,44}\) have reported a longitudinal increase in the NAA:Cr ratio of patients with ALS after short periods (≤3 weeks) of riluzole treatment; however, these 2 studies\(^{43,44}\) did not report absolute concentrations of the NAA and Cr metabolites, which makes direct comparison difficult. However, overall, these results do suggest the potential of \(^1\)H-MRS to establish Glx and GABA as surrogate markers of disease progression and treatment response, which might be useful in future pharmacological trials.

As in our study, almost all prior \(^1\)H-MRS studies have reported decreases in levels of NAA or NAA:Cr ratios, particularly in the motor cortex. The finding of elevated mI levels in the motor cortex and subcortical white matter is thought to represent increased numbers of glial cells.\(^8\) Although mI has not been investigated to the same extent as NAA, prior studies\(^{47-49}\) have reported mI (or mI:Cr ratio) elevations in the motor cortex. Interestingly, only a few studies have measured Glx, with 2 studies reporting elevations in Glx as reported by increased Glx:Cr ratios in the medulla in the study by Pioro et al\(^46\) and increased Glx:Cr ratios in the motor cortex in the study by Han and Ma.\(^{47}\) Of the prior studies that measured Glx, it is important to note that riluzole status, which is a potential mediating factor (as the present study would indicate), was either not mentioned\(^{43,46-48}\) or unclear.\(^{44}\)

As reported before, the limitations of \(^1\)H-MRS (including the MEGA-PRESS technique) include its inability to differentiate between the intracellular and extracellular contribution of the metabolites, its potential macromolecular component contributions, and the relatively large voxel sizes required. Our study includes the GABA data of 10 patients with ALS and 9 riluzole-naive patients with ALS, indicating greater disability, although the UMN disease burden was not significantly different between these 2 groups. Direct causality between riluzole treatment and effect on brain metabolites cannot be established given the cross-sectional nature of our study. A longitudinal imaging trial enrolling patients at initial diagnosis would be required to better establish the response of brain metabolites to treatment, as well as to probe ALS central nervous system changes, including the direct relationship between GABA and Glx over time.

In conclusion, reductions in GABA levels in the motor cortex of patients with ALS were observed, as well as elevations of Glx in riluzole-naive patients with ALS compared with riluzole-treated patients with ALS and healthy controls. The results support the hypothesis that an imbalance between excitatory and inhibitory neurotransmitters is an important factor in the pathogenesis of ALS, as well as the antiglutamatergic basis for the effects of riluzole. These findings also support the potential of \(^1\)H-MRS to establish Glx and GABA measurements as clinically relevant markers of disease, although additional research efforts are needed to better understand the findings reported herein.

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Diagnostic Accuracy of Diffusion Tensor Imaging in Amyotrophic Lateral Sclerosis: A Systematic Review and Individual Patient Data Meta-Analysis

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Rationale and Objectives: There have been a large number of case-control studies using diffusion tensor imaging (DTI) in amyotrophic lateral sclerosis (ALS). The objective of this study was to perform an individual patient data (IPD) meta-analysis for the estimation of the diagnostic accuracy measures of DTI in the diagnosis of ALS using corticospinal tract data.

Materials and Methods: MEDLINE, EMBASE, CINAHL, and Cochrane databases (1966–April 2011) were searched. Studies were included if they used DTI region of interest or tractography techniques to compare mean cerebral corticospinal tract fractional anisotropy values between ALS subjects and healthy controls. Corresponding authors from the identified articles were contacted to collect individual patient data. IPD meta-analysis and meta-regression were performed using Stata. Meta-regression covariate analysis included age, gender, disease duration, and Revised Amyotrophic Lateral Sclerosis Functional Rating Scale scores.

Results: Of 30 identified studies, 11 corresponding authors provided IPD and 221 ALS patients and 187 healthy control subjects were available for study. Pooled area under the receiver operating characteristic curve (AUC) was 0.75 (95% CI: 0.66–0.83), pooled sensitivity was 0.68 (95% CI: 0.62–0.75), and pooled specificity was 0.73 (95% CI: 0.66–0.80). Meta-regression showed no significant differences in pooled AUC for each of the covariates. There was moderate to high heterogeneity of pooled AUC estimates. Study quality was generally high. Data from 19 of the 30 eligible studies were not ascertained, raising possibility of selection bias.

Conclusion: Using corticospinal tract individual patient data, the diagnostic accuracy of DTI appears to lack sufficient discrimination in isolation. Additional research efforts and a multimodal approach that also includes ALS mimics will be required to make neuroimaging a critical component in the workup of ALS.

Key Words: Amyotrophic lateral sclerosis; diffusion tensor imaging; magnetic resonance imaging; diagnostic imaging; diagnostic accuracy; meta-analysis.

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Amyotrophic lateral sclerosis (ALS) is a fatal degenerative motor neuron disease involving the motor cortex, corticospinal tract (CST), and spinal anterior horn neurons. Clinical presentation of the disease is variable, contributing diagnostic uncertainty and delay (1). More than 40% of ALS patients undergo inappropriate medical treatment, including surgery (2). Electromyography can help confirm the diagnosis of lower motor neuron involvement. There is a high interest in developing upper motor neuron diagnostic biomarkers to facilitate an accurate diagnosis at an earlier stage (3–5).

A promising biomarker for ALS is diffusion tensor imaging (DTI), an advanced magnetic resonance imaging (MRI) application. Fractional anisotropy (FA), a scalar measurement of water diffusivity, is a key DTI metric. FA reductions have been reported in diseases that degrade white matter tracts, including ALS (6). Although several studies have reported FA decreases in ALS patients, only a few have addressed test accuracy measures with relative small subject numbers (7–13). We have recently completed a group-level meta-analysis of test accuracy measures of DTI for the diagnosis of ALS (14). However, individual patient data (IPD) meta-analysis approaches are generally considered superior to group-level approaches because more rigorous statistical methods can be employed, including covariate adjustment (15,16). Our study objective was to compare ALS patients who underwent DTI to healthy controls to determine diagnostic accuracy measures of FA using IPD meta-analysis techniques.

METHODS

Eligibility Criteria

We conducted MEDLINE (1966–April 2011), EMBASE (1999–April 2011), CINAHL (1999–April 2011), and Cochrane (2005–April 2011) searches. Search keywords included: amyotrophic lateral sclerosis, Lou Gehrig’s, magnetic resonance imaging, diagnostic imaging, diagnostic tests, diffusion tensor imaging, or fractional anisotropy. Full electronic search for MEDLINE is presented in Appendix Table 1. There were no language restrictions. A manual search of reference lists from identified articles was performed.

Eligible studies fulfilled the following criteria: 1) human studies and 2) use of DTI region of interest (ROI) or tractography techniques to compare brain mean FA values along the CST between ALS subjects and healthy controls (HC). We excluded voxel-based morphometry analysis studies and case studies.

Selection and Quality Assessment

Two authors (B.R.F., M.P.) independently assessed each abstract for inclusion and retrieved full publications for further evaluation, independently reviewed each article, and reached final consensus for inclusion. The same reviewers independently assessed study quality based on Quality Assessment of Diagnostic Accuracy Studies criteria (17). Each of these criteria were scored as “yes,” “no,” or “unclear.” Any disagreements between reviewers were resolved by discussion and consensus.

Data Extraction and Synthesis

Two authors (B.R.F., M.P.) abstracted the following information: author, journal, publication year, number of subjects, demographics and clinical subject characteristics, MRI field strength, DTI parameters, and FA analysis method. Corresponding authors from the identified publications were e-mailed three times separated by 2-week intervals to determine willingness to contribute IPD. Requested data included mean FA values for CST per subject with corresponding age, gender, disease duration (for ALS subjects) and Revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) score (for ALS subjects). To minimize heterogeneity, we used only average CST or internal capsule (IC) FA data even if studies interrogated other brain regions.

Retrospective Study

We retrospectively identified 14 patients (9 males, age 61.9 ± 9.7 years) who met El Escorial criteria (18) for definite or probable ALS and underwent 3T diagnostic brain MRI (Philips Achieva, Best, Netherlands). Fourteen age- and gender-matched HCs (8 males, age 58.4 ± 6.4 years) were included. Our institutional review board approved all study protocols (HUM00050553). Diffusion-weighted imaging was obtained using multiple shot spin–echo technique (repetition time/echo time = 7075/62 ms, 2 mm isotropic resolution, b values = 0, 800 s/mm², 15 gradients). Data processing was performed using ExploreDTI v4.8.2 employing motion and eddy current correction. Fiber tracking used the standard deterministic stream method with seed ROIs in the posterior limb of IC and pons using detailed white-matter atlases. Mean FA values of right and left CST were averaged. Results are listed as Foerster.

Statistical Analysis

Study level analysis: analysis occurred at the individual participant level using Stata version 12.0 (StataCorp). We applied binormal receiver operating characteristic (ROC) curve analysis and the Youden index using a linear mixed model to assign individual study cut points to calculate sensitivity and specificity. The prevailing hypothesis is that FA reduction along the CST in ALS is due to loss of fiber integrity/axonal degeneration (10,19). Thus, by convention, we assigned “positive” disease status for FA values below the individual study cut point and “negative” disease status for FA values above the individual study cut point. ROC curves
and areas under the ROC curve (AUC) were generated for each study. 

Meta-analysis model: using midas and the metan modules, we generated 1) summary ROC curve based on bivariate binomial regression of study-specific estimates of true-positive, true-negative, false-positive, and false-negative data (20,21) and 2) forest plots for AUC, sensitivity, and specificity including 95% confidence intervals (CI). We performed AUC meta-regression analysis using a binormal mixed model with individual covariate analyses for age, gender, disease duration, and ALSFRS-R scores. We also calculated a nonadjusted AUC for the respective study data sets.

We assessed heterogeneity using the \( I^2 \) statistic, a measure of variation between studies resulting from study differences rather than chance error. \( I^2 \) values <25% are considered low and \( I^2 \) values >75% are considered high (22). We evaluated publication bias using a funnel plot and linear regression of AUC versus the corresponding study’s standard error measurement, with \( P < .05 \) indicating significant asymmetry.

RESULTS

Study Selection

We provide the number of studies screened, assessed for eligibility, and included for review with reasons for exclusions at each stage in Figure 1. We collected individual patient data from 11 of 30 identified studies.

Study Characteristics

Study characteristics of the studies are summarized in Table 1 with additional study details in Appendix Table 2 (7,8,13,19,23–29). Studies enrolled 221 ALS subjects and 187 HCs. Equipment, DTI parameters, and analysis methods varied across studies. All studies based ALS diagnosis on the El Escorial criteria (18). Age and gender covariate data were available in 10 studies, ALSFRS-R covariate data were available in nine studies, and disease duration covariate data were available in eight studies.

Meta-Analysis of Sensitivity, Specificity, and AUC

Figure 2 shows the summary ROC curve. Figures 3 and 4 show forest plots for AUC and sensitivity/specificity measures, respectively. The pooled AUC was 0.75 (95% CI: 0.66–0.83), the pooled sensitivity was 0.68 (95% CI: 0.62–0.75), and the pooled specificity was 0.73 (95% CI: 0.66–0.80).

AUC Meta-analysis Using Covariates

Table 2 shows results of the meta-analysis incorporating each of the subject covariate information including age, gender, ALSFRS-R score, and disease duration for the studies in which the data were available. Adjusted confidence intervals overlapped with nonadjusted confidence intervals for each of the covariates.

Predictive Values

Using pooled estimates, positive likelihood ratio was 2.52 and negative likelihood ratio was 0.44. Using Bayesian techniques, we show posttest probabilities of disease after negative and positive DTI results using different disease pretest probabilities in Figure 5.

Study Quality, Publication Bias, and Heterogeneity

Study quality was generally high across the studies (Fig 6). Funnel plot and regression tests (\( P = .001 \)) demonstrate evidence of significant publication bias (Fig 7). Between-study AUC heterogeneity was at least moderate (\( I^2 = 73\% \)). Between-study sensitivity heterogeneity was low (\( I^2 = 14\% \)) and between-study specificity heterogeneity was moderate (\( I^2 = 35\% \)).

DISCUSSION

Conventional MRI findings in ALS are neither sensitive nor specific (30). Therefore, there has been great interest in using advanced neuroimaging methods such as DTI as diagnostic ALS biomarkers. Quantitative meta-analysis is necessary to properly evaluate the potential of new technologies to serve as diagnostic tests, particularly in the setting of imaging studies, which include relatively small numbers of subjects. Based on the available IPD, the meta-analysis results suggest that the mean FA within the CST provides relatively low diagnostic accuracy as an independent test for ALS.
The pooled DTI test accuracy measures including sensitivity, specificity, and AUC are relatively modest for the diagnosis of ALS. From a clinical perspective, these results are better illustrated using the Bayesian plot of posttest probabilities given different pretest disease probabilities. For example, a patient with a 0.50 pretest probability of disease based on clinical suspicion has a 0.68 posttest probability of ALS with a positive DTI test result and a 0.33 posttest probability of ALS with a negative DTI test result. Our retrospective dataset of 14 ALS subjects fell in the mid-range of the test accuracy measurements. Based on the confidence intervals, there were no significant differences between the covariate adjusted and nonadjusted meta-regression analysis. AUC values for the covariate adjusted and nonadjusted analyses were lower than the “overall” summary AUC estimate from the entire 12 datasets. This reflects the exclusion of the study cohorts that had greater test discrimination because of the lack of the respective covariate data.

The pooled DTI test accuracy measures including sensitivity, specificity, and AUC are relatively modest for the diagnosis of ALS. From a clinical perspective, these results are better illustrated using the Bayesian plot of posttest probabilities given different pretest disease probabilities. For example, a patient with a 0.50 pretest probability of disease based on clinical suspicion has a 0.68 posttest probability of ALS with a positive DTI test result and a 0.33 posttest probability of ALS with a negative DTI test result. Our retrospective dataset of 14 ALS subjects fell in the mid-range of the test accuracy measurements. Based on the confidence intervals, there were no significant differences between the covariate adjusted and nonadjusted meta-regression analysis. AUC values for the covariate adjusted and nonadjusted analyses were lower than the “overall” summary AUC estimate from the entire 12 datasets. This reflects the exclusion of the study cohorts that had greater test discrimination because of the lack of the respective covariate data.

Our meta-analysis raises a number of important issues. The high level of AUC heterogeneity reflects the variability between studies likely the result of differences in patient populations, MRI equipment, and imaging protocols including different TE and b-values (31) as well as analytical approaches. Consensus guidelines for ALS MRI studies such as those proposed by the Neuroimaging Symposium in ALS are needed (6). Even if more uniform studies are

### Table 1. Individual Study Characteristics

<table>
<thead>
<tr>
<th>Study Number</th>
<th>Author</th>
<th>MRI B Value</th>
<th>Number of Directions</th>
<th>Analysis Method</th>
<th>Brain Region</th>
<th>Average Disease Duration (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bartels (23)</td>
<td>2.9 T</td>
<td>1000</td>
<td>ROI-tractography</td>
<td>CST</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>2</td>
<td>Ciccarelli (24)</td>
<td>1.5 T</td>
<td>1150</td>
<td>Tractography</td>
<td>CST</td>
<td>21 ± 16</td>
</tr>
<tr>
<td>3</td>
<td>Cosottini (19)</td>
<td>1.5 T</td>
<td>1000</td>
<td>ROI-visual</td>
<td>CST</td>
<td>33 ± 20</td>
</tr>
<tr>
<td>4</td>
<td>Cosottini (7)</td>
<td>1.5 T</td>
<td>1000</td>
<td>ROI-visual</td>
<td>CST</td>
<td>17 ± 13</td>
</tr>
<tr>
<td>5</td>
<td>Ellis (25)</td>
<td>1.5 T</td>
<td>620</td>
<td>ROI-visual</td>
<td>CST</td>
<td>27 ± 26</td>
</tr>
<tr>
<td>6</td>
<td>Filippini (8)</td>
<td>3.0 T</td>
<td>1000</td>
<td>Tractography</td>
<td>CST</td>
<td>49 ± 38</td>
</tr>
<tr>
<td>7</td>
<td>Foerster</td>
<td>3.0 T</td>
<td>800</td>
<td>Tractography</td>
<td>CST</td>
<td>25 ± 15</td>
</tr>
<tr>
<td>8</td>
<td>Metwalli (26)</td>
<td>3.0 T</td>
<td>1000</td>
<td>ROI-visual</td>
<td>IC</td>
<td>26 ± 15</td>
</tr>
<tr>
<td>9</td>
<td>Pyra (13)</td>
<td>1.5 T</td>
<td>1000</td>
<td>ROI-visual</td>
<td>IC</td>
<td>25 ± 17</td>
</tr>
<tr>
<td>10</td>
<td>Roccatagliata (27)</td>
<td>1.5 T</td>
<td>1000</td>
<td>ROI-visual</td>
<td>IC</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>11</td>
<td>Senda (28)</td>
<td>3.0 T</td>
<td>700</td>
<td>ROI-tractography</td>
<td>IC</td>
<td>20 ± 9</td>
</tr>
<tr>
<td>12</td>
<td>Wang (29)</td>
<td>3.0 T</td>
<td>1000</td>
<td>ROI-tractography</td>
<td>IC</td>
<td>20 ± 20</td>
</tr>
</tbody>
</table>

CST, average corticospinal tract; IC, internal capsule; MRI, magnetic resonance imaging; ROI, region of interest.

*Indicates extra-motor regions also evaluated.

†Indicates other motor tract levels also evaluated.

**Figure 2.** Area under summary receiver operating characteristic (SROC) curve for diffusion tensor imaging fractional anisotropy values. Each circle represents an individual study result. Dashed circle represents 95% prediction interval of summary sensitivity (SENS) and specificity (SPEC). AUC, area under the curve.

**Figure 3.** Forest plot of area under the curve (AUC) for amyotrophic lateral sclerosis diagnosis using diffusion tensor imaging fractional anisotropy values.
performed, this study suggests that DTI in isolation lacks sufficient diagnostic power. An integrated functional, as well as a structural, advanced MRI approach offers promise in this regard (32). Although our results from this IPD meta-analysis are not significantly different from our previously published group-level meta-analysis (14), the IPD statistical methods allow for greater statistical flexibility and power both in terms of adjusting for patient’s characteristics and determining optimal cut points (33). As a result, IPD is considered the best meta-analysis methodology (34) and thus these reported results add significant credibility to our prior published findings.

Our study has several limitations. We used only data from published manuscripts in this meta-analysis. Data from 19 of the 30 eligible studies were not ascertained, raising the possibility of selection bias. Six of the studies had funding support; two of these six studies had AUC values less than 0.50, suggesting a limited role of funding bias. Several of the datasets did not contain all of the covariates. More than half of the studies did not have a representative disease spectrum of what would be expected in clinical practice, and it must be acknowledged that the differentiation of ALS patients from healthy individuals is not the clinical conundrum facing the clinician. However, DTI may have a role as a diagnostic test for those patients who do not yet have clinically definite signs of ALS if the test accuracy measures are sufficiently high. Several of the studies did not meet the 20 direction acquisition standard necessary for robust FA estimation adding variability to the results (35). Given the provided datasets and our protocol, we focused on the IC and the average CST mean FA measures to minimize heterogeneity. A few of the studies analyzed other specific motor tract regions, including Pyra et al (13), who found the greatest discriminatory power in the precentral gyrus (AUC = 0.79). We did not perform a meta-analysis of these other motor regions because of data paucity. Furthermore, this study did not consider the detection of extra-motor DTI changes, which may provide greater diagnostic accuracy when combined with CST measures. We were also unable to investigate other summary FA measures that have greater disease discrimination, such as the top quartile mean FA (24).

In conclusion, this IPD meta-analysis of test accuracy suggests that DTI of the CST, in isolation, provides limited diagnostic test accuracy for ALS patients versus healthy controls. Adjusting for age, gender, functional status, and disease duration did not significantly change the pooled AUC measures. There is a need for more uniform neuroimaging studies of ALS combining multiple advanced methods, implementing new brain pattern recognition...

### Table 2. AUC Measures with Covariates

<table>
<thead>
<tr>
<th>Included study numbers*</th>
<th>Age</th>
<th>Gender</th>
<th>ALSFRS-R</th>
<th>Disease Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1, 2, 5–12</td>
<td>1, 2, 5–12</td>
<td>1–4, 6, 7, 9, 10, 12</td>
<td>1, 2, 4, 5, 7, 9, 10, 12</td>
</tr>
<tr>
<td>Nonadjusted AUC</td>
<td>0.58 (0.32–0.84)</td>
<td>0.58 (0.32–0.84)</td>
<td>0.65 (0.47–0.84)</td>
<td>0.61 (0.36–0.85)</td>
</tr>
<tr>
<td>Adjusted AUC</td>
<td>0.58 (0.31–0.85)</td>
<td>0.58 (0.32–0.85)</td>
<td>0.58 (0.40–0.77)</td>
<td>0.61 (0.37–0.86)</td>
</tr>
</tbody>
</table>

ALSFRS-R, Revised Amyotrophic Functional Rating Scale; AUC, area under curve.

*From Table 1.

### Figure 4.

Forest plots of sensitivity (SENS) (a) and specificity (SPEC) (b) for diagnosis of amyotrophic lateral sclerosis using diffusion tensor imaging fractional anisotropy values.

### Figure 5.

Posttest probabilities after diffusion tensor imaging (DTI) for hypothetical populations with different disease pretest probabilities.

had funding support; two of these six studies had AUC values less than 0.50, suggesting a limited role of funding bias. Several of the datasets did not contain all of the covariates. More than half of the studies did not have a representative disease spectrum of what would be expected in clinical practice, and it must be acknowledged that the differentiation of ALS patients from healthy individuals is not the clinical conundrum facing the clinician. However, DTI may have a role as a diagnostic test for those patients who do not yet have clinically definite signs of ALS if the test accuracy measures are sufficiently high. Several of the studies did not meet the 20 direction acquisition standard necessary for robust FA estimation adding variability to the results (35). Given the provided datasets and our protocol, we focused on the IC and the average CST mean FA measures to minimize heterogeneity. A few of the studies analyzed other specific motor tract regions, including Pyra et al (13), who found the greatest discriminatory power in the precentral gyrus (AUC = 0.79). We did not perform a meta-analysis of these other motor regions because of data paucity. Furthermore, this study did not consider the detection of extra-motor DTI changes, which may provide greater diagnostic accuracy when combined with CST measures. We were also unable to investigate other summary FA measures that have greater disease discrimination, such as the top quartile mean FA (24).

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techniques, and involving specific ALS mimics to enable meaningful clinical implementation of MRI.

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REFERENCES

APPENDIX

TABLE 1. MEDLINE Keyword Search (1966-April 2011)

<table>
<thead>
<tr>
<th>Study</th>
<th>Author</th>
<th>Journal</th>
<th>Year</th>
<th>Number of ALS Patients</th>
<th>ALS Age, y (Mean ± SD)</th>
<th>Number of HC Subjects</th>
<th>HC Age, y (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bartels (23)</td>
<td>Neuromuscul Disord</td>
<td>2008</td>
<td>12 M:10 F</td>
<td>61 ± 7</td>
<td>5 M:8 F</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>Cosottini (19)</td>
<td>Radiology</td>
<td>2005</td>
<td>14 M:4 F</td>
<td>64 ± 7</td>
<td>4 M:8 F</td>
<td>65 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>Cosottini (7)</td>
<td>J Comput Assist Tomogr</td>
<td>2010</td>
<td>14 M:4 F</td>
<td>61 ± 8</td>
<td>2 M:14 F</td>
<td>65 ± 6</td>
</tr>
<tr>
<td>6</td>
<td>Filippini (8)</td>
<td>Neurology</td>
<td>2010</td>
<td>16 M:8 F</td>
<td>59 ± 12</td>
<td>17 M:7 F</td>
<td>58 ± 12</td>
</tr>
<tr>
<td>7</td>
<td>Foerster</td>
<td></td>
<td></td>
<td>8 M:6 F</td>
<td>61 ± 9</td>
<td>8 M:6 F</td>
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<td>Brain Res</td>
<td>2010</td>
<td>10 M:2 F</td>
<td>56 ± 11</td>
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<td>6 M:8 F</td>
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ALS, amyotrophic lateral sclerosis; F, female; HC, healthy control; M, male; SD, standard deviation.
25 years of neuroimaging in amyotrophic lateral sclerosis

Bradley R. Foerster, Robert C. Welsh and Eva L. Feldman

Abstract | Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease for which a precise cause has not yet been identified. Standard CT or MRI evaluation does not demonstrate gross structural nervous system changes in ALS, so conventional neuroimaging techniques have provided little insight into the pathophysiology of this disease. Advanced neuroimaging techniques—such as structural MRI, diffusion tensor imaging and proton magnetic resonance spectroscopy—allow evaluation of alterations of the nervous system in ALS. These alterations include focal loss of grey and white matter and reductions in white matter tract integrity, as well as changes in neural networks and in the chemistry, metabolism and receptor distribution in the brain. Given their potential for investigation of both brain structure and function, advanced neuroimaging methods offer important opportunities to improve diagnosis, guide prognosis, and direct future treatment strategies in ALS. In this article, we review the contributions made by various advanced neuroimaging techniques to our understanding of the impact of ALS on different brain regions, and the potential role of such measures in biomarker development.


Introduction

More than 100 years have passed since Jean-Martin Charcot first described amyotrophic lateral sclerosis (ALS).1 The disease affects motor neurons in the primary motor cortex, brainstem and spinal cord, and results in both upper motor neuron (UMN) and lower motor neuron (LMN) signs.2 The underlying pathophysiology is not well understood, and effective treatments are still needed for this fatal neurodegenerative disease.3 Furthermore, the presentation and natural history of ALS varies considerably from patient to patient, which can contribute to diagnostic uncertainty.4 Other motor neuron diseases, including progressive muscular atrophy (PMA; LMN signs only) and primary lateral sclerosis (PLS; UMN signs only), must also be considered in the differential diagnosis. Imaging has had a tremendous impact in the work-up of nervous system pathology, particularly for stroke, cancer and demyelinating diseases. By contrast, neurodegenerative diseases including ALS tend to involve more-insidious pathological processes and present with less clear-cut clinical diagnoses and imaging findings.

Early reports suggested that structural changes in ALS could be detected using MRI, including hyperintense T2-weighted signal in the corticospinal tract (CST).5 Such MRI findings can, however, also be seen in healthy controls.6 As a result, the main role of conventional MRI in ALS management is to exclude alternative diagnoses—such as an upper cervical cord lesion—that can mimic ALS and present with UMN and LMN signs. Over the past 25 years, considerable interest has developed in the use of advanced neuroimaging methods to study CNS changes in ALS.

Advanced MRI techniques allow investigation of the nervous system for atrophy and alterations in microstructure, biochemistry, neural networks, metabolism and neuronal receptors. In this Review, we first introduce four MRI modalities as well as various PET radioligands. We then discuss how these techniques have been applied to increase our understanding of changes in different brain regions in ALS. We propose that advanced neuroimaging techniques have considerable potential for translation into diagnostic and therapeutic biomarkers. In addition, we support implementation of a multimodality imaging approach to further our understanding of this heterogeneous and complex neurodegenerative disease.

Advanced neuroimaging techniques

Structural MRI

Structural MRI (sMRI) techniques enable detailed analysis of focal atrophy and regional grey and white matter.7 Typical high-resolution, single-contrast images are three-dimensional images acquired at 1-mm resolution in each dimension to ensure accurate structural demarcation. For some subcortical structures, tissue identification (segmentation) is best achieved through a combination of high-resolution sequences.8 In general, two classes of volume analysis are used: voxel-based morphometry (VBM), which measures relative grey and white matter volumes in specific brain regions;9 and surface-based morphometry (SBM), which measures cortical thickness.10,11

Competing interests

The authors declare no competing interests.
Advanced neuroimaging techniques noninvasively evaluate brain structure, chemistry, neural network connections, metabolism, and receptor distribution in neurodegenerative diseases.

Nervous system changes in amyotrophic lateral sclerosis (ALS) involve the motor cortex, corticospinal tract, corpus callosum, frontal lobes, basal ganglia, thalamus, brainstem and cervical spinal cord.

Neuroimaging in ALS provides evidence of neuronal loss, white matter tract disruption, alterations in neural networks, γ-aminobutyric acid system dysfunction, and changes in brain metabolism.

Advanced neuroimaging techniques provide unique opportunities to more fully characterize and classify the different motor neuron disease subtypes.

ALS is a heterogeneous disease, and neuroimaging studies generally include small numbers of patients with long disease duration, which could limit the generalizability of results.

MRI and PET show promise for development of ALS biomarkers, although additional research is required to translate these technologies for clinical application.

**Diffusion tensor imaging**

Diffusion tensor imaging (DTI) quantifies Brownian motion of water molecules in biological tissues. By measuring both rate and preferred axis of water diffusion, DTI can evaluate integrity of white matter tracts, which can be degraded in neurological diseases. Fractional anisotropy (FA) is a scalar measurement of the degree of a preferred axis of water diffusion, and decreases as the diffusion of water becomes less restricted to a single axis. Mean diffusivity (MD) ignores nonuniformity of the diffusivity and averages water diffusivity properties from all three axes. Additional diffusivity measures can be investigated using DTI, including radial diffusivity (diffusion perpendicular to the myelin sheath), and axial diffusivity (diffusion parallel to the myelin sheath). Data analysis can be performed using region-of-interest approaches, which evaluate a specific brain location; whole-brain voxel-wise approaches such as tract-based spatial statistics; or tractography analyses, which can be used to characterize white matter tracts.

**Proton magnetic resonance spectroscopy**

Proton magnetic resonance spectroscopy (1H-MRS) uses the slight differences in the magnetic field produced by electrons and nuclei in a localized region of tissue, so as to resolve and quantify certain tissue metabolites. The most commonly investigated metabolites are: N-acetylaspartate (NAA), a marker of neuronal integrity; choline (Cho), a marker of cell membrane turnover; and creatine (Cr), a marker of energy metabolism. Other brain metabolites include: myo-inositol, a marker of gial cells; glutamate and glutamine (collectively termed Glx); and γ-aminobutyric acid (GABA). Metabolite quantification can be reported in the form of metabolite ratios or absolute concentrations. Metabolite ratios are used because standardization of the metabolite of interest relative to Cr or Cho corrects for variability that can arise, including magnetic field inhomogeneity and volume loss. Advances in 1H-MRS post-processing techniques, however, have facilitated absolute quantification of metabolites, which is generally considered to be more informative.

**Resting-state functional connectivity MRI**

Resting-state functional connectivity MRI (rs-fcMRI) uses intrinsic random fluctuations of brain activity to investigate relationships between brain regions. Detection of patterns of coherent brain activity led to the concept of specific neural networks in the brain.

An early study demonstrated that, at rest, blood oxygen level-dependent signals (an indirect measure of neuronal activity) were synchronized between the motor cortices of each hemisphere, representing an intrinsic connection between these two cortical regions. Use of activation-based functional MRI to elucidate neural correlates related to task execution—such as cognitive, motor and emotional tasks—demonstrated similar findings to the PET literature discussed here, and has been reviewed elsewhere.

**PET**

PET uses radioactive isotopes to localize and quantify brain metabolic activity or specific brain receptors, which can be altered in disease processes. PET agents used to investigate CNS changes in ALS include [18F]fluorodeoxyglucose (FDG), a glucose analogue that is used to measure metabolism; [11C]-6-fluorodopa, a dopamine precursor; [11C]-PK11195, which binds to the translocator protein and is considered a marker of neuroinflammation; [11C]-flumazenil, which binds to the GABA A receptor; [11C]-WAY100635, which binds to the serotonergic 5-hydroxytryptamine receptor 1A; and [11C]-deprenyl, which binds to monoamine oxidase B.

**Advanced neuroimaging in ALS**

**Motor cortex**

**Structural MRI**

A study that used manual methods to evaluate volumetric loss did not show evidence of substantial atrophy of the motor cortex of patients with ALS, but did demonstrate 25% volume loss in patients with PLS. A study conducted prior to the advent of VBM that used automated group image analysis indicated loss of white matter underneath the motor cortex in patients with bulbar-onset ALS but not in those with limb-onset ALS. Conversely, a subsequent study using VBM reported no loss of volume in the motor cortices of patients with ALS. Owing to relatively small samples of healthy controls, both of these studies might lack sufficient statistical power to detect cortical changes. The majority of studies implementing VBM techniques have found grey matter atrophy in the motor cortex of patients with ALS. SBM studies have consistently found reductions in cortical thickness in the ALS motor cortex. Longitudinal SBM studies have reported progressive cortical thinning over 9 months.

**Proton magnetic resonance spectroscopy**

The majority of 1H-MRS studies have described reductions in NAA:Cr or NAA:Cho ratios or NAA levels in the motor cortex of patients with ALS, which suggests reduced neuronal integrity in this brain region. Evidence is conflicting as to whether these changes occur...
early in clinically evident disease: one study reported decreases in NAA:Cr ratios at a mean disease duration of 6.5 months,\textsuperscript{47} whereas another study reported no significant differences in NAA levels in patients diagnosed with ALS within the past year.\textsuperscript{48}

Lower NAA:Cr ratios have been reported across motor neuron disease phenotypes, with similar changes in patients with PLS or ALS, and less-dramatic reductions in patients with PMA.\textsuperscript{38} Substantially lower NAA:Cr ratios were reported in the motor cortex of patients with bulbar-onset compared with limb-onset ALS.\textsuperscript{49} A longitudinal study detected decreases in NAA levels over 6 months in patients with PMA but not in those with early-stage ALS.\textsuperscript{44} Other studies, which included ALS patients with longer disease duration, described longitudinal reductions in NAA in the motor cortex of ALS patients at 3 months,\textsuperscript{41} 6 months\textsuperscript{45} and 12 months.\textsuperscript{39} A number of studies have also explored correlations between NAA and clinical scales, and have reported significant associations between NAA levels and revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) scores,\textsuperscript{37,38,47,51} UMN disease burden,\textsuperscript{37,44,52} Norris Scale scores,\textsuperscript{36,42} finger-tapping rate,\textsuperscript{38} hand strength,\textsuperscript{38} disease duration,\textsuperscript{47} and rate of disease progression.\textsuperscript{34} Taken together, these results point to a reduction in neuronal integrity in the motor cortex in ALS.

Levels of other brain metabolites in the motor cortex have also been investigated, although not to the same extent as NAA. Early studies found no significant differences in Glx or Glu levels,\textsuperscript{45} or reported decreased Glu levels in patients with ALS compared with controls.\textsuperscript{40} A more recent study described elevations in Glx:Cr and Glu:Cr ratios in patients with ALS compared with controls.\textsuperscript{46} Increased Cho levels\textsuperscript{40} and decreased Cr levels\textsuperscript{40} have been described, but these findings were not replicated in other reports.\textsuperscript{36,41,44} Elevated myo-inositol levels or myo-inositol:Cr ratios have been detected in the motor cortex in patients with ALS\textsuperscript{40,43,51} or PLS,\textsuperscript{38} but not in early-stage ALS.\textsuperscript{48} The exact mechanism of myo-inositol alteration in ALS is uncertain, but might reflect an increased number of glial cells, or increased expression of the myo-inositol transporter on astrocytes.\textsuperscript{51} Using ‘H-MRS spectral editing techniques, we recently showed reduced GABA levels in patients with ALS compared with controls, suggesting that GABAergic dysfunction could have an important role in ALS neurodegeneration (Figure 1).\textsuperscript{51}

Resting-state functional connectivity MRI

Several studies have described decreased motor network (primary motor cortices and supplementary motor area) connectivity,\textsuperscript{24–36} or a trend towards decreased motor network connectivity,\textsuperscript{57} in ALS. One study also reported a correlation between disease burden and motor network connectivity: patients who were more affected by ALS had a more strongly interconnected network than did patients with lower disease burden.

A study that used the full precentral gyri to define the sensorimotor cortices detected increased connectivity of this region with nonmotor areas in patients with ALS compared with controls, but did not observe a change in connectivity between the motor cortex of each hemisphere.\textsuperscript{58} A combination of rs-fcMRI and DTI techniques revealed increased connectivity of the motor network in ALS patients with greater disease burden.\textsuperscript{59} This increased motor cortex connectivity was detected in regions abutting white matter areas that showed loss of integrity, as measured by DTI (Figure 2). As such, the observed increase in connectivity could be a manifestation of loss of interhemispheric inhibition,\textsuperscript{60} and is consistent with the decreased GABA concentration in the motor cortex of patients with ALS.\textsuperscript{55}

Taken together, these studies have shown motor network changes in ALS. A possible scenario to explain the observed changes could be that early in the disease process, when most function is still preserved, functional connectivity begins to decrease. With increased disease burden, however, loss of interhemispheric inhibition starts to manifest, resulting in increased connectivity. Longitudinal studies of connectivity and changes in GABA levels in ALS are required to investigate these findings further.

Figure 1 | Advanced imaging of the motor cortex in ALS. a,b | T1-weighted MRI scans showing single voxel placement centred on the left motor cortex (yellow boxes) in the sagittal (part a) and axial (part b) projections. c | Representative magnetic resonance spectroscopy spectrum from the left motor cortex using MEGA-PRESS spectral editing technique. d | GABA levels in the motor cortex are decreased in patients with ALS compared with controls. Horizontal bars indicate the mean. Abbreviations: ALU, arbitrary institutional unit; ALS, amyotrophic lateral sclerosis; GABA, γ-aminobutyric acid; Glx, glutamate and glutamine; HC, healthy control; NAA, N-acetylaspartate. Permission obtained from The American Academy of Neurology © Foerster, B. R. et al. Neurology 78, 1596–1600 (2012).
ALS compared with healthy controls (P < 0.05 corrected for multiple comparisons) in primary sensorimotor and premotor cortex, anterior and motor cingulate areas, frontal and central operculum, and the thalamus. Abbreviation: ALS, amyotrophic lateral sclerosis. Permission obtained from Oxford University Press

PET

PET has revealed reductions in cerebral blood flow in the motor cortex and alterations in neuronal activation in patients with ALS compared with controls. Patients had increased cerebral blood flow and activation in the contralateral motor cortex and adjacent association areas during a single-handed motor task, which may be due to compensatory adaptation and/or impairment of neuronal inhibitory tone.

Increased microglial activation in the motor cortex of patients with ALS was demonstrated using the PET ligand 11C-PK11195, a marker of neuroinflammation. Increased binding of 11C-PK11195 was also evident in the pons, dorsolateral prefrontal cortex, and thalamus. Two studies have used 11C-flumazenil to study both sporadic ALS and familial-type ALS caused by homozygous Asp90Ala mutation of superoxide dismutase 1 (SOD1), which tends to have a less aggressive disease course than sporadic disease. The studies showed reduced 11C-flumazenil binding in the motor cortex and motor association areas in sporadic ALS compared with controls. Reductions in 11C-flumazenil binding in familial-type ALS, however, were localized to the left frontotemporal region and anterior cingulate gyrus. Only patients with sporadic ALS demonstrated increased cortical excitability.

Implications of imaging findings

Advanced neuroimaging studies have demonstrated substantial changes in the motor cortex of patients with ALS. Structural imaging and 1H-MRS have provided evidence of neuronal degeneration, through measures showing reduced cortical thickness and reductions in NAA. 1H-MRS and PET have indicated compromise of the GABAergic system, and rs-fcMRI has suggested reduced neuronal inhibitory function. These results suggest an interneuron-targeted pathology involving loss of cortical inhibition and resulting hyperexcitability, perhaps through glutamate excitotoxicity.

Corticospinal tract

Diffusion tensor imaging

A number of studies have demonstrated reduced FA, increased MD, and increased radial diffusivity in the CST of patients with ALS. Reduced FA values in the CST and corpus callosum have been described in a small cohort of patients who did not have UMN signs at the time of the scan but later progressed to full ALS, providing evidence that white matter tract degradation can be detected prior to onset of clinical signs.

A study of patients with early-stage ALS, PMA or PLS generally found the lowest FA values in the PLS subgroup, with more-modest reductions in the ALS subgroups and the least extensive reductions in the PMA subgroup. A study that enrolled ALS patients with longer disease duration, however, found approximately twice the reduction of FA values in patients with ALS versus patients with PLS. These findings suggest that disease staging can affect DTI measurements in ALS.

Other studies have demonstrated no significant difference in FA values in patients with PMA or PLS compared with controls. Compared with patients with limb-onset ALS, patients with bulbar-onset ALS have greater decreases in FA and greater increases in MD relative to healthy controls. These studies reported increased MD values in patients with bulbar-onset ALS compared with healthy controls, but no significant differences in MD values in patients with limb-onset ALS compared with healthy controls.

FA values in the CST have been shown to correlate with UMN and ALSFRS-R scores. Two studies have shown that ALS patients with higher FA values have a more protracted disease duration, perhaps owing to differences in disease subtype. However, many DTI studies in ALS have not shown significant correlations between DTI metrics and clinical measures. Patients with ALS caused by homozygous Asp90Ala SOD1 mutations had similar CST FA values to healthy controls, whereas patients with sporadic ALS had lower CST FA values, indicating different disease mechanisms between this familial type and sporadic ALS. Some studies have reported decreased FA, increased MD, and increased axial diffusivity in the posterior limb of the internal capsule (PLIC). The PLIC contains densely packed CST fibres and might, therefore, be highly sensitive to DTI alterations.

Some longitudinal studies have demonstrated reductions in FA in the CST over time, whereas others have not. Using DTI, researchers demonstrated impairment of the motor network, involving the white matter tracts and their associated cortical regions, in patients with ALS, which could indicate spread of disease from the motor cortex into associated motor regions. Other DTI studies found the greatest changes in diffusivity in rostral, as opposed to caudal, regions of the CST.
the CST. Together, these findings suggest anterograde corticomotor neuronal degeneration.\textsuperscript{71,74} However, not all imaging data, including those from 1\textsuperscript{H}-MRS-based studies, support rostral-predominant changes,\textsuperscript{85} so the possibility remains that ALS disease processes advance retrogradely from the spinal anterior horns, as well as anterogradely from the motor cortex.\textsuperscript{95}

Evidence exists that patients with PLS have rostral-predominant changes in white matter integrity in the CST, whereas patients with ALS have caudal-predominant changes.\textsuperscript{72} In addition, reductions in FA in white matter adjacent to the motor cortex were greater in patients with PLS than in those with ALS, and greater reductions in FA were present in the PLIC in ALS versus PLS. FA values in the cerebral peduncle, however, did not differ between these two motor neuron diseases.\textsuperscript{97} Similar findings have been reported in patients with limb-onset ALS (more-rostral FA involvement) versus patients with bulbar-onset ALS (more-caudal FA involvement) at baseline, with increasing involvement of the CST in both groups over time.\textsuperscript{89} These results might be explained in part by the different site of onset of different motor neuron diseases.\textsuperscript{97} Proton magnetic resonance spectroscopy

A few studies have evaluated metabolite changes in the CST in ALS. Decreased NAA:Cr ratios were present in the subcortical white matter and periventricular white matter along the CST in patients with ALS compared with controls, although significant changes were not evident in the PLIC.\textsuperscript{86} Another study found reduced NAA:Cr and NAA:Cho ratios in the precentral gyrus and corona radiata in patients with ALS compared with controls, without significant changes in the PLIC or cerebral peduncles, suggesting attenuation of metabolite alterations in more-caudal parts of the CST.\textsuperscript{85} Reductions in NAA levels and elevations in Cho levels, as averaged over the CST, have also been described in ALS, as have reductions in NAA levels in the left PLIC.\textsuperscript{97} Using whole-brain 1\textsuperscript{H}-MRS techniques, a recent study reported reduced NAA levels along the CST.\textsuperscript{76} Correlations between NAA and clinical measures have been inconsistent.\textsuperscript{85,78,96}

Resting-state functional connectivity MRI

One study, although not explicitly measuring connectivity in the CST, investigated resting-state connectivity in the presence or absence of CST involvement, as determined by DTI.\textsuperscript{58} The left sensorimotor cortex had more widespread connectivity when DTI measures of the CST were preserved.

Implications of imaging findings

Overall, results from DTI and 1\textsuperscript{H}-MRS studies of the CST indicate disruption and degeneration of white matter in this region. The significance of alterations in DTI metrics has been the subject of much discussion in the literature.\textsuperscript{99} Axonal degeneration and disorganization\textsuperscript{99} is thought to give rise to alterations in water diffusivity\textsuperscript{100} in the setting of ALS, and the FA reductions have been posited to reflect gliosis, increased extracellular matrix, Wallerian degeneration, and/or axonal loss.\textsuperscript{68,81,101}

**Corpus callosum**

**Structural MRI**

Few studies have explicitly addressed changes in the corpus callosum in ALS. Manual measurement methods revealed reductions in the size of the corpus callosum in patients with this disease compared with healthy controls,\textsuperscript{102} whereas a VBM-based study showed increased corpus callosum size in patients with ALS.\textsuperscript{25} For our own study, we aligned each participant’s acquisition space to a common orientation, to avoid image misregistration errors that can occur through use of group spatial normalization in VBM.\textsuperscript{103,104} We found no change in the cross-sectional area of the corpus callosum in individuals with ALS. Notably, many studies have investigated global structural involvement in ALS through use of VBM or SBM analyses, and only a handful indicated morphological involvement of the corpus callosum.

**Diffusion tensor imaging**

DTI has been used to explore changes in the genu, body and splenium of the corpus callosum. The genu connects the inferior frontal lobes and anterior temporal lobes; the body connects the remaining regions of the frontal lobes as well as the parietal lobes; and the splenium connects the occipital lobes.

Several studies have documented FA reductions in the middle posterior body of the corpus callosum in ALS,\textsuperscript{71,72,75,77,78,101} as well as increases in MD,\textsuperscript{77} axial diffusivity\textsuperscript{79} and radial diffusivity\textsuperscript{71,76,77} (Figure 3). FA values of the middle posterior regions of the corpus callosum correlated negatively with percentage of contralateral involuntary movements, suggesting loss of neuronal inhibitory influence in ALS.\textsuperscript{103} FA values in the corpus callosum also correlated positively with ALSFRS-R\textsuperscript{71,105} and UMN scores.\textsuperscript{73,101}

FA of the body, genu and splenium of the corpus callosum was found to be decreased in patients with ALS compared with controls.\textsuperscript{87} Using a different cohort, the same study reported decreased FA in the body of the corpus callosum in patients with PLS versus patients with ALS or healthy controls. Similarly, FA in the body and splenium of the corpus callosum was found to be decreased in patients with PLS compared with patients with ALS or PMA.\textsuperscript{80} Longitudinal decreases in FA in the body of the corpus callosum have been described in patients with limb-onset or bulbar-onset ALS, and in patients with PMA. Patients with bulbar-onset ALS or PMA also showed longitudinal decreases in FA in the genu of the corpus callosum, which might contribute to the cognitive dysfunction observed in these patients.\textsuperscript{80}

**Implications of imaging findings**

DTI findings in the middle posterior body of the corpus callosum, similar to the CST results, show substantial reductions in FA. The middle posterior body of the corpus callosum connects the motor and motor association cortices. As such, the DTI results support the
hypothesis that disease propagates along structural connections. The direct contribution of structural tract changes in the corpus callosum and CST to functional alterations in ALS is unclear, although early findings suggest that white matter alterations are associated with increased functional connectivity.

**Frontal lobe**

**Structural MRI**

An early study of patients with ALS found a reduction of underlying white matter volume but not cortical surface area in the anterior frontal lobes. Frontal lobe involvement has also been reported using VBM methods. One study using SBM analysis found extensive cortical thinning in frontal brain regions, including the inferior part of the precentral sulcus, inferior and middle frontal sulci, and medial orbital sulcus.

Several studies have explored the link between structural changes in the frontal lobe and cognition in patients with ALS. A VBM study found frontal lobar atrophy in ALS patients, with the greatest effect in patients with ALS–frontotemporal dementia (FTD). Other studies have demonstrated reductions in white matter, grey matter or both, with greatest effect seen in cognitively impaired patients with ALS. An SBM study revealed extensive cortical thinning in bilateral frontal and temporal regions. In addition, performance on a recognition sorting test correlated with cortical thickness of the left prefrontal and parietal regions.

A few longitudinal studies have found changes in the frontal lobe in patients with ALS. One study that used tensor-based morphometry (an analytical method for longitudinal VBM) found more-rapid loss of frontal grey matter in patients with more-rapidly progressing disease at 9-month follow-up. Cortical thinning of the right-side pars triangularis was associated with disease progression, and showed a trend towards continued thinning in the left lateral orbital frontal region at 3–10-month follow-up. Decreased grey matter volume in the left insula and right temporopolar cortex, and decreased white matter volume in the right inferior frontal gyrus were reported at baseline, with increasingly pronounced bilateral involvement of the frontal and temporal lobes at 6-month follow-up.

**Proton magnetic resonance spectroscopy**

Both NAA:(Cr+Cho) and NAA:Cho ratios in the combined cortex of the bilateral frontal and parietal lobes were reduced in patients with probable or definite ALS (diagnosed according to El Escorial Criteria) compared with healthy controls. These reductions were not as dramatic as those present in the motor cortex. Another study found trends for reductions in NAA and elevations of myo-inositol levels in the mesial prefrontal cortex. Overall, the NAA:myo-inositol ratio was significantly decreased in patients with ALS, but did not correlate with verbal fluency or cognitive performance. Another study reported decreases in the NAA:Cr ratio in the frontal lobe, and these changes correlated with achievement on the Wisconsin Card Sorting Test. Other areas that have not shown substantial metabolite changes in ALS versus controls include the parietal lobe and occipital lobe, including the cuneus gyrus.

**Diffusion tensor imaging**

Reduced FA and increased MD values have been described in several frontal white matter regions in patients with ALS compared with controls. One DTI study described more-global reductions in FA values compared with other DTI studies, including involvement of frontal, temporal and parietal white matter. Cognitive testing scores correlated with FA values in white matter tracts located in frontotemporal regions, as well as in the corpus callosum and CST. Apathy testing scores correlated significantly with FA and MD values, as well as cortical volumes, in the frontal lobe. One study reported FA decreases in white matter of the frontal gyrus and dorsolateral prefrontal gyrus of patients with bulbar-onset but not limb-onset ALS. These differences could help to explain the more prominent cognitive impairment that tends to occur in bulbar-onset ALS compared with limb-onset ALS. The
same study also found longitudinal FA reductions in frontal white matter of patients with PMA, which might contribute to the memory and executive dysfunction observed in this subtype of motor neuron disease. By contrast, a subsequent study demonstrated FA decreases in subcortical white matter of the frontal gyrus of limb-onset ALS patients but not in those with bulbar onset.62 Neither study included neuropsychological testing, and further research efforts are required to better understand the relationship between cognitive function and DTI measures in the different ALS subtypes.

**PET**

PET studies have shown reductions in frontal lobe cerebral blood flow and oxygen metabolism in ALS patients with dementia compared with healthy controls.114 Similarly, reductions in frontal lobe cerebral blood flow were observed in patients with ALS who had impaired verbal fluency.115 An 18F-FDG PET study found that patients with bulbar-onset ALS had significantly lower verbal fluency scores and reduced metabolism in the frontal cortex compared with limb-onset ALS patients and healthy controls.116 This result suggests that the various ALS phenotypes differ in both cognitive dysfunction and metabolic profiles. Compromised verbal fluency measures have been shown to correlate with reduced 11C-flumanezil binding in the right inferior frontal gyrus, superior temporal gyrus, and anterior insula.117 11C-WAY100635 binding was globally decreased in the cortex of patients with sporadic ALS, with the most substantial changes manifesting in fronto-temporal regions.118 A similar pattern of reduced binding of 11C-WAY100635 was present in patients with homoyzygous Asp90Ala mutations in SOD1, although to a lesser extent than in patients with sporadic ALS. These results suggest that patients with Asp90Ala SOD1 ALS have less cortical damage than do patients with sporadic ALS.

**Implications of imaging findings**

Cognitive and executive functional changes in patients with ALS have been well-documented in the clinical literature. Neuroimaging findings provide corroborative evidence of frontal lobe changes in ALS, in that many of the imaging features correlate with cognitive test scores. Advanced neuroimaging methods reveal important structural, biochemical, metabolic and receptor alterations in the frontal lobe. These results are particularly interesting in light of the recent discovery of the C9orf72 hexanucleotide repeat expansion, which is most commonly found in patients with familial FTD and/or ALS.119 Neuroimaging techniques may be able to provide a better understanding of the continuum and overlap of ALS and FTD, and could help to explain the differences in cognitive impairment between bulbar-onset and limb-onset ALS.

**Deep grey nuclei**

**Structural MRI**

Few volumetric changes involving the deep grey nuclei have been described in the ALS literature, despite a reasonably large number of sMRI studies. A VBM study described atrophy in the right basal ganglia in ALS, with the most prominent atrophy in patients with rapid disease progression.113 Volume loss was detected in the left posterior thalamus of patients with ALS or ALS–FTD.24

**Diffusion tensor imaging**

DTI evaluation of the deep grey nuclei of patients with ALS revealed decreased FA and increased MD in the basal ganglia and thalamus, as well as significant negative correlations between several DTI measures and scores on cognitive and fine-motor-rate tests.120 Additional studies have described reduced FA in the thalamus of patients with ALS versus controls.66,25 One longitudinal study found baseline FA reductions in the thalamus of patients with ALS compared with healthy controls, and reductions in FA in the thalamus over time in patients with bulbar-onset or limb-onset ALS.40

**Proton magnetic resonance spectroscopy**

Reductions in NAA and elevations of Cho in both the basal ganglia and thalamus in patients with ALS versus controls have been described.121 NAA levels and the NAA:Cho ratio in the basal ganglia correlated positively with finger-tap rate, foot-tap rate, and bulbar muscle movement rate. A subsequent study reported no differences in the NAA:Cho ratio in the thalamus of patients with ALS versus controls, although substantial decreases in the NAA:Cho ratio were detected in the midcingulate cortex.24

**PET**

A PET study found a negative correlation between 6-fluorodopa uptake in the striatum and disease duration in patients with ALS who lacked extrapyramidal signs, providing evidence for substantia nigral cellular loss in this disease and suggesting that ALS might share disease mechanisms with Parkinson disease.122

**Resting-state functional connectivity MRI**

Only one rs-fcMRI study has reported a change in connectivity in subcortical structures in patients with ALS.39 Increased connectivity between the motor network and the thalamus was identified, implying damage to thalamocortical projections of the motor pathway.

**Implications of imaging findings**

The above findings emphasize the concept that ALS affects multiple CNS structures. Although the studies are limited in number, evidence exists to support loss of neuronal integrity in the deep grey nuclei. The components of the deep grey nuclei have important connections to the motor network and prefrontal cortices, and seem to be altered in ALS.

**Brainstem**

**Diffusion tensor imaging**

One of the first ALS studies to focus on the pyramidal tract in the brainstem reported decreased FA at the level of the pons and medulla in patients versus controls.58 By contrast, no disease-associated differences in FA or MD were detected in these brainstem regions in another study, although they were evident at the level of the
cerebral peduncle. Other reports have found reduced FA in the pyramid of the medulla, which correlated with UMN scores.

Proton magnetic resonance spectroscopy
Decreased NAA:Cr ratios and increased Glx:Cr ratios were detected in the medulla of patients with ALS compared with control individuals. The Glx:Cr ratio showed a significant negative correlation with ALSFRS-R bulbar subscores in patients with bulbar-onset disease. Reductions in brainstem levels of NAA have also been detected in patients with ALS, although no significant changes in Glx were observed. A more recent study did not find significant changes in NAA:Cr or NAA:Cho ratios in the medulla orpons in patients with early-onset ALS, although disease duration did show a significant negative correlation with NAA:Cr ratios in the medulla.

PET
A large 18F-FDG PET study found evidence of hypermetabolism in the midbrain andpons of patients with ALS. Another study showed increased uptake of the PET ligand 11C-l-deprenyl in the pons and supratentorial white matter in ALS, providing support for involvement of astrocytosis in ALS pathogenesis.

Implications of imaging findings
Advanced neuroimaging studies demonstrate structural and metabolite changes in the brainstem in ALS. DTI studies of the brainstem are subject to greater variability than are studies of other brain regions owing to decussation of fibre tracts in this region. However, significant reductions of FA that correspond to the CST results discussed above have been demonstrated in the brainstem of patients with ALS. Overall, results from 1H-MRS studies provide evidence of neuronal degeneration in the brainstem similar to that in the motor cortex. PET findings support the presence of metabolic, neuroinflammatory and reactive changes in the brainstem in ALS.

Cervical spinal cord
Diffusion tensor imaging
In ALS, DTI studies have shown reduced FA and increased radial diffusivity in the spinal cord, particularly in the distal cervical cord. In addition, cross-sectional spinal cord area was reduced, and FA values correlated with ALSFRS-R scores. Focal atrophy of the spinal cord in ALS correlated with muscle deficits. One study also demonstrated focal FA reductions and increased radial diffusivity in dorsal spinal cord columns, which suggests involvement of spinal sensory pathway in ALS. A longitudinal study demonstrated reductions in spinal cord FA and cross-sectional area and increased MD at 9-month follow-up.

Magnetic resonance spectroscopy
MRS studies have focused on the upper cervical cord to minimize the challenges of anatomy and motion artefacts. Reduced NAA:Cr and NAA:myo-inositol ratios have been reported in this region in patients with ALS and in presymptomatic carriers of SOD1 mutations. One study found increased myo-inositol:Cr ratios, which were not confirmed in the other MRS studies. NAA:myo-inositol ratios correlated with FVC and ALSFRS-R scores, as well as with rates of decline in these scores.

Implications of imaging findings
Both DTI and MRS studies of the cervical cord in ALS demonstrate structural and metabolite changes that are concordant with those seen in the brain. Further advances in neuroimaging techniques will enable the challenges of investigating this important nervous system structure—which is integrally involved in the pathophysiology of ALS—to be addressed.

Biomarker potential of imaging
Substantial interest has been expressed in the potential to translate research-driven advanced imaging protocols into clinically relevant imaging tools. Given the heterogeneous clinical presentation among patients with ALS, neuroimaging tests to assist diagnosis are desirable.

An important caveat is that most neuroimaging studies involve small numbers of patients who tend to have long disease duration, which could result in skewed sampling given the variable nature of this disease. This situation might explain some of the discrepant neuroimaging results in the literature, and can limit generalizability of the findings. To explore the potential of DTI to diagnose ALS, we recently performed a meta-analysis.
and concluded that current DTI techniques lack sufficient diagnostic accuracy to be implemented clinically (Figure 4). As such, additional research efforts are needed for diagnostic biomarker development.136

An attractive option for development of a diagnostic algorithm with sufficient accuracy lies in combining various advanced neuroimaging modalities and/or laboratory tests. International efforts towards this goal have produced a consensus statement paper from the Neuroimaging Society in ALS to facilitate neuroimaging biomarker development for this disease.131

More-advanced statistical techniques, such as machine-learning methods, will have an important role in analysis of the resulting rich data sets, to facilitate development of a multimodal neuroimaging biomarker.132 Another important step to assess the capacity of imaging biomarkers to predict conversion to ALS would involve enrolment of neurology patients who present with only LMN signs, as well as those with a genetic predisposition to ALS, in longitudinal trials. A multimodality neuroimaging approach could also be useful for development of therapeutic biomarkers to more fully investigate nervous system changes in response to potential new treatments.

Imaging markers for prognostication

The average life expectancy of patients with ALS is 2–4 years, but a subgroup of patients survive for more than 10 years.133 Clinical predictors of shorter survival time include older age at onset, bulbar onset, and respiratory muscle weakness.134 Neuroimaging may be able to provide important additional information to help determine prognosis. One 1H-MRS study demonstrated that lower NAA:Cho ratios in the motor cortex were predictive of shorter survival time in patients with ALS.126 Using DTI, another study showed that lower FA in the CST predicted shorter survival.128 FA values in the PLIC correlate with rate of disease progression, with patients with lower FA values having faster disease progression.89 Although the number of studies is small, these results suggest that advanced neuroimaging methods could provide important prognostic information to help guide clinical decisions and stratify patients for clinical research trials.

Conclusions and future directions

Neuroimaging has provided important insight into the pathophysiology of ALS in living patients. Compelling imaging evidence exists of neurodegeneration in many nervous system regions including the motor cortex, frontal lobe, CST, brainstem and cervical spinal cord. Furthermore, neuroimaging findings suggest involvement of the corpus callosum and basal ganglia, both of which have important connections to the motor system. Imaging studies also support the clinical findings of frontal lobe involvement in ALS and its overlap with FTD. We believe that neuroimaging will play an important part in furthering our understanding of the role of the recently discovered C9orf72 mutation in ALS pathogenesis. Advanced neuroimaging also demonstrates similarities and differences between the motor neuron disease phenotypes in terms of brain metabolism, biochemistry and structural alterations, which should contribute to elucidation of disease aetiologies across the spectrum of motor neuron diseases.

1H-MRS, rs-fcMRI and PET studies have provided corroborating evidence of GABAergic dysfunction in ALS, suggesting the therapeutic potential of strategies to restore normal inhibitory tone. DTI studies have demonstrated evidence of white matter degradation in the frontal lobes, corpus callosum and CST in patients with ALS, although additional research is needed to fully understand these findings. PET studies have shown CNS metabolic alterations in ALS, and have provided evidence of neuroinflammation and astrocytosis in the disease process.

Advanced neuroimaging techniques provide a unique opportunity to assess disease pathophysiology early and noninvasively and, therefore, offer promise for biomarker development. The need has emerged, however, for a multimodality approach to better characterize patients and to improve our understanding of the disease processes. In the ALS neuroimaging community, there is increasing interest in implementation of such a strategy. In addition, longitudinal trials with enrolment of patients at earlier stages of presentation are needed to better understand how the various imaging metrics change over time. MRI and PET technologies will undoubtedly continue to evolve, and are expected to make important contributions to the diagnosis and treatment of ALS.

Review criteria

Literature searches were performed in PubMed using the search terms “amyotrophic lateral sclerosis”, “ALS”, “magnetic resonance spectroscopy”, “diffusion tensor imaging”, “positron emission tomography”, “resting-state functional magnetic resonance imaging”, “functional connectivity”, “fcMRI”, “voxel-based morphometry”, “VBM”, “FreeSurfer”, “grey matter change”, “cortex change”, “MRI”, and “magnetic resonance imaging” from January 1990 to February 2013. We searched the reference lists of retrieved papers to identify additional articles. Only full-text papers written in English were considered.


Intraspinal Stem Cell Transplantation in Amyotrophic Lateral Sclerosis: A Phase I Trial, Cervical Microinjection, and Final Surgical Safety Outcomes

**BACKGROUND:** The first US Food and Drug Administration clinical trial for a stem cell-based treatment of amyotrophic lateral sclerosis has now been completed.

**OBJECTIVE:** Primary aims assessed the safety of a direct microinjection-based technique and the toxicity of neural stem cell transplantation to the ventral horn of the cervical and thoracolumbar spinal cord. Results from thoracolumbar-only microinjection groups have been previously published. Cervical and cervical plus thoracolumbar microinjection group perioperative morbidity results are presented.

**METHODS:** Eighteen microinjection procedures (n = 12 thoracolumbar [T10/11], n = 6 cervical [C3-5]) delivered NSI-566RSC (Neuralstem, Inc), a human neural stem cell, to 15 patients in 5 cohorts. Each injection series comprised 5 injections of 10 μL at 4-mm intervals. The patients in group A (n = 6) were nonambulatory and received unilateral (n = 3) or bilateral (n = 3) thoracolumbar microinjections. The patients in groups B to E were ambulatory and received either unilateral (group B, n = 3) or bilateral (group C, n = 3) thoracolumbar microinjections. Group D and E patients received unilateral cervical (group D, n = 3) or cervical plus bilateral thoracolumbar microinjection (group E, n = 3).

**RESULTS:** Unilateral cervical (group D, n = 3) and cervical plus thoracolumbar (group E, n = 3) microinjections to the ventral horn have been completed in ambulatory patients. One patient developed a postoperative kyphotic deformity prompting completion of a laminoplasty in subsequent patients. Another required reoperation for wound dehiscence and infection. The solitary patient with bulbar amyotrophic lateral sclerosis required perioperative reintubation.

**CONCLUSION:** Delivery of a cellular payload to the cervical or thoracolumbar spinal cord was well tolerated by the spinal cord in this vulnerable population. This encouraging finding supports consideration of this delivery approach for neurodegenerative, oncologic, and traumatic spinal cord afflictions.

**KEY WORDS:** Amyotrophic lateral sclerosis, Cell therapy, Intraspinal microinjection, Spinal cord injection, Stem cell

**ABBREVIATIONS:** ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised Amyotrophic Lateral Sclerosis Functional Rating Scale; FDA, US Food and Drug Administration; MEP, motor evoked potential; POD, postoperative day; SSEP, somatosensory evoked potential

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal’s Web site (www.neurosurgery-online.com).
procedures delivering NSI-566RSC to the ventral horn with either unilateral or bilateral cell injections. The first patient received treatment in January 2010, and the final microinjection procedure took place in August 2012. All procedures were completed at Emory University Hospital in Atlanta, Georgia.

Cervical Microinjection Outcomes

We have recently reviewed the multiple completed and ongoing domestic and international trials delivering cell-based therapies to the spinal cord. These trials included the treatment of ALS or alternative afflictions (eg, spinal cord injury, spinal muscular atrophy). A broad spectrum of delivery approaches (eg, intravascular, intrathecal, direct microinjection), cell-based therapies (eg, autologous vs allogeneic), and strategies for immunosuppression (eg, none, single agent, or multitarget) have been attempted. The design of the current trial was influenced by each of these previous experiences. First, we used a patient-stabilized microinjection platform that was tested in preclinical large animal studies. Second, an allogeneic cellular graft with supportive preclinical efficacy data was used as the payload. Third, a multitarget immunosuppressant regimen was used. Finally, we used a risk escalation paradigm of trial design. In this approach, thoracolumbar microinjections were performed before attempts at cervical intervention, and nonambulatory patients received grafts before ambulatory patients. Progression to cervical microinjection cohorts required an interim analysis by the FDA with demonstration of safety in nonambulatory and ambulatory thoracolumbar microinjection patients.

Interim results for safety and functional outcomes were previously published for thoracolumbar-only microinjection patients. These results support the safety of serial bilateral thoracolumbar microinjection in ambulatory patients and do not show acceleration of disease related to the injection procedure. Additionally, there was an early finding of clinical improvement in 1 patient. This article presents the perioperative morbidity data for ambulatory patients who underwent serial unilateral cervical and cervical + bilateral thoracolumbar microinjection. This represents 6 of the 15 enrolled patients, comprising 2 of the 5 cohorts. Finally, the experiences gained through both our extensive preclinical work and through completion of this phase I trial are used as a foundation to discuss relevant considerations for future efforts to translate cell-based therapies to the human spinal cord.

PATIENTS AND METHODS

Trial Design

The trial design has been published and is briefly reviewed. This open-label phase I safety trial was based on a "risk escalation" paradigm in which successive patient cohorts have both improved functional capacity and are subjected to a progressive element of risk to neurological function. There were 5 patient cohorts. Progression between groups (A to E) and ultimate trial completion required interval demonstration of procedural safety. Thoracolumbar-only microinjections were completed in groups A to C. Group A (n = 6) patients received unilateral or bilateral microinjection (n = 3 each) and were nonambulatory. Group B and C (n = 3 each) patients were ambulatory and received unilateral (B) or bilateral (C) microinjection. Interim results for the primary (safety) outcome and secondary (functional) outcomes for groups A to C have been published. Group D (n = 3) and Group E (n = 3) comprised ambulatory patients, who received unilateral cervical or unilateral cervical + bilateral thoracolumbar microinjection, respectively. Group D and E safety outcomes are reported here. Secondary trial end points have assessed disease outcome-related measures and will be reported separately.

Cell Suspension

NSI-566RSC (Neuralstem, Inc; Rockville, MD) is a human fetal neural stem cell line obtained from a fetal spinal cord of approximately 8 weeks gestational age. A tissue area corresponding to the lower cervical/upper thoracic cord was obtained and separated from associated meninges and dorsal root ganglia. Neural stem cells were isolated and propagated. This expanded cell line has been validated through both in vitro preclinical studies and in the superoxide dismutase 1 familial ALS small-animal model. It has been demonstrated to prolong motor neuron survival and improve overall superoxide dismutase 1 small-animal survival while expressing a largely GABAergic phenotype. Completion of in vitro and small-animal safety studies has demonstrated a lack of tumorigenicity; microarray and immunohistochemical analyses have shown low levels of human leukocyte antigen expression and a lack of immunoreactivity to ABO antigens. Further, the cell line tested negative for several disease-causing agents, including bacteria, mycoplasma, and multiple known viral pathogens. The cell suspension is prepared to a final concentration of 1 × 10⁶ cells/μL at 2 to 8°C in a Cryovial with a volume of 0.5 to 1 mL. Cellular graft preparation upheld appropriate FDA and National Institutes of Health guidelines. For full details regarding NSI-566RSC cell line characteristics, immunogenic profile, preclinical evaluation, or adherence to National Institutes of Health and FDA guidelines, please see the previous full descriptions.

Enrollment Criteria

Enrolled patients satisfied the El Escorial criteria for a diagnosis of ALS, ruling out other major categories of neurological disease. They were deemed safe candidates for surgical intervention. Further, enrolled patients underwent a rigorous institutional review board-reviewed process emphasizing the source of the implanted cells and that participation did not carry an expectation of prolonged survival. A detailed set of enrollment inclusion and exclusion criteria may be found at the following website: http://www.clinicaltrials.gov/ct2/show/NCT01348451. Further details regarding patient selection, recruitment, and the informed consent process may be found in reference. Table 1 provides basic demographic and functional data for patients from groups D and E.

Immunosuppression

Enrolled patients are planned to receive lifelong multitarget immunosuppression, consistent with the current standard of care for solid organ transplantation. Development of a postoperative infection or an attributable immunosuppressant-related toxicity constitute the discontinuation criteria. For full details regarding the immunosuppressant regimen, please see either the interim primary or secondary outcome trial results or a separately published description of the study protocol. An abbreviated description of the regimen is presented. Methylprednisolone 125 mg IV (SoluMedrol, Pfizer, Inc; New York City, New York) was administered at
2 hours before incision and over a subsequent 28-day oral taper, with a dose reduction each week (60 mg, 40 mg, 20 mg, 10 mg). Basiliximab 20 mg IV (Simulect, Novartis, East Hanover, New Jersey) was administered twice, first at the time of dural opening and again on postoperative day 4 (POD4). The 2 remaining agents continue to be administered on an ongoing basis. Tacrolimus 0.1 mg/kg per day (Prograf Fujisawa Healthcare, Inc, Deerfield, Illinois) is given with twice daily dosing, beginning on POD1. Mycophenolate mofetil 500 mg by mouth (Cellcept, Roche Laboratories Inc, Nutley, New Jersey) is first given orally twice per day on POD 1 and is titrated to a final dose of 1 g given twice daily by POD14.

### Patient Positioning and Neuromonitoring

Padded chest rolls and a Mayfield head holder were used for the cervical microinjection approach, as shown in Figure 1A. The patient’s head was placed in mild capital flexion, and tape was placed on the shoulders to improve fluoroscopic visualization during the operative exposure. Figure 1B demonstrates generous hair clipping below the level of the external occipital protuberance to accommodate the rostral microinjection platform posts. Neuromonitoring was used to allow assessment of somatosensory evoked potentials (SSEPs) during microinjection. The trial protocol required termination of injection if SSEP values fall to 50% of baseline without recovery within a 30-minute period. Surgical site preparation included the use of Hibiclens (4% chlorhexidine gluconate) followed by Betadine scrub and paint.

### Surgical Technique and Microinjection

Fluoroscopy was used to obtain positive confirmation of the appropriate levels for both thoracolumbar (T10/11) and cervical (C3-5) exposure. A standard midline posterior approach was used with completion of a T10/11 or C3-5 laminectomy. Secondary to the development of a progressive postoperative kyphosis in trial patient 13 (group D, patient 1), subsequent microinjection patients (16-18, respectively, trial patients 10, 12, and 11) received a cervical laminoplasty. After completion of the bony decompression, the microinjection platform base, shown in Figure 2A-C, was attached to a custom self-retaining retractor system. Figure 2A demonstrates the microinjection platform base. Figure 2B demonstrates incorporation of an

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**TABLE 1. Cervical Microinjection Demographic Data**

<table>
<thead>
<tr>
<th>Group</th>
<th>Trial Patient NO.</th>
<th>Operation No.</th>
<th>Age, Sex</th>
<th>Disease Duration</th>
<th>Surgery Date*</th>
<th>Length of Stay, Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>D1 13</td>
<td>13</td>
<td>50, M</td>
<td>3 y, 1 mo</td>
<td>11/18/2011</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>D2 14*</td>
<td>14</td>
<td>54, F</td>
<td>2 y, 9 mo</td>
<td>2/29/2012</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>D3 15</td>
<td>15</td>
<td>35, F</td>
<td>2 y, 8 mo</td>
<td>4/18/2012</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>E1 10</td>
<td>16</td>
<td>50, M</td>
<td>8 y, 7 mo</td>
<td>6/13/2012</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>E2 12</td>
<td>17</td>
<td>56, M</td>
<td>2 y, 11 mo</td>
<td>7/20/2012</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>E3 11</td>
<td>18</td>
<td>40, M</td>
<td>2 y, 2 mo</td>
<td>8/21/2012</td>
<td>6</td>
</tr>
</tbody>
</table>

*Group E patients had a previous lumbar microinjection surgery date. The date provided represents the cervical microinjection date.

*Patient 14 is documented to have bulbar predominant amyotrophic lateral sclerosis.

*Group E patients previously underwent bilateral thoracolumbar microinjection as participants in group C. The trial patient number reflects the initial numbering from group C.
integrated retractor system. In Figure 2C, the rostral posts, rostral platform base, and rail system were added. At this point, dural opening and tack-up were completed. Images of the microinjector and floating cannula have been previously demonstrated.\textsuperscript{6,10} Five sequential unilateral injections were completed (rostrocaudal spacing 4 mm) with the precalibrated MINI-PD microINJECTOR 8 pump (Tritech Research, Inc, Los Angeles, California). Injection targeting to the ventral horn was anatomically based and entails penetration 1 to 2 mm medial to the dorsal rootlet entry zone, to a depth of 4 to 5 mm. The final depth was based upon preoperative imaging. The anatomic targeting approach was discussed in detail in the interim safety outcomes article\textsuperscript{10} and was based on significant preclinical experience with microinjections in swine. The cannula was introduced into the spinal cord in rigid confirmation. Appropriate depth was indicated when the microinjection needle tip flange was flush with the spinal cord. At this point, the rigid outer cannula was drawn back. This allowed the needle tip and connected Silastic tubing to “float” with ventilation-associated cord excursion and cardioballistic-associated spinal cord pulsation. The injection process was recorded with the operative microscope. This allowed precise postsurgical graft site localization. Reflux was minimized by observance of a 60-s pause following injection completion before cannula withdrawal. An online supplement to the interim safety trial results\textsuperscript{10} demonstrates a video of the microinjection process, and may be found here: http://links.lww.com/NEU/A454. In this article, a new video is provided that highlights the increased degree of spinal cord excursion during cervical microinjection (see Video 1, Supplemental Digital Content 1, http://links.lww.com/NEU/XXXX, which details the cervical microinjection process and cervical cord excursion). This was largely attributable to patient movement during ventilation. A video demonstrating the entire surgical approach and microinjection procedure may be found in reference.\textsuperscript{14} A description of the evolutionary development of the microinjection platform and cannula hardware may be found here.\textsuperscript{6} Dural closure was completed with a running 4-0 Nurolon suture. Fascia was closed with 20 Prolene, subcutaneous layer closed with inverted interrupted 2-0 Vicryl, and skin with running 20 Ethilon.

**Postoperative Outcome Measures**

The primary trial objective has been to assess the safety of cellular delivery to the human spinal cord. This was assessed by collection of adverse events that may be considered as either procedurally related or graft-related. Interim primary outcome measure results may be found here.\textsuperscript{10} The former are attributable to the surgical intervention (eg, postoperative kyphosis, surgical site infection), whereas the latter are attributable to the presence of an indwelling cellular graft within the spinal cord (eg, graft rejection, cell proliferation). Secondary outcome measures include quantitative and semiquantitative measures of ALS progression. Those reported herein include percent predicted vital capacity and revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) scores. Interim secondary outcome results may be found here.\textsuperscript{11} In addition to adverse event and outcome measure collection, postoperative magnetic resonance imaging (MRI) ($t = 1, 6, 12$ months) was obtained to evaluate possible tumorigenesis or immune activation. Finally, histological analysis has been completed on deceased trial participants to evaluate for viable graft. Imaging and histological data will be separately presented.

**RESULTS**

**Patient Demographic Information**

A total of 6 (4 male, 2 female) patients underwent unilateral cervical microinjection. Each received a series of 5 unilateral injections. Patient age ranged from 35 to 56. Half ($n = 3$) of these patients (group E) had undergone previous bilateral lumbar microinjection with a series of 5 injections per side ($n = 10$ total thoracolumbar injections). A summary of patient demographics may be found in Table 1. Postoperative follow-up for groups D and E is ongoing. Trial patient 14 (group D patient 2) was diagnosed with bulbar-predominant ALS. Two patients in this trial are female (group D patients 2, 3; Trial patients 14, 15). All group D and E patients received a minimum of 7 months of follow-up from cervical microinjection.

**Intraoperative Findings**

Table 2 summarizes the intraoperative findings for the patients receiving cervical microinjection. SSEPs and motor evoked
potentials (MEPs) were recorded on all patients. Clinical decisions were not made based on MEP values, because these have not been previously documented in ALS patients. No changes in SSEPs or MEPs were noted throughout any of the cervical microinjection procedures. A total of 30 spinal cord penetrations and injections were completed in these 6 patients. Two episodes of mild venous tract hemorrhage were noted following injection needle removal (6.7%; 2 in 30 injections), with minimal oozing from a cord penetration site. This was well controlled with temporary application of Gelfoam and micropattie placement. No bleeding persisted following Gelfoam and micropattie placement. Neither intraoperative spinal cord swelling when seen under direct visualization nor postoperative morphological cord changes on MRI imaging obtained at 3 and 6 months were observed. The operative time for group E (298, 374, 313 minutes) was longer than observed for group D (234, 269, 252 minutes). This is attributable to laminoplasty hardware placement, cervical wiring, and the additional care required to maintain the C3-5 posterior elements out of the operative field during microinjection in group E patients.

Postoperative Neurological Findings

The length of postoperative inpatient stay ranged from 4 to 6 days. All patients were nonventilated and ambulatory at preoperative baseline by time of discharge. All patients were extubated in the operating room. One patient (group D, patient 2; trial patient 2) required reintubation and respiratory support. This patient, diagnosed with bulbar predominant ALS, was extubated on POD 2. No patients demonstrated evidence of significant bowel or bladder dysfunction. Figure 3 provides perioperative percent vital capacity (Figure 3A, C) and ALSFRS-R (Figure 3B, D). Preoperative baselines are compared with the first measured perioperative outcomes. Aside from the bulbar ALS patient, who is the only patient receiving cervical microinjection to have died to date (t = 6 months postoperatively), no patients demonstrated decrement of the ALSFRS-R or percent vital capacity measures. Further, no patients developed a focal neurological worsening in the perioperative period. Adverse events are summarized in Table 3.

Postoperative Kyphotic Deformity

All trial patients have completed postoperative surveillance imaging up to a minimum time point of 6 months postoperatively. A single patient (group D, patient 1; trial patient 13) has developed a progressive postoperative kyphotic deformity, as shown in Figure 4. Figure 4A demonstrates a preoperative baseline. Figure 4B and C, respectively, demonstrate postoperative months 3 and 6. Figure 4D is a lateral plain radiograph at 6 months. This patient has, to date, elected not to undergo further surgical intervention. Subsequent trial patients (group E, patients 1-3; trial patients 10-12) received a cervical laminoplasty following completion of the microinjection procedure. Figure 5 demonstrates preoperative (Figure 5A) and 3-month postoperative images (Figure 5B,C) in a patient who received a cervical laminoplasty.
Surgical Reintervention

A single group E patient (group E patient 3; trial patient 11) required reoperation for superficial wound dehiscence and infection 2 weeks postoperatively. Of note, this patient had already been on chronic immunosuppression as a previous member of group C. With consultation from the Infectious Disease service, superficial wound irrigation and debridement with operative reclosure was performed. Intraoperative cultures were positive for a polymicrobial infection. The patient received a peripherally inserted central catheter and an outpatient course of intravenous antibiotics. The patient subsequently underwent a second reoperation, wound culture, and hardware removal secondary to the presence of ongoing neck pain. No purulence or evidence of deep infection was noted during the deep wound exploration and hardware removal. Tacrolimus was temporarily discontinued in this patient as a result of temporary acute renal failure that developed after the initiation of intravenous antibiotics.

DISCUSSION

This article presents the perioperative cervical microinjection safety outcomes for an intraspinal microinjection approach designed to deliver a cellular payload to the ventral horn of the ALS spinal cord. When combined with recently published perioperative safety data for delivery to the thoracolumbar spine.
the primary outcome measure findings from this open-label phase I trial support the safety of a targeted direct microinjection approach to the already compromised and vulnerable ALS spinal cord. Specifically, the ALS spinal cord has shown an ability to tolerate up to 15 microinjections (5 unilateral cervical and 10 bilateral thoracolumbar; 150 μL total volume; 1.5 million delivered cells) without evidence of intraoperative (eg, SSEP decline) or postoperative neurological sequelae. Further, preclinical large-animal data have supported the safety of the maximum number of tested injections, 40 cervical microinjections (20/side) (N.M.B., J.R., unpublished data). When the provided intraoperative videos for thoracolumbar (http://links.lww.com/NEU/A454) and cervical microinjection procedures (see Video 1, Supplemental Digital Content 1, http://links.lww.com/NEU/XXXX, which details the cervical microinjection process and cervical cord excursion) were compared, ventilation-associated spinal cord excursion was observed to be significantly more pronounced in the cervical spinal cord.

| Table 3. Perioperative Adverse Events for Microinjection Groups D and E* |
|--------------------------|---------|-----------------|--------------------------|
| Adverse Event Category   | Group No., Patient No. | Severityb | Duration, Days | Comments |
| Operative                | E, 3    | 3               | 45            | Re-Op 1: superficial wound washout |
|                         | D, 2    | 2               | 10            | Re-Op 2: Deep Cx and hardware removal |
| Non-operative            | E, 1    | 2               | 36            |                                      |
|                         | E, 3    | 3               | 64            |                                      |
|                         | D, 1    | 3               | 53            |                                      |
|                         | D, 2    | 2               | 10            |                                      |
|                         | D, 3    | 3               | 53            |                                      |
|                         | E, 3    | 2               | 69            |                                      |
|                         | E, 2    | 2               | 45            |                                      |
|                         | D, 1    | 3               | 88            | Attributable to progressive kyphosis |
|                         | E, 3    | 4               | 30            | Attributable to progressive kyphosis |
|                         | D, 1    | 2               | 88            | Attributable to progressive kyphosis |
|                         | D, 3    | 2               | 43            |                                      |
| Muscle spasm            | E, 3    | 2               | 45            |                                      |
|                         | E, 2    | 2               | 45            |                                      |
| Incisional              | E, 1    | 2               | 36            |                                      |
|                         | E, 3    | 3               | 64            |                                      |
|                         | D, 1    | 3               | 53            |                                      |
|                         | D, 2    | 2               | 10            |                                      |
|                          | D, 3    | 3               | 53            |                                      |
|                          | E, 3    | 2               | 69            |                                      |
|                          | E, 2    | 2               | 45            |                                      |
|                          | D, 1    | 3               | 88            | Attributable to progressive kyphosis |
|                          | D, 2    | 2               | 41            |                                      |
|                          | D, 2    | 2               | 30            |                                      |
|                          | D, 3    | 2               | 53            |                                      |
|                          | D, 1    | 2               | Ongoing       |                                      |
|                          | E, 3    | 1               | 2             |                                      |
|                          | E, 1    | 1               | 2             |                                      |
| Cervical kyphosis       | D, 1    | 2               | Ongoing       |                                      |
| Bowel/bladder           | E, 3    | 1               | 2             |                                      |
| Urinary retention       | D, 3    | 2               | 53            |                                      |
| Constipation            | E, 1    | 1               | 1             |                                      |
| Motor                   | D, 1    | 2               | Ongoing       | Attributable to progressive kyphosis |
| Grind in neck           | D, 2    | 2               | 5             | Required reintubation; Dx bulbar ALS |
| Laryngeal edema         | D, 3    | 2               | 14            |                                      |
| Shoulder pain           | E, 2    | 1               | 4             |                                      |
| Nausea/vomiting         | D, 3    | 2               | 27            |                                      |
| Hiccups                 | E, 2    | 2               | 13            |                                      |
| Popliteal vein thrombosis| E, 2   | 2               | 50            | Subsequent imaging demonstrated recanalization and clot regression |

*ALS: amyotrophic lateral sclerosis; Re-Op: reoperation; Cx: correction; Dx: diagnosis; ADLs: activities of daily living; inc sep: incisional separation; N/V: nausea/vomiting; IVF: ileus; TF: transitory femoral pulse; inc sep: incisional separation; osteomyelitis; possible fascial disruption. |
Incorporation of the floating cannula, capable of accommodating for this movement, appears crucial to the lack of morbidity observed in human and large-animal cervical spinal cord microinjections. Although the presented thoracolumbar and cervical perioperative safety data are encouraging, several issues require further consideration. Broadly, these include (1) management strategies for observed postoperative kyphotic instability, (2) approaches to mitigate perioperative risk (eg, ALS variants, chronic immunosuppression), (3) methods to further tailor immunosuppressant requirements for future trials, and (4) strategies to improve postinjection graft identification.

The rapid development of a progressive postoperative kyphotic deformity in 1 of 6 patients highlights questions both as to the cause as well as to future management considerations. Published literature in the treatment of spondylotic myelopathy supports a high rate (≈20%) of postsurgical kyphosis following multilevel cervical laminectomy in the adult population.15-17 This has been observed to increase with a concomitant preoperative loss of cervical lordosis.15 Biomechanical studies expectedly support a transition of forces from the removed posterior elements to the adjacent facet joints.18 Although operative facet joint compromise and known loss of preoperative cervical lordosis may contribute to progressive deformity, it remains unclear as to whether ALS-associated erector spinae neuromuscular dysfunction may also be contributory. “Dropped head syndrome,” progressive cervical kyphosis, is a recognized clinical sign in ALS (1.3%)19,20 and has been attributed to erector spinae neuromuscular dysfunction. However, these data do not reflect the development of progressive deformity in ALS patients that had previously received a multilevel cervical laminectomy. It may be that performance of a multilevel laminectomy in ALS patients is more destabilizing than in patients without neuromuscular dysfunction. In this setting, a preoperative loss of cervical lordosis and the presence of underlying progressive neuromuscular dysfunction may both serve as risk factors for the development of postoperative kyphosis. We recommend obtaining preoperative static and dynamic imaging (eg, flexion-extension radiographs) to evaluate for any evidence of instability.16 Future cervical microinjection procedures in ALS patients will include careful preservation of the facet joints and consideration of lateral mass instrumented fusion for patients with preoperative evidence of dynamic subluxation or loss of cervical lordosis. A laminectomy or laminoplasty may be sufficient in the absence of dynamic instability and with adequate preoperative cervical lordosis.

Perioperative complications also included 1 patient who required reintubation in the postoperative anesthesia care unit, and 1 patient who required reoperation for superficial wound dehiscence with associated infection. The patient who required perioperative intubation (group D, patient 2; trial patient 14) was the only individual who carried an ALS variant diagnosis (eg, bulbar predominant ALS). All other patients carried a diagnosis of classical ALS. In this trial, the enrollment (inclusion/exclusion)
criteria did not distinguish between presenting forms of ALS. Future studies should consider the baseline dysfunction of the pharyngeal musculature in bulbar ALS to be a risk factor for compromised perioperative airway protection. This could be accommodated with plans to electively leave the patient intubated in the immediate postoperative period. Further, it may be reasonable to exclude this ALS variant from near-term prospective studies, because patients with bulbar ALS are less likely to benefit from direct delivery of a cell-based therapeutic to the spinal cord. The patient who required reoperation for wound dehiscence/infection and subsequently for hardware removal (group E, patient 3; trial patient 11) received previous bilateral thoracolumbar microinjection. As a result, this patient had been on a chronic multiagent immunosuppressant regimen. Future efforts could mitigate this risk by (1) more closely spacing the staged operations, (2) performing the thoracolumbar and cervical operations simultaneously (if both are being performed), or (3) providing an immunosuppressant holiday (reduced or withheld immunosuppressants) during the immediate perioperative healing period.

As a universally fatal neurodegenerative disease, there are no effective therapeutic interventions for ALS. Although the delivery of a cellular graft to achieve neuroprotective and neurorestorative endpoints is an attractive goal, little is known about the immunological environment of the human spinal cord. Therefore, the use of immunosuppression in the maintenance of a viable cellular graft is both an important consideration and holds an uncertain future role. Preclinical studies by Geron, Inc. and our group were unable to demonstrate a T-cell proliferative response to a delivered cellular graft. However, on the basis of the solid organ transplantation literature, we have incorporated a conservative multiagent regimen. Tailoring or withholding of immunosuppressants (immunosuppressant holiday) will require an improved mechanistic understanding of the spinal cord postengraftment immunological microenvironment. Preclinical studies will be necessary to elucidate (1) the role of these agents in maintenance of viable cellular grafts in the spinal cord, as well as (2) the specific immunogenic mechanisms by which the grafts trigger a host response. This will complement data that exist in the solid organ transplantation literature and will ultimately result in the development of more tailored immunosuppressant regimens and cellular grafts with reduced immunogenicity. The terminal nature of ALS, and the fact that some trial patients have been unable to tolerate maintenance on the full

**FIGURE 5.** Technique revision, laminoplasty. Given the observation of induced postsurgical kyphotic deformity in the early cervical microinjection patients, an alteration in surgical technique was introduced for group E patients. During dissection, the posterior tension band was preserved. Next, a bilateral laminectomy was performed but with preservation of the posterior tension band caudally at C5/6. To perform the visualization necessary to perform unilateral injection into the cervical spinal cord, the posterior elements of C3-C5 were placed behind the microinjection platform retractor blade. At the conclusion of microinjection and dural closure, the posterior elements were secured in place with bilateral laminoplasty plates and posterior wiring. The posterior elements of C3 were not included in this construct because the diminutive size of the bony structure precluded wiring. A, a preoperative T2-weighted midsagittal cervical MRI. B, C, three-month postoperative MRI and lateral plain film are shown.
immunosuppressant regimen, indicates that preliminary data for the role of a multitarget immunosuppressant regimen in maintenance of a cellular graft may be provided by postmortem histological evaluation of the spinal cord graft sites. The long-term goal of these studies will be to minimize the number of agents and duration necessary for postgraftment immunosuppression when delivering a cellular graft for the treatment of ALS or alternate indications.

Six patients have died to date while enrolled in this trial. This includes 1 patient who underwent cervical microinjection. The deceased cervical microinjection patient (group D, patient 2; trial patient 14) carried a diagnosis of bulbar ALS. This patient died at 6 months postoperatively. Whereas the patient succumbed to disease progression, respiratory failure, and pneumonia, perioperative functional measures were worsened in this patient. A contribution of either surgery cannot be excluded in this patient with an aggressive ALS variant. In each deceased patient, graft has been identified through a combination of precise graft site localization and the use of advanced detection techniques. Accurate graft site localization was accomplished through the use of video recordings and photographs on the intraoperative microscope. Figure 6A demonstrates a specific “vascular fingerprint” on the dorsum of the spinal cord surface as recorded by the operative microscope. Figure 6B shows a postmortem view of the same tissue, illustrating that the exact site of each cord penetration and graft can be effectively localized with the aid of this vascular fingerprint. Histological graft identification in preclinical studies used species-specific markers (as human cells were delivered into swine) as well as fluorescence in situ hybridization to Y-chromosome specific markers. This was possible because graft cells were male (XY) and recipient animals were female (XX). Although 2 enrolled patients are female, the remaining 13 are male. This has provided a new and ongoing challenge toward the identification of a human cellular graft when delivered to the human spinal cord. A full histological description of graft viability and detection techniques will be separately reported.

CONCLUSION

Even the compromised ALS spinal cord is tolerant to serial targeted microinjections to the cervical and thoracolumbar spinal cord. Demonstration of procedural safety in this vulnerable population may prevent the need to re-create a “risk escalation” paradigm in future trials. This is especially relevant for ALS because the potential for postoperative spinal instability should be considered in preoperative planning. Patients with either bulbar predominant ALS or on chronic immunosuppression may be susceptible to elevated periprocedural risk. Near-term preclinical studies are needed to further develop (1) an ability to refine the role for immunosuppressants, (2) imaging modalities capable of detecting postimplantation graft localization and viability, and (3) improved techniques to detect human allogeneic grafts in a human recipient spinal cord. We have recently received FDA approval to progress into a phase II trial. This will be needed to define a dose-limiting toxicity threshold, a maximum tolerated dose for the ALS spinal cord. Further, this effort will assess the range of modifiable infusion parameters that may be tolerated (eg injection number, sites injected, total dose delivered). Once defined, subsequent multicohort dose-ranging trials designed to assess therapeutic efficacy can be undertaken. The vulnerable nature of the ALS spinal cord may serve as an ideal setting to create a conservative estimate of spinal cord tolerance when generalizing biological therapeutics delivery approaches to alternate indications. Long-term efforts will focus on the validation of a targeted direct microinjection approach for application to the spectrum of spinal cord afflictions.

Disclosures

Neuralstem, Inc, provided financial assistance for microinjection platform construction and is funding the phase I clinical trial that is described herein. Dr Boulis has received an inventor’s fee for the microinjection platform and floating cannula. He is also eligible for royalties associated with future licensing of these technologies. Dr Glass has received research funding from Neuralstem, Inc. Dr Feldman has received support from Novartis Pharmaceuticals for services as a consultant. The other authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices described in this article.


**Acknowledgments**

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Review

Diabetic cardiac autonomic neuropathy: Insights from animal models

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Abstract

Cardiac autonomic neuropathy (CAN) is a relatively common and often devastating complication of diabetes. The major clinical signs are tachycardia, exercise intolerance, and orthostatic hypotension, but the most severe aspects of this complication are high rates of cardiac events and mortality. One of the earliest manifestations of CAN is reduced heart rate variability, and detection of this, along with abnormal results in postural blood pressure testing and/or the Valsalva maneuver, are central to diagnosis of the disease. The treatment options for CAN, beyond glycemic control, are extremely limited and lack evidence of efficacy. The underlying molecular mechanisms are also poorly understood. Thus, CAN is associated with a poor prognosis and there is a compelling need for research to understand, prevent, and reverse CAN.

In this review of the literature we examine the use and usefulness of animal models of CAN in diabetes. Compared to other diabetic complications, the number of animal studies of CAN is very low. The published studies range across a variety of species, methods of inducing diabetes, and timescales examined, leading to high variability in study outcomes. The lack of well-characterized animal models makes it difficult to judge the relevance of these models to the human disease. One major advantage of animal studies is the ability to probe underlying molecular mechanisms, and the limited numbers of mechanistic studies conducted to date are outlined. Thus, while animal models of CAN in diabetes are crucial to better understanding and development of therapies, they are currently under-used.

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Clinical Aspects of CAN in Diabetes

Autonomic innervation is one of the primary control mechanisms regulating heart rate and overall cardiac output (Pop-Busui, 2010). In CAN, these autonomic nerve fibers in the heart are damaged (Maser et al., 2003), resulting in a disruption of cardiac control (Spallone et al., 2011). CAN is detected in approximately 20% of diabetic individuals, increasing to 35%–65% in older patients with prolonged duration of diabetes (Spallone et al., 2011).

Clinically, CAN manifests itself in resting tachycardia, exercise intolerance, orthostatic hypotension, and reduced heart rate variability (HRV) (Vinik et al., 2003). A major meta-analysis showed a significantly increased mortality rate in diabetic patients with CAN compared to those without (Maser et al., 2003). There is also evidence for a link between CAN and cardiovascular mortality (Astrup et al., 2006; Spallone et al., 2011). Abnormalities in HRV and QT indices (measured by electrocardiogram (ECG)) serve as strong predictors of mortality independent of any other risk factors a diabetic patient may have (Lykke et al., 2008; Ziegler et al., 2008). However, some patients with CAN appear asymptomatic, with many of them remaining asymptomatic until CAN is in its most severe stages (Abd El Dayem et al., 2011; Low et al., 2004). CAN is also thought to contribute to high
mortality risk through its relationship to silent myocardial ischemia (SMI). SMI is present in 20% of those with CAN and only present in 10% of those diabetic patients without CAN (Spallone et al., 2011). Patients with CAN have a high risk of major cardiac events, and those with both CAN and SMI have an even higher risk (Valensi et al., 2001).

Glycemic control, duration of diabetes, age, and hypertension are key factors that contribute to the development of CAN (Stella et al., 2000; Witte et al., 2005). Although some patients show no signs of CAN, there are indicators that put individuals at higher risk for CAN onset and progression. In the case of type 1 diabetes, poor glycemic control increases risk (Spallone et al., 2011). The same is true for type II diabetes, but risk is also affected by conditions such as dyslipidemia and obesity (Spallone et al., 2011). Studies have also indicated that smoking, high levels of HDL cholesterol, cardiovascular disease, waist circumference, and use of high blood pressure medications can affect one's risk for developing CAN (Spallone et al., 2011).

Diagnosing CAN is not yet a definitive process, but in general CAN progression can be characterized through various tests. These include HRV tests, postural blood pressure testing, and Valsalva maneuvers, which require the patients to exhale while maintaining pressure (Valensi et al., 2001), testing a complex set of reflexes involving both sympathetic and parasympathetic pathways to the heart and baroreceptors in the chest and lungs (Abd El Dayem et al., 2011; Vinik et al., 2003). It has been determined that one abnormal cardio-vagal test is enough to identify the possible presence of CAN or early CAN, but two abnormal cardio-vagal tests are required to confirm CAN in a patient (Spallone et al., 2011). Once CAN is confirmed, its progression into severe and advanced stages is determined by the presence and severity of orthostatic hypotension as well as more abnormal heart rate test results (Spallone et al., 2011). A reduction in HRV is one of the earliest signs of CAN, and is recommended to be evaluated in all patients with diabetes (Vinik et al., 2003). Beginning HRV monitoring immediately upon diagnosis of diabetes provides a baseline so that repeat tests can be taken at one year intervals (Vinik et al., 2003), serving as the earliest indicator and predictor of CAN onset (Abd El Dayem et al., 2011).

Once CAN is identified, there is no immediate treatment other than to monitor progression and to manage complications of CAN. However, several approaches to combat the effects of CAN are currently being studied. Certain angiotensin converting enzyme inhibitors (ACE inhibitors) have been shown to have beneficial effects on preventing cardiac autonomic complications of diabetes (Kontopoulos et al., 1997), but there are contradictory results (Malik et al., 1998) and more follow-up studies are needed (Vinik et al., 2003). Similar results for improving the effects of CAN have been found with beta-blockers (Ebbehoj et al., 2002; Vinik et al., 2003). Moreover, alpha-lipoic acid (ALA) taken orally was shown to improve CAN slightly by improving HRV (Ziegler et al., 1997). Further studies were performed to show the same type of effects of ALA from the use of vitamin E and C-peptide (Manzella et al., 2001; Spallone et al., 2011). Nevertheless, many more studies need to be done in order to confirm positive effects of these therapeutic agents. Despite the need to discover effective drugs and therapeutic methods to improve the prognosis of CAN and its complications, most of the current success comes from tight metabolic and glycemic control, as well as being aware of and managing potential symptoms and risk factors (Spallone et al., 2011; Vinik et al., 2003).

Studies Using Animal Models of CAN in Diabetes

Animal studies related to diabetic autonomic neuropathy in the heart to date largely fall into one of three categories: 1) visualization of the anatomy of the cardiac autonomic nerves by immunohistochemistry (IHC) or electron microscopy (EM); 2) functional properties of the nerves (norepinephrine (NE) concentration in the tissue, NE release, or uptake of a radiolabeled NE mimetic); or 3) functional effects on the heart (changes in heart rate and HRV). Cardiac contractility, and subsequently, cardiac output, are also likely to be affected by changes in the function of autonomic nerves during diabetes. However, it is not easy to differentiate between diabetes-induced changes in cardiac autonomic nerves and the well-documented effects of diabetic cardiomyopathy, in which the major effect is directly on the cardiomyocytes themselves. Diabetic cardiomyopathy has been extensively reviewed elsewhere (Boudina and Abel, 2010; Poornima et al., 2006), and studies examining diabetes-induced changes in contractility will not be discussed here unless specifically targeted at examining the role of the neuropathy.

Characterization of CAN in Animal Models

One of the aims of characterizing CAN in animal models is to better understand the disease pathology. In order to do this it is crucial to be able to determine in what ways the animal models are similar to the human disease, and in what ways they are different. However, as explained above, the diagnosis of CAN in humans is not by a single definitive test but by a combination of several suggestive tests. Many of these human tests require communication with and cooperation from the patient, for example remaining still during HRV testing, undergoing tilt-table testing for orthostatic hypotension, or using specialized equipment; following specific instructions during the Valsalva maneuver. These are clearly not possible in animal studies, making it difficult to assess the relevance of any characterization in animals to human disease. However, there are many characterization studies that can be done in animals that are impossible in humans because they require terminal procedures. For example, characterizing the anatomy of the damaged cardiac nerves by IHC or EM requires removal and processing of the heart. While this may be possible in post-mortem studies in humans, animal studies allow for detailed characterization under strictly controlled conditions. Another major advantage to animal studies is that the characteristics of CAN may be determined over the timescale of disease progression.

Regardless of whether or not characterization of CAN in animals will reveal anything new about the human disease, a second, extremely important reason to characterize diabetic CAN in these animals is to determine if and how they may be useful as models of the disease state, since having a variety of even partially-relevant animal models of CAN allows us to probe the mechanisms underlying the disease through intervention studies (for example, using pharmaceutical tools or genetic manipulation). These studies are where the real advantages of animal models become apparent, but they are only useful if the models have been well-characterized and shown to be relevant to human disease.

Compared to other diabetic complications, such as nephropathy, retinopathy, and even peripheral sensory neuropathy, the numbers of animal studies examining diabetic CAN are very low. Those studies that do exist span a wide range of species, methods of induction of diabetes, timescales, and methods of assessment. A summary of the relevant publications is shown in Table 1.

A handful of studies have quantified innervation of hearts using IHC. A consistent finding has been a reduction in ventricular sympathetic staining: in db/db mice at 6 months of age (Tessari et al., 1988); in STZ-treated mice after 6 months of diabetes (Kellogg et al., 2009); in BioBreeding/Ottawa Karlsburg (BB/KO) rats after 6 months of diabetes (Schneider et al., 2010); and in alloxan-treated rabbits 20 weeks post-treatment (Wang et al., 2012). In parallel to the IHC, Wang et al. also showed a reduction in tyrosine hydroxylase (TH) expression by quantitative PCR. Mabe and Hoover (2011) looked at the atrial innervation using markers of both sympathetic (TH) and parasympathetic (choline acetyltransferase, ChAT, and vesicular acetylcholine transporter, VACHT) nerves. They found an increase in VACHT-positive neurons in the sinoatrial (SA) node of STZ-treated mice after 8 and 16 weeks of diabetes, but no changes in innervation of the atria. In contrast to this finding, Yang and Chon (2011) showed reduced synaptophysin-positive neurons (a marker of general nerves) in the SA node of Akita mice at 4 months of age. However, the differences in strain of mouse, cause of diabetes, and type of staining, not to mention the low number of studies,
Table 1
Animal studies of cardiac autonomic neuropathy in diabetes.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Species</th>
<th>Method of diabetes induction</th>
<th>Diabetes Type</th>
<th>Intervention Method of neuropathy assessment</th>
<th>Time of neuropathy assessment</th>
<th>Outcome</th>
<th>Effect of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Tomlinson and Yusof, 1983]</td>
<td>1983</td>
<td>Rat</td>
<td>Alloxan</td>
<td>Type 1</td>
<td>None</td>
<td>EM</td>
<td>7-8 months</td>
<td>Abnormal noradrenergic nerves in the RA</td>
</tr>
<tr>
<td>(Tessari et al., 1988)</td>
<td>1988</td>
<td>Mouse</td>
<td>db/db</td>
<td>Type 2</td>
<td>Ganglioside mixture</td>
<td>IHC (catecholamines) NE concentration (HPLC) HR changes in isolated atria after transmural stimulation</td>
<td>6 months of age</td>
<td>↓ sympathetic nerve density ↓ NE content ↓ tachycardia induced by transmural stimulation</td>
</tr>
<tr>
<td>(Kofo-Abayomi and Lucas, 1988)</td>
<td>1988</td>
<td>Rat</td>
<td>STZ</td>
<td>Type 1</td>
<td>None</td>
<td>HR in isolated atria Intramural nerve stimulation in isolated atria in the presence of propranolol (“vagal stimulation”) or atropine (“sympathetic stimulation”)</td>
<td>6 weeks after STZ</td>
<td>↓ HR ↑ sensitivity to “vagal stimulation” ↔ sensitivity to “sympathetic stimulation”</td>
</tr>
<tr>
<td>(Schmid et al., 1999)</td>
<td>1999</td>
<td>Rat</td>
<td>STZ</td>
<td>Type 1</td>
<td>None</td>
<td>Radiolabelled tracer ([11C]HED) NE concentration</td>
<td>6 and 9 months diabetes</td>
<td>↓ tracer retention in proximal myocardium ↓ tracer retention in distal myocardium ↔ NE concentration</td>
</tr>
<tr>
<td>(Mesangeau et al., 2000)</td>
<td>2000</td>
<td>Pig</td>
<td>STZ</td>
<td>Type 1</td>
<td>None</td>
<td>HR (BP telemetry) HRV (RRSD and respiratory component of frequency variation)</td>
<td>3 and 6 months of diabetes</td>
<td>↑ HR (at 6 months) ↓ HRV (at 3 and 6 months)</td>
</tr>
<tr>
<td>(Semenuk et al., 2002)</td>
<td>2002</td>
<td>Mouse</td>
<td>db/db</td>
<td>Type 2</td>
<td>h-GLUT4 overexpression</td>
<td>HR (measured in conscious mice by Echo)</td>
<td>6, 12 weeks of age</td>
<td>↓ HR</td>
</tr>
<tr>
<td>(Lo Guidice et al., 2002)</td>
<td>2002</td>
<td>Rat</td>
<td>STZ</td>
<td>Type 1</td>
<td>Acetyl-L-carnitine</td>
<td>HR and HRV (implanted ECG)</td>
<td>6 weeks diabetes</td>
<td>↓ HR ↓ HRV</td>
</tr>
<tr>
<td>(Shotton et al., 2003)</td>
<td>2003</td>
<td>Rat</td>
<td>STZ</td>
<td>Type 1</td>
<td>Antioxidant α-lipoic acid (LA) and γ-linolenic acid (EPO)</td>
<td>NE concentration in RA</td>
<td>8 weeks of diabetes</td>
<td>↓ NE concentration</td>
</tr>
<tr>
<td>(Howarth et al., 2005)</td>
<td>2005</td>
<td>Rat</td>
<td>STZ</td>
<td>Type 1</td>
<td>None</td>
<td>HR (EEG telemetry) HRV (SDNN and LF/HF power)</td>
<td>4, 8, 12, 16, and 22 weeks post-STZ</td>
<td>↓ HR ↓ HRV ↓ LF/HF power ratio</td>
</tr>
<tr>
<td>(Gross et al., 2008)</td>
<td>2008</td>
<td>Mouse</td>
<td>NOD</td>
<td>Type 1</td>
<td>None</td>
<td>HR (BP telemetry)</td>
<td>4 weeks of diabetes</td>
<td>↓ HR</td>
</tr>
<tr>
<td>(Kusmic et al., 2008)</td>
<td>2008</td>
<td>Mouse</td>
<td>STZ</td>
<td>Type 1</td>
<td>None</td>
<td>Radiolabelled tracer ([123I]MIBG)</td>
<td>7 months</td>
<td>Faster cardiac washout of tracer</td>
</tr>
<tr>
<td>(Howarth et al., 2008)</td>
<td>2008</td>
<td>Rat</td>
<td>Goto-Kakizaki</td>
<td>Type 2</td>
<td>None</td>
<td>HR (EEG telemetry) HRV (SDNN)</td>
<td>2, 7, and 15 months of age</td>
<td>↓ HR ↓ HRV (at 2 and 7 months)</td>
</tr>
<tr>
<td>(Goncalves et al., 2009)</td>
<td>2009</td>
<td>Mouse</td>
<td>db/db</td>
<td>Type 2</td>
<td>HR (BP telemetry) HRV (LF HR oscillation (ms^2)) Sympathetic tone (HR response to metoprolol) Parasympathetic tone (HR response to atropine)</td>
<td>12 weeks of age</td>
<td>↑ HR ↓ HRV ↓ sympathetic tone ↓ parasympathetic tone</td>
<td></td>
</tr>
<tr>
<td>(Kellogg et al., 2009)</td>
<td>2009</td>
<td>Mouse</td>
<td>STZ</td>
<td>Type 1</td>
<td>None</td>
<td>HR (BP telemetry) HRV (BP telemetry)</td>
<td>6 months of diabetes</td>
<td>↓ sympathetic nerve density ↑ HR</td>
</tr>
<tr>
<td>(Senador et al., 2009)</td>
<td>2009</td>
<td>Mouse</td>
<td>db/db</td>
<td>Type 2</td>
<td>Cyclooxgenase-2 gene inactivation (COX-2-/- mice)</td>
<td>IHC in LV (TH) HR (Echo)</td>
<td>8, 14 weeks of age</td>
<td>↓ HR ↔ HRV</td>
</tr>
<tr>
<td>(Senador et al., 2009)</td>
<td>2009</td>
<td>Mouse</td>
<td>db/db</td>
<td>Type 2</td>
<td>Cyclooxgenase-2 gene inactivation (COX-2-/- mice)</td>
<td>HR and HRV (BP telemetry)</td>
<td>8, 14 weeks of age</td>
<td>↓ HR ↔ HRV</td>
</tr>
</tbody>
</table>
make it difficult to draw conclusions from these studies about the effect of diabetes on cardiac innervation.

Two studies examined nerve phenotypes at high magnification using electron microscopy. The first (Tomlinson and Yusof, 1983) showed abnormalities in the noradrenergic nerves of right atria from alloxan-treated rats after 7–8 months of diabetes. The second (Schneider et al., 2010) showed slight to moderate ultrastructural alterations of autonomic nerve fibers and related Schwann cells in BB/OK rats after 6 months of diabetes.

Three studies in STZ-induced diabetic rats examined NE concentrations within the heart. Schmid et al. (1999) showed a decrease in NE concentration in the apex of the heart after 9 months of diabetes. Shotton et al. (2003) examined the right atria and showed a decrease in NE concentration after just 8 weeks of diabetes. In contrast, Sviglerova et al. (2011) showed a slight increase in NE concentrations in the atria after 1 month of diabetes, no difference between control and diabetic groups at 2 and 4 months, and another increase after 22 months. Since these studies were all in the same animal model (STZ rats), the differences cannot be explained by inherent model differences. However, the low number of studies and the different time-points used make it impossible to determine the usual time-course of NE concentration changes in this rat model. In the alloxan-treated rabbit model, Wang et al. (2012) found reduced NE content in the LV after 20 weeks of diabetes.

One method of assessing sympathetic nerve function is by measuring the uptake and/or washout of radiolabeled catecholamines/catecholamine analogs. Kusmic et al. (2008) used this approach to show faster washout of tracer in STZ-induced diabetic mice after 7 months of diabetes. They proposed that this was due to impaired NE reuptake into neurons via the NE transporter. Schmid et al. (1999) showed a reduction in tracer retention in the distal but not the proximal ventricular myocardium of their STZ-induced diabetic rats at 6 months. By 9 months both the distal and proximal regions were impaired.

A relatively large number of the studies reviewed here examined one or both of these parameters, using a variety of methods to capture the heart rate. The gold-standard method is implantable ECG telemetry, because it provides ECG recordings in conscious, unsedated animals in a stress-free environment (often their home cages). Just...
four studies, to our knowledge, have used ECG telemetry to study heart rate and HRV in diabetic animal models. They used a range of diabetic models: STZ-induced diabetic rats after 6 weeks of diabetes (Lo Giudice et al., 2002); STZ rats after 4–22 weeks (Howarth et al., 2005); Goto–Kakizaki rats at 2, 7, and 15 months of age (Howarth et al., 2008); and Akita mice at 4 months of age (Yang and Chon, 2011). The results were consistent across the studies, with all four showing reduced heart rate and decreased HRV in the diabetic models. Two other studies used an alternative method to record ECG in conscious mice, employing a device in which the animals stand on a small platform with built-in electrodes, so that the ECG is recorded when 3 limbs are in contact with the electrodes. One (Mabe and Hoover, 2011) showed reduced heart rate and reduced high-frequency HRV in mice with STZ-induced diabetes from 2 to 14 weeks post-injection, while the other (Sviglerova et al., 2011) showed resting bradycardia in STZ-induced diabetic rats after 1, 2, 4, and 22 months. One additional study (VanHoose et al., 2010) measured heart rate using ECG recording, this time in anesthetized Zucker diabetic fatty (ZDF) rats. In contrast to the previously-mentioned ECG studies, they showed an increased heart rate in the diabetic rats, measured at 12 and 19 weeks old. The discrepancy between studies may be due to the presence of anesthetic or the different causes of diabetes in the models.

It is also possible to measure heart rate, and subsequently heart rate variability, from recordings of blood pressure, though this does not allow for differentiation between SA node-driven beats and other arrhythmic events. Several studies have utilized implantable telemetry devices to measure blood pressure and from this assess heart rate in diabetic animals. Two showed decreased heart rate in diabetic models (in NOD mice after 4 weeks of diabetes (Gross et al., 2008), and in db/db mice at 8 and 14 weeks of age (Senador et al., 2009)); and two showed an increase in heart rate (in db/db mice at 12 weeks of age (Goncalves et al., 2009) and in STZ-induced diabetic pigs after 6 months (Mesangeau et al., 2000)). As well as showing increased heart rate, both Goncalves et al. and Mesangeau et al. showed decreased HRV, while Senador et al. showed no difference in HRV between their diabetic and control mice. A study measuring blood pressure in anesthetized STZ mice after 30 days of diabetes also showed a decrease in heart rate in the diabetic animals (Lin et al., 2010).

Another method that allows heart rate to be calculated is echocardiography. Again, there is variability across studies using this technique: Semeniuk et al. (2002) showed decreased heart rate in db/db mice at 6 and 12 weeks of age, while Kellogg et al. (2009) showed an increased heart rate in STZ mice after 6 months of diabetes.

The variation between studies in terms of effects of diabetes on heart rate is difficult to explain. Ten of the studies discussed (including those measuring ECG, blood pressure, and echocardiogram) report a decrease in heart rate, whereas 4 report an increase. While there are many differences between the studies in terms of species, methods, and time-scales, there is no one factor that could explain this split in outcomes. It may, therefore, be influenced by a complex interaction of different factors. Of note is the fact that the majority of the animal studies showed reduced heart rate in diabetes, while the clinical phenotype in humans is an increased, tachycardic heart rate (Pop-Busui, 2012). This may be explained by the fact that in humans the heart rate is under tight control by the parasympathetic system, so any dysfunction in these nerves would lead to an increased heart rate. In small animals, on the other hand, the heart rate is thought to be predominantly under sympathetic control (Jansen and Smits, 2002). It has also been postulated that the animal housing temperature may influence the autonomic nervous system (Karp, 2012) (with colder conditions leading to higher sympathetic drive), so this is another variable that may influence the outcome of the animal studies. Housing temperature is not usually stated in publications, but perhaps this would be a useful addition to methods sections.

Two of the previously-mentioned studies measuring heart rate by ECG telemetry also examined autonomic tone using autonomic-blocking drugs. Sviglerova et al. (2011) measured the heart rate response to β-adrenergic receptor blockade (metipranolol), following administration of atropine (a muscarinic receptor antagonist), as a measure of sympathetic tone. They showed an almost completely abolished heart rate response in the diabetic rats at 1, 2, and 4 months, but restoration to control levels after 22 months (when there was partial spontaneous recovery from diabetes). In contrast, Mabe and Hoover (2011) showed no difference between diabetes and control in heart rate response to atropine or atenolol. Mabe and Hoover conducted an additional experiment in which the vagus nerve was stimulated directly in anesthetized mice with limb-lead ECG recording. Here they showed a significantly suppressed negative chronotropic response to vagal stimulation in diabetic mice at 16 (but not 8) weeks. Goncalves et al. (2009) also studied autonomic tone by measuring heart rate responses to metoprolol (a β-adrenergic receptor antagonist) and atropine. They showed an increased autonomic and decreased parasympathetic tone in 12-week-old db/db mice.

Kofo-Abayomi and Lucas (1988) studied autonomic nerve function in isolated atria. They first showed that spontaneous atrial beating rate was lower in STZ diabetic atria (after 6 weeks of diabetes) than in controls. They then paced at a constant rate and, measuring contractile force, showed that diabetic atria were more sensitive to “vagal stimulation” (mimicked by intramural nerve stimulation in the presence of propranolol). This fits with their hypothesis that there is increased vagal activity at this relatively early stage of diabetes. There was no difference between the groups upon intramural nerve stimulation in the presence of atropine (“sympathetic stimulation”). Tessari et al. (1988) conducted a similar experiment studying heart rate changes in isolated atria after transmural stimulation in the presence of atropine. However, they showed that 6 month old db/db mice had a reduced tachycardic response to the stimulation. The difference in outcome between the two studies may be explained by the difference in time points (6 months vs. 6 weeks), species (mouse vs. rat) and/or method of inducing diabetes (db/db type 2 model vs. STZ type 1 model).

One concern about the characterization of these models is the lack of multi-time-point studies required to study the progression of the disease. Of the 23 studies reviewed here, only 9 looked at multiple time-points in diabetes, with just 4 having more than 2 time-points in the study.

Overall, the most striking aspects of the publications characterizing CAN in diabetic animal models are the very low number of total publications and the variability in the methods used, timescales examined and, crucially, the outcomes.

**Investigating Mechanisms Underlying CAN in Diabetes**

As mentioned above, using intervention studies to investigate mechanisms underlying disease is where animal studies have a real advantage over human studies. Several of the studies mentioned above, in addition to characterizing changes occurring during diabetes, also investigated the effects of an intervention (i.e. a drug or a genetic modification). By blocking or enhancing a specific molecular pathway, these studies can reveal if that pathway is important in disease progression.

As part of a study examining the effectiveness of the antioxidant α-lipoic acid and the free fatty acid γ-linolenic acid (found in evening primrose oil) in preventing autonomic neuropathy in different organs in STZ-diabetic rats, Shotton et al. (2003) examined NE concentrations in the right atrium. They found that α-lipoic acid prevented the diabetes-induced reduction in NE, but that γ-linolenic acid failed to prevent it. This finding supports a role for oxidative stress in the mechanism underlying CAN.

Matsuki et al. (2010) also studied the effects of reducing cardiac oxidative stress, in this case by treatment with fluvastatin, on sympathetic neuropathy. They showed that the diabetes-induced decrease...
in radiolabeled tracer uptake in STZ-treated rats was attenuated by fluvastatin treatment.

A third study (Schneider et al., 2010) investigated the role of oxidative stress in diabetic CAN. They did this by studying the effects of Ginkgo biloba extract (EGB761), a radical scavenger, on CAN in BB/OK rats after 6 months of diabetes. EGB761 prevented the diabetes-induced ultrastructural changes observed by EM in autonomic nerves, as well as the reduction in LV nerve staining, but had no effect on the reduction in 123I-MIBG tracer uptake into the heart. Thus, the results of all three of these studies point towards a link between cellular oxidative stress and CAN in diabetes, which is similar to that previously shown for other diabetic complications (Vincent et al., 2004).

Kellogg et al. (2009) investigated the role of glucose-mediated upregulation of the cyclooxygenase pathway (which produces prostaglandins and downstream inflammatory signals) in diabetic CAN. They showed that knockout of the COX-2 gene in STZ-treated mice completely prevented the diabetes-induced reduction in left ventricular sympathetic nerve density and increase in heart rate. The authors provide evidence supporting the hypothesis that this may be due to the reduction of oxidative stress, inflammation, and fibrosis in the myocardium.

Lo Giudice et al. (2002) examined the effects of acetyl-L-carnitine (ALC) on autonomic neuropathy in STZ-diabetic rats. ALC helps to facilitate transport of long-chain fatty acids across the inner mitochondrial membrane for β-oxidation (Williamson and Arrigoni-Martelli, 1992), and there is evidence that ALC treatment is beneficial in peripheral neuropathies (Ito et al., 1994; Stevens et al., 1996). ALC partly reduced the diabetes-induced bradycardia and reduction in HRV in the rats.

Tessari et al. (1988) looked for a protective effect of a bovine brain ganglioside mixture on cardiac autonomic nerve function. Previous reports had shown a protective effect of gangliosides on peripheral sensorimotor neuropathies (Norido et al., 1982, 1984). This study showed that ganglioside treatment restored cardiac NE content to control levels and protected against diabetes-induced impairment of tachycardic response to sympathetic nerve stimulation in isolated atria.

Importantly, there have also been mechanistic studies that have shown negative results (i.e., the molecular mechanism under investigation was not shown to be linked to diabetes-induced CAN). Kofo-Abayomi and Lucas (1988) studied treatment with myoinositol (in the drinking water) on autonomic function in the hearts of their 6-week STZ diabetic rats. The hypothesis was that myoinositol depletion in the diabetic nerves reduces Na+/K+ ATPase activity and subsequently depresses membrane transport and impulse transmission. Myoinositol treatment increased myoinositol concentration in both sciatic nerve and atrial tissue, but did not reverse the diabetes-induced increase in sensitivity to vagal stimulation in isolated atria.

Senador et al. (2009) investigated whether the angiotensin II receptor antagonist losartan could reverse the autonomic changes, as well as hypertension, seen in db/db mice. Using blood pressure telemetry they showed that the reduction in heart rate seen in db/db mice at 8 and 14 weeks of age was not reversed by losartan treatment.

Semeniuk et al. (2002) used a mouse overexpressing the human transgene for the insulin-regulatable glucose transporter (hGLUT4) crossed with the db/db mouse to study the role of GLUT4 in diabetic cardiomyopathy. In diabetic db/db mice there is altered cardiac metabolism, with increased fatty acid oxidation and reduced glucose utilization compared to controls (Belke et al., 2000). The hypothesis being tested was that by overexpressing hGLUT4, and restoring cardiac metabolism in the db/db GLUT4 mice to normal, the hearts would be protected against diabetic cardiomyopathy. Echocardiography was used to assess cardiac contractile function, but heart rate was also measured. The results showed that the contractility was improved in db/db GLUT4 mice compared to db/db, but that the reduction in heart rate was not reversed. This indicates that overexpressing GLUT4 can at least partially rescue the diabetic cardiomyopathy, but that it has no effect on the diabetic autonomic neuropathy that is likely to underlie the reduction in heart rate.

While the studies outlined above provide insights into aspects of the mechanisms underlying CAN, there are very few of them, and the research is far from comprehensive. Thus, although it seems to be widely assumed that the mechanisms underlying CAN are the same as those underlying peripheral sensory neuropathy, the studies that would provide the evidence for this have not been conducted.

In addition to revealing mechanisms of disease, animal models can be useful in pre-clinical trials to determine efficacy of potential therapeutics. Again, this requires good, well-characterized animal models and a thorough understanding of how the models are different to the human situation. We feel that the current lack of animal studies is a major issue in the field, particularly since new treatments can and should be developed in pre-clinical animal studies before being tested in patients. More animal research in this area would greatly increase understanding of the mechanisms underlying CAN and allow desperately-needed better treatments to be developed.

Conclusions

We have outlined a range of studies utilizing animal models to examine different aspects of diabetic CAN and its underlying mechanisms. From the currently available literature, we conclude that the best animal model for the study of mechanisms underlying CAN in type 1 diabetes is the STZ-treated rat for drug studies and the STZ-treated mouse for genetic manipulation studies. For type-2 diabetes, we conclude that the db/db mouse model is the best. All three of these models have consistently shown reduction in autonomic nerve function at the level of heart rate control. They have also shown relatively consistent damage to cardiac neurons by IHC, reduction in norepinephrine concentration, and/or disruption of radiolabeled tracer transport. However, we feel that the field as a whole would benefit from better characterization of animal models of diabetic CAN. As it stands currently, the low numbers of studies and the variability between studies make it very difficult to assess the normal characteristics and progression of the disease and without this it is very difficult to study the underlying disease mechanisms. Having a range of well-characterized models would allow researchers to pick the most appropriate model for a specific question. In addition, demonstration of a key mechanism in a number of different animal models provides much stronger evidence than if the mechanism is only shown to be relevant in one specific animal model. Again, this requires a range of well-characterized models. In conclusion, CAN is a serious and common complication of diabetes that has been under-studied. Further work in animals, initially characterizing the models and then studying molecular mechanisms, will be invaluable in advancing the field and identifying new therapeutic targets.

Literature Search Terms

We searched PubMed for articles on CAN that utilized diabetic animal models using the following search terms: “Cardiac Autonomic Neuropathy Diabetes” and “Mouse”, “Rat”, “Rabbit”, “Dog”, “Cat” or “Pig”.

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Decreased glycolytic and tricarboxylic acid cycle intermediates coincide with peripheral nervous system oxidative stress in a murine model of type 2 diabetes

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Abstract

Diabetic neuropathy (DN) is the most common complication of diabetes and is characterized by distal-to-proximal loss of peripheral nerve axons. The idea of tissue-specific pathological alterations in energy metabolism in diabetic complications-prone tissues is emerging. Altered nerve metabolism in type 1 diabetes models is observed; however, therapeutic strategies based on these models offer limited efficacy to type 2 diabetic patients with DN. Therefore, understanding how peripheral nerves metabolically adapt to the unique type 2 diabetic environment is critical to develop disease-modifying treatments. In the current study, we utilized targeted liquid chromatography–tandem mass spectrometry (LC/MS/MS) to characterize the glycolytic and tricarboxylic acid (TCA) cycle metabolomes in sural nerve, sciatic nerve, and dorsal root ganglia (DRG) from male type 2 diabetic mice (BKS.Cg-m+/-Leprdb; db/db) and controls (db/+). We report depletion of glycolytic intermediates in diabetic sural nerve and sciatic nerve (glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate (sural nerve only), 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, and lactate), with no significant changes in DRG. Citrate and isocitrate TCA cycle intermediates were decreased in sural nerve, sciatic nerve, and DRG from diabetic mice. Utilizing LC/electrospray ionization/MS/MS and HPLC methods, we also observed increased protein and lipid oxidation (nitrotyrosine; hydroxyoctadecadienoic acids) in db/db tissue, with a proximal-to-distal increase in oxidative stress, with associated decreased aconitase enzyme activity. We propose a preliminary model, whereby the greater change in metabolomic profile, increase in oxidative stress, and decrease in TCA cycle enzyme activity may cause distal peripheral nerves to rely on truncated TCA cycle metabolism in the type 2 diabetes environment.

Key Words

- diabetes
- neuropathy
- metabolomics
- sural nerve
- sciatic nerve
- dorsal root ganglia

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Introduction

According to statistics published by the Centers for Disease Control (2011), ~8% of the US population has diabetes mellitus, with 1.9 million new cases diagnosed in 2010. Although both type 1 diabetes and type 2 diabetes are characterized by impaired insulin signaling and hyperglycemia, the two disease forms are distinct. Type 2 diabetes, accounting for 95% of all cases (CDCP 2011), is a metabolic disease characterized by high pancreatic insulin production and insulin resistance in muscle, fat, and liver. The remaining 5% of individuals have type 1 diabetes (CDCP 2011), which is characterized by autoimmune destruction of the pancreas and loss of insulin production.

Diabetic neuropathy (DN) is the most common complication of diabetes and over time affects ~60% of all patients with the disease (Vincent & Feldman 2004). DN is characterized by progressive, length-dependent loss of peripheral nerve axons in a stocking and glove (distal-to-proximal) pattern (Said 2007), resulting in pain, decreased sensation, and eventually complete loss of sensation. In the USA, DN is the leading cause of diabetes-related hospital admissions and non-traumatic amputations (Edwards et al. 2008, Feldman 2008). Despite the morbidity associated with DN, there is currently no treatment available to patients suffering from DN other than glycemic control, largely due to our incomplete understanding of disease mechanisms. In order to gain insight into DN pathogenesis and develop disease-modifying treatments, we have recently started to investigate the role of aberrant energy metabolism in the peripheral nervous system. We hypothesize that DN arises from tissue-specific metabolic reprogramming, resulting in alterations in fuel utilization that lead to dysfunction of the tissue.

Glucose is the major substrate for peripheral nerves, although they do not rely on insulin for its uptake (Greene & Winegrad 1979). In rodent models of type 1 diabetes, peripheral nerve glucose content is elevated (Thurston et al. 1995, Kishi et al. 1999, Obrosova et al. 2005b) and oxidative stress is increased within the sciatic nerve and the dorsal root ganglia (DRG) containing the sensory neuron cell bodies (Vincent et al. 2004). It is also established that all mechanisms known to be contributory to the onset and progression of DN are in some way related to oxidative stress (Vincent et al. 2011). Based on the findings primarily in cultured endothelial cells, oxidative stress was thought to be related to hyperglycemia-induced overproduction of superoxide $\left(\text{O}_2^{-}\right)$ by the mitochondrial electron transport chain (Brownlee 2001). Thus, it was proposed that increased glycolytic flux leads to increased hyperglycemia-derived electron donors from the tricarboxylic acid (TCA) cycle (FADH$_2$ and NADH), which generates a high mitochondrial membrane potential, facilitating the reduction of $\text{O}_2$ to $\text{O}_2^{^-}$ (Brownlee 2001, Tomlinson & Gardiner 2008). Indeed, a study by Thurston et al. (1995) in the mid 1990s reported an increase in nerve glycolytic intermediates in long-term type 1 diabetes in rats. Recent reports, however, suggest downregulation of glycolytic intermediates in complication-prone tissues in type 1 diabetes, including peripheral nerves (Gardiner et al. 2007, retina (Ola et al. 2006), and lens (Obrosova & Stevens 1999). Recent metabolomic studies in models of type 1 diabetes reported downregulation of key TCA cycle and mitochondrial proteins (Akude et al. 2011) and enzyme activities (Chowdhury et al. 2010) in DRG, with decreased mitochondrial $\text{O}_2^{^-}$ production (Akude et al. 2011). Conversely, Schwann cells grown under hyperglycemic conditions demonstrated upregulation of the TCA cycle and mitochondrial proteins (Zhang et al. 2010). Critically, these data were produced in models of type 1 diabetes.

While the mechanisms underlying type 1 diabetes and type 2 diabetes are distinct, it is generally held that DN is due to hyperglycemic damage, regardless of the type of diabetes (Callaghan et al. 2012). However, DN is more common in patients with type 2 diabetes (Young et al. 1993), and DN in type 2 diabetic patients is less amenable to tight glycemic control, indicating differing underlying pathogenic mechanisms (Callaghan et al. 2012). Despite the significant progress in our understanding of altered peripheral nerve metabolism in models of type 1 diabetes, fundamental differences between type 1 diabetes and type 2 diabetes confer a critical need to understand how peripheral nerves adapt to the unique type 2 diabetic environment (hyperglycemia, hyperinsulinemia, and hyperlipidemia). We hypothesize that measures of oxidative stress are increased in a proximal-to-distal gradient in peripheral nerves in type 2 diabetes, consistent with DN pathogenesis. This will be reflected in a decrease in the glycolytic and TCA cycle metabolomes and related enzymes moving proximally to distally from the DRG to the sciatic nerve and its terminal branches, including the sural nerve.

In the current study, we utilized liquid chromatography–tandem mass spectrometry (LC/MS/MS) and LC-electrospray ionization (ESI)–MS/MS to explore glycolytic and TCA cycle metabolomic changes within sural nerve, sciatic nerve, and DRG of the BKS.Cg-m+/+Lepr$^{db}$
(db/db) mouse model of type 2 diabetes. We present steady-state metabolomics data demonstrating decreased glycolytic intermediates in the sural and sciatic nerves and a decrease in TCA cycle intermediates in DRG, sciatic nerve, and sural nerve, complementing the published data in peripheral nerves in type 1 diabetes. We confirm that these changes occur concurrently with decreased aconitase enzyme activity as well as increased protein and lipid oxidation in the sciatic nerve and DRG. We present the first evidence that oxidative stress is more pronounced distally in peripheral nerves from a mouse model of type 2 DN. Finally, we propose a preliminary model whereby these changes may cause the sciatic nerve to rely on truncated TCA cycle metabolism in the type 2 diabetes environment.

Materials and methods

Diabetic mice

Male type 2 diabetic (BKS.Cg-m+/+Leprdb, db/db) and control (db/+ ) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). A mutation in the leptin receptor of the db/db mouse results in hyperphagia, severe obesity, hyperinsulinemia, and hyperglycemia beginning at ~4 weeks of age (Jackson Laboratories, http://jaxmice.jax.org/jaxmicedb/html/model_66.shtml). At 24 weeks of age (20 weeks of diabetes), db/db mice exhibit insensitivity to mechanical and thermal stimuli, along with slowed nerve conduction velocities and reduced intra-epidermal nerve fiber (IENF) density (Sullivan et al. 2007, Cheng et al. 2009). Animals were maintained at the University of Michigan in a pathogen-free environment and cared for following the University of Michigan Committee on the Care and Use of Animals guidelines. Mice were given continuous access to food (Purina 5001, Purina Mills LLC, St Louis, MO, USA) and water.

Blood glucose (6-h fasting) and body weight were measured every 4 weeks to document the onset and duration of diabetes. One drop of tail blood was analyzed using a standard glucometer (One Touch Profile, LIFESCAN, Inc., Milpitas, CA, USA). At the end of the experimental period, glycated hemoglobin (Ghβ) was measured using the Helena Laboratories Test Kit (Glyco-Tek Affinity Column Method).

Tissue harvest

Mice were killed by sodium pentobarbital overdose at 24 weeks of age, followed by transcardial PBS perfusion to remove residual contaminating blood. This method was chosen over CO2/cervical dislocation (recommended by Stevens et al. (2000)) as CO2/cervical dislocation is not compatible with transcardial perfusion, a step that was necessary to remove blood artifact and for collaborative use of the other tissues. The left sural and sciatic nerves and lumbar DRG were dissected, briefly rinsed in double-distilled water, frozen by immersion in liquid nitrogen, and stored at −80 °C until metabolomic analysis was conducted (eight db/+ and db/db DRG; nine db/+ and db/db sural nerve and sciatic nerve). The right sciatic nerve and lumbar DRG were dissected and immediately submerged in ice-cold antioxidant buffer, rapidly frozen by immersion in liquid nitrogen, and stored at −80 °C for quantification of end products of oxidative damage (six db/+ and db/db DRG; 12 db/+ and db/db sciatic nerve). The right sciatic nerve from a separate cohort of mice was dissected, cut in thirds, and the proximal and distal thirds prepared as earlier for quantification of oxidative damage (six db/+ and six db/db). The left sciatic nerve and lumbar DRG were used for determination of aconitase enzyme activity as per manufacturer’s instructions (Cayman Chemical Company, Ann Arbor, MI, USA).

Although the method of killing confers a greater time from death to tissue harvest than CO2/cervical dislocation, the timings were standardized across all mice (sural and sciatic nerves 8 min, DRG 15 min), allowing comparative results within this study.

Targeted metabolomic analysis by LC/MS/MS

Frozen tissue samples were extracted with 150 μl of chilled 8:1:1 methanol:chloroform:water containing 13C-labeled glycolysis and TCA cycle standards. Samples were sonicated on ice for 20 s (20% duty cycle, 20% maximum power), held at 4 °C for a further 5 min, and centrifuged at 15 000 g for 5 min at 4 °C. Supernatant was transferred to autosampler vials and directly analyzed by LC/MS.

Chromatographic separation of 18 targeted glycolytic and TCA intermediates was performed based on the methods of Lorenz et al. (2011). Briefly, hydrophilic interaction liquid chromatography was performed using a Phenomenex Luna NH2 column (3 μm, 150×1 mm i.d.) on an Agilent 1200 RRLC coupled to an Agilent 6410 triple quadrupole mass spectrometer. Mobile phase A was 5 mM ammonium acetate in water, adjusted to pH 9.9 with ammonium hydroxide. Mobile phase B was acetonitrile. The initial gradient was a 10-min linear ramp from 20 to 100% B, followed by a 3-min hold at 100% B. The mobile phase was returned to 20% B and held for 17 min for re-equilibration before the next run.
The instrument was operated in multiple reaction monitoring (MRM) mode using MS/MS transitions previously optimized by analysis of authentic standards. The following parameters were implemented: flow rate was 0.07 ml/min, column temperature 25 °C, injection volume 20 µl, spray voltage 4.0 kV in negative ion mode, desolvation gas flow rate 10 l/min, desolvation gas temperature 325 °C, and nebulizer pressure 40 psi.

The ratio of each metabolite peak area to that of the closest-matching 13C-labeled standard was calculated. Metabolite concentration was determined using calibration curves generated from known concentrations of authentic standards and equal concentrations of 13C-labeled compounds as were present in the samples. Concentrations were normalized to total protein content of the tissue sample, determined by the Bradford-Lowry method using the reagents and protocol supplied by BioRad Laboratories. Final values are expressed as picomole/microgram tissue. Intermediates of the pathways not included in the LC/MS/MS output were due to lack of commercial availability of standard, insufficient concentration to reach detection limits, or degradation issues preventing accurate analysis by the current method.

Oxidative stress measures

DRG and sciatic nerve samples were analyzed for nitrated protein (3-nitrotyrosine) and oxidized lipids (hydroxyoctadecadienoic acids (HODEs)) as described previously (Wiggin et al., 2008, Vivekanandan-Giri et al., 2011). Tissue was homogenized at 4 °C in antioxidant buffer (100 µM diethylenetriaminepentaacetic acid, 50 µM butylated hydroxytoluene, 1% (v/v) ethanol, 10 mM 3-amino-1,2,4-triazole, and 50 mM sodium phosphate buffer (pH 7.4)) to prevent ex vivo oxidation, frozen, and thawed. Protein was precipitated with ice-cold trichloroacetic acid (10% v/v), collected by centrifugation, washed with 10% trichloroacetic acid, and delipidated twice with 1,2,4-triazole, and 50 mM sodium phosphate buffer (pH 7.4) to prevent ex vivo oxidation, frozen, and thawed.

Isotopically labeled internal standards were added and samples were hydrolyzed in 4 M methane sulfonic acid at 8 C for 24 h under argon as described previously (Pennathur et al., 2004, 2005). HODEs were quantified by reverse-phase C-18 HPLC analysis of triphenylphosphine-reduced lipid extracts after base hydrolysis. The protein content of tissue pellets was determined by a modified Lowry protein assay using BSA as a standard (Pennathur et al., 2004). Amino acids were isolated from the acid hydrolysate using a solid-phase C-18 column (Supelco; Pennathur et al., 2004, 2005) and quantified by MRM using isotope dilution LC/ESI/MS/MS as described previously (Vivekanandan-Giri et al., 2011). Results were normalized to tyrosine content, the precursor of 3-nitrotyrosine.

Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 (GraphPad Software). Comparisons between groups were performed using either one-way ANOVA with Tukey posttest for multiple comparisons or an unpaired t-test, as applicable. Assumptions about the Gaussian distribution of data and rules for transformation of nonnormative data were made as described previously (Russell et al., 1999). Significance was assigned when P<0.05.

Results

Metabolomic intermediates are decreased in sural nerve, sciatic nerve, and DRG tissue in db/db mice

Diabetes was confirmed in db/db mice by significant elevations in body weight, blood glucose, and GHb compared with their age-matched db/+ controls (P<0.001; Table 1). To begin to characterize the changes in glycolytic and TCA cycle intermediates within peripheral nervous system tissue of the db/db mouse, we performed targeted LC/MS/MS metabolomic analysis on sural nerve, sciatic nerve, and DRG extracts (Table 2). At 24 weeks of age (20 weeks of diabetes), there was a significant decrease in four of the five measured glycolytic intermediates within both diabetic sural nerve and sciatic nerve compared with db/+ controls (glucose-6-phosphate/fructose-6-phosphate (G6P/F6P), 62, 45%; 3-phosphoglycerate/2-phosphoglycerate (3PG/2PG), 75, 75%; and lactate phosphate/fructose-6-phosphate (G6P/F6P), 62, 45%; 3-phosphoglycerate/2-phosphoglycerate (3PG/2PG), 75, 75%; and lactate (LAC), 46, 56% respectively), with additional diabetic decrease in fructose-1,6-bisphosphate (FBP) content of sural nerve (63%; Table 2). By contrast, diabetes had no effect on measured glycolytic intermediates in DRG

Table 1 Body weight, fasting blood glucose, and GHb for db/+ and db/db mice at 24 weeks of age. Data are mean ± S.E.M.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Weight (g)</th>
<th>Blood glucose (mg/dl)</th>
<th>GHb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 weeks db/+ (9)</td>
<td>32.0 ± 1.0</td>
<td>203 ± 9</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>24 weeks db/db (9)</td>
<td>52.6 ± 4.8*</td>
<td>672 ± 67*</td>
<td>13.7 ± 0.4*</td>
</tr>
</tbody>
</table>

*P<0.001 vs db/+. Nine db/+ and db/db.
(Table 2). Among the TCA cycle metabolites, citrate (CIT) and isocitrate (ICIT) were significantly lower in all three tissues from db/db mice compared with db/+ controls, with a greater decrease seen in sural nerve and sciatic nerve (48, 50% sural nerve; 55, 50% sciatic nerve; and 34, 50% DRG). Sural nerve, sciatic nerve, and DRG content of the remaining measured TCA cycle intermediates were not significantly altered with diabetes (Table 2). These observations indicate that type 2 diabetes affects the TCA cycle metabolome of sural nerve, sciatic nerve, and DRG, as well as the glycolytic metabolome of the sural nerve and sciatic nerve, and suggest that the effect is greater in the peripheral nerve than the DRG.

### Protein oxidation and lipid peroxidation markers are elevated in sciatic nerve and DRG tissue in db/db mice

Oxidative stress is a major mechanism of hyperglycemia-induced DN in humans and rodents, particularly through the oxidation of proteins and lipids (Vincent & Feldman 2004, Vincent et al. 2004, 2009b). Hydrogen peroxide (H$_2$O$_2$) and O$_2^*$ react with nitrite, derived from NO, to produce peroxynitrite in peripheral nerves in experimental diabetes (Obrosova et al. 2005a). 3-Nitrotyrosine accumulates when peroxynitrite oxidizes tyrosine (Corbett et al. 1992, van der Veen & Roberts 1999, Du et al. 2002). To confirm whether protein oxidation was elevated in the db/db peripheral nervous system in vivo, we used isotope dilution LC/ESI/MS/MS to quantify levels of protein-bound 3-nitrotyrosine in sciatic nerve and DRG tissue from the db/+ and db/db mice (Fig. 1A). Nitrotyrosine in tissues of the peripheral nervous system was significantly increased by diabetes (sciatic nerve nitrotyrosine 2.3-fold increase, $P<0.001$; DRG nitrotyrosine 1.7-fold increase, $P<0.05$). To determine whether lipid peroxidation was also elevated, we used HPLC to quantify HODEs in lipid extracts from the sciatic nerve and DRG of db/+ and db/db mice (Fig. 1A). The sciatic nerve and DRG samples from the db/db mice contained significantly more HODEs than those from the db/+ mice (sciatic nerve HODEs 2.4-fold increase, $P<0.001$; DRG HODEs 1.6-fold increase, $P<0.01$). This increase in oxidized lipids was greater in sciatic nerve than in DRG ($P<0.05$).

The data presented in Fig. 1A are those from whole sciatic nerve. Owing to the greater observed effect of diabetes on the metabolome of sural nerve and sciatic nerve than DRG (Table 2), and the peripheral presentation of signs of DN in rodents (Sullivan et al. 2007) and in humans (Edwards et al. 2008), we explored whether oxidative stress is greater distally in the sciatic nerve by repeating the analyses for 3-nitrotyrosine and HODEs on proximal and distal segments of the sciatic nerve from db/+ and db/db mice (Fig. 1B and C). There were no differences in oxidative stress measures between proximal and distal segments of db/+ control nerves. Oxidized lipids (HODEs) were 2.3- and 2.6-fold greater in proximal and distal db/db sciatic nerve respectively compared with those of db/+ mice ($P<0.05$). Furthermore, diabetic sciatic nerve HODEs were 1.6-fold greater distally than proximally ($P<0.05$; proximal db/+ $= 112 \pm 15$, proximal db/db $= 252 \pm 35$, distal db/+ $= 152 \pm 16$, distal db/db $= 391 \pm 56$ pmol/mg protein; Fig. 1B). Nitrated protein was 2.3- and 2.2-fold greater in proximal and distal db/db sciatic nerve respectively compared with those of db/+ mice (proximal db/+ $= 216 \pm 54$, proximal db/db $= 505 \pm 57$ μmol/mol tyrosine, $P<0.01$; distal db/+ $= 335 \pm 45$, proximal db/db $= 735 \pm 90$ μmol/mol tyrosine, $P<0.05$).

### Table 2 Quantified LC/MS/MS metabolomic data from db/+ and db/db mouse sural nerve, sciatic nerve, and DRG. Data are mean picomole/microgram protein for each group ± s.e.m.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>24 weeks db/+ sural nerve</th>
<th>24 weeks db/db sural nerve</th>
<th>24 weeks db/+ sciatic nerve</th>
<th>24 weeks db/db sciatic nerve</th>
<th>24 weeks db/+ DRG</th>
<th>24 weeks db/db DRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P/F6P</td>
<td>16.3±4.9</td>
<td>6.2±3.1*</td>
<td>10.9±1.9</td>
<td>6.0±1.1*</td>
<td>5.5±1.0</td>
<td>6.8±1.2</td>
</tr>
<tr>
<td>FBP</td>
<td>3.8±0.6</td>
<td>1.4±0.3†</td>
<td>9.7±1.6</td>
<td>6.2±1.3</td>
<td>7.1±0.9</td>
<td>6.3±0.9</td>
</tr>
<tr>
<td>3PG/2PG</td>
<td>0.4±0.1</td>
<td>0.1±0.03*</td>
<td>2.2±0.4</td>
<td>0.8±0.2†</td>
<td>0.1±0.03†</td>
<td>0.1±0.02†</td>
</tr>
<tr>
<td>PEP</td>
<td>0.4±0.1</td>
<td>0.1±0.02†</td>
<td>0.4±0.1</td>
<td>0.1±0.02†</td>
<td>0.1±0.03†</td>
<td>0.1±0.03†</td>
</tr>
<tr>
<td>LAC</td>
<td>136.8±19.0</td>
<td>73.3±19.7*</td>
<td>220.7±45.8</td>
<td>96.1±14.4*</td>
<td>149.8±14.7</td>
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<tr>
<td>CIT</td>
<td>8.3±1.0</td>
<td>4.3±0.6†</td>
<td>49.7±6.9</td>
<td>22.2±4.4*</td>
<td>8.5±0.8</td>
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<tr>
<td>ICIT</td>
<td>0.2±0.03</td>
<td>0.1±0.03*</td>
<td>0.6±0.1</td>
<td>0.3±0.1*</td>
<td>0.2±0.02</td>
<td>0.1±0.01*</td>
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<tr>
<td>SUC</td>
<td>1.4±0.05</td>
<td>1.1±0.2</td>
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<td>4.7±0.8</td>
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</tr>
<tr>
<td>FUM</td>
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<td>3.6±0.1</td>
</tr>
<tr>
<td>MAL</td>
<td>8.0±0.9</td>
<td>6.2±1.0</td>
<td>16.9±2.5</td>
<td>11.5±2.0</td>
<td>10.9±0.9</td>
<td>10.6±0.7</td>
</tr>
</tbody>
</table>

*P<0.05; †P<0.01 vs db/+; ‡P<0.001 vs db/+ and db/db DRG; nine db/+ and db/db sciatic nerve and sural nerve; all data from male mice. G6P/F6P, glucose-6-phosphate/fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; 3PG/2PG, 3-phosphoglycerate/2-phosphoglycerate; PEP, phosphoenolpyruvate; LAC, lactate; CIT, citrate; ICIT, isocitrate; SUC, succinate; FUM, fumarate; MAL, malate.
distal db/db 739 ± 142 µmol/mol tyrosine, P < 0.05). Nitrated protein content was not significantly altered proximally to distally in diabetic sciatic nerve (Fig. 1C).

Together, these observations indicate that type 2 diabetes increases both protein and lipid oxidation in db/db mouse sciatic nerve and DRG. The diabetic increase in lipid peroxidation is greater in sciatic nerve than in DRG, and the extent of this oxidation is greater distally in sciatic nerve.

Aconitase enzyme activity is decreased in sciatic nerve and DRG tissue in db/db mice

Aconitase catalyzes the conversion of CIT to ICIT in the TCA cycle and is the most sensitive TCA cycle enzyme to reactive oxygen species (ROS) inhibition (Tretter & Adam-Vizi 2000). Work by Tretter & Adam-Vizi (2000) established that when aconitase is inhibited, a segment of the TCA cycle between α-ketoglutarate (α-KG) and oxaloacetate still functions via anaplerosis of glutamate in to the TCA cycle. To begin to understand the lack of change in TCA cycle intermediates other than CIT and ICIT, we measured aconitase enzyme activity in sciatic nerve and DRG from the db/+ and db/db mice (Fig. 2). Aconitase activity was 1.6-fold greater in DRG than sciatic nerve in control (db/+ ) mice (DRG 1.50 ± 0.06; sciatic nerve 0.96 ± 0.10 µM NADPH/mg protein per min, P < 0.01), with this tissue difference maintained in the db/db mice (DRG 0.91 ± 0.12; sciatic nerve 0.46 ± 0.06 µM NADPH/mg protein per min P < 0.05). Type 2 diabetes was associated with a decrease in aconitase activity, with a greater effect in sciatic nerve than DRG (DRG 0.39-fold decrease, P < 0.01; sciatic nerve 0.51-fold decrease, P < 0.01).

Discussion

Recent metabolomic studies in models of type 1 diabetes reported downregulation of key TCA cycle and mitochondrial proteins (Akude et al. 2011) and enzyme activities (Chowdhury et al. 2010) in DRG and upregulation of TCA cycle and mitochondrial proteins in Schwann

Figure 1

Increased oxidative stress in diabetic sciatic nerve and DRG. (A) Fold changes in oxidative stress measures from whole sciatic nerve and DRG were calculated from the ratio of db/db to db/+ . The nitrated protein content and HODEs were increased in whole sciatic nerve and DRG from diabetic mice compared with those from db/+ controls (P < 0.05; **P < 0.01; and ***P < 0.001 vs db/+ ). The diabetic fold increase in oxidized lipids was greater in sciatic nerve than in DRG (P < 0.05). Data expressed as mean fold-change ± S.E.M.; six db/+ and db/db DRG; 12 db/+ and db/db sciatic nerve. There were no changes in oxidative stress measures between proximal and distal segments of db/+ control sciatic nerves (B and C). (B) Oxidized lipids (HODEs) were elevated in proximal and distal db/db sciatic nerve respectively compared with those of db/+ mice. Diabetic sciatic nerve HODEs were greater distally than proximally. (C) The nitrotyrosine-to-tyrosine ratio was elevated in proximal and distal db/db sciatic nerve respectively compared with those of db/+ mice. Data expressed as mean ± S.E.M.; six db/+ and db/db sciatic nerve. *P < 0.05 and **P < 0.01. SCN, sciatic nerve; DRG, dorsal root ganglia; P, proximal; D, distal; NT, 3-nitrotyrosine. All data from male mice.
cells (Zhang et al. 2010). Differences in metabolic profiles, prevalence of DN, and efficacy of glycemic control on DN in type 1 diabetes and type 2 diabetes suggest that therapeutic strategies based on models of type 1 diabetes may offer limited efficacy to the type 2 diabetic patient with DN (Callaghan et al. 2012). There is therefore a critical need to understand peripheral nerve-specific metabolic changes in type 2 diabetes, and their role in nerve injury, to develop disease-modifying treatments. To this end, we evaluated changes in the glycolytic and TCA cycle metabolomes of key components of the peripheral nervous system in the db/db mouse model of type 2 diabetes. We performed targeted LC/MS/MS metabolomic analysis on sural nerve, sciatic nerve, and DRG from db/db mice. To confirm that ROS is elevated in the db/db peripheral nervous system, we used both LC/ESI/MS/MS and HPLC to measure nitrated protein and peroxidated lipid content respectively. We observed decreased glycolytic and TCA cycle intermediates in the db/db tissue, with concurrent increases in protein and lipid oxidation. Additionally, our data suggest a greater metabolic impact of diabetes distally, a proximal–distal increase in the severity of oxidative stress, and a proximal–distal decrease in aconitase enzyme activity in the db/db nerve.

Oxidative stress is strongly implicated in DN pathogenesis, particularly through the oxidation of proteins and lipids (Vincent & Feldman 2004, Vincent et al. 2004, 2009b). To confirm whether protein and lipid oxidation are elevated in the db/db peripheral nervous system in vivo, we quantified levels of protein-bound 3-nitrotyrosine in tissue extracts, and HODEs in lipid extracts respectively from the sciatic nerve and DRG of db/+ and db/db mice. Nitrotyrosine and HODEs were significantly elevated in the db/db peripheral nerve and DRG (Fig. 1), consistent with previous work showing that diabetes increases neuronal oxidized protein (Vincent et al. 2004, 2005, Obrosova et al. 2005a, Pennathur et al. 2005, Wiggins et al. 2008) and lipids (Vincent et al. 2004, 2005, Wiggins et al. 2008). Elevated oxidized proteins and oxidized lipids in distal diabetic sciatic nerve compared with distal control sciatic nerve indicate greater oxidative stress at peripheral nerve extremities in diabetes (Fig. 1B and C), paralleling the dying-back axonopathy observed in animal models (Sullivan et al. 2007) and human patients (Edwards et al. 2008). The diabetic elevation in lipid peroxidation (HODEs) was greater in the sciatic nerve than in the DRG and increased moving distally along the db/db sciatic nerve (Fig. 1). There are at least two possible explanations for these findings. First, the increased HODEs as a measure of oxidative stress could simply represent the greater lipid content in sciatic nerve compared with DRG (data not shown) or, alternatively, the increase is a true reflection of impaired axonal function with secondary impairment of axon/Schwann cell interactions leading to Schwann cell injury. To our knowledge, these are novel data that oxidative stress increases proximally-to-distally in the neuropathic diabetic peripheral nerve. DN predominantly occurs in a distal symmetrical pattern, with skin denervation (reduced IENF density; Said 2007, Sullivan et al. 2007) increasing with diabetes duration (Shun et al. 2004), and proximal-to-distal graded loss of myelinated fiber density observed in diabetic patients (Sullivan et al. 2003). The current data suggest that there may be a link between this proximal-to-distal graded loss of myelinated fiber density and a graded increase in oxidative stress. In addition to its role in nitration of proteins (Corbett et al. 1992, van der Veen & Roberts 1999, Du et al. 2002, Obrosova et al. 2005a), peroxynitrite formation is required for myelin–lipid peroxidation in in vitro myelin suspensions (van der Veen & Roberts 1999); the greater fold increases observed in sciatic nerve than in DRG lipid peroxidation measures (Fig. 1A) may reflect oxidation of myelin in the peripheral nerve.
Aconitase catalyzes the conversion of CIT to ICIT in the TCA cycle and is the most sensitive TCA cycle enzyme to ROS inhibition (Tretter & Adam-Vizi 2000). To begin to understand the specificity of the change in CIT and ICIT TCA cycle intermediates, we measured aconitase enzyme activity in sciatic nerve and DRG from the db/+ and db/db mice. The diabetes-related depletion of aconitase activity (Fig. 2) is consistent with our previous report in cultured DRG neurons (Vincent et al. 2005). The greater diabetic impairment observed in sciatic nerve than DRG is likely related to greater oxidative stress-mediated aconitase inhibition (Gardner & Fridovich 1992, Gardner et al. 1995, Tretter & Adam-Vizi 2000) in the sciatic nerve. This is contradictory to our previous in vitro observation that DRG neurons are more susceptible to oxidative stress than non-myelinating Schwann cells (Vincent et al. 2009c). It is important to note that our 2009 study investigated the effects of mild, acute oxidative stress induced by high glucose and H2O2 in cells derived from immature rat embryos and pups. We did not assess the effects of myelinite-lipid peroxidation, or high free fatty acids and elevated ox-LDL, both of which are associated with type 2 diabetes and linked to oxidative stress-mediated injury in these cells (Vincent et al. 2009a, Suzuki et al. 2011).

LAC production via the reversible LAC dehydrogenase-catalyzed reaction from pyruvate, the last metabolic intermediate of glycolysis, is intimately linked to the NAD+/NADH cellular redox status. Further flux experiments are required, but the pattern of glycolytic and TCA cycle intermediates in the sural nerve and sciatic nerve in diabetic mice compared with controls suggests that the observed significant depletion of sural nerve and sciatic nerve LAC (Table 2) is related to inhibition of glycolysis. These data are in contrast with findings of elevated LAC in sciatic nerve from type 1 diabetic streptozotocin (STZ)-treated rats (Obrosova et al. 1999, Stevens et al. 2000). Our finding that LAC levels were unchanged in db/db DRG is in agreement with reports in STZ-diabetic lens (Obrosova et al. 1998, Obrosova & Stevens 1999). Work by Stevens et al. (2000) highlighted concerns over anesthetic-driven LAC elevation to introduce artifact into the data. In addition, it should be noted that the above studies by Obrosova and Stevens were performed in a model of type 1 diabetes in rats and over shorter diabetes duration (3–6 weeks).

Our finding that G6P and F6P were decreased in sciatic nerve, with additional decrease in FBP in sural nerve, in db/db mice at 24 weeks of age (Table 2), is in contrast to findings in STZ diabetic lens (Obrosova et al. 1998, Obrosova & Stevens 1999). As G6P and F6P are derived from the same pool in the current MS approach, we cannot determine the contribution from each intermediate to the observed measurements. Whether there is a true decrease in both F6P and upstream G6P or a decrease in F6P due to redirection of G6P into the polyol (or other) pathways remains unclear.

Hexokinase saturation and maximal glycolytic flux is one proposed mechanism underlying the accumulation of nerve glucose and its direction into the polyol pathway in diabetes (Tomlinson & Gardiner 2008). Gardiner et al. (2007) observed diminished hexokinase activity in DRG from STZ diabetic rats. Studies on excised rat retinas (Ola et al. 2006) concluded that glycolysis and glucose metabolism downstream of hexokinase are not elevated by hyperglycemia or type 1 diabetes but that intermediates of alternative glucose metabolism, such as those of the polyol pathway, are increased. This is in agreement with conclusions of studies by the Greene group in STZ diabetic rat sciatic nerve (Obrosova et al. 1999, Stevens et al. 2000). Thus, it is possible that glucose is being preferentially directed into the polyol pathway in the db/db sural nerve and sciatic nerve, away from energy-producing glycolysis.

Decreased steady-state concentrations of metabolites in the lower segment of glycolysis (3PG/2PG; PEP) in db/db sural and sciatic nerves compared with controls agrees with previously published reports in the STZ diabetic lens (Obrosova et al. 1998, Obrosova & Stevens 1999; levels of these intermediates were similar between control and diabetic DRG in the current study), which concluded that sites of regulation are at glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reaction and downstream (e.g. enolase and pyruvate kinase). This conclusion is supported in the current model by our recent report of downregulation of the gene encoding enolase (Eno2) in db/db sciatic nerve at 24 weeks of age (Pande et al. 2011), the fact that GAPDH is vulnerable to oxidative damage (Du et al. 2000), and greater markers of lipid and protein oxidative damage in distal sciatic nerve compared with DRG (Fig. 1). In the more distal sural nerve, data suggest additional upstream sites of glycolysis inhibition (e.g. hexokinase,
phosphoglucone isomerase, and phosphofructokinase), with a greater diabetic decrease in metabolic intermediates in sural nerve compared with sciatic nerve (Table 2). In addition, slow axonal transport, responsible for the transport of glycolytic enzymes, is impaired in db/db diabetes at 20–24 weeks of age (Vitadello et al. 1983, 1985). Decreased peripheral enzyme availability coupled with increased distal oxidative stress may be related to the greater distal metabolomic changes observed in the current study.

Our observed decreases in CIT and ICIT (Table 2) are consistent with work by Akude et al. reporting down-regulation of key TCA cycle proteins, including CIT synthase, in DRG from 22-week STZ diabetic rats (Akude et al. 2011). One question arising from the TCA cycle metabolomic data is the lack of change in additional intermediates other than CIT and ICIT. Aconitase, catalyzing the CIT to ICIT reaction in the TCA cycle (Fig. 3), is inhibited by ROS, including O$_2^-$ (Gardner & Fridovich 1992, Gardner et al. 1995) and H$_2$O$_2$ (Tretter & Adam-Vizi 2005). When aconitase is fully inhibited by H$_2$O$_2$ in nerve terminals, $\alpha$-KG dehydrogenase ($\alpha$-KGDH; catalyzing the $\alpha$-KG to succinyl-CoA reaction) remains functional and a segment of the TCA cycle ($\alpha$-KG to oxaloacetate) is maintained by glutamate, which is converted to $\alpha$-KG via transamination (see Tretter & Adam-Vizi (2005)) for comprehensive explanation). ROS-mediated inhibition of aconitase (Fig. 2) may activate this truncated TCA in diabetic sural nerve, sciatic nerve, and DRG (Fig. 3), explaining the maintenance of levels of succinate, fumarate, and malate in the face of decreased CIT and ICIT observed in this study. This truncated segment of the TCA cycle may function in the absence of glucose (Yudkoff et al. 1994, Erecinska et al. 1996). In this respect, a state of oxidative stress when aconitase is completely inhibited but $\alpha$-KGDH remains active is similar to a glucose-free state. Additionally, $\alpha$-KGDH is itself a source of ROS production (see Gibson et al. (2010) for review of $\alpha$-KGDH in neurodegeneration), the level of which increases when $\alpha$-KG is utilized as a fuel source over glucose in isolated brain synaptosomes (Tretter & Adam-Vizi 2004). The greater decrease in CIT and ICIT observed in db/db sural and sciatic nerves (compared with DRG) may be due to reduced anaplerosis of intermediates from upstream glycolysis to the TCA cycle. This may be interpreted as a glucose-deficient state, activating the $\alpha$-KG-utilizing truncated TCA cycle pathway, further contributing to ROS production, and potentially contributing to the greater degree of oxidative stress observed in diabetic sciatic nerve than in DRG (Fig. 1A). Contribution to these pathways from an altered diabetic lipid metabolome should be considered but has not been explored in the current study.

Collectively, our data and those of the Fernyhough, LaNoue, and Obrosova groups support the emerging idea of tissue-specific alterations in energy metabolism in diabetic complications-prone tissues such as the peripheral nerve (Gardiner et al. 2007, Chowdhury et al. 2010, Akude et al. 2011), retina (Ola et al. 2006), and lens (Obrosova et al. 1998, Obrosova & Stevens 1999). For the first time, we expand this knowledge to neuropathy in a type 2 model of diabetes, additionally demonstrating different metabolomic profiles within specific tissues of the peripheral nervous system. The greater metabolomic changes in sural nerve and sciatic nerve than DRG may reflect increased oxidative stress and subsequent inhibition of key metabolic enzymes, including aconitase. However, our static measurements of metabolites are not direct indicators of flux through a pathway: these data are the beginnings of a full characterization of bioenergetic alterations in diabetic peripheral nerve and are necessary to develop effective disease-modifying treatments for DN.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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The Role of Oxidative Stress in Nervous System Aging

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Abstract

While oxidative stress is implicated in aging, the impact of oxidative stress on aging in the peripheral nervous system is not well understood. To determine a potential mechanism for age-related deficits in the peripheral nervous system, we examined both functional and morphological changes and utilized microarray technology to compare normal aging in wild-type mice to effects in copper/zinc superoxide dismutase-deficient (Sod¹/²) mice, a mouse model of increased oxidative stress. Sod¹/² mice exhibit a peripheral neuropathy phenotype with normal sensory nerve function and deficits in motor nerve function. Our data indicate that a decrease in the synthesis of cholesterol, which is vital to myelin formation, correlates with the structural deficits in axons, myelin, and the cell body of motor neurons in the Sod¹/² mice at 30 months and the Sod¹/² mice at 20 months compared with mice at 2 months. Collectively, we have demonstrated that the functional and morphological changes within the peripheral nervous system in our model of increased oxidative stress are manifested earlier and resemble the deficits observed during normal aging.

Introduction

Neuromuscular system function declines with age and manifests as sarcopenia [1]. Skeletal muscle atrophy and weakness lead to the loss of functional mobility and independence for many older adults [2]. Age-related changes in the central nervous system are well documented and include neuronal loss, demyelination, and deficits in cognitive function; however, little has been reported concerning age-related changes in the peripheral nervous system beyond a decline in nerve conduction velocities (NCVs) [3]. A clear understanding of the mechanisms underlying age-related changes in the peripheral nervous system is necessary to fully understand and prevent the decline in neuromuscular function that often accompanies aging.

The oxidative stress or free radical theory of aging, proposed by Denham Harman, suggests that free radicals cause oxidative damage to proteins, DNA, and lipids, and that this damage accumulates over time [4,5,6]. Oxidative stress is the result of an imbalance between pro-oxidants and antioxidants [5]. To date, both invertebrate and vertebrate models have been generated in which one or more antioxidants are either ablated or over-expressed; however, the role of oxidative stress in aging vertebrates, including rat, mouse, and human, remains unclear, likely due to the complexity of the aging process [7].

All cells contain multiple enzymes that target and neutralize free radicals. Superoxide dismutase (SOD) partners with another antioxidant enzyme, catalase, to defend against oxidative damage by converting the free radical pro-oxidant superoxide anion into molecular oxygen and hydrogen peroxide. There are three mammalian forms of SOD: cytoplasmic copper/zinc or SOD1, mitochondrial manganese or SOD2, and extracellular or SOD3. SOD1-deficient (Sod¹/²) mice appear normal at birth, but over time exhibit increased levels of oxidative stress in muscle and plasma, display a chronic peripheral neuropathy, accelerated age-associated hind limb muscle mass loss, neuromuscular junction degeneration, muscle weakness, and a 30% reduction in lifespan [8,9,10,11]. Furthermore, we previously reported that denervation in the muscles of the Sod¹/² mice resulted in fiber loss and muscle atrophy [12]. Mice lacking SOD2 (Sod²/²) die shortly after birth from cardiomyopathy and neurodegeneration [13]; whereas Sod²/² mice are viable until adulthood with no change in lifespan and exhibit elevated markers of oxidative damage and increased sensitivity to oxidative stress [14]. Mice lacking SOD3 (Sod³/³) are healthy and exhibit a normal lifespan [15]. Thus, given the severity of the muscle phenotype and resemblance to human aging, the Sod¹/² mouse model is more informative than models with other SOD mutations for studying the effects of oxidative stress on aging in the peripheral nervous system. The aim of this study is to better understand the contribution of oxidative stress in peripheral nervous system aging. Here, we accomplish this aim by correlating functional and morphological changes in sensory and motor neurons with microarray and bioinformatics analyses of nerve during normal aging in Sod¹/+ mice and in Sod¹/² mice, a model with increased oxidative stress.
**Materials and Methods**

Oxidative damage was assessed in DRG neurons of *Sod1* mice by A) quantitatively assessing the autofluorescence of lipofuscin, and by western immunoblotting of B) nitrated protein (nitrotyrosine; NT) and C) oxidative lipid degradation (malondialdehyde; MDA). The *Sod1**/+** mice at 20 mo, *Sod1**−/−** mice at 20 mo, and *Sod1**+/+** mice at 30 mo are represented by white, black, and gray bars, respectively. **p < 0.01 and ***p < 0.001 compared to age-matched *Sod1**+/+** mice at 20 mo; n=4.

![Figure 1. Assessment of oxidative damage in the cell body of sensory neurons.](image)

**Thermal Thresholds**

Tail flick analgesia was measured according to previously published protocols [17]. Briefly, for the tail flick assay, mice were placed in an acrylic holder atop a tail flick analgesia meter (ITC Life Science, Woodland Hills, CA) with the tail in contact with an adjustable red light emitter (range 60°–170°C). The time from activation of the beam to the animal response is detected and electronically recorded.

**Nerve Conduction Studies**

Measures of NCV were performed at 2, 8, 20, and 30 mo of age per our published protocols [17]. Mice were anesthetized with Avertin (200 mg/kg) and body temperature was maintained at 34°C using a warming lamp and dermal temperature probe. The platinum needle electrodes (ViaSys, Madison, WI) were cleaned between measurements using 70% alcohol. Tail distal motor latency (TDML) is an orthodromic measurement determined by stimulating the proximal 30 mm segment of the tail. Latency was measured from the initial onset of the compound muscle action potential. The sural NCV, a measure of sensory nerve function, was determined by recording in the dorsum of the foot and stimulating antidromically with supramaximal stimulation at the ankle and recorded at the dorsum of the foot. NCV was calculated by dividing the distance by the take-off latency of the sensory nerve action potential. The sciatic-tibial motor NCV (SMNCV) was determined by recording in the dorsum of the foot and orthodromically stimulating with supramaximal stimulation first at the ankle then at the sciatic notch. Latencies were measured from the initial onset of the compound muscle action potential. Final NCV was calculated by dividing the difference of the ankle from the notch distance by the difference in the ankle and notch latencies.

**Tissue Processing**

For microarray analysis, one SCN was stored in RNAlater (Ambion, Inc., An Applied Biosystems Buiness, Austin, TX) at −80°C until use for RNA isolation and gene expression analysis. For western immunoblotting, the tissue was snap frozen in liquid nitrogen and stored at −80°C until use for western immunoblotting. For lipofuscin analysis, light or transmission electron microscopy, animals were intracardially perfused with 15 ml of phosphate buffered saline (PBS 0.1 M, pH 7.2) and 30 ml 2% paraformaldehyde prior to dissection. For light and transmission electron microscopy, L5 spinal cord and ventral root were dissected and post-fixed overnight in 4% paraformaldehyde/2.5% glutaraldehyde. Tissue was processed by the Microscopy
Imaging Laboratory (MIL; University of Michigan, Ann Arbor, MI).

**Lipofuscin Analysis**

For lipofuscin analysis, sections were heated on a slide warmer for 10 min, hydrated in PBS for 5 min, and coverslipped with Prolong anti-fade mounting medium containing DAPI. The images were captured using a Spot-RT camera (Diagnostic Instruments Inc., Sterling Heights, MI) attached to a Nikon Microphot-FXA microscope. The resulting images of lipofuscin were analyzed for intensity using MetaMorph software (Universal Imaging Corp., West Chester, PA).

**Western Immunoblotting**

Western immunoblotting was performed as previously described [18]. SCN, lumbar spinal cord, and DRG were homogenized in tissue protein extraction reagent (Pierce, Rockford, IL) containing a protease inhibitor cocktail (Calbiochem, San Diego, CA). The lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Tris-buffered saline with Tween-20 supplemented with 5% non-fat dry milk was used to decrease non-specific binding and dilute the following primary antibodies: nitrotyrosine (NT), malondialdehyde (MDA) (both from Abcam, Cambridge, MA), cleaved caspase-3 (Millipore, Billerica, MA), 7-dehydrocholesterol reductase (DHCR7; Abcam), 24-dehydrocholesterol reductase (DHCR24; Cell Signaling, Danvers, MA), and mevalonate diphospho decarboxylase (Santa Cruz, Dallas, Texas). The signal was visualized using LumIGLO enhanced chemiluminescence reagent (Cell Signaling, Danvers, MA).

**Figure 2. Assessment of oxidative damage in the motor neuron micro-environment.** Oxidative damage was assessed in the spinal cord of Sod1 mice by A) quantitatively assessing the autofluorescence of lipofuscin, and by western immunoblotting of B) nitrated protein (NT) and C) oxidative lipid degradation (MDA). The Sod1+/+ mice at 20 mo, Sod1−/− mice at 20 mo, and Sod1+/+ mice at 30 mo are represented by white, black, and gray bars, respectively. D) Apoptosis was assessed in the motor neurons of Sod1 mice by cleaved caspase-3 western immunoblotting. Densitometry revealed an increase in cleaved caspase-3 in the in Sod1−− mice at 20 mo compared to the Sod1+/+ mice at 20 mo. *p<0.05, **p<0.01; n=4 for all groups.

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nescence 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).

RNA Preparation

Total RNA was isolated from one SCN (the other SCN was used for western immunoblotting as described above) using the RNeasy Mini Kit (QIAGEN, Inc., Valencia, CA), including an on-column deoxyribonuclease digestion, following the manufacturer’s protocol. RNA quality and quantity were assessed by microfluid electrophoresis using an RNA 6000 Pico LabChip on a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Samples with a minimum RNA Integrity Number of seven were used for microarray hybridization [19].

Affymetrix Microarrays

Samples meeting the RNA quality criteria were analyzed by microarray (Sod1+/+ mice at 8 mo (n = 6) or 20 mo (n = 5); Sod1−/− mice at 8 mo (n = 6), 20 mo (n = 6), and 30 mo (n = 5)). Total RNA (75 ng) 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 were performed by the University of Michigan Comprehensive Cancer Center Affymetrix and Microarray Core Facility (University of Michigan, Ann Arbor, MI) using the Affymetrix GeneChip Mouse Genome 430 2.0 Array. The intensities of the target hybridizations to their respective probe features were detected by laser scan of the array. Image files were generated by Affymetrix GeneChip software (MAS5).

The Affymetrix raw data files (CEL files) were analyzed using a local copy of GenePattern, a bioinformatics platform from the Broad Institute [20]. The samples were Robust Multi-array Average (RMA) normalized using the BrainArray Custom Chip Definition File (CDF) version 15 [21]. Microarray quality was assessed using the AffyAnalysisQC R package (http://arrayanalysis.org/) with Bioconductor [22]. Arrays deemed as potential outliers by two or more quality assessment modules in the AffyAnalysisQC package were excluded.

Microarray Data Analysis

Identification of differentially expressed genes (DEGs). DEGs were obtained either between genotypes (Sod1+/+ and Sod1−/−) at the same age or between different ages (2, 20, and 30 mo) within each genotype. Differential expression of genes was determined using the Intensity-Based Moderated T-statistic (IBMSTR) test [23] with a false discovery rate (FDR)<0.05.

Figure 3. Behavioral and functional measures of sensory and motor nerve function in Sod1 mice. A) The behavioral tail flick response to a thermal stimulus in the Sod1+/+ mice at 2, 8, 20, and 30 mo (n = 7, 16, 10, and 7, respectively) and Sod1−/− mice at 2, 8 and 20 mo (n = 4, 21, and 8, respectively) is a measure of both sensory and motor nerve function. B) Sensory nerve function was assessed using sural nerve conduction velocity (NCV) in the Sod1+/+ mice at 8, 20, and 30 mo (n = 13, 9, and 12, respectively) and Sod1−/− mice at 8 and 20 mo (n = 10 and 7, respectively). Motor nerve function was examined by measuring C) Tail distal motor latency (TDML) in the Sod1+/+ mice at 2, 8, 20, and 30 mo (n = 8, 21, 12, and 9, respectively) and Sod1−/− mice at 2, 8 and 20 mo (n = 6, 17 and 4) and by measuring D) sciatic motor NCV in the Sod1+/+ mice at 2, 8, 20, and 30 mo (n = 7, 15, 7, and 9, respectively) and Sod1−/− mice at 2, 8 and 20 mo (n = 6, 19 and 6). *p<0.05, **p<0.01, and ***p<0.0001 compared to age-matched Sod1+/+ mice; #P<0.01 and ##p<0.0001 compared to Sod1+/+ mice at 20 mo.

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The numbers of identified DEGs at each comparison are noted under DEGs in age (horizontal) and genotype (vertical) group comparisons. Obtained in each comparison. IBMT FDR 5% was used to determine significantly over-represented biological functions. A heat-map was generated using top 10 most over-represented biological functions in each DEG set, clustered based on the significance values (log-transformed P-values).

**Literature survey of cholesterol-related DEGs.** To prioritize the DEGs for protein-assay validation, we surveyed the literature using a literature mining tool to examine the relevance and importance of our DEGs to the enriched biological function, cholesterol metabolism. SciMiner (http://jdrf.neurology.med.umich.edu/SciMiner) [25], our in-house web-based literature mining tool, was used to identify genes and how often they appear in the cholesterol- and aging-related literature, defined by a PubMed query of “aging AND cholesterol”. As of 09/12/2012, the PubMed result queried in 5,257 papers, from which SciMiner identified 1,179 genes. The number of papers for each gene was statistically evaluated using a Fisher’s Exact Test against the complete PubMed abstracts to determine its relative importance to the topic [26]. Table S1 includes the number of papers and p-value from the statistical test for each DEG, if included in the literature-mined 1,179 genes.

**Real-time qRT-PCR**

The expression of eight of the down-regulated DEGs related to cholesterol/sterol metabolism and two DEGs that were up-regulated with a fold change greater than 4 was confirmed by real-time qRT-PCR using the same samples as the microarray. Reverse transcription was performed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR amplification and SYBR Green fluorescence detection were performed using the Applied Biosystems StepOnePlus Real-time PCR System (Life Technologies Corporation, Carlsbad, CA). The fluorescence threshold value (C_T) was calculated using StepOne system software. The mRNA levels were normalized to an endogenous reference gene (glyceraldehyde-3-phosphate dehydrogenase (Gapdh)) and then relative to a control group (C_T) and expressed as 2^{-\Delta\Delta C_T}. The average was calculated from two runs per sample.

**Analysis of Axons and Myelin**

The spinal cord ventral roots were processed by the MIL (University of Michigan, Ann Arbor, MI) for transmission electron microscopy (TEM). The samples were rinsed in PBS and post-fixed in 1% osmium tetroxide. After rinsing again in PBS and distilled H_2O, the samples were stained in aqueous 3% uranyl acetate. The samples were dehydrated in ethanol, propylene oxide, and infiltrated with Spurr’s resin. After polymerizing for 24 h at 60°C, the samples were sectioned using an ultra-microtome at 500 nm for thick sections and 90 nm for thin sections. Images were captured on a Philips CM-100 TEM with a digital camera.

The area and equivalent diameter of the inner axon and the axon (including the myelin) was determined using MetaMorph image analysis software (Molecular Devices Corporation, Sunnyvale, CA). A blinded observer assessed at least 3 images per animal from the proximal and distal ventral root. The area of myelin was calculated by subtracting the inner axonal area from the total axonal area.

**Analysis of Cell Body of Motor Neurons**

For light microscopy, sections of the lumbar spinal cord (1 μm) were stained with toluidine blue to determine size and number of motor neurons per our published protocols [27]. Bright field images were captured on a Nikon Microphot FXA microscope with a SPOT-RT digital camera. The resulting images were

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**Figures**

Figure 4. Assessment of gene expression in the sciatic nerve during aging. Alterations in the gene expression in the sciatic nerve during normal aging (Sod1+/+ mice at 2 mo and 30 mo) and during increased oxidative stress (Sod1−/− mice at 2 mo and 20 mo), were assessed using Affymetrix microarray technology. A) A comparison of the data sets for differential gene expression and the number of DEGs obtained in each comparison. IBMT FDR 5% was used to determine DEGs in age (horizontal) and genotype (vertical) group comparisons. The numbers of identified DEGs at each comparison are noted under the arrows. B) A Venn-diagram of the 2 mo/30 mo and 2 mo/20 mo Sod1−/− mouse comparison (normal aging) and the 2 mo/20 mo Sod1−/− mouse comparison (increased oxidative stress). C) The five DEG sets, including the two original DEG sets (denoted as 2moKO_20mKO_ALL (406 DEGs) and 2mWT_30mWT_ALL (1,904 DEGs)) and three subsets (common DEGs denoted as SOD1-KO-Only (254 DEGs) or SOD1-WT-Only (1,752 DEGs)) from Figure 3B, were subject to functional enrichment analysis using DAVID. Benjamini-Hochberg (BH)-corrected P-values of the top 10 most significant functional terms are represented in a heat-map with –log_{10}(BH-corrected P-value) as color index and number. doi:10.1371/journal.pone.0068011.g004

**Functional annotation and enrichment analyses.** The Database for Annotation, Visualization and Integrated Discovery (DAVID) [24] was used to identify over-represented biological functions and pathways among the DEGs. The p-values for annotation terms in DAVID were determined using a modified Fisher’s Exact Test, and terms with a Benjamini-Hochberg corrected P-value<0.05 were selected as significantly over-represented biological functions. A heat-map was generated using top 10 most over-represented biological functions in each DEG set, clustered based on the significance values (log-transformed P-values).
analyzed for area (μm²) using MetaMorph. For TEM, the spinal cord was processed by MIL and imaged as described above for the ventral roots.

Statistical Analysis

Data analyses were performed using Prism, version 5 (GraphPad Software, Inc., La Jolla, CA). Assumptions about Gaussian distribution of data were made using the D’Agostino & Pearson omnibus normality test. Groups were compared using the ANOVA test using either age or genotype as the independent variable. The t-test was used when appropriate and for the comparison of Sod1²⁻² mice at 20 mo to Sod1⁺/⁺ mice at 30 mo. All values are reported with standard error of the mean.

Results

Levels of Oxidative Damage in Sensory and Motor Neurons

To determine whether the systemic increase in oxidative stress leads to oxidative damage in the cell bodies of sensory neurons (dorsal root ganglia; DRG) and in the motor neuron micro-environment (spinal cord) in Sod1²⁻² and Sod1⁻/⁻ mice, we quantitatively assessed oxidative damage by lipofuscin autofluorescence and western immunoblotting of nitrated proteins and lipid peroxidation, which is oxidative lipid degradation. We did not observe a significant increase in oxidative damage with lipofuscin, nitrated proteins, or lipid peroxidation between the Sod1⁻/⁻ mice and the Sod1⁺/⁺ mice at 8 mo (Figure S1). The levels of lipofuscin in sensory neurons were increased 1.3-fold in the Sod1²⁻² mice at 20 mo (not significant), and 1.4-fold in the Sod1⁺/⁺ mice at 30 mo compared with the Sod1⁺/⁺ mice at 20 mo (Figure 1A). A 2-fold increase in nitrated proteins, indicative of nitrosative stress, and a 1.7-fold increase in lipid peroxidation was revealed in sensory neurons of the Sod1⁻/⁻ mice at 20 mo compared to the Sod1⁺/⁺ mice at 20 mo and 30 mo (Figures 1B and C, respectively). Because oxidative stress is known to induce programmed cell death in a number of cell types, including sensory neurons [28], we utilized western immunoblotting to examine the cleavage of caspase-3, an indicator of apoptosis. We did not observe a significant difference in cleaved caspase-3 in sensory neurons in the Sod1²⁻² mice at 20 mo or the Sod1⁺/⁺ mice at 30 mo (data not shown).

We also examined oxidative damage in the motor neuron micro-environment by quantitatively assessing lipofuscin, nitrated proteins, and lipid peroxidation. We did not observe a significant increase in oxidative damage with lipofuscin, nitrated proteins, or lipid peroxidation between the Sod1⁻/⁻ mice and the Sod1⁺/⁺ mice at 8 mo (Figure S2). The levels of lipofuscin in the motor neuron micro-environment were increased 1.3-fold in the Sod1⁻/⁻ mice at 20 mo compared with 2 mo mice, and 1.4-fold in the Sod1⁺/⁺ mice at 30 mo compared with 2 mo mice. Western immunoblotting densitometry was used to validate the protein expression levels of two genes that were differentially expressed and encode enzymes in the cholesterol/sterol synthesis pathway, C) DHCR24 and D) MVD; *p<0.05 and **p<0.01.

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Figure 5. qRT-PCR verification of the expression levels of the DEGs in the sciatic nerve. The log₂ transformation of the fold-change of the relative mRNA expression levels in the 10 DEGs selected for qRT-PCR (black bars) parallels the direction of changes in the microarray (white bars) in A) normally aged mice, the Sod1⁻/⁻ mice at 20 mo compared with 2 mo mice, and B) in a model of increased oxidative stress, the Sod1⁻/⁻ mice at 30 mo compared with 2 mo mice. Western immunoblotting densitometry was used to validate the protein expression levels of two genes that were differentially expressed and encode enzymes in the cholesteral/sterol synthesis pathway, C) DHCR24 and D) MVD; *p<0.05 and **p<0.01. 

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or 20 mo (20.7 ± 0.001, **p < 0.0001, and *p < 0.0001 compared to Sod1+/+ mice at 20 mo.

To assess functional changes in motor nerve function, motor latency and NCV were evaluated. Tail distal motor latency (TDML; ms) was comparable in the Sod1+/− and Sod1+/+ mice at 2 mo (2.9 ± 0.13 and 2.7 ± 0.06, respectively). The latency was significantly increased in the Sod1+/− mice at 8 mo (3.2 ± 0.08) and 20 mo (3.4 ± 0.42) compared to the Sod1+/+ mice at 8 mo (3.2 ± 0.08) and 20 mo (2.8 ± 0.10). The tail flick latency significantly increased with age in the Sod1+/− and Sod1+/+ mice at 8 mo compared to the 2 mo, and Sod1+/− mice at 30 mo compared to the 20 mo (Figure 3A). Thermal analgesia is a spinal reflex and requires proper function of both motor and sensory components. To assess specific differences in these neuronal populations, we first characterized the sensory nerve function using sural NCV (m/s). Sural NCV was not significantly different in the Sod1+/− mice at 8 mo (20.1 ± 1.7) or 20 mo (20.7 ± 1.7) compared to the Sod1+/+ mice at 8 mo (22.9 ± 1.1) or 20 mo (22.0 ± 1.5); however, the Sod1+/− mice at 30 mo (17.2 ± 1.2) had a sensory deficit compared with the Sod1+/+ mice at 20 mo (Figure 3B).

To assess changes in nerve function, we assessed thermal analgesia in the Sod1+/− and Sod1+/+ mice using tail flick latency, as a greater latency represents nerve dysfunction. Tail flick latencies (s) were not significantly different in the Sod1+/− and Sod1+/+ mice at 2 mo (5.6 ± 0.06 and 4.9 ± 0.15, respectively), but were significantly increased in the Sod1+/− mice at both 8 mo (4.0 ± 0.18) and 20 mo (4.3 ± 0.42) compared to the Sod1+/+ mice at 8 mo (3.2 ± 0.08) and 20 mo (2.8 ± 0.10). The tail flick latency significantly increased with age in the Sod1+/− and Sod1+/+ mice at 8 mo compared to the 2 mo, and Sod1+/− mice at 30 mo compared to the 20 mo (Figure 3A). Thermal analgesia is a spinal reflex and requires proper function of both motor and sensory components. To assess specific differences in these neuronal populations, we first characterized the sensory nerve function using sural NCV (m/s). Sural NCV was not significantly different in the Sod1+/− mice at 8 mo (20.1 ± 1.7) or 20 mo (20.7 ± 1.7) compared to the Sod1+/+ mice at 8 mo (22.9 ± 1.1) or 20 mo (22.0 ± 1.5); however, the Sod1+/− mice at

Sensory and Motor Nerve Function

Figure 6. Structural deficits in the axons and myelin of motor neurons in the Sod1 mice. Transmission electron microscopy (TEM) of the ventral roots from A) Sod1+/+ mice at 20 mo, B) Sod1+/− mice at 20 mo, and C) Sod1+/+ mice at 30 mo revealed thinning myelin (arrow) and delaminating myelin (arrow head). Scale bar = 10 μm. The area of D) axons and E) myelin in the ventral root was measured by light microscopy in the Sod1+/− mice at 20 and 30 mo (n = 9, 3, and 5, respectively) and Sod1+/+ mice at 20 mo (n = 19, and 10). **p < 0.001, ***p < 0.0001, and *p < 0.0001 compared to Sod1+/+ mice at 20 mo.

Sod1+/+ mice at 20 mo (Figure 2C). The cleavage of caspase-3 was increased in the motor neuron environment in the Sod1+/− mice at 20 mo compared to the Sod1+/+ mice at 20 mo (Figure 2D). These data suggest that oxidative stress is more detrimental to motor neurons than sensory neurons.
Mechanisms Contributing to Deficits in the Peripheral Nerve

To examine the potential mechanisms underlying the contribution of oxidative stress to age-related deficits in the peripheral nervous system, we employed microarray technology and bioinformatics approaches to systematically analyze the changes in the gene expression profiles in the SCN, which contain peripheral axons. Figure 4A illustrates the comparisons between age (normal aging in the Sod1+/+ mice and Sod1−/− mice) and genotype (Sod1+/+ and Sod1−/−) groups and the number of DEGs determined by IBMT (see Table S1 for a complete list of the DEGs). During normal aging, only 48 genes are differentially expressed in the 20 mo compared with the 2 mo Sod1+/+ mice; however, 1,904 genes were differentially expressed in the 30 mo compared with the 2 mo Sod1+/+ mice. At 2 mo, only 9 genes were significantly different between Sod1+/+ and Sod1−/− mice, suggesting that the lack of Sod1 does not affect early development. The changes in gene expression in the peripheral nerve mediated by oxidative stress includes 406 DEGs in the 20 mo compared with the 2 mo Sod1−/− mice.

To determine potential mechanisms for oxidative stress-mediated effects in the peripheral nerve, we focused on the 2 mo/30 mo Sod1+/+ mouse comparison and the 2 mo/20 mo Sod1−/− mouse comparison. Approximately one-third of the DEGs in the Sod1−/− mouse overlapped with the Sod1+/+ mice (Figure 4B). A functional enrichment analysis was performed to identify significantly over-represented biological functions in terms of Gene Ontology and pathway terms of these DEGs, and a heatmap was mapped to summarize the most significantly over-represented functions (Figure 4C). The common DEGs between the two sets were highly enriched with processes related to cholesterol and sterol metabolism.

To validate the microarray expression data, qRT-PCR was performed on eight DEGs related to cholesterol and sterol metabolism (all down-regulated) and two DEGs related to inflammation due to the high up-regulated fold change (>4): 7-dehydrocholesterol reductase, Dhcr7 (−1.41-fold change in Sod1+/+ and −1.30-fold change in Sod1−/− mice); farnesyl diphosphate farnesyl transferase 1, Ffpt1 (−1.86-fold change in Sod1+/+ and −1.50-fold change in Sod1−/− mice); hydroxysteroid (17-beta) dehydrogenase 7, Hsd17b7 (−2.18-fold change in Sod1+/+ and −1.77-fold change in Sod1−/− mice); low density lipoprotein receptor, Ldlr (−1.75-fold change in Sod1+/+ and −1.42-fold change in Sod1−/− mice); 24-dehydrocholesterol reductase, Dhcr24 (−2.77-fold change in Sod1+/+ and −1.79-fold change in Sod1−/− mice); mevalonate (diphospho) decarboxylase, Mvd (−2.33 fold change in Sod1+/+ and −1.66-fold change in Sod1−/− mice); sterol-C4-methyl oxidase-like, Scmoul (−2.07-fold change in Sod1+/+ and −1.75-fold change in Sod1−/− mice); sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisiae), Sc5d (−2.31-fold change in Sod1+/+ and −1.35-fold change in Sod1−/− mice); toll-like receptor 7, Tlr7 (4.11-fold change in Sod1+/+ and 1.62-fold change in Sod1−/− mice); chemokine (C-X-C motif) ligand 14, Cxcl14 (7.02-fold change in Sod1+/+ and 2.66-fold change in Sod1−/− mice). All ten tested genes demonstrated significant differential expression and the fold changes paralleled the directionality of down- or up-regulation in the microarray results (Figures 5A and B).
To confirm the biological relevance of the cholesterol/sterol metabolism-related down-regulated DEGs during normal aging in the Sod1<sup>+/+</sup> mice and aging with increased oxidative stress in the Sod1<sup>−/−</sup> mice, we evaluated the protein levels of two DEGs—DHCR24 (end of the pathway) and MVD (beginning of the pathway), which were significantly over-represented in the literature based on our text mining tool (SciMiner) results [25].

The relative protein levels of DHCR24 decreased by 56% during normal aging in the Sod1<sup>+/+</sup> mice at 30 mo compared with the Sod1<sup>+/+</sup> mice at 2 mo (0.72 ± 0.54 and 1.6 ± 0.22, respectively), and by 52% in the Sod1<sup>−/−</sup> mice at 20 mo compared with the Sod1<sup>−/−</sup> mice at 2 mo (0.72 ± 0.082 and 1.5 ± 0.30, respectively; Figure 5C).

Similarly, the relative protein levels of MVD decreased by 55% during normal aging in the Sod1<sup>+/+</sup> mice at 30 mo compared with the Sod1<sup>+/+</sup> mice at 2 mo (0.72 ± 0.05 and 1.61 ± 0.25, respectively), and by 62% in the Sod1<sup>−/−</sup> mice at 20 mo compared with the Sod1<sup>−/−</sup> mice at 2 mo (0.76 ± 0.13 and 2.0 ± 0.54, respectively; Figure 5D).

**Structural Deficits in Axons, Myelin, and the Cell Body of Motor Neurons**

Abnormalities in cholesterol metabolism can lead to abnormalities in axons and myelin [29,30]. Thus, the deficits we observed in...
the NCV may be indicative of abnormalities in axons or myelin. Analysis of the spinal cord ventral root did not reveal a significant difference between the axonal area or myelin area in the Sod1+/− mouse at 8 mo compared with the Sod1+/+ mouse at 8 mo (data not shown). On the other hand, structural changes in the Sod1+/− mouse at 20 mo included alterations in axonal size and de laminating myelin, and structural changes in the Sod1+/+ mouse at 30 mo demonstrated alterations in axonal size and thinning myelin (Figure 6A-C). Quantitative analysis of the axonal area in the ventral root revealed a 1.3-fold decrease in the Sod1+/− mouse at 20 mo and a 1.6-fold decrease the Sod1+/− mouse at 30 mo compared with the Sod1+/+ mice at 20 mo (Figure 6D). Although the area of myelin was not significantly different in the Sod1+/− mice at 20 mo compared with the Sod1+/+ mice at 20 mo, the area of myelin decreased 1.3-fold in the Sod1+/+ mice at 30 mo compared with the Sod1+/− mice at 20 mo (Figure 6E).

A deficit in the axon or myelin is not necessarily indicative of cell body damage. Therefore, we assessed anatomical alterations in the cell body of motor neurons utilizing light microscopy. Analysis of the lumbar spinal cord did not reveal any difference in area or total number of motor neurons in the Sod1+/− mice at 8 mo compared with the Sod1+/+ mice at 8 mo (data not shown); however, analysis of the aged mice revealed an increase in the area of motor neurons in the Sod1+/− mice at 20 mo compared to the Sod1+/+ mice at 20 mo and 30 mo (Figure 7A-D), with a trending decrease in the total number of L5 lower motor neurons in the Sod1+/− mice at 20 mo and a significant decrease in the Sod1+/+ at 30 mo compared to the Sod1+/− at 20 mo (Figure 7E).

To further explore the anatomical alterations in the cell body of motor neurons we also utilized transmission electron microscopy (TEM; Figure 8); TEM of motor neurons in the Sod1+/− mice at 20 mo compared with the Sod1+/+ mice at 20 mo revealed a lack of distinct Nissl bodies associated with the endoplasmic reticulum, darkening of the cytoplasm, disorganized organelles, and formation of vacuoles. Similar to the Sod1+/− mice at 20 mo, although less pronounced, TEM of motor neurons in the Sod1+/+ mice at 30 mo also revealed a lack of Nissl bodies associated with the endoplasmic reticulum, darkening of the cytoplasm, disorganized organelles, and the formation of vacuoles compared with the Sod1+/− mice at 20 mo.

Discussion

Increasing oxidative stress is implicated in normal aging, sarcopenia, and decreased neuromuscular function. Building on our previous observation that denervation of the muscle resulted in abnormalities in gene expression in the peripheral (sciatic) nerve, in which Schwann cells are the primary cellular component [34], from 2 mo to 30 mo in the Sod1+/− mouse and from 2 mo to 20 mo in the Sod1+/− mouse. Our data are in general agreement with a previous study, which demonstrated greater dysfunction in the ventral root (motor) compared to the dorsal root (sensory) axons in the Sod1+/− mice [11]. The motor nerve function deficits in our Sod1+/− model of increased oxidative stress resembles deficits observed during normal aging in the Sod1+/− mouse, suggesting that oxidative stress plays a role in the age-related decline in motor function.

NCV deficits in the Sod1+/− mice may be due to either axonal degeneration or demyelination of the peripheral nerve. We used microarray technology, to correlate the functional deficits with alterations in gene expression in the peripheral (sciatic) nerve, in which Schwann cells are the primary cellular component [34], from 2 mo to 30 mo in the Sod1+/− mouse and from 2 mo to 20 mo in the Sod1+/− mouse. Schwann cells support the preservation and function of peripheral nerve axons [35]. Bioinformatics analyses revealed that the most significantly enriched processes that overlapped in these two DEG sets are associated with cholesterol/sterol metabolism. A recent study demonstrated that abnormal lipid metabolism leads to axonal degeneration [36]. Cholesterol synthesis is vital for peripheral nervous system function, especially for axonal support and myelination by Schwann cells [37]. The transcriptional down-regulation of genes involved in the cholesterol synthesis pathway leads to myelin degeneration [38]. Furthermore, during loss of axonal contact with the motor endplate causes Schwann cells to reduce expression of the two major myelin proteins of the peripheral nervous system, myelin protein zero (P0) and peripheral myelin protein 22 (Pmp22). Interestingly, P0 is down-regulated in both the Sod1+/− mouse at 30 mo compared to 2 mo and in the Sod1+/− mice at 20 mo compared to the 2 mo, Pmp22 is down-regulated in the Sod1+/− mice at 30 mo compared to 2 mo but not in the Sod1+/− mice at 20 mo compared to the 2 mo. Thus, abnormalities in cholesterol metabolism may lead to abnormalities in both axons and myelin [29,30].

The survival of motor axons is dependent upon the presence of SOD1 [33]. Thus, it is not surprisingly that there is a loss of innervation in the hindlimb muscle in the Sod1+/− mice [12,33]. This loss of innervation provides evidence of axonal degeneration. The reduction we observed in axonal area in the motor axons of the ventral root in the Sod1+/− and Sod1+/+ mice at 30 mo is in agreement with previous work by Flood and colleagues [99] in the Sod1+/− mice [11], and provides additional evidence of axonal degeneration. Although demyelination observed in the normally aged Sod1+/−, we did not observe a significant difference in the area of myelin in the Sod1+/− mice. These data suggest that axonal degeneration rather than myelin degeneration occurs during aging associated with increased levels of oxidative damage.
Aberrant cholesterol metabolism is observed in neurodegenerative diseases [39]. A recent report linked aberrant cholesterol metabolism to mitochondrial dysfunction, axonal degeneration, and neuropathy [36]. The ultrastructural evaluation of the cell body of motor neurons in the Sod1−/− mice compared with the Sod1+/+ mice revealed the presence of abnormally shaped mitochondria. A previous study reported mitochondrial deficits in the Sod1−/− mice [40]. Thus, the loss of muscle fibers, atrophy, and axonal degeneration observed in the Sod1−/− mice and during normal aging [12] correlates with deficient cholesterol synthesis. A reduction in the levels of molecules involved in cholesterol trafficking and intracellular accumulations of cholesterol, and its precursors and metabolites were observed in neurons exposed to chronic levels of oxidative stress [41]. Hence, it is possible that the Schwann cells adapt to chronic levels of oxidative stress associated with aging by reducing cholesterol metabolic pathways; however, further studies are required to confirm this interaction.

If oxidative stress is solely responsible for aging, then the following three conditions must also be true: 1) oxidative damage increases with age, 2) manipulations to decrease oxidative stress would result in decreased cellular damage and an increased lifespan, and 3) manipulations that increase lifespan would result in a decrease in oxidative stress [42]. Over-expression of Sod1, a manipulation to decrease oxidative stress, increases the lifespan in Drosophila [43]; however, the same result was not produced in mice [0,44]. Although genetic manipulations in animal models that produce an increased lifespan appear to correlate with a reduction in oxidative stress/damage [42], oxidative stress is not solely responsible for aging. This is underscored by the fact that administration of antioxidants in clinical trials have either failed to provide a beneficial effect or significantly increased mortality [45]. Oxidative stress does, however, play a major role and increases with age in both human tissue and animal models [42].

Our study reveals that although the systemic presence of increased oxidative stress in the Sod1−/− mice is sufficient to cause functional deficits, structural abnormalities correlate with oxidative damage. The Sod1−/− mice are functionally normal at 2 mo of age but display an early onset of decreased motor function by 8 mo similar to the deficits associated with normal aging in the Sod1−/− mice at 30 mo. Studies investigating the differences in antioxidant capacities between motor and sensory neurons may provide useful information for understanding the differences in the sensitivity to oxidative damage. A previous study reported that Schwann cells express different motor and sensory phenotypes that regulate axonal support and regeneration [46]; thus, studying the potential differences in these properties in peripheral nerve may provide insight into why motor axons are more susceptible to oxidative damage than sensory axons. Furthermore, our future studies will evaluate the adaption response of Schwann cells in vitro to chronic oxidative stress. This is necessary to fully elucidate the role of cholesterol pathways in aging nervous system. Collectively, our data indicate that oxidative stress mediates motor nerve function deficits in the aging peripheral nervous system and that these deficits are due to abnormalities in axonal degeneration.

Supporting Information

Figure S1 Assessment of oxidative damage in the cell body of sensory neurons in young Sod1 mice. Oxidative damage was assessed in dorsal root ganglia neurons of 8 mo Sod1 mice by A) quantitatively assessing the autofluorescence of lipofuscin and western immunoblotting, and densitometry analysis of B) nitrated proteins (nitrotyrosine, NT) and C) oxidative lipid degradation (malondialdehyde, MDA). Sod1+/+ and Sod1−/− mice are represented by light gray and black/white strip bars, respectively; n≥4. (TIFF)

Figure S2 Assessment of oxidative damage in the motor neuron micro-environment in young Sod1 mice. Oxidative damage was assessed in the spinal cord of 8 mo Sod1 mice by A) quantitatively assessing the autofluorescence of lipofuscin and western immunoblotting, and densitometry analysis of B) nitrated proteins (NT) and C) oxidative lipid degradation (MDA). Sod1+/+ and Sod1−/− mice are represented by light gray and black/white strip bars, respectively; n≥4. (TIFF)

Table S1 List of DEGs. Fold-changes are given if the gene is a DEG. DEG/Set count indicates the number of DEG sets (out of 6 sets in total) in which the corresponding gene has been identified as a DEG. The total number of DEGs in each set is denoted in red. The two columns highlighted in yellow are the primary DEG sets used in the present study. Any DEGs that were also identified in the cholesterol- and aging-related literature are noted in the last three columns. #Paper; the number of papers in which the corresponding gene was identified by ScMiner; Enrichment: the fold-enrichment of the frequency (the number of papers with the corresponding gene/total number of cholesterol- and aging-related literature) compared to the frequency of the gene in the whole PubMed abstracts; BH P-value: Benjamini-Hochberg corrected p-value of the Fisher’s exact test to test the significance of the gene in the cholesterol- and aging-related literature. The genes are sorted by ‘DEG count’, and the fold-changes in the ‘2mKO_20mKO’ and ‘20mWT_30mWT’ sets. (PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: CS-R SVB ELF. Performed the experiments: CS-R JH JMH JRD PKJ. Analyzed the data: CS-R JH JMH JRD PKJ. Contributed reagents/materials/analysis tools: CS-R JH SVB ELF. Wrote the paper: CS-R. Revised the manuscript: CS-R JRH JMH JRD PKJ SVB ELF.

References

Increased Axonal Regeneration and Swellings in Intraepidermal Nerve Fibers Characterize Painful Phenotypes of Diabetic Neuropathy

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‡College of Literature, Science, and the Arts, University of Michigan, Ann Arbor, Michigan.

Abstract: We examined changes in intraepidermal nerve fibers (IENFs) to differentiate patients with diabetic neuropathy (DN) and diabetic neuropathic pain (DN-P) from those with DN without pain (DN-NOP). Punch skin biopsies were collected from the proximal thigh (PT) and distal leg (DL) of normal subjects, patients with type 2 diabetes without evidence of DN (DM), or DN-P and DN-NOP patients. Protein gene product 9.5-positive (PGP1) immunohistochemistry was used to quantify total IENF, and growth-associated protein 43 (GAP43) for regenerating IENF. Compared to normal subjects and patients with type 2 diabetes without evidence of DN, both DN-P and DN-NOP have reduced PGP1 IENF densities in DL and PT. Although GAP43 IENF densities were also reduced in DL for both DN-P and DN-NOP, the GAP43 IENF densities in PT of DN-P remained at the control levels. Higher GAP43/PGP ratios were detected in DN-P compared to DN-NOP in the DL and PT. In parallel, increased numbers of axonal swellings per PGP1 fiber (axonal swelling/PGP) were detected in DN-P compared to normal subjects, patients with type 2 diabetes without evidence of DN, and DN-NOP in the DL. These axonal swellings were positive for tropomyosin-receptor-kinase A and substance P, suggesting that they are associated with nociception. 

Perspective: Among patients with DN, the ratios of GAP43/PGP and axonal swelling/PGP are likely to differentiate painful from painless phenotypes.

Key words: Diabetic pain, intraepidermal nerve fiber, peripheral neuropathy.
without pain (DN-NOP). First, we examined protein gene product 9.5-positive (PGP+) IENF density (IENFD) using a standard protocol for quantifying IENFD in patients. Results from patients with DN-P\(^4\) were compared to normal subjects (NS), patients with diabetes without neuropathy (DM), and patients with DN-NOP. We next examined the degree of axonal regeneration by measuring the ratio of growth-associated protein 43 (GAP43), a marker for axonal regeneration, to PGP+ IENFs. Finally, the density of axonal swelling, a morphologic change associated with neuropathy, was measured using a previously published protocol by Lauria et al.\(^{13}\) Our results suggest that ratios of GAP43/PGP and axonal swelling/PGP could be useful biomarkers for characterizing DN-P.

**Methods**

**Patient Populations**

Skin samples were collected from NS, DM, DN-P, and DN-NOP patients (Table 1) participating in a longitudinal cohort study on DN (The Utah Diabetic Neuropathy Study [neuropathy@hsc.utah.edu]). Subjects fulfilling the pre-specified criteria for each group were randomly selected from the full Utah Diabetic Neuropathy Study cohort. All diabetic patients underwent a quantitative neurologic history and examination and a series of confirmatory neurophysiologic tests to diagnose and characterize neuropathy. Subjects were characterized based on 6 attributes: symptoms, signs (based on The Utah Early Neuropathy Scale >4), nerve conduction studies, quantitative sudomotor axon reflex testing, quantitative sensory testing for vibration detection and cold detection thresholds, and IENFD. Subjects with 0/6 abnormal attributes were classified as without neuropathy, subjects with 3 or more abnormal attributes as probable neuropathy, and subjects with 1 or 2 abnormal attributes as possible neuropathy.\(^{18}\) All DN patients in the current study had probable neuropathy for less than 5 years. Patients with a visual analog pain score (VAS) greater than 5 out of 100 were considered part of the DN-P group. All the DN-P patients had features of neuropathic pain (burning, tingling, electric-like, cramping, or aching pain) in both feet. Hemoglobin A1c (HbA1c) levels were measured for each patient. The study received approval by the institutional review board of the University of Utah, and written informed consent was obtained from all subjects.

**Skin Biomarkers Associated With Painful Diabetic Neuropathy**

**Human Skin Biopsy**

All subjects underwent 3-mm punch skin biopsies at the distal leg (DL; 10 cm above the lateral malleolus, ankle) and the proximal lateral aspect of the thigh (PT; 20 cm below the anterior iliac spine).\(^{11}\) Samples were immediately fixed in 0.75 M l-lysine solution (pH 7.4) with 2% paraformaldehyde and 0.05 mM sodium periodate for 12 to 24 hours at 4°C, cryoprotected in phosphate-buffered saline with 20% glycerol, and sectioned with a sliding microtome into 50-μm-thick free-floating sections before being processed for immunohistochemistry.

**Intraepidermal Nerve Fiber Analysis**

Three randomly selected sections were stained with each marker as previously described.\(^{15}\) Sections were incubated at 4°C for 16 to 24 hours with the following primary antibodies: PGP (1:1000; Millipore, Billerica, MA), Trk A (1:500; R&D Systems, Minneapolis, MN), SP (1:500; Abcam, Cambridge, MA), transient receptor potential cation channel subfamily V member 1 (TRPV1, 1:1000; Alomone labs, Jerusalem, Israel), and GAP43 (1:500; AbD Serotec, Raleigh, NC). Sections were then rinsed 3 times in phosphate-buffered saline and incubated with secondary antiserum conjugated with appropriate fluorophores (AlexaFluor 594 or 647; Invitrogen, Carlsbad, CA). Finally, sections were rinsed and mounted with ProLong Gold antifade reagent (Invitrogen). Fluorescent images were collected on an Olympus FluoView 500 confocal microscope using a 40 x 1.2 oil immersion objective at a resolution of 1040 x 1040 pixels. The optical section thickness was 0.5 μm. Forty images per stack were flattened using the MetaMorph (Molecular Devices, Sunnyvale, CA, version 6.14) arithmetic option.

IENFD data were presented as the mean number of fibers crossing the dermal/epidermal junction per linear millimeter of epidermis from all 6 skin sections.\(^{5}\) Axonal swelling was defined as a PGP+ globular axonal structure with a diameter >2 times that of the diameter of the attached axons. Axonal swelling densities were determined by calculating the mean number of PGP+-axonal swellings in the epidermis and subepidermal plexus per linear millimeter of epidermis from all 6 skin sections.\(^{13}\) All studies for the quantification of IENF and axonal swelling densities were performed in a blinded fashion. Sections were incubated with primary antiserum alone, and secondary antiserum alone to ensure specificity.

---

**Table 1. Demographic and Clinical Features of the Study Population**

<table>
<thead>
<tr>
<th></th>
<th>NS</th>
<th></th>
<th>DM</th>
<th></th>
<th>DN-P</th>
<th></th>
<th>DN-NOP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>61</td>
<td>53.5–65.5</td>
<td>57.5</td>
<td>52.3–64.8</td>
<td>58</td>
<td>48–61.5</td>
<td>59</td>
<td>52–61</td>
</tr>
<tr>
<td>Female/male</td>
<td>9/8</td>
<td></td>
<td>9/11</td>
<td></td>
<td>6/10</td>
<td></td>
<td>10/9</td>
<td></td>
</tr>
<tr>
<td>Mean HbA1c (%)</td>
<td>N/A</td>
<td></td>
<td>5.95</td>
<td>5.7–6.6</td>
<td>5.8</td>
<td>5.4–6.8</td>
<td>6.5†</td>
<td>6.0–8.0</td>
</tr>
<tr>
<td>Pain score (0–100)</td>
<td>0 (0–0)</td>
<td></td>
<td>0</td>
<td>0–0</td>
<td>50.0*</td>
<td>33.0–69.0</td>
<td>0</td>
<td>0–0</td>
</tr>
<tr>
<td>Months of diabetes</td>
<td>N/A</td>
<td></td>
<td>57</td>
<td>25–99.8</td>
<td>72</td>
<td>38–107.5</td>
<td>120†</td>
<td>60–156.0</td>
</tr>
</tbody>
</table>

*\(^{P < .0001}\) compared to DM and DN-NOP.
†\(^{P < .05}\) compared to DM and DN-P.
Nonspecific immunolabeling detected in either condition was eliminated during counting.

Data Presentation and Statistical Analyses
All data are presented as group medians with interquartile ranges (25th–75th percentile) and analyzed with a Mann-Whitney test. A P value less than .05 was considered statistically significant.

Results

Demographic and Clinical Characteristics
The demographic and clinical features of the study population are demonstrated in Table 1. Gender and age distribution were similar between groups. When comparing the clinical features between groups, the DN-NOP group had higher HbA1c levels than the DM and DN-P groups (P < .05 for both pairs). The mean pain score in the DN-P group was 50.0, which was significantly higher than pain scores of 0 in the NS controls, DM, and DN-NOP groups (P < .0001 for all pairs). Typically, DN-P patients presented with burning, tingling, prickling, and/or sharp stabbing foot pain. Additionally, the duration of diabetes was significantly longer in the DN-NOP group compared to the DM and DN-P groups (P < .05, Table 1).

Neurophysiologic tests, including the measurements of peroneal motor response proximal conduction velocity, peroneal motor response amplitude, sural sensory amplitude, quantitative sensory testing for cold and vibration detection thresholds, quantitative sudomotor axon reflex testing, and the Utah Early Neuropathy Scale, were performed for the diagnosis of DN. All measurements were consistent with the presence of neuropathy (Table 2). There was no significant difference in these parameters between the DN-P and DN-NOP groups.

Table 2. Parameters of Neuropathy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NS</th>
<th>DM</th>
<th>DN-P</th>
<th>DN-NOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophysiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMCV (m/s)</td>
<td>N/A</td>
<td>46.4</td>
<td>40.5***</td>
<td>41.8***</td>
</tr>
<tr>
<td>PMA</td>
<td>N/A</td>
<td>2.9**</td>
<td>2.3–4.0</td>
<td>3.0*</td>
</tr>
<tr>
<td>SSA</td>
<td>N/A</td>
<td>11.4</td>
<td>4.6**</td>
<td>3.0**</td>
</tr>
<tr>
<td>QST</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDT (%)</td>
<td>N/A</td>
<td>27.0</td>
<td>97.0****</td>
<td>97.0****</td>
</tr>
<tr>
<td>VDT (%)</td>
<td>N/A</td>
<td>62.5</td>
<td>90.0**</td>
<td>95.0****</td>
</tr>
<tr>
<td>QSART</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foot</td>
<td>N/A</td>
<td>1.19</td>
<td>0.38*</td>
<td>0.72</td>
</tr>
<tr>
<td>Distal leg</td>
<td>N/A</td>
<td>1.22</td>
<td>0.17****</td>
<td>0.12–0.74</td>
</tr>
<tr>
<td>Proximal thigh</td>
<td>N/A</td>
<td>0.73</td>
<td>0.21</td>
<td>1.05</td>
</tr>
<tr>
<td>UENS</td>
<td>0</td>
<td>0.0–2.0</td>
<td>9.0****</td>
<td>8.0**</td>
</tr>
</tbody>
</table>

Abbreviations: PMCV, peroneal motor response proximal conduction velocity; PMA, peroneal motor response amplitude; SSA, sural sensory amplitude; CDT and VDT, cold and vibration detection thresholds; QSART, quantitative sudomotor axon reflex testing; QST, quantitative sensory testing; UENS, Utah Early Neuropathy Scale.

Measurements From Distal Leg Skin
Although the DN-P subjects have pain in the feet, the current protocol for measuring IENFD involves collecting skin biopsies from 10 cm above the lateral malleolus (defined as DL) and 20 cm below the anterior iliac spine (defined as PT) as described previously. To evaluate the length-dependent changes in IENFs for patients with neuropathy, we performed immunohistochemistry for PGP and GAP43 on skin biopsies from both the DL (Table 3) and PT (Table 4) of patients from each group.

In the DL (Table 3), PGP+ IENFDs were significantly reduced in the DN-P and DN-NOP groups, compared to the NS and DM groups. There was no significant difference between the 2 DN groups. To examine the degree of axonal regeneration, GAP43 immunohistochemistry was performed (Fig 1). Although the GAP43+ IENFDs were significantly lower in both DN groups compared to the NS and DM groups, those of the DN-P group were higher than those of the DN-NOP group (P < .05). We then calculated the GAP43/PGP ratios to reflect the degree of axonal regeneration per IENF. Using this approach, GAP43/PGP ratios in DN-P were similar to those of the NS group and were significantly higher than DN-NOP (P < .001).

An axonal swelling is defined as an enlargement of an axonal segment with a diameter greater than 2 times that of the axons attached to it. The IENF axonal swellings were PGP+ globular structures that were distributed along the axons (Fig 2). Using the method described by Lauria and colleagues, we calculated the number of PGP+ axonal swellings per IENF (axonial swelling/PGP ratio) and observed a significant increase in axonal swelling/PGP ratio in the DL in DN-P patients compared to NS, DM, and DN-NOP patients (Table 3).

Measurements From Proximal Thigh Skin
In contrast to the DL, PGP+ IENFDs were not significantly different among the NS, DN-P, and
DN-NOP groups in the PT (Table 4). GAP43+ IENFDs were lower in the DN-NOP group compared to NS, DM, and DN-P groups. GAP43+ IENFD of DN-P patients remained at the NS and DM levels. The DN-NOP group presented with lower GAP43 IENFD (P < .0001) compared to the other 3 groups. In addition, GAP43/PGP levels of patients in the DN-NOP group were significantly reduced compared to those in the NS (P < .0001), DM (P < .001), and DN-P (P < .0001) groups. The DN-NP group had a higher ratio of axonal swellings/PGP in the PT compared to the NS (P < .001) and DN-NOP (P < .001) groups.

**Immunological Characterization of Axonal Swellings**

To further characterize the axonal swellings detected in IENFs in our DN-P skin samples, we performed double immunofluorescent studies for PGP and common pain markers, including Trk A, SP, TRPV1, and GAP43 (Fig 2). As demonstrated in Fig 2, most of these axonal swellings were positive for PGP (Fig 2A, arrow and arrowhead), Trk A (Fig 2B, arrow and arrowhead), SP (Fig 2D, arrow and arrowhead), and TRPV1 (Fig 2E, arrow and arrowhead). The percentages of GAP43+ axonal swellings that contained Trk A and SP immunoreactivity were quantified for DN-P and DN-NOP as demonstrated in Fig 2G. DN-P had higher percentages of Trk A+ and SP+ axonal swellings compared to those of DN-NOP (P < .05).

**Discussion**

Neuropathic pain from distal polyneuropathy is a common symptom that affects at least 40 to 50% of patients who suffer from diabetic neuropathy. However, only a subgroup of patients develops painful phenotypes (DN-P), whereas others with neuropathy have no pain (DN-NOP). Here, we present data to support the use of skin biopsy to study the distinct pathomechanisms of DN-P and DN-NOP. Our results indicate that PGP+ IENFD measurement, the most commonly used methodology for analyzing IENFs, may not be useful for specifically identifying DN-P among patients with DN. Instead, analysis of GAP43+ fibers and axonal swellings could provide further understanding of the underlying pathomechanisms that are specific to DN-P.

Our sample size was determined by the amount of available skin samples at the time of the study. Even with randomization, we detected higher HbA1C values and longer duration of diabetes in the DN-NOP group compared to DN-P. These findings suggest that increased severity and duration of diabetes could be confounding factors that contribute to the development of insensate neuropathy. This finding is supported by our animal data that suggest that mechanical allodynia in db/db mice is developed in the early phase, whereas loss of sensation occurs at later stages of diabetic neuropathy. However, follow-up studies are necessary to determine if DN-NP patients develop an insensate phenotype over time.

### Table 3. Measurements of IENF and Axonal Swelling Densities in DL Skin Biopsies

<table>
<thead>
<tr>
<th></th>
<th>NS</th>
<th>DM</th>
<th>DN-P</th>
<th>DN-NOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP+ IENFD (fiber/mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>9.76</td>
<td>10.7</td>
<td>2.62**</td>
<td>1.15–7.63</td>
</tr>
<tr>
<td>25th–75th percentile</td>
<td>6.08–13.15</td>
<td>6.87–15.5</td>
<td>1.07–8.83</td>
<td>5.62**</td>
</tr>
<tr>
<td>GAP43+ IENFD (fiber/mm)</td>
<td>3.97</td>
<td>5.12</td>
<td>1.64*</td>
<td>0.34–4.08</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
<td>1.04–7.99</td>
<td>0.35***</td>
</tr>
<tr>
<td>25th–75th percentile</td>
<td></td>
<td></td>
<td>0.80–1.55</td>
<td>2.5–8.07</td>
</tr>
<tr>
<td>GAP43/PGP</td>
<td>0.43</td>
<td>0.5</td>
<td>0.34†</td>
<td>0.15**</td>
</tr>
<tr>
<td>Median</td>
<td>0.00–2.96</td>
<td>0.05–1.75</td>
<td>0.27–0.47</td>
<td>0.09–0.20</td>
</tr>
<tr>
<td>25th–75th percentile</td>
<td></td>
<td></td>
<td>0.80†</td>
<td>2.3–6.3</td>
</tr>
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</table>

### Table 4. Measurements of IENF and Axonal Swelling Densities in PT Skin Biopsies

<table>
<thead>
<tr>
<th></th>
<th>NS</th>
<th>DM</th>
<th>DN-P</th>
<th>DN-NOP</th>
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</thead>
<tbody>
<tr>
<td>PGP9.5+ IENFD (fiber/mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>13.90</td>
<td>16.69</td>
<td>12.17†</td>
<td>11.01†</td>
</tr>
<tr>
<td>GAP43+ IENFD (fiber/mm)</td>
<td>11.22</td>
<td>11.73</td>
<td>9.99</td>
<td>0.41***</td>
</tr>
<tr>
<td>Median</td>
<td>7.90–15.21</td>
<td>5.43–14.60</td>
<td>5.32–14.05</td>
<td>0.32–0.58</td>
</tr>
<tr>
<td>25th–75th percentile</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAP43/PGP</td>
<td>0.74</td>
<td>0.67</td>
<td>0.82†</td>
<td>0.03**</td>
</tr>
<tr>
<td>Median</td>
<td>0.66–0.84</td>
<td>0.41–0.83</td>
<td>0.75–0.88</td>
<td>0.03–0.06</td>
</tr>
<tr>
<td>25th–75th percentile</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axonal swelling/PGP</td>
<td>1.20</td>
<td>4.29*</td>
<td>5.62**</td>
<td>2.16†</td>
</tr>
<tr>
<td>Median</td>
<td>0.46–2.65</td>
<td>1.15–7.63</td>
<td>2.55–8.07</td>
<td>1.22–3.16</td>
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<tr>
<td>25th–75th percentile</td>
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</table>

*P < .05, **P < .01, ***P < .001, ****P < .0001 compared to NS.
|P < .05, | |P < .0001 compared to DM.
|P < .001, | |P < .0001 compared to DN-P.
Our data suggest that measurement of PGP-IENFDs, the most widely used method in current practice for small-fiber neuropathy, is not a useful method for the diagnosis of DN-P. In support of our current data, loss of PGP-IENFs in animals and humans with DN-P from both type 1 and type 2 diabetes have been reported in the literature. Sorensen and colleagues specifically studied the relationship among pain severity and loss of PGP-IENFs in the DL of patients with both types of diabetes. They concluded that the severity of PGP-IENF loss is associated only in patients with neuropathic pain with no objective signs of neuropathy, suggesting that this information could be useful to acknowledge early stages of DN-P. In our study, both DN-P and DN-NOP patients had significant features of neuropathy. However, we did not detect a significant correlation between IENFD loss and the severity of pain (data not shown).

GAP43 is a neuronal membrane protein involved in axonal growth and regeneration. Our data demonstrated an increased GAP43/PGP ratio in DN-P, suggesting increased regeneration of IENFs in DN-P skin. Axonal regeneration is a well-known feature of DN; however, the link between axonal regeneration and pain is still unclear. Previously, we reported increased NGF-mediated nerve

![Figure 1. GAP43+ IENFs in DN-P. Representative images of GAP43 immunohistochemistry on skin biopsies from DN-P (A) and DN-NOP (B) patients. Prominent GAP43+ fibers (arrows) were detected in DN-P, but not in DN-NOP. Bar = 10 μm. Abbreviation: Epi, epidermis.](image1)

![Figure 2. Immunohistochemical characterization of axonal swellings from DN-P skin samples. (A–C) Axonal swellings (arrows and arrowheads) were identified along IENFs (arrowheads) and in the subepidermal plexus (arrows). Most of these structures were positive for both PGP (A, C) and Trk A (B, C). Bar = 50 μm. (D–F) Some axonal swellings in DN-P were positive for both SP (arrow, D) and TRPV1 (arrow, E), or either one of these pain markers (D–F, arrowheads). Bar = 25 μm. Abbreviation: Epi, epidermis. (G) Percentages of PGP+ axonal swellings that contain Trk A and SP immunoreactivity in DN-P and DN-NOP. *P < .05.](image2)
regeneration in IENFs of db/db mice during the period of mechanical allodynia. In that study, anti-NGF antiserum treatment reduced axonal regeneration and pain behavior, suggesting that axonal regeneration mediates painful phenotypes. In support of our hypothesis, Britland et al reported that increased axonal regeneration is detected in painful phenotypes in sural nerve biopsies of DN. Fantini and colleagues reported colocalization of GAP43 and Trk A in human skin, suggesting the regeneration of nociceptive nerve fibers. The current study confirms our previous findings and supports our hypothesis that targeting NGF-mediated nerve regeneration could be a viable therapeutic strategy for treating DN-P associated with type 2 diabetes.

In the current study, we detected increased axonal swellings in IENFs, previously reported as early axonal changes of sensory neuropathy. These axonal swellings could be associated with an increased risk of developing sensory symptoms in suspected small fiber neuropathy. Similar to our current results, the report by Lauria and colleagues demonstrated higher axonal swelling/PGP ratios in the DL of patients with painful neuropathy from a variety of causes, including diabetes, in comparison to healthy controls. Nonetheless, we observe similar findings at both the PT and DL in patients with type 2 diabetes in the DN-P group.

The current results demonstrate that axonal swellings in DN-P are positive for Trk A, SP, and TRPV1. Previously, axonal swellings from neuropathy were characterized by Lauria and colleagues, whose results indicated that the swellings contain components of microtubules and ubiquitin-associated proteins. In addition, Ebenezer and colleagues studied the microstructure of axonal swellings using electron microscopy and reported that axonal swellings contain an accumulation of mitochondria, vesicular organelles, and neurofilaments. These reports suggest that axonal swelling could be a result of defective axonal transport, a common feature of diabetic neuropathy.

Acknowledgments
The authors thank Dr. Brian Callaghan and Dr. Junguk Hur for assisting with statistical analysis; and John Hayes and Chelsea Lindblad for technical assistance. This work utilized the Morphology and Image Analysis Core of the Michigan Diabetes Research and Training Center funded by NIH (5P60 DK20572) from the National Institute of Diabetes & Digestive & Kidney Diseases.

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Identification of Factors Associated With Sural Nerve Regeneration and Degeneration in Diabetic Neuropathy

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Brian C. Callaghan, MD
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Eva L. Feldman, MD, PhD

OBJECTIVE—Patients with diabetic neuropathy (DN) demonstrate variable degrees of nerve regeneration and degeneration. Our aim was to identify risk factors associated with sural nerve regeneration in patients with DN.

RESEARCH DESIGN AND METHODS—Demographic, anthropometric, biochemical, and anatomical data of subjects with DN from a 52-week trial of acetyl-L-carnitine were retrospectively examined. Based on the change in sural nerve myelinated fiber density (ΔMFD%), subjects were divided into three groups: regenerator (top 16 percentiles, n = 67), degenerator (bottom 16 percentiles, n = 67), and intermediate (n = 290), with dramatically increased, decreased, and steady ΔMFD%, respectively. ANOVA, Fisher exact test, and multifactorial logistic regression were used to evaluate statistical significance.

RESULTS—ΔMFD% were 35.6 ± 17.4 (regenerator), −4.8 ± 12.1 (intermediate), and −39.8 ± 11.0 (degenerator). HbA1c at baseline was the only factor significantly different across the three groups (P = 0.01). In multifactorial logistic regression, HbA1c at baseline was also the only risk factor significantly different between regenerator (8.3 ± 1.6%) and degenerator (9.2 ± 1.8%) (odds ratio 0.68 [95% CI 0.54–0.85]; P < 0.01). Support Vector Machine classification using HbA1c demonstrated 62.4% accuracy of classifying subjects into regenerator or degenerator. A preliminary microarray experiment revealed that upregulated genes in the regenerator group are enriched with cell cycle and myelin sheath functions, while downregulated genes are enriched in immune/inflammatory responses.

CONCLUSIONS—These data, based on the largest cohort with ΔMFD% information, suggest that HbA1c levels predict myelinated nerve fiber regeneration and degeneration in patients with DN. Therefore, maintaining optimal blood glucose control is likely essential in patients with DN to prevent continued nerve injury.

Twenty-five million Americans, or >8% of the population, have diabetes, and 1.9 million new cases were diagnosed in 2010 (1). In the U.S., the total cost for management of diabetes in 2007 was 218 billion USD (1). Complications of diabetes, including diabetic neuropathy (DN), nephropathy, and retinopathy, often have a significant impact on quality of life. DN is the most common diabetes complication; 60–70% of diabetic patients develop DN (2). DN is responsible for >60% of nontraumatic lower-limb amputations (1,3). Management of DN-related complications accounts for an estimated 27% of the total cost of diabetes treatment (3).

The most common type of DN is distal symmetric polyneuropathy. It affects the longest axons in the extremities first and progresses proximally in a stocking-glove pattern with increasing severity and duration of diabetes (2). The sural nerve is one of the most frequently affected nerves in DN. Although overall sural myelinated fiber density (MFD) decreases with age, the nerve itself can regenerate, making the grafting of sural nerves into other injured nerves possible (4). Axonal regeneration is a natural response of the body to compensate for damage caused by diabetes, but incomplete or unsuccessful regeneration may constitute a critical component in DN progression (5).

Our laboratory maintains a unique repository of human sural nerve biopsies harvested as part of a double-blind placebo-controlled clinical trial testing acetyl-L-carnitine (ALC) efficacy for DN (6,7). ALC treatment alleviated pain symptoms but had no effect on sural nerve conduction velocities (NCVs), amplitudes, or MFD (6). Our initial demographic analyses of these participants revealed that elevated serum triglycerides measured at trial onset correlated with DN progression after correcting for baseline DN severity and clinical factors, such as sex, age, duration and types of diabetes, insulin treatment, ALC treatment, and HbA1c (7). A subsequent study identified 532 differentially expressed genes (DEGs) between progressive and nonprogressive DN, highly enriched with immune response and lipid metabolism (8). Our previous studies focused on the loss of absolute MFD over the course of a 52-week clinical trial, resulting into two groups of patients (a regressor group with ≥500 fibers/mm² MFD loss and a nonregressor group with ≤100 fibers/mm² MFD loss). While reexamining these data, we observed that ~43% of the subjects gained MFD over 52 weeks. Although modest regeneration has been documented in DN (9), no study has investigated critical factors affecting nerve regeneration in DN.

In the current study, we reexamined this DN cohort with MFD data available to identify critical factors that may impact sural nerve regeneration, focusing on subjects with the greatest gain or loss of MFD. As patients at different ages and
HbA1c predicts peripheral nerve change in DN

Table 1—Characteristics of subjects and statistical evaluation results

<table>
<thead>
<tr>
<th></th>
<th>Degen</th>
<th>Inter</th>
<th>Regen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total subjects</td>
<td>67</td>
<td>290</td>
<td>67</td>
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<tr>
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<tr>
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<td>No</td>
<td>31</td>
<td>109</td>
<td>35</td>
</tr>
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<td>Diabetes type</td>
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<tr>
<td>Type 2</td>
<td>51</td>
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<tr>
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<td>53.6</td>
<td>53.6</td>
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<tr>
<td>BMI (kg/m²)</td>
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<td>29.5</td>
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<tr>
<td>Diabetes duration (years)</td>
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<td>Cholesterol (mmol/L)</td>
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<td>Albumin (mmol/L)</td>
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<td>Hematocrit (fraction)</td>
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<td>0.5</td>
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<td>O'Brien neuropathy score</td>
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<td>3,427.6</td>
<td>3,427.6</td>
</tr>
<tr>
<td>MFD baseline (fibers/mm²)</td>
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<td>2,949.2</td>
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<td>MFD 52 weeks (fibers/mm²)</td>
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<td>MFD change (fibers/mm²)</td>
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<tr>
<td>MFD percent change (%)</td>
<td>-39.8</td>
<td>-48.8</td>
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</tbody>
</table>

Degen, degenerator; Inter, intermediate; Regen, regenerator. *Significant, P < 0.05.

The primary outcome measure was ΔMFD% at week 52. Three outlier subjects with >200% increase in MFD were excluded from any further analyses. In the remaining 424 subjects (ΔMFD% range -78.6 to 87.7%), 183 subjects (42.9%) demonstrated positive ΔMFD%. Based on ΔMFD%, the subjects were divided into three groups: regenerator (top 16 percentiles equivalent to beyond 1 SD from the mean); degenerator (bottom 16 percentiles), and intermediate (remaining subjects).

Neuropathy evaluations
Electrophysiological measurements, including bilateral sural NCV and amplitude, peroneal NCV, and amplitude on the dominant side and median motor and
sensory NCV and amplitude on the non-dominant side, were performed the baseline and completion of the trial to generate an O’Brien neuropathy score (6). These measurements were done in triplicate, and the median value was used.

**Computational classifier for regenerator and degenerator**

Computational classifiers of regenerator and degenerator were generated and evaluated using ORANGE (http://orange.biolab.si/), an open-source, component-based data-mining and machine learning software suite (11). Seven classification algorithms (Naive Bayes, Logistic Regression, k Nearest Neighbors, Classification Tree, CN2 rules, Support Vector Machine [SVM], and Random Forest) available for binary class prediction were used with 20-fold cross-validation sampling to classify the subjects as regenerator or degenerator based on the demographic, anthropometric, and biochemical data.

**Microarray data analysis**

Based on the new grouping, we reanalyzed the previously published microarray data set (8) and an additional batch of unpublished microarray data (n = 35 and n = 33, respectively). These 68 microarrays included samples from 14 degenerators, 7 regenerators, and 45 intermediates. Microarrays were normalized using Robust Multiarray Average (12), and the batch effect was corrected using the distance-weighted discrimination method (13). Intensity-based moderated T statistics (14) was used to determine the DEGs among the groups. Owing to the small number of available microarrays (7 regenerators), a nominal P value of 0.05 without multiple testing corrections was used as the cutoff for DEGs.

The identified DEGs were further analyzed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) (15,16), to determine overrepresented biological functions in terms of Gene Ontology (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/). LRpath (http://lrpath.ncbi.org/), a logistic regression-based genome enrichment testing tool, was also used in our analysis. LRpath accepts statistical significance values from all genes on the tested array and does not require a predefined DEG set (17).

**Correlation between regeneration cluster density and MFD change**

Electron microscopy (EM) was performed on the baseline and/or 52-week biopsies of approximately half of the subjects (n = 219) immediately after the termination of the 52-week trial. The number of regenerating nerve clusters were counted, and the density of regenerating clusters was calculated (6). In the current study, we correlated the ΔMFD% over 52 weeks with changes in the density of regenerating clusters for subjects with both baseline and 52-week biopsies examined by EM (n = 168).

**Statistical analysis**

Variable differences between the groups were analyzed with the Fisher exact test for categorical variables and ANOVA with Bonferroni post hoc tests for continuous variables. Multifactorial logistic regression between the regenerator and degenerator groups was performed to evaluate the effect of multiple factors including age, sex, ALC treatment, diabetes type, diabetes duration, HbA1c, insulin treatment, BMI, triglyceride, cholesterol, albumin, hematocrit, and O’Brien neuropathy rank-sum score (7,18). The statistical significance level was set at 0.05. For statistical analyses, R, version 2.15.2 (http://cran.r-project.org/), was used. Data are means ± SD or percentage unless otherwise stated.

**RESULTS**

**Group classification**

Based on the ΔMFD%, subjects were divided into three groups: regenerator (n = 67), degenerator (n = 67), and intermediate (n = 290). The mean ΔMFD% ± SD were 35.6 ± 17.4 (regenerator), −4.8 ± 12.1 (intermediate), and −39.8 ± 11.0 (degenerator). Table 1 summarizes the demographic, anthropometric, and biochemical characteristics for all subjects, comparing subjects from regenerator, degenerator, and intermediate groups by ANOVA and Fisher exact tests.

At baseline, there were no differences between groups in age, sex, diabetes duration or type, BMI, triglyceride, or total cholesterol. There were also no significant differences between groups in the number of subjects randomized to ALC or subjects treated with insulin. In addition, there were no differences between MFD at baseline between degenerator and regenerator, but MFD was significantly higher at baseline in the intermediate group (P = 8.34E-08). The groups differed in the O’Brien neuropathy rank-sum score, based on the electrophysiological measurements, with the lowest score observed in the degenerator group (2,839.8 ± 1,167.5) and the highest score in the regenerator group (3,427.6 ± 1,132.7) and intermediate (3,563.4 ± 1,119.7) groups (P = 1.88E-05). Among the other evaluated risk factors shown in Table 1, HbA1c at baseline was the only risk factor significantly different across the three groups: regenerator (8.3 ± 1.6%), degenerator (9.2 ± 1.8%), and intermediate (8.8 ± 1.7%) (P = 0.01).

To further understand potential reasons that would drive regeneration or degeneration, we next compared the two extreme groups (degenerator vs. regenerator). Again, HbA1c level was the only significantly different biochemical factor (Bonferroni-corrected P = 0.01) between the two groups, while all other variables listed were not
HbA1c predicts peripheral nerve change in DN

Predicted Class: Degenerator

Figure 1—Receiver operating characteristic of the classifiers. The seven machine learning classification algorithms available in ORANGE were evaluated for classifying DN patient regenerator and degenerator groups. Classifiers were trained on the HbA1c levels of the subjects from these two groups, and testing was performed in 20-fold cross-validation. CN2, Clark Niblett 2; FP, false positive; kNN, k-nearest neighbor; TP, true positive.

significantly different (Table 1). The multivariable logistic regression analysis also confirmed that the HbA1c level at baseline was the only significantly different factor (odd ratio [OR] 0.68 [95% CI 0.54–0.85]; P < 0.01) with other variables adjusted (Table 2). The regenerator group included more females and more patients with type 1 diabetes; however, the differences were not statistically significant. Interestingly, although these two groups had similar baseline MFD (2,949.2 ± 1,504.5 [degenerator] and 3,100.0 ± 1,634.9 [regenerator]; P = 1), the baseline O’Brien score was significantly lower in the degenerator group compared with the regenerator group (Table 1) (2,839.8 ± 1,167.5 vs. 3,427.6 ± 1,132.7 respectively, P = 0.01).

Computational classifier for regenerator and degenerator

A machine-learning approach was used to test whether risk factors may predict the classification category outcome of participants with DN. Among the seven machine learning algorithms using a 20-fold cross-validation, two algorithms (SVM and logistic regression) achieved a classification accuracy (CA) >60%, with logistic regression being the best classifier (CA = 62.7%). Figure 1 illustrates receiver operating characteristic curves of the evaluated classifiers and indicates that SVM and logistic regression are the best classifiers. The addition of other factors resulted in degraded classification performance; however, O’Brien neuropathy score slightly improved the classifiers, with SVM achieving the highest CA of 64.2%.

Microarray data analysis

To examine the gene expression profiles that are significantly different between the two extreme groups, two batches of human sural nerve microarray datasets were combined (one published [8]). Intensity-based moderated T statistics identified a total of 490 DEGs between regenerator (n = 7) and degenerator (n = 15) at a nominal P value of 0.05 without multiple testing corrections. Supplementary Table 1 lists the 10 most upregulated and 10 most downregulated DEGs. Multiple immune-related genes such as CD177 molecule (CD177) (19), human leukocyte antigen (HLA) complex group 4 (HCG4), and chemokine (C-X-C motif) ligand 10 (CXCL10) (20) are upregulated in regenerator, indicating possible activation of multiple immune cell types such as neutrophils (19) and natural killer cells (20).

Table 3 lists the top 20 concepts (gene sets defined by biological functional terms such as Gene Ontology terms) identified by LRpath that have a significantly lower false discovery rate (FDR) for differential gene expression. Although some immune activation gene markers (CD177 and CXCL10) were markedly upregulated in regenerator, LRpath suggests that genes associated with immune response (FDR = 2.23E-08), defense response (FDR = 3.73E-07), and inflammatory response (FDR = 6.16E-04) were generally downregulated in regenerator. The top concepts upregulated in regenerator included condensed chromosome (FDR = 4.47E-04) and transmission of nerve impulse (FDR = 5.44E-04). Supplementary Table 2 lists all the significant genes in these 20 top concepts. DAVID, another gene set enrichment analysis tool, identified biological functional terms significantly overrepresented in the 490 DEG set. A heat map (Supplementary Fig. 1) was generated to summarize the most significant functions and indicates that the genes upregulated in regenerators were highly enriched with cell cycle, suggesting active regeneration.

Correlation with regeneration clusters

Approximately one-third (n = 168) of the study subjects had their baseline and 52-week biopsies examined by EM, and the Pearson correlation coefficient between ΔMFD% and the regeneration fiber density change was 0.33 (P < 0.001). When the analysis is limited only to the regenerator and degenerator groups, the correlation coefficient was 0.35 (P = 0.014 with n = 48). Although the correlation is not strong, the data suggest that the change in MFD is partially reflected in the decreased level of nerve regeneration. We also
examined whether HbA1c level is correlated with the absolute density and the change of the regenerating clusters. The correlation between baseline HbA1c and the changes in regenerating clustering density was 0.02 for all 168 subjects; however, this correlation became −0.15 with the analysis limited to the regenerator and degenerator groups (n = 48). Although this does not reach the statistical significance cutoff, the negative correlation values suggest that there is a trend for decreased regeneration cluster density over 52 weeks with a higher baseline HbA1c level. However, no linear correlation was observed between the baseline HbA1c level and the baseline regeneration cluster density (correlation coefficient = 0.01 in both sets using all subjects and the two extreme sets). These results suggest that the HbA1c level at a certain time point may not be predictive of the absolute level of the regenerating cluster density but may partially predict the changes in the regenerating cluster and MFD over time.

**CONCLUSIONS**—Previous analyses by our group of human sural nerve biopsies harvested as part of a double-blind, placebo-controlled, 52-week trial of ALC for DN (6,7) revealed that elevated serum triglycerides measured at trial onset correlate with DN progression (7) and that the alterations in immune response and lipid metabolism genes are also associated with progressive DN (8). Further examination of these data, however, revealed that MFD improved in ∼43% of the subjects, and although modest regeneration has been documented in DN (9), no study has investigated critical factors affecting nerve regeneration in DN. In the current study, we examined demographic, anthropometric, and biochemical data of these subjects to identify the potential risk factors that correlate with myelinated nerve fiber regeneration and degeneration. We found that HbA1c was the only factor significantly associated with regeneration and degeneration. In fact, the baseline HbA1c level alone was able to correctly classify 62.7% of the subjects as a regenerator or degenerator.

This study is an extension of the previously published analysis of the same cohort and was pursued in an attempt to better understand factors contributing to nerve fiber degeneration associated with DN. The previous study used the absolute loss of MFD over the course of the 52-week clinical trial as the classifier, resulting in three groups of patients (progressor group with ≥500 fibers/mm² of MFD loss, nonprogressor group with ≤100 fibers/mm² of MFD loss, and intermediate group for the remainders) (7,8). Therefore, the nonprogressor group in the previous study comprised all of the regenerator subjects from the current study, as well as a large portion of the intermediate subjects. Furthermore, the present subject groups were selected using the ΔMFD% as the classifier rather than absolute MFD change, as patients at different ages and with different durations of diabetes tend to have substantially variable levels of baseline MFD. Thus, the percent change, rather than absolute change of MFD, was used to evaluate the effects of several important clinical factors shown to contribute to DN progression.

Peripheral nerves undergo spontaneous regeneration upon injury; however, the risk factors in diabetes affecting nerve regeneration and degeneration are not clearly understood. Although the overall MFD decreases with age, the nerve itself may regenerate either spontaneously or in response to external stimuli (21). Axonal regeneration actively takes place as a natural compensatory response to damage caused by diabetes, but incomplete or unsuccessful regeneration may constitute a critical component of DN progression (3). We anticipated that genes related to axonal regeneration or cell growth would be more actively expressed in the
regenerator compared with the degenera-
tor group.

Microarray analyses confirmed that
those genes involved in cell cycle func-
tions are highly upregulated in regen-
erator compared with degenerator. Biologi-
cal functions associated with neu-
ron projection and myelin sheath were
highly enriched in those genes upregu-
lated in regenerator compared with the
intermediate group. These functions were
not significantly overrepresented in the
DEGs between degenerator and regener-
ator, which is probably due to the low
power of detecting differential expression
with a limited number of samples. The
results should be only considered as
preliminary, and more samples need to be
processed to increase the statistical power.
It should also be noted that Schwann cells
are major contributors to the mRNA in the
sural nerve biopsies, with a small contribu-
tion coming from axons, epineural fi-
broblasts, adipocytes, vascular endothelial
cells, and immune cells such as macro-
phages. Therefore, the gene expression
changes observed by microarray are most
likely to represent the changes in Schwann
cells in response to diabetes.

Another interesting finding is the fact
that in spite of similar MFD at baseline in
the degenerator and regenerator groups,
the degenerator group had a much lower
O’Brien neuropathy score. The O’Brien
scores are accepted methods to quantify
multiple electrophysiological measure-
ments obtained from nerve conduction
studies (NCSs). NCSs assess mostly large
myelinated nerve fiber function and are still
considered by most as the gold standard
end point for DN in clinical trials (22).
Our findings suggest that nerve fiber func-
tion as assessed by NCS may be decreased
before an anatomical loss of myelinated
fibers and can be used in combination with
other factors (such as HbA1c) to predict
nerve fiber degeneration.

Fiber regeneration delays have been
observed in both the tibial (largely motor)
and sural (sensory) distal sciatic branches
after both sciatic nerve crush injury and
complete sciatic nerve transection in
streptozocin-treated mice, a type 1 di-
abetes animal model (23). Interestingly,
macrophage invasion was associated with
the delay in this model, supporting a po-
tential mechanism for impaired regenera-
tion due to abnormal macrophage
participation in nerve repair (23); how-
ever, the role of macrophages in nerve re-
pair is still controversial (24–26).

According to our microarray data,
microphage differentiation had a FDR of
0.018. Although only one gene, THO
complex 5 (THOC5), was deemed a sig-
nificant gene according to this concept,
the overall changes of genes related to
macrophage differentiation and other similar
terms, such as macrophage activation,
were found to be downregulated in regen-
erator by LPath. More studies on the po-
tential role of macrophages will be necessary
to elucidate the exact mechanisms.

Study limitations include that blood
chemistry data were only available at
baseline and no subsequent measuring
was done during or at the end of the trial.
Therefore, controlling for in-trial changes
in the covariates analyzed was not possi-
bile. It is possible that the overall HbA1c
levels changed during the trial and data
regarding lifestyle or diet changes were
not available. In addition, even though
the study cohort is the largest one avail-
able to date with ΔMFD% information,
we may have lacked sufficient power to
detect meaningful effects for all risk fac-
tors. Although diabetes type did not
have a statistically significant effect on re-
generator and degenerator classification
in the current study, future separate anal-
yses of type 1 and 2 diabetic subjects may
be informative and will be pursued.

In the current study, we evaluated
potential biomarkers and gene expression
profiles of the sural nerve biopsies from
the largest available DN patient cohort
with ΔMFD% information in order to as-
certain factors associated with nerve re-
generation and degeneration in DN. The
data suggest that HbA1c levels are sig-
nificantly associated with the nerve regener-
ation and degeneration and may be
predictive of future sural peripheral nerve
regeneration. The microarray data suggest
that immune and inflammatory responses
may play a crucial role in nerve regenera-
tion and degeneration. Although the ex-
act mechanisms must still be elucidated,
these data indicate that optimal blood
glucose control in patients with DN is
likely to impact sural nerve regeneration
and that the immune response may play
an important role in this process.

Acknowledgments—This work was sup-
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to this article were reported.

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Long-Chain Acyl Coenzyme A Synthetase 1 Overexpression in Primary Cultured Schwann Cells Prevents Long Chain Fatty Acid-Induced Oxidative Stress and Mitochondrial Dysfunction

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Abstract

Aims: High circulating long chain fatty acids (LCFAs) are implicated in diabetic neuropathy (DN) development. Expression of the long-chain acyl-CoA synthetase 1 (Acsl1) gene, a gene required for LCFA metabolic activation, is altered in human and mouse diabetic peripheral nerve. We assessed the significance of Acsl1 upregulation in primary cultured Schwann cells. Results: Acsl1 overexpression prevented oxidative stress (nitrotyrosine; hydroxyoctadecadienoic acids [HODEs]) and attenuated cellular injury (TUNEL) in Schwann cells following 12 h exposure to LCFAs (palmitate, linoleate, and oleate, 100 μM). Acsl1 overexpression potentiated the observed increase in medium to long-chain acyl-carnitines following 12 h LCFA exposure. Data are consistent with increased mitochondrial LCFA uptake, largely directed to incomplete beta-oxidation. LCFAs uncoupled mitochondrial oxygen consumption from ATP production. Acsl1 overexpression corrected mitochondrial dysfunction, increasing coupling efficiency and decreasing proton leak. Innovation: Schwann cell mitochondrial function is critical for peripheral nerve function, but research on Schwann cell mitochondrial dysfunction in response to hyperlipidemia is minimal. We demonstrate that high levels of a physiologically relevant mixture of LCFA induce Schwann cell injury, but that improved mitochondrial uptake and metabolism attenuate this lipotoxicity. Conclusion: Acsl1 overexpression improves Schwann cell function and survival following high LCFA exposure in vitro; however, the observed endogenous Acsl1 upregulation in peripheral nerve in response to diabetes is not sufficient to prevent the development of DN in murine models of DN. Therefore, targeted improvement in Schwann cell metabolic disposal of LCFA may improve DN phenotypes. Antioxid. Redox Signal. 00, 000–000.

Introduction

Diabetic neuropathy (DN) is a prevalent complication of diabetes and affects ~60% of the 26 million people with prediabetes and diabetes in the United States (6, 32). The consequences of DN, including chronic pain or loss of sensation, recurrent foot ulcerations, and amputation, are responsible for significant morbidity and high economic impact (10). Dyslipidemia is a recognized risk factor for the development of DN (1, 30, 40). Lipid profiles are commonly abnormal early in the course of type 2 diabetes and correlate with the onset of early DN (7). While glucose-induced oxidative stress is a well-studied mechanism underlying the pathogenesis of DN (16, 19, 26, 36–38), recent data from both diabetic subjects and murine models of type 2 diabetes strongly suggest a role for dyslipidemia and lipid-mediated oxidative stress in the onset and progression of DN (30, 34). The goal of our research is to understand how both glucose- and lipid-mediated oxidative stress lead to injury in cells of the peripheral nervous system, resulting in DN. Our hope is to...
ultimately discover mechanism-based therapies that can prevent this injury cascade and ameliorate the signs and symptoms of DN (33, 35–38).

Schwann cells are the support cells of the peripheral nervous system and are required for peripheral nerve health, maintenance, and recovery from injury. Schwann cell-specific knockout of the mitochondrial transcription factor A gene (Tfam) in mice (29) induces peripheral nerve disease that strikingly resembles that seen in mouse models of DN (25), highlighting the necessity of normal Schwann cell mitochondrial function for long-term support of peripheral axons (8).

Our laboratory has completed a series of microarray studies on human and mouse diabetic peripheral nerves (13, 21), primarily assessing gene expression changes within Schwann cells. We have found increased mitochondrial long-chain acyl-CoA synthetase 1 (Acsl1), carnitine palmitoyltransferase 1a (Cpt1a), carnitine palmitoyltransferase 1b (Cpt1b), and carnitine/acyl-carnitine translocase (CACT) gene expression in sciatic nerves (SCN) from a murine model of type 2 diabetes with DN, the leptin receptor-deficient db/db mouse model (21), expressing in cultured Schwann cells (Supplementary Fig. S2). In response to incubation in high LCFA (∼5 mM), Schwann cells increase Acsl1 protein expression by 2.7-fold (∼5 mM) in culture media, Schwann cells increase Acsl1 protein expression by 2.7-fold (∼5 mM) in culture media.

In the current study, we examined mitochondrial metabolism, oxidative stress, and cellular injury in response to a high LCFA environment in primary Schwann cells. We report that high levels of a physiologically relevant mixture of saturated, monounsaturated, and polyunsaturated LCFA induce mitochondrial dysfunction and oxidative stress in primary Schwann cells. Acsl1 overexpression significantly improves mitochondrial function, ameliorates oxidative stress, and restores Schwann cell viability. We conclude that Acsl1 overexpression improves Schwann cell function and survival in an in vitro high LCFA environment. However, endogenous Acsl1 upregulation in the db/db mouse SCN is not sufficient to prevent the development of DN in the complex and chronic in vivo diabetic environment. Our data support the growing body of literature that lipotoxicity is a pathomechanism underlying DN and suggest that therapeutically targeting Schwann cell metabolic disposal of LCFAs could provide a novel therapy for DN.

**Results**

db/db mice exhibit hypertriglyceridemia, nerve-specific oxidative stress, and Acsl1 protein upregulation

A mutation in the leptin receptor of the db/db mouse results in hyperphagia, severe obesity, hyperlipidemia, hyperinsulinemia, and hyperglycemia beginning at ∼4 weeks of age (Jackson Laboratories; 000642). Significant increases in oxidative modification were observed in db/db mouse SCN extracts compared with those of age-matched controls, as evidenced by increased nitrotyrosine (nitrosylated proteins) and increased hydroxyoctadecadienoic acids (HODEs) (lipid peroxidation) (Supplementary Fig. S1A, B; Supplementary Data are available online at www.liebertpub.com/ars). Significant elevation of fasting plasma triglycerides in db/db mice at 8 and 24 weeks of age compared with age-matched db/+ controls ($p<0.01$) was also confirmed, with this diabetic elevation significantly increased between 8 and 24 weeks of age ($p<0.01$) (Supplementary Fig. S1C). We performed fast protein liquid chromatography (FPLC) fractionation to compare the plasma lipoprotein profiles and confirmed these triglycerides are predominantly held within the VLDL class of lipoproteins (Supplementary Fig. S1D). VLDL triglycerides are comprised of LCFAs and are thus substrates for Acsl1, leading us to explore the significance of regulated Acsl1 gene expression in the db/db SCN. To verify that the upregulated mRNA is translated to a biological protein, we performed western immunoblotting on homogenized whole SCN (Fig. 1). Acsl1 protein expression was significantly increased in db/db SCN compared with age-matched db/+ SCN (8 weeks, 3.5-fold increase, $p<0.05$; 24 weeks, 4.0-fold increase, $p<0.01$). Together, these data confirm that the db/db type 2 diabetes mouse model exhibits altered triglyceride profiles and oxidative modifications that are correlated with Acsl1 gene and protein regulation.

**Incubation in high LCFA increases Acsl1 protein expression in primary cultured Schwann cells**

To examine the relationship between LCFA metabolism and Acsl1 expression in vitro, we used primary Schwann cell cultures. We confirm that Schwann cells express Acsl1 protein under basal media conditions (5.5 mM glucose) (Supplementary Fig. S2). In response to incubation in high LCFA (+ 100 μM) media, Schwann cells increase Acsl1 protein expression by 2.7-fold ($p<0.001$) (Fig. 2). These data confirm that Acsl1 protein is expressed in cultured Schwann cells (Supplementary Fig. S2) and increased in response to high LCFA (Fig. 2).

**LCFA-induced increases in mitochondrial transport genes and metabolites of beta-oxidation are potentiated by Acsl1 overexpression**

We next examined the effects of lentiviral overexpression of Acsl1 on specific responses to LCFA. Overexpression was
confirmed by visual confirmation of simultaneously-expressed mCherry fluorescence (data not shown) and via western immunoblotting (A). Pixel density of the Acs11 band in each condition was normalized to the corresponding GAPDH band (B). Data are mean±SEM for four nerves per group. *p<0.05, **p<0.01 versus age-matched db/+.

Mitochondrial LCFA metabolism first requires transport of Acs11-activated LCFA into the mitochondria, and the rate-limiting enzyme in this process is Cpt1. Real-time PCR (RT-PCR) was performed for Acs11 and the three known Cpt1 isoforms. Exposure to LCFA increased mRNA levels of Acs11, Cpt1a, Cpt1b, and Cpt1c compared with control media (Fig. 3).

Further, Acs11 overexpression significantly increased these LCFA-induced changes in Cpt1a and Cpt1b transcription (p<0.05) (Fig. 3). Once the activated LCFA (acyl-CoAs) have entered the mitochondrial matrix, they undergo consecutive turns of beta oxidation, removing two carbons in the form of acetyl-CoA with each cycle. The consecutively shortened acyl-CoA can be completely converted to acetyl-CoA through beta-oxidation cycling, or can be directed to form acyl-carnitines at any stage. To begin to assess the metabolic fate of Acs11-activated LCFA, we performed directed liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS)

**FIG. 1.** Acs11 protein expression is increased in db/db SCN at 8 and 24 weeks of age. The expression of Acs11 protein in 10 µg homogenized SCN from control (db/+ ) and diabetic (db/db) mice was determined with western immunoblotting (A). Pixel density of the Acs11 band in each condition was normalized to the corresponding GAPDH band (B). Data are mean±SEM for four nerves per group. *p<0.05, **p<0.01 versus age-matched db/+. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SCN, sciatic nerve.

**FIG. 2.** Incubation in high LCFA increases Acs11 protein expression in primary cultured Schwann cells. Schwann cells were incubated in control defined media, defined media containing +100 µM LCFA (for a total concentration of 100 µM) for 12 h. Cells were harvested and a western immunoblot was performed using 20 µg Schwann cell protein lysate (A). Pixel density of the Acs11 band in each condition was normalized to the corresponding GAPDH band (B). High LCFA (FA) increased Acs11 expression 2.7-fold above control media. Data are mean±SEM, n=3 replicate cultures, ***p<0.001 versus control. LCFA, long chain fatty acids.
for acetyl-CoA, acetyl-carnitine, and medium to long-chain acyl-carnitines on control and Acsl1-overexpressing Schwann cells incubated with 100 μM LCFA for 12 h (control + FA or Acsl1 + FA, respectively). High LCFA were associated with a significant increase in Schwann cell acetyl-CoA (p < 0.001), acetyl-carnitine (p < 0.05), and medium to long-chain acyl-carnitines (dodecanoylcarnitine, C12, p < 0.05; myristoylcarnitine, C14, p < 0.01; palmitoylcarnitine, C16, p < 0.001) (Fig. 4A, B, F–H). This effect of high LCFA is further increased by Acsl1 overexpression, with all measured metabolites significantly greater in the Acsl1 + FA Schwann cells compared with the control + FA cells (p ≤ 0.05 for all metabolites) (Fig. 4). Together, these findings confirm that Acsl1 potentiates LCFA mitochondrial transport and has an effect on LCFA metabolism.

Measurement of mitochondrial function in primary cultured Schwann cells

To determine the impact of these changes on mitochondrial bioenergetic function, the bioenergetic profile of control and Acsl1-overexpressing Schwann cells incubated with 100 μM LCFA for 12 h was determined using the XF24 Analyzer following the sequential addition of oligomycin (ATP synthase inhibitor), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (uncoupling protonophore), and antimycin (complex III inhibitor) (Fig. 5).

The oxygen consumption rate (OCR) remaining following antimycin A addition (1 μM) gives a measure of non-mitochondrial respiration. Nonmitochondrial Schwann cell oxygen consumption is unaffected by LCFA treatment or Acsl1 overexpression (derived from Fig. 5A, B) (Control 3.3 ± 0.3; Control + FA 3.4 ± 0.4; Acsl1 3.4 ± 0.5; Acsl1 + FA 2.9 ± 0.4 pMoles/min/μg protein). LCFA treatment was associated with significant decreases in coupling efficiency (Fig. 5F), respiratory control ratio (Fig. 5G) and spare respiratory capacity (Fig. 5E). Acsl1 overexpression significantly attenuated this LCFA-induced effect on coupling efficiency (Fig. 5F) (p < 0.05). Extracellular acidification rate (ECAR) (a measure of glycolysis) was not significantly different between groups, indicating no effect of LCFA or Acsl1 overexpression on glycolysis (data not shown). These data suggest an improvement in basal mitochondrial efficiency (less proton leak, more ATP-linked respiration) in LCFA-treated Acsl1 cells compared with LCFA-treated control cells.

To allow us to assess the implications of these changes on dynamic cellular bioenergetics, these oxygen consumption data are expressed in terms of maximal respiratory capacity (FCCP response) in the respective groups (Fig. 6). Comparison of charts between untreated and LCFA-treated control cells (Fig. 6A, B) shows a 23% increase in proton leak (14% to...
Acsl1 overexpression does not increase mitochondrial mass

To assess the contribution of adaptive effects on mitochondrial biogenesis to the improved mitochondrial bioenergetic profile, we examined changes in mtDNA expression as a measure of mitochondrial biogenesis. Twelve hours LCFA treatment significantly decreased mtDNA expression, with no significant effect of Acsl1 overexpression (Supplementary Fig. S5). These data suggest that the improvements in bioenergetic parameters are not due to an increase in mitochondrial mass.

Acsl1 overexpression protects against LCFA-induced oxidative stress and cellular injury in primary cultured Schwann cells

Finally, to assess whether Acsl1 is a modifier of LCFA-induced lipotoxicity, we examined the effects of Acsl1 overexpression on oxidative stress and cell viability changes in response to LCFA using CellROX Green Reagent, FPLC quantification of nitrotyrosine (nitrosylated proteins) and HODEs (lipid peroxidation), and the TUNEL assay. CellROX Green Reagent was used to assess cellular reactive oxygen species (ROS) production in control (empty vector) and Acsl1-overexpressing (Acsl1) Schwann cells. Thirty \( \mu \text{M} \) LCFA increased CellROX signal in control cells after 1 h (Fig. 7). This LCFA treatment effect was attenuated with Acsl1 overexpression (Supplementary Fig. S5). These data suggest that the improvements in mitochondrial mass are not due to a decrease in oxidative stress and cellular injury.

37%), with a decrease in spare respiratory capacity of a similar magnitude (43% to 21%). The ATP-linked oxygen consumption is maintained in terms of maximal cellular respiratory capacity (33% to 29%). Comparison of data between LCFA-treated control and Acsl1-overexpressing cells (Fig. 6B, D) shows that Acsl1 overexpression normalizes proton leak (37% to 18%), with a decrease in spare respiratory capacity of a similar magnitude (43% to 21%). The ATP-linked oxygen consumption is maintained in terms of maximal cellular respiratory capacity (33% to 29%).

FIG. 4. LCFA-induced increase in Schwann cell mitochondrial metabolites is potentiated by Acsl1 overexpression. Control and Acsl1-overexpressing Schwann cells were incubated in defined media and defined media containing \( +100 \mu \text{M} \) fatty acids (Control + FA and Acsl1 + FA, respectively) for 12 h. Cells were snap frozen with liquid nitrogen and directed LC/MS/MS was performed for the presented metabolites. LCFAAs (100 \( \mu \text{M} \)) significantly increased Schwann cell acetyl-CoA (A), acetyl-carnitine (B), and medium to long-chain acyl-carnitines (F–H). Acsl1 overexpression significantly increased this LCFA-mediated response (A–H). Data are mean \pm SEM. * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \) versus Ctrl; \( \dagger p<0.01 \), \( \ddagger p<0.001 \) versus Ctrl + FA; \( \ddagger\ddagger p<0.01 \), \( \dagger\dagger\dagger p<0.001 \) versus Ctrl + FA.

**A.** Acetyl-CoA (nanomoles/cell count)

**B.** Acetyl-carnitine (C2) (nanomoles/cell count)

**C.** Hexanoyl carnitine (C6) (nanomoles/cell count)

**D.** Octanoyl carnitine (C8) (nanomoles/cell count)

**E.** Decanoyl carnitine (C10) (nanomoles/cell count)

**F.** Dodecanoyl carnitine (C12) (nanomoles/cell count)

**G.** Myristoyl carnitine (C14) (nanomoles/cell count)

**H.** Palmitoyl carnitine (C16) (nanomoles/cell count)

**Acsl1** overexpression and with 3 h pretreatment in antioxidants that target global cellular oxidative stress (Fig. 7). This pro-oxidant effect of LCFA was maintained following 12 h in high LCFA media (100 \( \mu \text{M} \) LCFA), with nitrotyrosine and HODEs levels increased by 3.5-fold \( ( p<0.05 \) and 2.3-fold \( ( p<0.01 \) respectively in control Schwann cells (Fig. 8A, B). This LCFA-induced increase in markers of protein and lipid oxidative stress and cellular injury in primary cultured Schwann cells
stress did not occur in Acsl1-overexpressing cells; levels of nitrotyrosine and HODEs in LCFA-treated Acsl1-overexpressing cells and untreated control cells were not significantly different (Fig. 8A, B). One hundred μM LCFA was associated with almost 100% TUNEL positivity in control cells ($p < 0.001$ compared with untreated control cells), while in Acsl1-overexpressing Schwann cells only 15% of LCFA-treated cells were TUNEL positive ($p < 0.001$ compared with untreated Acsl1-overexpressing cells) (Fig. 8C). These data suggest that the LCFA-induced increase in oxidative stress in control Schwann cells is injurious and can be blocked by antioxidant pretreatment.

**FIG. 5.** Measurement of mitochondrial function in primary cultured Schwann cells using the XF24 Analyzer. OCR was measured at basal level and with the sequential addition of oligomycin (1.25 μM), FCCP (300 nM), and antimycin (1 μM) to control (A) and Acsl1-overexpressing (B) Schwann cells following a 12 h incubation in +100 μM LCFA (+FA). OCR levels are normalized to μg protein. The OCR measurements in (A) and (B) are plotted in (C) and (D) as a percentage of baseline respiration. Basal OCR (E), coupling efficiency (F), respiratory control ratio (G), and spare respiratory capacity (H) were calculated after subtracting nonmitochondrial respiration as described in (I). Data are mean±SEM of 3 replicate cultures, with 10 replicate measures (10 wells) per condition per experiment; *$p < 0.05$, **$p < 0.01$ versus Ctrl; †$p < 0.05$ versus Ctrl + FA; ‡$p < 0.05$, §§$p < 0.01$, §§§$p < 0.001$ versus Acsl1. FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; OCR, oxygen consumption rate.
Discussion

High circulating triglycerides and LCFAs are implicated in the development and progression of DN (1, 7, 34, 40). Microarray studies have allowed us to explore the role of hyperlipidemia-driven gene changes in the diabetic peripheral nerve (13, 21). Given that Schwann cells are the primary cellular components of peripheral nerves (28) and Schwann cell dysfunction contributes to DN (8), we sought to understand the implications of our identified gene changes on Schwann cell function and survival. Post-hoc analysis of microarray data uncovered cross-species regulation of Acsl1 in both human and mouse diabetic peripheral nerves (13, 21). In a murine model of type 2 diabetes with elevated VLDL triglycerides, the db/db mouse (Supplementary Fig. S1D), we confirmed that Acsl1 gene upregulation translates to an increase in Acsl1 protein expression (Fig. 1). In parallel, we also observed significant increases in both protein and lipid oxidative modifications in SCN from db/db mice (Supplementary Fig. S1A, B), supporting our contention that oxidative stress is a major mechanism of hyperglycemia- and lipid-induced DN in humans and rodents, particularly through the oxidation of proteins and lipids (32, 34, 39). In the current study, we explored the biological significance of Acsl1 overexpression in DN and the intersection of Acsl1 activity and oxidative stress as an underlying mechanism in DN.

Acsl1 is required for metabolic activation of LCFAs, a process necessary for LCFA entry into mitochondrial beta-oxidation (17). We demonstrated that Acsl1 protein is expressed in primary Schwann cells in vitro (Supplementary Fig. S2), and that Acsl1 levels are increased in response to high LCFA treatment (Fig. 2). These data suggest that modulation of LCFA metabolism by Acsl1 in Schwann cells may be instrumental in maintaining normal nerve homeostasis and in response to elevated LCFA treatment, there is a compensatory increase in Acsl1. Given the increased levels of Acsl1 after LCFA treatment, we next explored whether this translated to increased mitochondrial uptake and metabolism of LCFA.

Mitochondrial LCFA metabolism first requires transport of Acsl1-activated LCFA into the mitochondria. We therefore overexpressed Acsl1 in primary Schwann cells (Supplementary Figs. S3 and S4) concurrent to LCFA treatment to investigate changes in LCFA transport. The transport proteins involved are Cpt1, Cpt2, and CACT, where Cpt1 is the rate-limiting enzyme of the complex (14). We observed
LCFA-induced transcriptional upregulation of Cpt1, which is potentiated by Acsl1 overexpression (Fig. 3). This is consistent with the finding that palmitic, oleic, and linoleic acids, either directly or in their activated acyl-CoA forms, each markedly induce Cpt1 gene expression in the pancreatic beta cell line INS-1 (3).

Once the activated LCFA (acyl-CoAs) have entered the mitochondrial matrix, they undergo consecutive cycles of beta oxidation. Each cycle shortens the acyl-CoA chain by two carbons in the form of acetyl-CoA, and produces reducing equivalents (NADH, FADH2) for oxidative phosphorylation. The acyl-CoAs can be completely catabolized to acetyl-CoA when reducing equivalents are required for ATP production, or they can be directed away from beta-oxidation and oxidative phosphorylation to form acyl-carnitines and acetyl-carnitine. We observed an increase in acetyl-CoA, acetyl-carnitine, and medium to long-chain acyl-carnitines following LCFA treatment (Fig. 4). Our data suggest that the LCFA are entering the mitochondrial matrix, and entering the beta-oxidation cycle. This is in agreement with the report that LCFA-induced Cpt1 gene upregulation leads to increased activity of Cpt1 and a higher LCFA oxidation capacity in INS-1 cells (3). However, after a limited number of turns (based on the species we see that is, medium to long-chain acyl-carnitines), the LCFA are not fully catabolized through beta-oxidation. Overexpression of Acsl1 leads to a potentiation of this response, suggesting that Acsl1 is not only promoting mitochondrial metabolism of LCFA, but is also increasing the direction of acyl-CoAs to acyl-carnitine production (Fig. 4). In parallel, we also observed that Acsl1 overexpression ameliorated LCFA-induced cellular injury (Fig. 8C), further supporting the idea that Acsl1 overexpression leads to an increase in incomplete beta-oxidation of LCFA and diverts unrequired energy substrates from oxidative phosphorylation to offer protection from electron transport chain overload (5).

Electron transport overload is associated with increased proton leak, resulting in mitochondrial uncoupling and reduced mitochondrial efficiency (5, 37). Therefore, we performed bioenergetic profiling to assess the impact of high LCFA treatment on mitochondrial function. We observed that 12 h LCFA treatment significantly decreased mitochondrial efficiency via uncoupling oxygen consumption from ATP production (Fig. 5). The resulting mitochondrial substrate overload is associated with increased mitochondrial dysfunction in a number of cell types (5, 26). Further, in primary Schwann cells, hyperglycemic conditions decrease...
of Acsl1-overexpressing cells were TUNEL positive. Data are mean as percentage TUNEL-positive cells.

Schwann cells, even in the presence of 100 µM FA; 100 µM FA for 12 h increased nitrotyrosine and HODEs by 3.50-fold and 2.3-fold, respectively. Nitrotyrosine and HODEs levels in Acsl1-overexpressing cells did not differ from untreated control Schwann cells, even in the presence of 100 µM LCFA (Acsl1 + FA). Cellular injury was quantitated by TUNEL assay and is plotted as percentage TUNEL-positive cells (C). After LCFA treatment, almost 100% of control cells were TUNEL positive, while only 15% of Acsl1-overexpressing cells were TUNEL positive. Data are mean ± SEM, n = 3 replicate cultures, *p < 0.05, **p < 0.01, ***p < 0.001 versus Ctrl, †p < 0.05, ††p < 0.01, †††p < 0.001 versus Ctrl + FA; ‡p < 0.001 versus Acsl1. HODEs, hydroxyoctadecadienoic acids.

mitochondrial efficiency via uncoupling oxygen consumption from ATP production, while also decreasing spare respiratory capacity (41). Under these hyperglycemic conditions there is a concurrent increase in the ECAR, suggesting that Schwann cells shift to glycolytic metabolism in a hyperglycemic environment (41). Importantly, we did not observe any significant changes in ECAR (data not shown) in the current study, indicating that Schwann cells did not compensate for the decrease in ATP-coupled mitochondrial respiration by increasing glycolysis.

Another measure of mitochondrial energetic status is spare respiratory capacity, which represents the cells ability to respond to changes in bioenergetic needs. We observed a decrease in the spare respiratory capacity of the Schwann cells following LCFA treatment (Fig. 5). A decrease in spare respiratory capacity limits the ability of cells to respond to changes in bioenergetic needs (4), suggesting that the LCFA-treated Schwann cells are less able to respond to changes in metabolic load. This decrease in spare respiratory capacity was also reported in hyperglycemia-treated Schwann cells (41); however, under hyperglycemic conditions, the cells have the potential to utilize glycolysis to produce ATP in response to changes in energy demands. Taken together, the increased incomplete beta-oxidation (Fig. 4), uncoupling of oxygen consumption from ATP production, and decreased spare respiratory capacity following LCFA treatment (Figs. 5 and 6) suggest a LCFA-induced mitochondrial bioenergetic crisis, which ultimately results in Schwann cell injury (Fig. 8C). Our data strongly suggest that Acsl1 is instrumental in modulating the Schwann cell response to the observed LCFA-induced lipotoxicity. Overexpression of Acsl1 normalized ATP-linked oxygen consumption and proton leak, and hence, coupling efficiency (Figs. 5 and 6). In addition, with respect to the increased incomplete beta-oxidation (Fig. 4), mitochondrial function was normal in Acsl1-overexpressing cells despite 12 h of LCFA-treatment when compared with LCFA-treated control cells (Figs. 5 and 6). These data suggest that mitochondria in Acsl1-overexpressing cells are able to direct surplus energy substrates to acyl-carnitines rather than direct the reducing equivalents to the electron transport chain, perhaps decreasing the energetic overload on the electron transport chain.

Finally, we hypothesized that overexpression of Acsl1 would decrease both oxidative stress and cellular injury in response to LCFA. As a first step, we examined the effects of LCFA treatment on empty vector control cells. In LCFA-treated control Schwann cells, we demonstrated an increase in Schwann cell ROS production after only 1 h exposure to LCFA (Fig. 7), and after 12 h exposure to 100 µM LCFA we observed oxidative damage to both proteins and lipids (Fig. 8A, B) and significant cellular injury (Fig. 8C). Our data in control Schwann cells are consistent with reports that overwhelming mitochondrial substrate load is associated with increased oxidative stress and cellular apoptosis (5, 26). Our data are also consistent with the report that palmitate is lipotoxic to immortalized cultured Schwann cells, with ROS production significantly and persistently elevated from 12 h onward following 150 µM palmitate exposure (20). Pretreatment of control Schwann cells with the antioxidant cocktail (N-acetylcysteine, alpha-lipoic acid, and catalase) blocked LCFA-mediated oxidative stress (Fig. 7). These data agree with our own previous reports that increasing endogenous antioxidant defense in Schwann cells prevents oxidative mediated damage (35).

As predicted, Acsl1 overexpression prevented LCFA-mediated oxidative stress in Schwann cells (Figs. 7 and 8A, B) and attenuated Schwann cell injury (Fig. 8C). These data further support our contention that Acsl1 overexpression diverts surplus energy away from oxidative phosphorylation, preventing mitochondrial overload, bioenergetics failure, and ROS and cellular oxidative damage. Alternatively, it could be that Acsl1 is promoting LCFA uptake from the cytosol into the mitochondria, with a corresponding decrease in cytosolic oxidative stress. This idea is supported by reports that oleate-
facilitated clearance of palmitate from the cytosol attenuates palmitate-induced oxidative stress, endoplasmic reticulum stress, and apoptosis in myocytes (18). The contribution of increased cytosolic clearance and decreased endoplasmic reticulum stress to our understanding of Acsl1 action on Schwann cells will be the focus of future experiments.

In summary, we report that Acsl1 overexpression normalized high LCFA-induced mitochondrial dysfunction and oxidative stress, significantly improving Schwann cell viability. We postulate that this is due to (i) increased mitochondrial LCFA activation and entry into the mitochondrial matrix, without direction of excess substrate to the electron transport chain and (ii) decreased cytosolic LCFA, reducing cellular oxidative stress. Our data suggest that Acsl1 upregulation may be a compensatory response to elevated LCFA in Schwann cells, and that extreme Acsl1 upregulation allows the cells to cope with high LCFA. However, endogenous upregulation is not sufficient to prevent lipotoxicity in either cultured Schwann cells or db/db mouse SCN. If this endogenous compensation can be amplified by drug intervention, targeted improvement in Schwann cell mitochondrial metabolic disposal of LCFA may have implications for altering DN phenotypes.

Materials and Methods

Materials

Chemicals were purchased from Sigma-Aldrich Corp. or Fisher Scientific unless otherwise stated.

Diabetic mice

Male type 2 diabetic (BKS.Cg-m+/Leprdb; db/db) and control (db/+ ) mice were purchased from Jackson Laboratories. A mutation in the lepin receptor of the db/db mouse results in hyperphagia, severe obesity, hyperinsulinemia, and hyperglycemia beginning at 4 weeks of age (Jackson Laboratories; 000642). At 24 weeks of age (20 weeks of diabetes), db/db mice exhibit insensitivity to mechanical and thermal stimuli, along with slowed nerve conduction velocities and reduced intraepidermal nerve fiber density Sullivan et al. (25). Animals were maintained at the University of Michigan in a pathogen-free environment and cared for following the University of Michigan Committee on the Care and Use of Animals guidelines. Mice were given continuous access to food (Purina 5001; Purina Mills LLC) and water.

Terminal metabolic phenotyping and SCN collection

Mice were euthanized by sodium pentobarbital overdose at 8 and 24 weeks of age (6 db/+ and db/db at 8 weeks; 6 db/+ and db/db at 24 weeks). The left and right SCN were dissected. The left SCN was frozen in liquid nitrogen and stored at −80°C until western immunoblotting was performed. The right SCN was prepared for quantification of markers of oxidative damage as previously described (12). Plasma was collected for total triglyceride and lipoprotein triglyceride measurements as previously described (11).

Acsl1 plasmid and lentivirus production

Total RNA was extracted from frozen mouse spinal cord from C57BL/6 mice using the RNeasy RNA extraction kit (Qiagen). cDNA was obtained by reverse transcription with the iScript cDNA synthesis kit (Bio-Rad) using 1 μg of total RNA in a 20 μl reaction according to the manufacturer’s protocol. The cDNA was diluted 1.5 with ddH2O. Acsl1 was amplified by PCR in a 50 μl reaction using 1 μl of the diluted mouse spinal cord cDNA, 2 μl of 10 mM dNTPs (Denville Scientific, Inc.) (final concentration 200 μM each), 5 μl of Phusion HF buffer, Phusion High-Fidelity DNA polymerase (New England BioLabs), and 50 pmol of forward and reverse primers: 5′-CGGATCCATGGAACTCATTGAGTGTGTCGTA-3′ (Integrated DNA Technologies). The following thermocycler protocol was used for the reaction: 98°C for 30 s, (98°C for 5 s, 55°C for 30 s, 72°C for 30 s) x 30 cycles; and a final extension of 72°C for 10 min. The amplified product was directionally cloned into BamHI and NotI of the pLVX-IRES-mCherry vector (Clontech). The construct was confirmed by sequencing at the University of Michigan Sequencing Core. 10× Lentiviral stocks were generated by the University of Michigan Vector Core.

Schwann cell culture and fatty acid preparation

Schwann cells were isolated from SCN of P3 rat pups as previously described (35). Cells were plated on Primaria surface-modified plates in Dulbeccos’s Modification of Eagle’s Medium (DMEM)/10% FBS. At confluence, fibroblasts were removed by complement lysis by using thy1.1 antibody and rabbit complement. Schwann cells were maintained in low glucose (1 g/L) DMEM containing 10% heat-inactivated FBS, 2 μM forskolin, 20 μg/ml bovine pituitary extract, and penicillin/streptomycin/neomycin (feed media).

For lentiviral transduction, Schwann cells were incubated in feed media containing 1× Acsl1-containing lentivirus construct or empty vector for 24 h. Cells were then washed twice with Hank’s balanced salt solution, fresh feed media was added, and cells were maintained for 7 days prior to treatment experiments. During optimization experiments, transduction efficiency was confirmed by visualization of mCherry fluorescence every 2 days, and a western immunoblot for Acsl1 protein was performed on day 7. Following confirmation that mCherry fluorescence correlated with Acsl1 overexpression, visual determination was used to confirm transduction efficiency in all subsequent experiments.

Prior to LCFA treatment experiments, Schwann cells were switched to defined media for 12 h (1:1 mix of low glucose [1 g/L] DMEM and Ham’s F12K media containing 10 μg/ml transferrin, 20 μM putrescine, 20 nM progesterone, and 30 nM sodium selenite). Mitochondrial uptake of activated LCFA is facilitated through the temporary replacement of the CoA group by carnitine (27). Carnitine (2 mM) and CoA (200 μM) were therefore added to culture media for the duration of high LCFA incubations. For CellROX, TUNEL, and XF24 Analyzer assays, Schwann cells were plated on poly-L-lysine-coated coverslips and XF24 Analyzer culture plates.

A single, 100× solution of linoleate, oleate, and palmitate (Nu-Chek Prep, Inc.) was prepared in 0.5% fatty acid-free bovine serum albumin (Equitech-Bio, Inc.) in low glucose DMEM heated to 37°C and added to culture wells for a final concentration of 100 μM. Treatment media were placed in a water bath at 37°C for 30 min before they were added to cells. LCFA treatment durations were 12 h unless otherwise stated.
Subcellular fractionation and western immunoblotting

Subcellular fractionation was performed based on the methods of Arnoult et al. (2). Schwann cells were harvested in isotonic mitochondrial buffer (MB: 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES [pH 7.5]) supplemented with the protease inhibitor mixture Complete (Roche Molecular Biochemicals) and homogenized for 30–40 strokes with a Dounce homogenizer. Samples were transferred to Eppendorf centrifuge tubes, homogenized for a further 20 strokes with a 28 gauge needle, and centrifuged (500 g, 5 min, 4°C) to remove nuclei and unbroken cells. The resulting supernatant was then centrifuged (10,000 g, 45 min, 4°C) to obtain the heavy membrane fraction enriched for mitochondria, and the resulting supernatant was collected as the cytosolic fraction. Cytosolic and heavy membrane fractions (30 and 10 µg of protein, respectively) were subject to western immunoblotting.

Western immunoblotting was performed as previously described (36–38). Polyclonal antibodies against long-chain acyl-CoA synthetase 1 (Acsl1; Pierce Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore), actin (Abcam), and anti-porin/voltage-dependent anion channel (VDAC; Millipore) were used.

Real-time PCR

Assessment of mRNA for specific mitochondrial proteins was performed by real-time PCR (RT-PCR) as previously described (31). Reverse transcription was performed using the iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR amplification and SYBR green fluorescence detection were performed using the iCycler iQ Real-time Detection System (Bio-Rad). The fluorescence threshold value (Ct) was calculated using iCycler iQ system software. The mRNA levels were normalized to an endogenous reference gene (GAPDH, encoding glyceraldehyde-3-phosphate dehydrogenase; ΔCt) and then relative to a control group (ΔΔCt), and were expressed as 2^−ΔΔCt. The average was calculated from two runs per sample.

Targeted metabolomic analysis by LC/MS/MS

Targeted metabolomic analysis was performed as previously described (12). Briefly, frozen Schwann cell samples were extracted with 150 µl of chilled 8:1:1 methanol:chloroform:water containing 13C-labeled standards and chromatographic separation of eight targeted metabolic intermediates was performed based on the methods of Lorenz et al. (15). The ratio of each metabolite peak area to that of the closest-matching 13C-labeled standard was calculated. Metabolite concentration was determined using calibration curves generated from known concentrations of authentic standards and equal concentrations of 13C-labeled compounds as were present in the samples. Concentrations were normalized to cell counts from TUNEL assays (number of cells per 20× objective field of view; four fields of view per coverslip; five coverslips per condition). Final values are expressed as nmol/cell count.

Mitochondrial respiration and extracellular acidification measures

The XF24 Extracellular Flux Analyzer (Seahorse Biosciences) was used to measure bioenergetic function in intact primary cultured Schwann cells. The XF24 culture plates allow simultaneous measurement of real-time OCR and ECAR (indirect assessment of glycolytic activity due to lactic acid production and proton extrusion) with negligible disruption to the cells. Bioenergetic measurements using the XF24 Analyzer require buffer-free media, thus, 1 h prior to mitochondrial respiration measurements, media was changed to unbuffered DMEM, supplemented with 1 mM sodium pyruvate and 5.5 mM D-glucose (pH 7.4). Based on initial optimization experiments, Schwann cells were seeded at 2.5 × 10^5 cells per well 24 h prior to treatments. For all experiments, optimal concentrations of respiratory chain inhibitors oligomycin and antimycin A were 1.25 and 1 μM, respectively. An FCCP (uncoupling protonophore) concentration of 300 nM was determined to give maximal flux without significant toxicity (data not shown). Following stable baseline OCR and ECAR measurements, bioenergetic profile experiments were performed by the sequential injection of oligomycin, FCCP, and antimycin A. Loop Start, Mix, Wait, and Measure times were 4, 3, 2, and 3 min, respectively. ATP-linked oxygen consumption, non-ATP-coupled mitochondrial oxygen consumption (proton leak), maximal respiratory capacity, and nonmitochondrial oxygen consumption were calculated from the response curves (4, 22). Additional bioenergetic parameters, including coupling efficiency, respiratory control ratio, and spare respiratory capacity, were derived from the response curves as described (4, 23).

Following mitochondrial respiration measurements, cells were harvested and the protein concentration of each well was determined for normalization of experimental rate values to protein content. Bioenergetic profile respiration experiments were repeated thrice, with 10 replicate measures (10 wells in the XF24 culture plate) per condition per experimental repeat.

Mitochondrial DNA quantification

Levels of mtDNA were measured by normalizing the mitochondrial gene (cytochrome b) to the nuclear gene (actin) as previously described (9, 31). A total of 10 ng genomic DNA was used for mtDNA and nuclear DNA markers.

Oxidative stress measures

SCN and Schwann cell samples were analyzed for nitrated protein (3-nitrotyrosine) and oxidized lipids (HODEs) as previously described (12).

CellROX Green Reagent (Invitrogen) is a cell-permeant dye that exhibits bright green photostable fluorescence upon oxidation by ROS and subsequent binding to DNA. CellROX was applied to Schwann cells at a final concentration of 5 μM for 30 min at 37°C. Cells were washed with PBS, fixed in 2% paraformaldehyde, and mounted with ProLong antifade mounting media containing dapi (Invitrogen). Green fluorescence was imaged using the 488 nm filter and 20× objective on a Nikon Microphot FXA fluorescent microscope.

TUNEL analysis

Cellular injury was assessed by counting the number of TUNEL-positive cells identified using the ApopTag Peroxidase In Situ Apoptosis Detection Kit as previously described (37, 38). Mean values were calculated from four fields of view.
per coverslip using a 20× objective, with five coverslips per condition.

**Statistical analysis**

Data analysis was performed using GraphPad Prism 5.0 (GraphPad Software). Comparisons between groups were performed using one-way ANOVA with Tukey post-test for multiple comparisons, two-way ANOVA with Bonferroni post-test for multiple comparisons, or an unpaired t-test, as applicable. Assumptions about the Gaussian distribution of data and rules for transformation of non-normative data were made as previously described (24). Significance was assigned when \( p < 0.05 \).

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**Author Disclosure Statement**

No competing financial interests exist.

**References**


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Abbreviations Used

AcsL1 = long-chain acyl-CoA synthetase 1
CACT = carnitine/acylcarnitine translocase
Cpt1 = carnitine palmitoyltransferase 1
Cpt2 = carnitine palmitoyltransferase 2
DMEM = Dulbecco’s Modification of Eagle’s Medium
DN = diabetic neuropathy
ECAR = extracellular acidification rate
FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
HODEs = hydroxyoctadecadienoic acids
LC/MS/MS = liquid chromatography triple quadrupole mass spectrometry
OCR = oxygen consumption rate
ROS = reactive oxygen species
SCN = sciatic nerve
VLDL = very low density lipoprotein
Hyperglycemia-Induced Tau Cleavage in \textit{in vitro} and \textit{in vivo}: A Possible Link Between Diabetes and Alzheimer’s Disease

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Abstract. Multiple lines of evidence link the incidence of diabetes to the development of Alzheimer’s disease (AD). Patients with diabetes have a 50 to 75% increased risk of developing AD. In parallel, AD patients have a higher than normal tendency to develop type 2 diabetes or impaired fasting glucose. Tau is the major component of neurofibrillary tangles, one of the hallmarks of AD pathology. The current study examined the effect of hyperglycemia on tau modification. Glucose treatment of rat embryonic cortical neurons results in concentration-dependent apoptosis and caspase-3 activation. These changes are well correlated with glucose time- and concentration-dependent tau cleavage. Ap treatment induces tau cleavage and when added together with glucose, there is an additive effect on caspase activation, apoptosis, and tau cleavage. Tau cleavage is partially blocked by the caspase inhibitor, ZVAD. Cleaved tau displays a punctate staining along the neurites and colocalizes with cleaved caspase-3 in the cytoplasm. Both type 1 and type 2 diabetic mice display increased tau phosphorylation in the brain. In agreement with the effects of glucose on tau modifications \textit{in vitro}, there is increased tau cleavage in the brains of ob/ob mice; however, tau cleavage is not observed in type 1 diabetic mouse brains. Our study demonstrates that hyperglycemia is one of major factors that induce tau modification in both \textit{in vitro} and \textit{in vivo} models of diabetes. We speculate that tau cleavage in diabetic conditions (especially in type 2 diabetes) may be a key link for the increased incidence of AD in diabetic patients.

Keywords: Alzheimer’s disease, diabetes, hyperglycemia, tau

INTRODUCTION

Alzheimer’s disease (AD) affects 4.5 million Americans and the incidence is expected to reach over 13 million by 2050 [1]. At the same time, over 20 million Americans have diabetes and this incidence is increasing by 5% per year [2]. Multiple studies report that patients with diabetes have a 50 to 75% increased risk of developing AD compared to age- and gender-matched control groups [3–7]. In parallel, a recent study of the Mayo Clinic AD Patient Registry reveals that 80% of AD patients have either type 2 diabetes or impaired fasting glucose [8].

Two of the most prominent pathological characteristics of AD are the accumulation of amyloid-β (Aβ) in extracellular plaques and the appearance of intracellular neurofibrillary tangles (NFT) containing hyperphosphorylated tau [9, 10]. Tau undergoes various post-translational modifications, hyperphosphorylation being the most prominent and studied change [10]. Mounting evidence suggests that not only the hyperphosphorylation, but also tau cleavage plays an important role in the progression of AD [11, 12]. N- and C-terminal fragmentation induces toxic tau aggregation in N2a cells [13]. When cleaved at Asp421 by caspase, tau assembles more rapidly and more extensively into tau filaments \textit{in vitro} [14], suggesting that cleaved tau enhances polymerization kinetics and serves as a nucleation center, promoting the pathologic assembly of tau filaments [15, 16]. Caspases...
MATERIALS AND METHODS

Antibodies and chemicals

Polyclonal antibodies against phosphorylated tau (pTau, pS199/202, pS396, pT231) were purchased from Biosource International (Camillo, CA). Anti-(pTau, pS199/202, pS396, pT231) were purchased from Cell Signaling (Beverly, MA). Anti-GAPDH antibodies were from Chemicon (Temecula, CA). Anti-tau46 (detecting total tau), and anti-GAPDH antibodies were from Millipore (Billerica, MA). Tau1 (recognizing dephosphorylated tau), Tau5 (for total tau), and anti-GAPDH antibodies were from Chemicon (Temecula, CA). Anti-tau46 (detecting total tau) was from Millipore (Billerica, MA). The antibody against cleaved caspase-3 was purchased from Cell Signaling (Beverly, MA).

Inhibitors of caspases, calpain, and the proteasome were purchased from Calbiochem (La Jolla, CA). D (+)-glucose and 2-deoxy-D-glucose (a non-metabolizable glucose analog) were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from either Sigma or Fisher Scientific (Fair Lawn, NJ).

Cortical neuron preparation

Cortical neurons were prepared from E15 embryos of Sprague Dawley rats. The cortex was dissected and dissociated using trypsin and plated in 12-well tissue culture plates coated with poly-L-lysine (PLL). For immunohistochemistry (IHC), cells were plated on glass cover slide coated with PLL in 24-well culture plates. Cells were maintained in feed media (Neurobasal media, Invitrogen, Grand Island, NY) supplemented with 1X B27 without antioxidant (Invitrogen), antibiotics (penicillin, streptomycin, and neomycin, Sigma), 2.5 μg/ml albumin, 10 μg/ml apotransferrin, 0.1 μg/ml biotin, 15 μg/ml D-galactose, 7 ng/ml progestrone, 16 μg/ml putrescine, 4 ng/ml selenium, 3 ng/ml β-estradiol, 4 ng/ml hydrocortisone, 3 μg/ml catalase, and 2.5 μg/ml SOD. Cells were cultured for 6 days before being used in experiments. Culture media was changed to treatment media (feed media without B27 and antibiotics) for 3–4 h prior to glucose, Aβ, and/or inhibitor treatment.

Induction of diabetes and mouse brain preparation

Wild type C57Bl/6J and ob/ob mice (B6.V-Lep+/−J, JAX Mice # 000632) were purchased from Jackson Laboratory (Bar Harbor, ME) and used as a model of type 2 diabetes. Mice were sacrificed at 12 weeks of age (approximately 8 weeks of diabetes). Type 1 diabetes was induced by streptozotocin (STZ) injection when mice (DBA/2, JAX Mice #000671) reached a weight of 25 g (~13 weeks old). STZ was injected at the concentration of 50 mg/kg for 5 consecutive days (http://www.diacomp.org/shared/protocols.aspx) [25]. Mice were sacrificed at 38 weeks of age (25 weeks of diabetes). At least 6 animals were used for each group. Fasting blood glucose levels were measured every 4 weeks using a standard Glucometer (One-Touch; 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.

The mice were euthanized per our published protocols with an overdose of sodium pentobarbital. For western blotting analyses, brains were cut in half and the cortex and hippocampus separated and then snap frozen in liquid nitrogen and stored at −80°C until use. For IHC, mice were perfused intracardially with 30 ml...
of 2% PLP (paraformaldehyde-lysine-periodate) following euthanasia. After perfusion, whole brains were removed and processed for the cryosection as previously described [22, 26]. Brain sections were mounted on SuperFrost glass slides (Fisher Scientific, Pittsburgh, PA) and stored at −20°C until use.

**Western immunoblotting**

Western immunoblotting was performed as described previously [27]. Cortical neuron cultures were lysed in RIPA buffer (Pierce) containing 1 μg/ml of aprotinin and leupeptin and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF). Lysates were collected, briefly sonicated, and centrifuged at 13,000 rpm for 15 min at 4°C. Mouse cortex and hippocampus were homogenized using a plastic pestle in a microcentrifuge tube in T-PER tissue protein extraction reagent (Pierce) containing aprotinin, leupeptin, and PMSF. Samples were briefly sonicated and centrifuged at 13,000 rpm for 20 min at 4°C and supernatants were collected. Protein concentration of the cortical neuron or brain lysates was measured by DC protein assay reagent (Bio-Rad Laboratory, Hercules, CA). The lysates were separated by SDS-PAGE and transferred to nitrocellulose (NC) membranes. After blocking with Superblock solution (Pierce) with 0.1% Tween-20, NC membranes were incubated with the appropriate primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase (Santa Cruz). The incubations with primary and secondary antibodies were carried out either at room temperature for 2 h or at 4°C overnight. The signal was visualized using enhanced chemiluminescence reagents (ECL, Amersham Bioscience, Piscataway, NJ) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) depending on the signal strength. Images were captured using the Chemidoc XRS system and analyzed by Quantity One software (Bio-Rad Laboratory). In some experiments, the NC membranes were incubated at 60°C for 15 min in stripping solution (2% SDS, 100 mM dithiothreitol, and 100 mM Tris, pH 6.8) whereupon they were utilized for immunoblotting with another antibody. All experiments were repeated at least 3 times and representative results are presented in the figures.

**Immunohistochemistry**

The slides with brain sections were heated on a slide warmer (55°C) for 10 min followed by hydration in PBS. Cortical neurons grown on cover slides were fixed with 2% paraformaldehyde (PFA) in PBS for 10 min. The slides were permeabilized and incubated in primary antibody diluted in PBS with 1% BSA/1% normal goat serum/0.1% Triton X-100 in a humidified chamber at room temperature overnight. After rinsing with PBS 3 times for 5 min each, the cells were incubated with the appropriate secondary antibody conjugated with Alexa Fluor 594 or 488 (Molecular Probes, Eugene, OR) for 2 h at room temperature. After rinsing with PBS, the samples were mounted with Pro-Long Gold anti-fade mounting media containing DAPI (Molecular Probes). The digital images were captured using a Spot-RT camera (Diagnostic Instruments Inc., Sterling Heights, MI) attached to a Nikon Microphot-FXA microscope.

**Measurement of apoptosis, TdT mediated dUTP-biotin nick end labeling (TUNEL)**

TUNEL staining labels fragmented DNA, which is a characteristic of apoptotic cells. Cortical neurons grown on glass cover slides were fixed in 4% paraformaldehyde prior to staining. Samples were labeled with digoxygenin-dUTP and followed by horseradish peroxidase-conjugated anti-digoxygenin antibody using a kit according to the manufacturer’s instructions (Intergen, Gaithersburg, MD).

**Measurement of apoptosis, Annexin V staining**

During apoptosis, phosphatidylserine, which is normally located on the cytoplasmic surface, translocates to the outer leaflet of the plasma membrane [28]. Annexin V binds to the exposed phosphatidylserine in a Ca2+-dependent manner [29]. Annexin V conjugated with Alexa Fluor 488 was purchased from Molecular Probes. After treatment, cells were labeled with Annexin V (1:20 dilution) in binding buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2) for 10 min at room temperature. The resulting fluorescence was measured using a Fluoroskan Ascent FL fluorimeter (Labsystems, Thermo Fisher Scientific Inc., Waltham, MA).

**RESULTS**

**Tau cleavage by high glucose and Aβ in primary rat embryonic cortical neurons**

Our laboratory demonstrated that hyperglycemia mimicking diabetes induces diabetic neuropathy in vivo [21, 23] as well as neuronal apoptosis in vitro
in both neuroblastoma cell lines and primary neuron cultures [26, 30, 31]. Recent reports demonstrate tau dysfunction in both type 1 and type 2 diabetic animal models [32–34], suggesting that tau modification is a link between increased AD in diabetic patients. Therefore, we examined whether high glucose treatment induces neuronal apoptosis and tau modification in vitro using primary rat embryonic cortical neurons as the model system. Consistent with our previous reports, glucose treatment resulted in a concentration-dependent increase in neuronal apoptosis (Fig. 1A) detected by TUNEL staining. Activation of the caspase family of cell death proteases is essential for most types of apoptosis [35]. We and others have shown that activation of caspase-3 by a variety of neurotrophic insults, including hyperglycemia, plays an important role in apoptosis in neuronal cells [30, 36]. In agreement with the apoptosis results, there was a glucose concentration-dependent increase in caspase-3 cleavage (i.e., activation) after 24 h of hyperglycemia (Fig. 1B). Treatment with an equimolar concentration of mannitol, however, failed to induce caspase-3 activation (Fig. 1C). Treatment of the cortical neurons with as much as 250 mM mannitol or 2DG resulted only minimal caspase-3 cleavage, suggesting that the effect of glucose was not the result of hyperosmotic stress.

We next examined tau modification during glucose-mediated apoptosis. Tau cleavage was detected by the appearance of cleavage products recognized by Tau1 immunoblotting. Treatment of the embryonic cortical neurons with 100 mM glucose resulted in a time-dependent increase in tau cleavage (Fig. 2A) with the appearance of the prominent ∼40 to ∼10 kDa (Fig. 2A, asterisks). The glucose concentration-dependent increase in tau cleavage was overexposed (Fig. 2A); however, the immunoreactivity was not as prominent when detected by the Tau5 antibody, or tau phosphorylation at the Ser199/202 residues, were not significantly changed at 48 h of glucose treatment. The effect of glucose on the appearance of cleaved tau fragments was also concentration-dependent up to 250 mM glucose treatment (Fig. 2B, arrow) and was well-correlated with apoptosis and caspase-3 cleavage (Fig. 1). In agreement with the effect on caspase-3 cleavage, mannitol treatment up to 250 mM had a minimal effect on the appearance of the 14 kDa tau fragment even though 40 kDa fragments were present (Fig. 2B, asterisks). Tau cleavage was also confirmed by TauC3 immunoblotting (Fig. 2C), which detects tau cleaved at Asp421.

Studies demonstrate that Aβ treatment results in apoptosis of embryonic cortical or hippocampal neurons [17, 37]. We next tested the hypothesis that hyperglycemia accelerates neuronal injury induced by Aβ. Treatment of cortical neurons with glucose or Aβ for 24 h resulted in concentration-dependent tau cleavage as seen by the decrease in Tau46 immunoreactivity (Fig. 3A, upper panel) and the appearance of cleaved fragments (Fig. 3A, arrowhead), and an increase in cleaved caspase-3. When the cells were treated with both glucose and Aβ, there was an additive effect on tau cleavage and caspase-3 activation (Fig. 3A). Even though there is a tendency for increased total tau levels by glucose and/or Aβ, the changes were not statistically significant (data not shown). In agreement with caspase-3 activation, cell death was also increased when examined by Annexin V staining, with an additive effect when glucose and Aβ were added together (Fig. 3B). Apoptosis was also measured by examining fragmented apoptotic nuclei after staining the cells with DAPI. The cells treated with glucose or Aβ displayed increased staining of fragmented nuclei (Fig. 3C, arrowheads), which became more frequent when both treatments were added together.

Caspases [11, 17], the proteosome [18], and calpain [19, 20] are all implicated in tau cleavage during AD progression. Therefore, we next examined the possible proteases responsible for tau cleavage during glucose/Aβ treatment using protease inhibitors. Glucose- and Aβ-mediated tau cleavage and caspase-3 activation were partially blocked by the pan-proteosome inhibitor, ZVAD, and almost completely blocked by IGF-1 (Fig. 4A), which was used as a positive control. This result suggests the involvement of other proteases. Calpain inhibitor III and lactacystin (proteosome inhibitor), however, could not prevent tau cleavage since they augmented caspase activation and cell death (Fig. 4B). A recent study also reported similar results with calpain inhibitor (MDL28170)-treated SH-SY5Y cells [38]. We also observed similar results using other calpain (ALLN) and proteosome (MG-132) inhibitors (data not shown).
Fig. 1. Glucose-mediated apoptosis of cortical neurons. A) E15 rat embryonic cortical neurons cultured in vitro for 6 days were treated with 0, 50, or 100 mM glucose for 24 h and apoptosis was measured using TUNEL staining. Arrows show the apoptotic cells. Glucose treatment significantly increased the number of TUNEL-positive cells. *, p < 0.05 by t-test. B, C) Cortical neurons were treated with the indicated concentrations of glucose (Glu), 2-deoxyglucose (2DG, 100 mM) or mannitol (Man, 100 mM) for 24 h. Cell lysates were prepared in RIPA buffer and immunoblotted with an antibody against cleaved caspase-3 (B) or total caspase-3 (C). The blots were stripped and reprobed with an anti-GAPDH antibody. These experiments were repeated at least 3 times and a representative result is shown.

Fig. 2. Tau cleavage by glucose treatment. A) E15 rat embryonic cortical neurons were treated with 100 mM glucose for 8, 24, or 48 h. Cell lysates were immunoblotted with the indicated antibodies. For Tau1 and Tau5 antibodies, the blots were exposed for a short time using ECL or a longer time using SuperSignal West Femto to examine various cleavage products (asterisks). Arrow indicates the major cleavage products of 14 kDa. B) Cortical neurons were treated with increasing concentrations of glucose or mannitol for 24 h. Tau cleavage was examined as in (A). Tau cleavage also confirmed by TauC3 antibody immunoblotting (C). The Western blots were repeated at least 3 times and representative results are shown.
We conducted IHC to better understand the subcellular changes of tau modification during glucose-mediated apoptosis. Embryonic cortical neurons grown on cover slips were treated with glucose, Aβ, or both for 24 h. The cells were fixed with 2% PFA and then stained with the TauC3 antibody to examine the distribution of cleaved tau. In agreement with the increase in apoptosis and caspase expression (Figs. 3 & 4), there was a decrease in the number of neurites in the cells treated with glucose or Aβ and it was more prominent when the cells were exposed to both glucose and Aβ (Fig. 5, insets). In contrast, there was a strong labeling of TauC3 in the cytoplasm of apoptotic neurons (Fig. 5, arrows). TauC3 staining also displayed a punctate pattern of labeling along the neurites (Fig. 5, arrowheads), which was more numerous when glucose and Aβ were treated together. We next examined the distribution of cleaved caspase-3 after glucose and Aβ treatment in the cortical neurons. There was increased immunostaining for cleaved caspase-3 in the cytoplasm when the cells were treated with glucose and Aβ. TauC3 IHC showed labeling in the cytoplasm (Fig. 6, arrows) as well as a punctate pattern along the neurites (Fig. 6, arrowheads), which were not detected in control cells. When the images were merged, we could detect the colocalization of cleaved caspase-3 with cleaved tau in the cytoplasm (Fig. 6, arrows). There was similar colocalization when the cells were treated with glucose or Aβ alone (data not shown). These results, along with the biochemical data, strongly suggest that caspase is the key protease involved in tau cleavage during glucose- and Aβ-mediated neuronal apoptosis.
Our results from the embryonic cortical neurons strongly suggest the direct role of hyperglycemia on tau modification. Recently we reported age-dependent tau phosphorylation and cleavage in type 1 (STZ-injected) and type 2 (db/db) diabetic mice [24]. Here we employ different models of diabetes to confirm our previous reports as well as the in vitro results.

B6.V-Lep<sup>−/−</sup> mice, commonly known as ob/ob, are used as a type 2 diabetes model. ob/ob mice express a homozygous mutation of leptin and their characteristics are well described in the Jackson laboratory web site (http://jaxmice.jax.org/strain/000632.html). Wild type (WT) C57Bl/6J mice are used as controls. We examined tau phosphorylation in 12 week old WT and ob/ob brains. We observed increased tau phosphorylation in the cortex of ob/ob mice compared to WT at several residues including Ser199/202, Ser262, and Thr231 (Fig. 7). We also detect a decreased immunoreactivity by Tau1 antibody, which detects dephosphorylated tau. Total tau levels, examined with Tau5 and Tau46 antibody, were not different between WT and ob/ob. We observed, however, a decrease in the mobility and increased diffusion of the tau band from ob/ob mouse brains in SDS-PAGE, which represent high Ser/Thr phosphorylation levels in these animals.

Mounting evidence suggests that not only the hyperphosphorylation, but also tau cleavage plays an important role in the progression of AD [11]. Therefore, we next examined whether tau cleavage is enhanced in ob/ob mice. The tauC3 antibody detects tau cleaved at Asp421 [14]. When the brain lysates were immunoblotted with the TauC3 antibody, we detected an increased expression of a band representing cleaved tau in the cortex of ob/ob mice (Fig. 7). In addition, we detected a smaller band by longer exposure of the Tau1 immunoblots, which may represent a cleaved fragment of tau. These results from TauC3 and Tau1 antibodies are in agreement with our previous report using db/db mice [24]. We observed similar changes in tau phosphorylation and cleavage in the hippocampus (data not shown).

STZ-injected DBA/2J mice were used as a type 1 diabetes model. Fasting glucose levels were significantly higher at 38 weeks of age (25 weeks of diabetes) in STZ injected groups (501.5±23.4 mg/dL) compared to control (112.6±23.4 mg/dL). In agreement with the published reports [24, 33, 34], we detected increased tau phosphorylation in the cortices of STZ-injected mice at all residues examined (Fig. 8). In contrast to ob/ob mice, however, we did not detect tau cleavage in STZ-injected mouse brains (data not shown).

**DISCUSSION**

The abnormal aggregation of tau into NFT is one of the hallmarks of AD [9]. Both the cleavage and hyperphosphorylation of tau lead to aggregation and NFT formation [11, 13]. When cleaved by caspases, tau aggregates more easily and extensively in vitro [14]. In the current report, we present results strongly suggesting that caspase-mediated cleavage of tau due to hyperglycemic conditions is a potential mechanism for the increased risk of AD in diabetic patients [6, 7].

Our laboratory has reported that hyperglycemia resembling the high blood glucose levels in diabetes results in neuronal apoptosis in vivo and in vitro [21–23]. Consistent with our theory, glucose treatment induced concentration-dependent apoptosis in rat embryonic cortical neuron cultures. Apoptosis was well-correlated with the appearance of cleaved caspase-3 (i.e., activation), suggesting the involvement of caspases during apoptosis. Equimolar concentrations of mannitol or 2DG did not induce caspase-3 activation, demonstrating that the effect of glucose is not due to hyperosmorality.
Fig. 5. TauC3 immunostaining of rat embryonic cortical neurons. E15 rat embryonic cortical neurons were grown on PLL-coated glass coverslips for 6 days and then treated with 100 mM glucose, 10 μM Aβ or both for 24 h. The cells were fixed with 2% PFA and stained for TauC3. Arrowheads indicate TauC3 staining along the neurites and arrows indicate staining in the cytoplasm. Insets: Cells were stained with anti-neurofilament for the neurites (red) and DAPI for the nuclei (blue). Arrows in insets indicate the neurites. The bar represents 100 μm.

Fig. 6. TauC3 is colocalized with cleaved caspase-3. E15 rat embryonic cortical neurons were treated without (top panel) or with 100 mM glucose + 10 μM Aβ (bottom 2 panels) for 24 h. The cells were simultaneously stained for TauC3 (green, first column) and cleaved caspase-3 (red, second column). Nuclei are stained with DAPI (blue). Arrowheads indicate the TauC3 staining along the neurites and arrows indicate the colocalization of TauC3 and cleaved caspase-3 in the cytoplasm. The bars represent 50 μm.
Fig. 7. Increased tau hyperphosphorylation and cleavage in 12 week old ob/ob mouse cortex. Cortex lysates from 12 week old wild type (WT) and ob/ob male mice were immunoblotted with the indicated antibodies for phosphorylated, total, or cleaved tau. Tau1 immunoblot was exposed for a short time to detect tau (de)phosphorylation or overexposed to detect tau cleavage (arrows). The blots were stripped and reprobed with the anti-GAPDH antibody as a loading control. We observe similar results using hippocampus lysates (data not shown).

Previous studies demonstrated modulation of tau phosphorylation and cleavage during apoptosis in cortical neurons [39, 40] and our current results clearly show that tau is altered by glucose-mediated apoptosis. Tau cleavage was glucose time- and concentration-dependent and well-correlated with the appearance of cleaved caspase-3 and apoptosis. Our IHC data of TauC3 staining further support glucose-mediated tau cleavage in cortical neurons. A similar pattern of the TauC3 immunostaining along neurites has been demonstrated in hippocampal/cortical neuron cultures during staurosporine-induced apoptosis [11]. Consistent with the apoptosis results, mannitol or 2DG did not induce tau cleavage, demonstrating a specific effect of glucose. To our knowledge, our data are the first to demonstrate glucose-mediated tau cleavage both in vitro and in vivo.

In our study, tau cleavage is demonstrated by the appearance of fragments using a Tau1 antibody both for in vitro (cortical neuron) and in vivo (ob/ob mouse brain). Tau cleavage was also confirmed by the increase in TauC3 immunoreactivity. Despite the significant

Fig. 8. Tau phosphorylation is increased in the cortices of STZ-injected mice. Brains were prepared from DBA/2J mice 25 weeks after STZ injection (38 weeks of age). The cortex was homogenized in T-PER buffer and the lysates were immunoblotted with the indicated antibodies. The relative density of phosphorylated tau over total tau (Tau5) was measured after immunoblotting. *p<0.05 and **p<0.01 by t-test.

Fig. 9. Model. Hyperglycemia, possibly along with IR (diabetes pathology) may initiate the apoptotic pathway, including caspase activation, and tau cleavage. This process may prime neurons to be more susceptible to Aβ insults (AD pathology). The combined effects of glucose and Aβ further facilitate the apoptotic pathway, generating toxic tau cleavage fragments which can augment the process by a positive feedback mechanism.
The current study does not elucidate the exact nature of the tau cleavage products; however, studies show the formation of various sizes of tau fragments during neuronal apoptosis [38, 52–54]. One study demonstrated the generation of a similar size tau fragment to our current study in hippocampal neurons during Aβ-mediated apoptosis using the same Tau1 antibody [55]. The cleavage fragments, when directly added to the culture, are toxic to the neurons and induce apoptosis [38, 55]. Truncated tau, without mutation or hyperphosphorylation, can facilitate NFT formation in vitro [16, 56]. Therefore, it is possible that glucose and Aβ induce the apoptotic pathway, generating tau fragments that facilitate the process by a feed-forward mechanism.

Several proteases including caspasas [11, 17], proteases associated with the proteasome [18], and calpains [19, 20] are implicated in tau cleavage. Tau cleaved at Asp421 by caspase-3 is detected in AD brains and neurons treated with Aβ [57, 58]. Calpain is abnormally activated in AD patients compared to age-matched controls [59] and colocalized with NFT in AD [60]. The role of the proteasome in tau degradation remains controversial [61, 62]. We demonstrate in this report the activation of caspase-3 during glucose and Aβ treatment, which show an additive effect when both are used together. Furthermore, our IHC data demonstrating the colocalization of cleaved tau with active caspase-3 strongly support the important role of caspase-3 in tau cleavage. Similar colocalization of active caspase-3 and cleaved tau has been reported from postmortem AD brains [41]. However, the pan-caspase inhibitor, ZVAD, did not completely inhibit caspase-3 activation or tau cleavage as much as IGF-I treatment did, suggesting the involvement of other proteases. Unfortunately, calpain or proteasome inhibitors augmented cell death and increased caspase-3 activation. A recent report by Calissano and colleagues showed similar results using a different calpain inhibitor, MDL28170 [38]. Therefore, we could not exclude the possibility of the contribution of calpains or the proteasome. These results support the idea that different and/or additional proteases may be operational in vivo.

In agreement with in vitro results, tau cleavage was also observed in the brains of the ob/ob mouse model of type 2 model of diabetes, but not in WT mice, confirming our recently published report [24]. Unlike the effect of glucose on cortical neuron culture, tau cleavage in ob/ob mouse brains was accompanied by increased tau phosphorylation. Our results are in agreement with several recent reports of AD-like changes in type 1 and type 2 diabetic animal models [32, 33, 63]; however, all studies so far focus on tau hyperphosphorylation. Our current and previous [24] results show clear evidence of increased tau cleavage in type 2 diabetic animals. In spite of the increased glucose level and tau phosphorylation, however, tau cleavage was not observed in STZ-injected DBA/2J (current study) or C57Bl/6J mice [24]. These results suggest that type 1 and type 2 diabetes affect tau modifications differently regardless of genetic background. The difference of tau cleavage in type 1 and type 2 diabetes may be due to the insulin resistance (IR) observed in type 2 diabetes. IR is the major feature of the cluster of related physiologic dysfunctions known as the metabolic syndrome (glucose intolerance, obesity, dyslipidemia, and hypertension) [64, 65]. IR and metabolic syndrome are major risk factors for the development of type 2 diabetes [66]. We recently published a series of manuscripts demonstrating the development of IR in neurons [67–69]. We proved that, like the peripheral tissues, neurons develop IR upon chronic hyperinsulinemia which is observed only in type 2 diabetes. We further demonstrated the neuronal IR disrupts Akt-mediated signaling, which is critical for cell survival and glucose transport [70, 71]. Therefore, even though our in vitro results clearly demonstrate that hyperglycemia is critical for tau...
cleavage with acute treatment, additional factors (such as IR) may be required for chronic long term effects in vivo for tau modification. Recent reports suggest that IR also affects amyloid-β protein precursor processing [72, 73].

In summary, we demonstrate the possible contribution of hyperglycemia on tau modification in vivo and in vitro. Our hypothesis is presented in Fig. 9. Hyperglycemic conditions in diabetes (along with IR and type 2 diabetes) may initiate the apoptotic pathway, including caspase activation, which leads to tau cleavage. This process may prime the neurons to be more susceptible for later Aβ insults. The combined effects of glucose and Aβ further facilitate the apoptotic pathway, generating toxic tau cleavage fragments, which can augment the process by a positive feedback mechanism. Recent reports demonstrate a clear correlation between AD and diabetes. Considering the steady increase in the number of diabetic and AD patients, it is becoming more important to elucidate the mechanisms linking these two diseases. Our results present a new mechanism for increased AD incidence in diabetic patients: hyperglycemia-induced tau cleavage, in combination with Aβ pathology, facilitates AD pathogenesis.

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Effects of triple antioxidant therapy on measures of cardiovascular autonomic neuropathy and on myocardial blood flow in type 1 diabetes: a randomised controlled trial

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Abstract

Aims/hypothesis We evaluated the effects of a combination triple antioxidant therapy on measures of cardiovascular autonomic neuropathy (CAN) and myocardial blood flow (MBF) in patients with type 1 diabetes.

Methods This was a randomised, parallel, placebo-controlled trial. Participants were allocated to interventions by sequentially numbered, opaque, sealed envelopes provided to the research pharmacist. All participants and examiners were masked to treatment allocation. Participants were evaluated by cardiovascular autonomic reflex testing, positron emission tomography with $[^{11}C]$meta-hydroxyephedrine ($[^{11}C]$HED) and $[^{13}N]$ammonia, and adenosine stress testing. Markers of oxidative stress included 24 h urinary F$_2$-isoprostanes. Diabetic peripheral neuropathy (DPN) was evaluated by symptoms, signs, electrophysiology and intra-epidermal nerve fibre density. Randomised participants included 44 eligible adults with type 1 diabetes and mild-to-moderate CAN, who were aged 46±11 years and had HbA1c 58±5 mmol/mol (7.5±1.0%), with no evidence of ischaemic heart disease. Participants underwent a 24-month intervention, consisting of antioxidant treatment with allopurinol, α-lipoic acid and nicotinamide, or placebo. The main outcome was change in the global $[^{11}C]$HED retention index (RI) at 24 months in participants on the active drug compared with those on placebo.

Results We analysed data from 44 participants (22 per group). After adjusting for age, sex and in-trial HbA1c, the antioxidant regimen was associated with a slight, but significant worsening of the global $[^{11}C]$HED left ventricle RI ($-0.010$ [95% CI $-0.020$, $-0.001$] $p=0.045$) compared with placebo. There were no significant differences at follow-up between antioxidant treatment and placebo in the global MBF, coronary flow reserve, or in measures of DPN and markers of oxidative stress. The majority of adverse events were of mild-to-moderate severity and did not differ between groups.

Conclusions/interpretation In this cohort of type 1 diabetes patients with mild-to-moderate CAN, a combination antioxidant treatment regimen did not prevent progression of CAN, had no beneficial effects on myocardial perfusion or DPN, and may have been detrimental. However, a larger study is necessary to assess the underlying causes of these findings.
Keywords Antioxidant therapy · Cardiovascular autonomic neuropathy · Myocardial blood flow · Randomised trial

Abbreviations
ALA α-Lipoic acid
CAN Cardiovascular autonomic neuropathy
CARTs Cardiovascular autonomic reflex tests
CFR Coronary flow reserve
CRP C-reactive protein
CVD Cardiovascular disease
DPN Diabetic peripheral neuropathy
IENFD Intra-epidermal nerve fibre density
LV Left ventricle
MBF Myocardial blood flow
MNSI Michigan Neuropathy Screening Instrument
NCS Nerve conduction studies
PET Positron emission tomography
QST Quantitative sensory testing
QSART Quantitative sudomotor axon reflex testing
RI Retention index
SOD Superoxide dismutase

Introduction

Death from cardiovascular disease (CVD) remains the main cause of excess mortality rates in patients with type 1 diabetes [1]. Cardiovascular autonomic neuropathy (CAN) is associated with silent myocardial ischaemia and predicts enhanced cardiac risk. The development of CAN is a function of complex interactions between degrees of glycaemic control, disease duration, age-related neuronal attrition, and systolic and diastolic blood pressure [2]. These promote progressive autonomic neural dysfunction in a fashion that parallels the development of peripheral neuropathy, e.g. beginning distally and progressing proximally. Our data [3] and those of others [4] have confirmed that there is a compensatory increase in cardiac sympathetic tone in response to subclinical peripheral denervation early in the progression of CAN in patients with type 1 diabetes. Sympathetic denervation follows later, beginning at the apex of the ventricles, and progresses towards the base.

Oxidative stress is implicated in the development and progression of the chronic complications of diabetes, including CAN [3, 5–8]. We have previously reported that indices of oxidative stress were correlated with cardiac sympathetic dysinnervation and impaired myocardial vascular responsiveness in type 1 diabetes patients [3].

The goal of the current study was to understand the interrelationships between left ventricle (LV) sympathetic dysinnervation, altered myocardial blood flow (MBF) regulation, impaired neurotrophism and oxidative stress in type 1 diabetes. We postulated that attenuation of oxidative stress would result in the prevention or reversal of CAN. Phase II randomised controlled trials using the antioxidant α-lipoic acid (ALA) had previously shown some favourable effects on indices of heart rate variability [9]. However, the reported effects of various individual antioxidants in preventing or reversing diabetic peripheral neuropathy (DPN) or CAN have been inconsistent and disappointing [2, 6, 7]. We postulated that the failure of single antioxidant agents reflected a failure to address the multiple pathways that generate oxidative stress and are thought to promote complications of diabetes.

In the current study, we used a combination antioxidant therapeutic regimen aimed to address three different steps in the generation of oxidative damage: (1) xanthine-oxidase inhibition with allopurinol (to prevent oxygen free radical formation); (2) scavenging of oxygen free radicals with ALA; and (3) poly (ADP-ribose) synthase inhibition with nicotinamide (to reduce the downstream consequences of oxidative stress). We hypothesised that if several pathways were targeted at the same time with a combined antioxidant regimen, this would more effectively attenuate oxidative stress than single agents, and prevent deficits in LV sympathetic innervation and deficits in myocardial vascular responsiveness in participants with type 1 diabetes and mild-to-moderate CAN. We also conducted a comprehensive analysis of the effects of this antioxidant cocktail on measures of DPN in these patients.

Methods

Study design and patient population

This trial was a prospective, randomised, double-blind, placebo-controlled parallel group study. Participants with type 1 diabetes with early complications were randomly assigned to a combination antioxidant regimen or to placebo. Inclusion criteria required a diagnosis of type 1 diabetes (WHO), age 18 to 65 years, HbA$_1c$ <7.5 mmol/mol (9%), stable diabetes control over the previous 3 months, the presence of mild non-proliferative diabetic retinopathy (at least level ≥20 in the Early Treatment of Diabetic Retinopathy Scale) [10] or of microalbuminuria, and the presence of CAN, defined as a distal defect in $[^{11}C]meta$-hydroxyephedrine ($[^{11}C]HED$)
retention involving at least 10% of the LV. These criteria were selected on the basis of previously obtained evidence describing mild deficits in LV $^{[11]}$C HED retention in participants with mild non-proliferative retinopathy or mild albuminuria in spite of normal cardiovascular reflex testing [3]. Participants with any of the following were excluded: pre-existent CVD (coronary artery disease, positive stress test, congestive heart failure, arrhythmias, ventricular structural abnormalities, valvular disease), uncontrolled hypertension, severe systemic or inflammatory diseases (which could be associated with an increased likelihood of retinopathy, nephropathy or neuropathy), previous kidney, pancreas or cardiac transplantation, pregnancy or lactation in women, history of drug or alcohol dependence, and use of any other medications known to interfere with the uptake or metabolism of catecholamines (including immunosuppressants, tricyclic antidepressants, monoamine oxidase inhibitors and cocaine).

**Intervention** Allopurinol (300 mg daily), ALA (600 mg twice daily) and nicotinamide (750 mg twice daily), or matched oral placebos were administered for 24 months. The administration of each individual active drug or placebo component was titrated in consecutive weeks (first ALA, then nicotinamide, finally allopurinol) such that the participant began receiving full therapeutic doses of all the medications 3 weeks post-randomisation. Participants were allocated to interventions by sequentially numbered, opaque, sealed envelopes provided to the research pharmacist. All investigators, clinic staff and participants were blinded to treatment group assignment.

Participants were evaluated at 3 month intervals and the following recorded: data on vital signs, results of physical examination, HbA1c values, compliance with study drugs, laboratory safety and adverse events. Follow-up evaluations were obtained immediately after completion of the 24 month treatment. All patients were required to stay on the study drug until the day of final evaluations.

All participants signed a written informed consent document and the University of Michigan Institutional Review Board approved the study.

**CAN evaluations**

Patients were evaluated for the presence of CAN using positron emission tomography (PET) imaging of the LV with $^{[11]}$C HED and cardiovascular autonomic reflex tests at baseline and follow-up.

Because autonomic function may be altered by various factors, all participants were required to fast and avoid caffeine, tobacco products, and prescription and over-the-counter medicines (except usual insulin regimen) for at least 8 h before CAN testing. Participants who experienced hypoglycaemia (defined as blood glucose $\leq 2.775$ mmol/l and/or signs/symptoms of hypoglycaemia) after midnight prior to the above examinations, as well as those with acute illness in the previous 48 h were rescheduled.

Evaluation of LV sympathetic innervation by PET imaging with $^{[11]}$C HED

All PET studies were performed on an ECAT ExactHR+PET scanner (Siemens Medical Solutions USA, Knoxville, TN, USA). Participants were positioned in the PET scanner gantry and 74 MBq $^{[13]}$N ammonia was injected i.v., followed by a 2 min brief scan to visualise the heart. This ‘scout’ $^{[13]}$N ammonia scan was used to adjust the bed position so that the heart was at the centre of the scanner’s field of view and to confirm lack of resting perfusion abnormalities in the participant’s LV. After 30 min, 740 MBq $^{[11]}$C HED was injected i.v. while a 40 min dynamic PET data acquisition sequence was started (22 image frames; $12\times 10$ s, $2\times 30$ s, $2\times 60$ s, $2\times 150$ s, $2\times 300$ s, $2\times 600$ s) as described [11, 12].

$^{[11]}$C HED retention analysis The LV wall in the eight short axis slices from the $^{[11]}$C HED study (encompassing the LV from apex to base) was divided into 60 sectors to generate 480 independent LV regions. The $^{[11]}$C HED radioactivity concentration measured in each sector in the final image frame (30–40 min) was normalised to the calculated integral of the total radioactivity in the blood pool throughout the PET study, and the $^{[11]}$C HED retention index (RI) (in [ml blood] min$^{-1}$ [ml tissue]$^{-1}$) was obtained for each LV sector, as previously described [11].

Polar maps of regional $^{[11]}$C HED retention were generated and visually inspected for $^{[11]}$C HED retention deficits. A quantitative measure of the degree of cardiac denervation in each participant was generated by statistically comparing the $^{[11]}$C HED RI value of each sector in the participant’s $^{[11]}$C HED polar map with the mean and SD of the RI data for that sector in our database of healthy non-diabetic participants (age range 20 to 78 years, $n=15$ men, $n=18$ women, $n=33$ total). Using this standard $z$ score analysis [12], sectors in the participant’s $^{[11]}$C HED polar map with RI values more than 2.5 SD below the healthy control mean value were considered to be regions with ‘abnormal’ $^{[11]}$C HED retention.

**Cardiovascular autonomic reflex tests**

The standardised cardiovascular autonomic reflex tests (CARTs) included the paced R-R response to deep breathing, the Valsalva manoeuvre and postural changes in blood pressure as described [13], and were performed with Viking Quest II (Nicolet, Middleton, WI, USA).

Evaluation of MBF and coronary flow reserve

Evaluation of dynamic MBF and coronary flow reserve (CFR), a measure of endothelial function, was done at baseline and...
follow-up, using [13N]ammonia at rest and during pharmaco-logically induced (i.v. adenosine) coronary vasodilatation as previously described [14].

DPN evaluations

DPN evaluations comprised the following: (1) assessment of symptoms using the Michigan Neuropathy Screening Instrument (MNSI), a validated neuropathy questionnaire [15]; (2) comprehensive neurological evaluation performed by board-certified neurologists; (3) nerve conduction studies (NCS) including the sural, peroneal and median nerves with standardisation for limb temperature as described [16]; (4) quantitative sensory testing (QST) for cold detection and vibration perception thresholds; and (5) quantitative sudomotor axon reflex testing (QSART). In addition, skin biopsies were obtained to assess intra-epidermal nerve fibre density (IENFD), a measure of small-fibre neuropathy, to capture earliest changes associated with DPN and to evaluate the effects of intervention. All these tests were done at baseline and follow-up. Skin biopsies were performed as described [17]. Briefly 3 mm skin samples were obtained from the ankle and proximal thigh on the non-dominant side after intradermal local anaesthesia with 1% (wt/vol.) lidocaïne. The tissue was fixed for 6 h in paraformaldehyde lysine phosphate before transfer to cryoprotectant and cutting into 50 μm sections using a freezing sliding microtome (Leica CM1850, Leica Microsystems, Buffalo Grove, IL, USA). Sections were stained with the pan axonal marker PGP 9.5 (Serotec, Raleigh, NC, USA).

Epidermal nerve fibres were counted using established criteria as described [17]. The final IENFD measurement was derived by taking the mean of four to six randomly selected individual sections.

Evaluation of systemic oxidative stress

Systemic oxidative stress was evaluated by measuring free F2-isoprostanes, a reliable biomarker for assessment of oxidative stress and lipid peroxidation in vivo, from 24 h urine sample collections, and quantified using gas chromatography–mass spectrometry as described by Liu et al [18]. Deuterated internal standard was added to the free F2-isoprostanes and solid-phase extraction was completed. A moiety of pentafluorobenzyl was then introduced to the molecule and the hydroxyl groups were capped by trimethylsilyl derivatisation. A selective-ion monitoring technique was used to analyse the derivatives of F2-isoprostanes and the internal standard; the ions monitored were m/z 569 and m/z 573, respectively.

Outcome measures

The primary outcome was the change in the global [11C]HED RI at 24 months in participants taking the active drug compared with those on placebo. Three secondary endpoints were also specified: endothelial dysfunction as measured by the global CFR, systemic oxidative stress as assessed by 24 h urinary free F2-isoprostanes and inflammation as assessed by high-sensitivity C-reactive protein (CRP). All other outcomes were specified as exploratory; these included changes in: (1) regional [11C]HED RI and regional CFR; (2) resting MBF; (3) cardiovascular autonomic reflex testing; (4) measures of DPN, defined either as clinically confirmed peripheral neuropathy requiring two positive responses among neuropathic symptoms (pain, numbness, paresthesia); and sensory signs and abnormal ankle reflexes, confirmed by NCS abnormalities involving two or more of the sural, peroneal and median nerves [16]; and (5) changes in IENFD.

Statistical analysis

Randomisation Block randomisation was based on a block size of 4, with stratification by sex and age, where age was divided into two strata: ≤45 vs >45 years old.

Sample size The SD of [11C]HED was assumed to be 0.0113, based on our previously published data of the magnitude of the RI deficit observed in the distal myocardial segments of patients with moderate to severe CAN [19]. With 15 participants per group, we estimated 80% power to detect a change of 0.015 between the mean [11C]HED RI of the placebo-treated and actively treated groups, when testing the null hypothesis using a two-tailed two-sample t test at a 5% level of significance. A 20% loss due to early withdrawals and/or non-evaluable measurements was assumed and, combined with the effect of stratification on analysis, resulted in the requirement to recruit 22 patients per treatment group.

Data analysis Data are shown as mean±SD. A general linear model (a more general form of ANOVA) was fitted to the data, with treatment group, sex and age strata as the independent factors. When the dependent variable was measured at 24 months, the baseline value was also included as a covariate. In addition, the analyses of all endpoints (e.g. PET and NCS) except blood pressure were adjusted for HbA1c; analyses of nerve conduction data were also adjusted for temperature of the limb. Differences between groups were considered significant at p≤0.05. For the significant tests we also report the estimated adjusted mean difference between the groups; i.e. antioxidant group-adjusted mean change – placebo group-adjusted mean change [20]. We report the analysis using data for participants who completed the trial, i.e. had measurements performed at baseline and 24 months. However, we repeated the analyses of the primary and secondary endpoints with zero differences imputed for participants who did not complete the trial; these analyses gave similar results to the reported analyses. The analyses were performed using SAS 9.2 (SAS Institute, Cary, NC, USA).
Results

Participants

We enrolled 44 participants with type 1 diabetes who met the inclusion criteria and were randomised to either antioxidant treatment or placebo (22 participants per group) (Fig. 1). These participants, 39% of whom were women and 94% of whom were whites, had a mean age of 46±11 years, with 26±12 years duration of diabetes and HbA1c of 58±5 mmol/mol (7.5±1.0%) at baseline. There were no significant differences in any of the demographic or other baseline characteristics of the

Table 1 Selected baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th>Placebo</th>
<th>Antioxidant</th>
<th>p value</th>
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<td>22</td>
<td>22</td>
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<tr>
<td>Age (years)</td>
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<td>27±12</td>
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<td>Triacylglycerol (mmol/l)</td>
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</table>

Data are shown as mean±SD or n

p values were computed using general linear model on ranked data adjusted for sex and age strata, except for age, which was adjusted only for sex, and for sex, which was estimated by Fisher’s exact test.
participants, except for weight, as summarised in Table 1. There were also no differences in HbA1c, systolic or diastolic blood pressure, resting heart rate or total cholesterol between groups at 12 (data not shown) and 24 months of treatment (Table 2). A total of 31 participants completed the 24 month intervention, 18 in the placebo and 13 in the antioxidant group (Fig. 1). The main reasons for dropout are shown in Fig. 1.

Effects of treatment on \([^{11}\text{C}]\text{HED RI}\)

At baseline there were no differences between groups in global (Table 2) or any regional \([^{11}\text{C}]\text{HED RI}\) (electronic supplementary material [ESM] Table 1). The antioxidant regimen was associated with a slight, but significant worsening (decreases) of the global \([^{11}\text{C}]\text{HED RI}\) change compared with placebo after adjusting for age, sex and HbA1c (\(p=0.045\)) (Table 2). No change from baseline was observed with placebo (Table 2).

Analyses of the regional \([^{11}\text{C}]\text{HED RI}\) also showed that the antioxidant treatment induced slightly greater decreases in the \([^{11}\text{C}]\text{HED RI}\) in the distal anterior (\(p=0.043\)), proximal lateral (\(p=0.043\)) and proximal anterior (\(p=0.03\)) segments compared with the placebo group (ESM Table 1). In subgroup analyses, no difference was seen in the global or regional \([^{11}\text{C}]\text{HED RI}\) between participants with a positive change in 24 h urinary F2-isoprostanes (a marker of oxidative stress) and those with a negative change (discussed further below).

| Table 2 | Effects of intervention on primary and secondary outcomes, and on other selected endpoints |
| --- | --- | --- | --- | --- |
| Outcome per time-point | Placebo\(^a\) | Antioxidant\(^b\) | \(p\) value | Antioxidant effect\(^c\) |
| Primary | | | | |
| Global \([^{11}\text{C}]\text{HED RI}\) (blood min\(^{-1}\) [ml tissue]\(^{-1}\)) | | | | |
| Baseline | 0.073±0.016 | 0.081±0.017 | 0.32 | |
| 24 months | 0.074±0.016 | 0.070±0.018 | 0.045 | −0.010 (−0.020, −0.001) |
| Secondary | | | | |
| Global coronary flow reserve | | | | |
| Baseline | 2.94±1.70 | 2.95±1.32 | 0.52 | |
| 24 months | 3.22±0.85 | 3.02±1.52 | 0.82 | 0.08 (−0.67, 0.84) |
| High-sensitivity CRP (nmol/l) | | | | |
| Baseline | 10.87±3.33 | 10.38±2.76 | 0.94 | |
| 24 months | 16.95±18.38 | 17.51±20.19 | 0.83 | 1.3 (−11.5, 14.1) |
| F2-Isoprostanes (ng/g creatinine) | | | | |
| Baseline | 2.21±1.36 | 2.35±1.44 | 0.87 | |
| 24 months | 2.09±1.12 | 2.92±1.99 | 0.24 | 0.78 (−0.55, 2.11) |
| Other | | | | |
| Systolic BP (mmHg) | | | | |
| Baseline | 129±12 | 132±15 | 0.42 | |
| 24 months | 124±17 | 120±23 | 0.37 | −6 (−20, 7) |
| Diastolic BP (mmHg) | | | | |
| Baseline | 75±8 | 74±10 | 0.93 | |
| 24 months | 72±10 | 70±11 | 0.82 | −1 (−8, 6) |
| Heart rate (bpm) | | | | |
| Baseline | 75±9 | 81±15 | 0.18 | |
| 24 months | 72±8 | 82±9 | 0.039 | 5 (0, 10) |
| Valsalva ratio | | | | |
| Baseline | 1.51±0.40 | 1.33±0.20 | 0.083 | |
| 24 months | 1.43±0.21 | 1.46±0.27 | 0.55 | 0.06 (−0.15, 0.26) |
| E/I ratio | | | | |
| Baseline | 1.21±0.15 | 1.17±0.15 | 0.74 | |
| 24 months | 1.15±0.07 | 1.49±1.13 | 0.13 | 0.46 (−0.15, 1.07) |
| 30:15 ratio | | | | |
| Baseline | 1.26±0.71 | 1.28±0.65 | 0.75 | |
| 24 months | 1.75±1.30 | 2.04±1.33 | 0.88 | 0.07 (−0.81, 0.94) |

All data shown as mean±SD, except where shown otherwise

\(^a\)\(n=22\) baseline, \(n=18\) follow-up; \(^b\)\(n=22\) baseline, \(n=13\) follow-up; \(^c\)with 95% CI

\(p\) values were computed using a general linear model adjusted at baseline for age strata, sex and HbA1c, and at 24 months for baseline value, age strata, sex and HbA1c; blood pressure was not adjusted for HbA1c (see Methods).

At \(p<0.05\), the antioxidant effect is the estimated adjusted mean difference between the antioxidant group and the placebo group.

bpm, beats per min

\(\odot\) Springer
Effects of treatment on measures of endothelial function (CFR) and MBF

At baseline and 24 months there were no differences between the antioxidant and placebo groups in the global CFR, a measure of endothelial function and secondary endpoint of this trial (Table 2).

The global and regional resting MBF were similar in participants in both groups at baseline and in the change between baseline and 24 months (ESM Table 1).

No between-group differences were observed in the global stress MBF at baseline \((p=0.48)\). The global stress MBF was decreased slightly under the antioxidant regimen at 24 months, compared with a slight increase in the placebo group; this difference did not reach statistical significance \((p=0.53)\). There were no between-group differences in regional stress MBF in the change between baseline and 24 months (ESM Table 1).

Effects of treatment on urinary F2-isoprostanes and CRP

No differences were observed between placebo and antioxidant groups, respectively, in the levels of 24 h urinary F2-isoprostanes at baseline or in the change between baseline and 24 months of treatment \((p=0.87\) and \(p=0.24\), respectively) (Table 2). Similarly, the antioxidant treatment had no effect on CRP levels (Table 2).

Table 3  Adverse events

<table>
<thead>
<tr>
<th>Event category</th>
<th>Placebo ((n))</th>
<th>Antioxidant ((n))</th>
<th>Total adverse events ((n))</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory</td>
<td>26 34 60</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>25 23 48</td>
<td>0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Musculoskeletal</td>
<td>18 21 39</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral infections</td>
<td>9 10 19</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>9 9 18</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ears, nose, throat</td>
<td>10 5 15</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allergy</td>
<td>8 6 14</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin rash</td>
<td>6 6 12</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurology</td>
<td>8 1 9</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gynaecology</td>
<td>1 7 8</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urogenital</td>
<td>4 4 8</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blurry vision</td>
<td>4 3 7</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression</td>
<td>1 4 5</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plastic surgery</td>
<td>3 1 4</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>0 3 3</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver enzyme elevation(^a)</td>
<td>1 1 2</td>
<td>1.00</td>
<td></td>
<td></td>
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<tr>
<td>Haemopoietic</td>
<td>0 1 1</td>
<td>1.00</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>137 141 278</td>
<td>0.86</td>
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</table>

\(^a\) Elevated <2.5 times

Discussion

In this cohort of type 1 diabetes patients with mild-to-moderate CAN, an antioxidant regimen designed to affect three different steps in the oxidative stress pathways did not prevent progression of CAN, had no beneficial effects on myocardial perfusion and may have been detrimental (as suggested by the decrease in global \(^{13}\)C HED RI and MBF). This antioxidant regimen also had no effect on other measures of CAN, such as cardiovascular reflex testing, or on wide-ranging measures of large- and small-fibre neuropathy.

The role of oxidative stress in the pathophysiology of diabetic microvascular complications, including CAN and DPN, has been amply discussed, and the evidence is compelling [3, 5–7, 21]. Moreover, various antioxidants have been shown to prevent or delay the progression of CAN and DPN in animal models [21–24]. For instance, treatment with the antioxidant ALA prevented the formation of reactive oxygen species, caspase-3 activation, nuclear DNA degradation and activation of the receptor for advanced glycation end-products, which have all been shown to promote the development of DPN [25]. The antioxidant allopurinol has been shown to have marked neural and vascular effects in a rat model of diabetes [26] and to attenuate the development of diabetic cardiomyopathy in the streptozocin-induced mouse model of type 1 diabetes [27]. In another established rat model of type 1 diabetes, nicotinamide was shown to be effective at reversing...
early DPN [28] and to be neuroprotective in combination with antioxidant melatonin [29]. Furthermore, in the experimental model study that provided a basis for the present clinical trial, our group demonstrated that ALA, allopurinol and nicotinamide had independent effects on oxidative stress and neuronal survival, as well as providing neural protection when used in combination [30].

Our choice of antioxidant regimen was based on the mechanism by which each antioxidant exerts its own effect on attenuating oxidative stress. ALA directly scavenges free radicals, recycles other natural antioxidants, protects peripheral nerves from lipid peroxidation and increases the activity of catalase and superoxide dismutase (SOD) [31], possibly resulting in the normalisation of impaired endoneural blood flow and nerve conduction velocity [31]. Further mechanisms of ALA include: (1) improving the antioxidant defence system through gene expression; (2) inhibiting nuclear factor κB; and (3) activating AMPK in skeletal muscles, with each of these factors having numerous effects [32]. Allopurinol inhibits the xanthine-oxidase pathway, thereby reducing the number of reactive oxygen species formed. Nicotinamide, a water-soluble form of vitamin B3, is a weak first-generation poly(ADP-ribose) polymerase inhibitor and a precursor of NAD⁺. Besides its antioxidant properties, it has been shown to improve energy status in ischaemic tissue, regulate neuronal calcium fluxes and inhibit apoptosis [28].

Despite the promising results in animal studies, studies of the effects of antioxidant therapy on measures of neuropathy in humans have been disappointing. The conclusions of a 4 month randomised control multicentre trial (DEKAN study) showed that ALA treatment (800 mg/day orally) of type 2 diabetes mellitus patient ‘may slightly improve CAN’ in this patient population [33]. Moreover, the data from seven clinical trials (ALADIN I, II, and III, SYDNEY, SYDNEY 2, ORPIL and NATHAN I) with a total of 1,551 patients treated with ALA (parenteral, oral or combined) for periods ranging from 3 weeks to 4 years are inconclusive [34–37]. Although some studies showed improvements in various symptoms scores, more objective measures were neutral at best. In another study done on 40 adolescents with type 1 diabetes, a controlled-release formulation of ALA did not have any significant effect on markers of oxidative damage or total antioxidant status [38].

A possible explanation for the low rate of success in previous studies is the complexity of mechanisms underlying increased oxidative stress in diabetes, and the fact that using single agents addressing a single pathway have been considered insufficient to effectively correct excess oxidative stress. A recent prospective, non-randomised, open-label study of 50 patients with diabetic neuropathy who were treated with ALA and SOD showed improved nerve conduction velocity and pain perception [39], providing some support for the rationale of our study. However, here we report that our antioxidant approach lacked effectiveness, with no detectable changes in the levels of 24 h urinary F2-isoprostanes, a marker of oxidative stress. This is unlikely to reflect a suboptimal dosing regimen, as therapeutic doses of all agents were used at levels at or above those used in previous clinical reports [34–37, 40]. It is therefore possible that a lack of penetration of the active drug regimen into the target tissues or cellular compartments may have contributed to the lack of efficacy. This raises the possibility that: (1) absorption of these agents was inefficient; and (2) unaccounted for metabolic interactions may have negatively affected the in vivo anti-oxidative properties of the agents when used in combination, possibly nullifying their individual effects. However, with the available data, we were not able to assess causality in this study.

Several other interventional studies using antioxidants have failed, with possible suggested explanations including inadequacy of the doses used, short duration of therapy or poor timing of initiation of the supplement [41]. Another possible reason for the failure of antioxidants to reduce diabetes-related complications is the vast array of mechanisms of glucotoxicity that are independent of oxidative stress. Thus Mooradian and Haas suggest that endoplasmic reticulum stress could explain a recent failure of antioxidant treatment to reduce diabetes-related complications [41].

An additional unexpected observation was the possible detrimental effect of the triple antioxidant on myocardial perfusion. This is in contrast to data obtained in various cohorts of patients with type 1 or type 2 diabetes when using these agents as monotherapy. Allopurinol [42] and nicotinamide [43] used as monotherapies have both been shown to improve endothelial function. However, importantly, allopurinol blocks purine degradation and may result in the accumulation of purine metabolites, including adenosine in the myocardium. This may have contributed to the slightly (although non-significant) higher resting MBF in participants treated with the antioxidant regimen, and to an impaired microvascular responsiveness to adenosine infusion.

Study limitations include the high rate of dropout, the limited power due to the relatively low number of patients, the relatively short duration of the study and the limited assessment of changes in oxidative stress markers. Since this study was designed and initiated, more sensitive techniques such as mass spectrometry have been developed to evaluate multiple oxidative pathway intermediates, which unfortunately, due to sample limitations, were not available for this study. However, this study is to date the first and only one to comprehensively assess the efficacy of a combined antioxidant approach that has been validated in experimental models of neuropathy [30] in participants with diabetes using highly sensitive state of the art technology to characterise CAN, MBF and DPN.

In summary, treatment with a triple antioxidant regimen consisting of ALA, allopurinol and nicotinamide for 24 months failed to prevent changes in oxidative stress and progression of
CAN, MBF deficits or DPN in this cohort of patients with type 1 diabetes, suggesting that traditional antioxidant approaches have limited efficacy in human diabetes even when used in combination. These findings are important for the design of future studies using new agents to target pathogenic mechanisms of CAN and DPN development in diabetes.

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**Duality of interest** The authors declare that there is no duality of interest associated with this manuscript.

**Contribution statement** RPB performed the study, provided a substantial contribution to the acquisition, analysis and interpretation of data, drafted the article and revised it critically for important intellectual content, and gave final approval of the version to be published. MJS provided a substantial contribution to study conception and design, and to the analysis and interpretation of data, as well as critically revising the manuscript for important intellectual content and giving final approval of the version to be published. DMR provided a substantial contribution to the acquisition, analysis and interpretation of data, revised the manuscript critically for important intellectual content, and gave final approval of the version to be published. EAW and MM provided a substantial contribution to the analysis of data, revised the manuscript critically for important intellectual content and gave final approval of the version to be published. CDP provided a substantial contribution to the acquisition of data, revised the manuscript critically for important intellectual content and gave final approval of the version to be published. MBB analysed all data, provided a substantial contribution to the interpretation of data, revised the manuscript critically for important intellectual content and gave final approval of the version to be published. ELF provided a substantial contribution to study conception and design, revised the manuscript critically for important intellectual content and gave final approval of the version to be published.

**References**

The Metabolic Syndrome and Neuropathy: Therapeutic Challenges and Opportunities

Brian Callaghan, MD and Eva Feldman, MD, PhD

The metabolic syndrome and neuropathy are common conditions, especially in the elderly, that are associated with significant morbidity. Furthermore, the metabolic syndrome is reaching epidemic proportions across the world. Current evidence supports the association of the metabolic syndrome and its individual components with neuropathy. Several clinical trials have demonstrated that treating hyperglycemia, a component of the metabolic syndrome, has a significant effect on reducing the incidence of neuropathy in those with type 1 diabetes. However, glucose control has only a marginal effect on preventing neuropathy in those with type 2 diabetes, suggesting that other factors may be driving nerve injury in these patients. Emerging evidence supports the metabolic syndrome as including risk factors for neuropathy. Interventions exist for treatment of all of the metabolic syndrome components, but only glucose control has strong evidence to support its use and is widely employed. Our understanding of the biology of metabolic nerve injury has rapidly expanded over the past several years. Mechanisms of injury include fatty deposition in nerves, extracellular protein glycation, mitochondrial dysfunction, and oxidative stress. Additionally, the activation of counter-regulatory signaling pathways leads to chronic metabolic inflammation. Medications that target these signaling pathways are being used for a variety of diseases and are intriguing therapeutic agents for future neuropathy clinical trials. As we move forward, we need to expand our understanding of the association between the metabolic syndrome and neuropathy by addressing limitations of previous studies. Just as importantly, we must continue to investigate the pathophysiology of metabolically induced nerve injury.

Obesity is a worldwide epidemic with a 100% increase in all-cause mortality. Between 1980 and 2008, the prevalence of obese individuals doubled, reaching greater than half a billion worldwide. Obesity is the central element underlying the metabolic syndrome (MetS), a clustering of 5 risk factors including obesity, insulin resistance, hypertension, hypertriglyceridemia, and dyslipidemia. MetS is principally responsible for the alarming increase in chronic diseases, chiefly diabetes, cardiovascular disease, neurodegenerative disease, and cancer. According to National Nutrition and Health Survey (NHANES) data from 1988–1994, 22% of the adult US population met criteria for MetS, with >40% of the elderly affected. Using the 1999–2002 NHANES data, the prevalence of MetS had climbed to a staggering 34.5%, and the current prevalence is approaching 50% (www.cdc.org). Like obesity, MetS is not just an American problem. India, Iran, Mexico, Ireland, Scotland, and Turkey are just some of the countries with >20% of their population affected by MetS. China, with the world’s largest population, has a rapidly increasing MetS prevalence of approximately 30%.

Peripheral neuropathy is a chronic and common disease, affecting 2 to 7% of the population, according to estimates from population-based studies in India and Italy. As with MetS, the prevalence rises in the elderly, with 15% affected according to a study that focused on a US population aged >40 years. Not only is neuropathy a widespread condition, but it is also quite disabling. Neuropathic pain affects approximately half of patients with diabetic neuropathy. Moreover, sensory deficits lead to balance difficulties and frequent falls, with resulting musculoskeletal injuries, including fractures. Neuropathy is also a risk factor for foot ulcerations and
lower extremity amputations, particularly in those with diabetes. All of these manifestations of neuropathy have a profound effect on an individual’s quality of life. Both neuropathy and MetS are frequently encountered conditions that disproportionately affect the elderly, with significant morbidity and mortality. When considering the discrete components of MetS, diabetes and prediabetes have the strongest evidence supporting a pathogenic link with neuropathy, but each of the other components also have evidence supporting their association with neuropathy in diabetic populations. Specifically, obesity has been shown by multiple investigators to be associated with neuropathy in diabetic patients. Isomaa and colleagues, Costa and colleagues, and the Metascreen investigators have independently shown that an individual with diabetes is more likely to have neuropathy if other components of MetS are present. In a study of 427 diabetic patients with mild to moderate diabetic neuropathy, elevated triglycerides correlated with loss of sural nerve myelinated fiber density, a direct anatomical measurement of neuropathy. In contrast, there was no association with glycemic control and neuropathy in this cohort. The most telling data are from several large clinical trials, all of which report that glycemic control alone is not enough to prevent type 2 diabetic patients from developing neuropathy. Furthermore, patients with normoglycemia and neuropathy have the same prevalence of MetS components as those with impaired glucose tolerance and neuropathy, and an even higher prevalence of MetS components than those with diabetes and no neuropathy. These results indicate that MetS and its components are likely to be important in nondiabetic populations as well. Given the clustering of MetS components, hypertension, hypertriglyceridemia, dyslipidemia, and particularly obesity are prime candidates to be the essential factors underlying the neuropathy present in patients with type 2 diabetes.

Modern Understanding of Biology

Up until this past decade, it was generally believed that the underlying cause of neuropathy was hyperglycemia, irrespective of the type of diabetes (1 or 2). The more plausible and current hypothesis is that MetS underlies the onset and progression of neuropathy and that obesity and its consequences are the driving factors leading to nerve injury.

The fundamental property of obesity is energy imbalance, with low energy expenditure compared to high caloric consumption. Excess nutrients are initially stored in “professional” metabolic tissues, such as fat, skeletal muscle, and liver. When the storage capacity of

![FIGURE 1: Signaling pathways underlying nutrient excess and metabolic neuropathy. Hyperglycemia and hyperlipidemia incite a feed-forward cycle of cellular damage with production of reactive oxygen species leading to cellular oxidative stress, mitochondrial dysfunction, and parallel endoplasmic reticulum (ER) stress. These changes not only lead to direct neuronal injury but also promote nutrient excess-mediated insulin resistance, initiating tissue inflammation, which in turn exacerbates insulin resistance and mediates injury cascades. There is leukocyte recruitment with the production of tissue-damaging inflammatory chemokines and activation of Jun N-terminal kinases (JNK) and inhibitor of nuclear factor Kb kinase B (IKKb) triggering further insulin resistance, inflammatory responses, and tissue damage. JNK and IKKb also mediate nuclear factor kappa B (NFkB) activation, leading to production of inflammatory and tissue-damaging signals. Collectively, these diverse but interlinked pathways reinforce a destructive cycle of cellular impairment and damage linking nutrient excess to metabolic neuropathy. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]](https://www.annalsofneurology.org)
these tissues is exceeded, bystander tissues such as the nervous system are subjected to excess nutrients with little ability to handle superphysiologic substrates, resulting in extrinsic and intrinsic cellular dysfunction. Extrinsic forces include fatty deposition in the nerve and extracellular protein glycation and oxidation. The hallmark of intrinsic dysfunction is metabolic imbalance with lipid and glucose dysregulation leading to mitochondrial dysfunction and subsequent oxidative and endoplasmic reticulum stress. Neurons also express receptors for low-density lipoproteins (LDLs), and elevated levels of oxidized LDLs, a hallmark of obesity and MetS, activate receptors for oxidized LDLs, such as lectinlike oxidized LDL receptor-1, to promote additional mitochondrial injury.

With ongoing energy imbalance, there is a vicious feed-forward cycle, activating counter-regulatory signaling pathways that converge to inhibit insulin signaling and promote chronic metabolic inflammation. These counter-regulatory pathways include extracellular regulated kinases, Jun N-terminal kinases (JNKs), inhibitor of nuclear factor κB kinase β, mammalian target of rapamycin (mTOR), and endoplasmic reticulum-to-nucleus signaling 1 (IRE1), each a potential target for mechanism-based intervention. Continued inflammation fosters neuronal insulin resistance and loss of insulin neurotropism, and engorged neural adipocytes secrete inflammatory chemokines capable of recruiting proinflammatory M1 macrophages to the already stressed nerve, intensifying neural injury. Systemic inflammation promotes hypertension, resulting in nerve ischemia, further promoting oxidative and nitrosative stress, aberrant neuronal and axonal mitochondrial function, energy deprivation, and nerve injury. The Figure depicts the intersection of MetS components with neuronal injury and the central role of inflammation.

**Currently Available Therapies**

The only component of MetS with an established treatment for the prevention of neuropathy is diabetes. Enhanced glucose control has been shown to decrease the incidence of neuropathy in patients with type 1 diabetes,
with little effect in those with type 2 diabetes (Table).\textsuperscript{46–49} In type 1 diabetes, enhanced glucose control can be achieved through diet, exercise, and insulin. Similar diet and exercise regimens with the addition of metformin, sulfonylureas, and other less common drugs provide improved glycemic control but little protection against neuropathy in type 2 diabetes. Diet and exercise in those with prediabetes and neuropathy has been shown to increase nerve fiber density, but no controlled clinical trial has been performed to confirm this finding.\textsuperscript{26} Furthermore, diet, exercise, and metformin reduce the incidence of diabetes in those with prediabetes, but the effect on the prevention of neuropathy is unclear.\textsuperscript{50} Although effective pharmaceutical treatments exist for hypertension, hypertriglyceridemia, and dyslipidemia, no studies have investigated the effect of these interventions on the prevention or improvement of neuropathy. Similarly, although diet and exercise programs and medications can be effective in the treatment of obesity, no current data exist on the effect of these interventions on peripheral neuropathy in this population. Importantly, diet and exercise regimens have the potential to treat MetS as a whole; however, compliance and long-term maintenance on these regimens are notoriously difficult. The good news is that there are many currently available treatments of MetS components. The bad news is that our only curative treatment or improvement of neuropathy in patients with diabetes, and this has little effect on neuropathy in patients with type 2 diabetes and MetS neuropathy.

**Therapeutic Pipeline in 2013**

The central role of inflammation in MetS and associated chronic clinical disorders has led to the recent development of mechanism-based therapies, which include small molecule kinase, chemokine and cytokine inhibitors, and genetically engineered recombinant proteins that target specific inflammatory receptors or ligands, as well as the use of older, more broadly based anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs such as salicylate. Although these therapies have not yet been used to abrogate the acquired inflammatory microenvironment in the peripheral nervous system, these approaches are in current experimental use in other chronic MetS diseases, including central nervous system neurodegenerative disorders.

For example, small molecule kinase inhibitors targeting JNK, mTOR, and IRE1 can attenuate inflammation and macrophage activation to provide neuroprotection in neurodegenerative conditions, including traumatic brain and spinal cord injury, Parkinson disease, multiple sclerosis, and Alzheimer disease.\textsuperscript{51,52} JNK inhibitors are currently in phase II clinical trials for inflammatory endometriosis and idiopathic pulmonary fibrosis, demonstrating the translational potential of this therapeutic strategy for metabolic neuropathy.\textsuperscript{52} Inhibitors of IRE1 are effective in the treatment of endoplasmic reticulum stress-associated diseases, including multiple myeloma.\textsuperscript{53,54} mTOR inhibitors are another popular therapeutic strategy, and the role of mTOR in cellular metabolism, autophagy, and survival has supported applications of mTOR inhibitors in breast cancer and spinal cord injury and as an antiaging treatment.\textsuperscript{55–57} Interestingly, the type 2 diabetes drug metformin functions by activating adenosine monophosphate–activated protein kinase, which in turn negatively regulates mTOR signaling, and indirect mTOR regulatory mechanisms of metformin have also been recently uncovered.\textsuperscript{56,58,59}

Attention to chemokines as a therapeutic target is also increasing, with evidence of critical implications of CC ligand 2 (Ccl2) and its receptor CCR2 in neuronal injury and multiple sclerosis.\textsuperscript{60,61} Small molecule inhibitors of Ccl2 and Ccl5 are currently in phase II trials for the treatment of diabetic nephropathy (www.clinicaltrials.gov; NCT01712061). Salsalate is reported to have significant glucose-lowering effects by blocking low-grade inflammation via inhibiting nuclear factor kappa B and consequently improving insulin sensitivity in multiple small trials and case reports.\textsuperscript{62} Goldfine and colleagues recently completed a large multicenter randomized trial, the Targeting Inflammation with Salsalate in Type 2 Diabetes trial, evaluating the effects of salsalate on serum inflammatory markers, insulin levels, and glucose control. They report that salsalate lowers hemoglobin A1C levels and improves glycemic control in patients with type 2 diabetes.\textsuperscript{63} Together, these approaches provide optimism that a novel and as yet untested therapeutic pipeline exists for neuropathy.

**Unmet Needs**

Although multiple studies have demonstrated associations between MetS and neuropathy, studies to date have largely focused on patients with diabetes, have utilized cross-sectional study designs, and have used inconsistent definitions of neuropathy. Furthermore, the proportion of patients with neuropathy that are attributed to MetS is also unclear. The high prevalence of MetS makes this syndrome a potentially large contributor to the development and progression of neuropathy in those with and without diabetes, but the degree of impact of MetS on neuropathy remains to be defined. Past studies investigating the association between specific MetS components and neuropathy have also yielded inconsistent results. For example, De Block et al did not find an association between obesity and neuropathy, whereas 3 other
investigators found a significant association. Identifying the particular components that drive neuropathy is essential in informing future clinical trials. We also have no information on the interactions between the different MetS components and neuropathy. It is possible that a specific combination of MetS components is needed to cause neuropathy or that the effects of the individual components are not additive but synergistic. Much also remains to be learned about the underlying causes and potential treatments of metabolic neuropathy, and we contend that targeting inflammation offers a novel and likely effective treatment strategy.

Possible New Directions for Research
An evolving literature indicates that type 1 and type 2 diabetes are substantially different diseases with disparate mechanisms. MetS and its individual components are potential explanations for this observation, with a much greater prevalence in those with type 2 diabetes. Future investigations are needed to define the underlying pathophysiologic differences between the 2 different types of diabetes, with a focus on MetS components and inflammation. This information would have significant implications for the development of new therapeutic agents in this area. There is also a need for epidemiologic studies that address some of the shortcomings of existing trials, such as studying patients with MetS with and without diabetes, utilizing longitudinal study designs, and employing rigorous definitions of neuropathy. This information has the potential to give further evidence that there is a causal relationship between MetS and neuropathy. We also must define the impact of MetS on neuropathy, the role of its individual components, and the interactions between them. Enhancing our knowledge of the underlying scientific mechanisms and epidemiology of metabolic neuropathy has the potential to rapidly lead to clinical trials, as all MetS components have currently available treatments. Hopefully, this new knowledge will also help us develop novel therapeutics with the potential to prevent, halt, or reverse this common, disabling disease.

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Dr. S. S. Jacoby contributed to critical review of the manuscript.

Authorship
B.C. and E.F. participated in the literature review and writing of the manuscript.

Potential Conflicts of Interest
Nothing to report.

References


Expenditures in the elderly with peripheral neuropathy

Where should we focus cost-control efforts?

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Eva L. Feldman, MD, PhD
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Summary
To optimize care in the evaluation of peripheral neuropathy, we sought to define which tests drive expenditures and the role of the provider type. We investigated test utilization and expenditures by provider type in those with incident neuropathy in a nationally representative elderly, Medicare population. Multivariable logistic regression was used to determine predictors of MRI and electrodiagnostic utilization. MRIs of the neuroaxis and electrodiagnostic tests accounted for 88% of total expenditures. Mean and aggregate diagnostic expenditures were higher in those who saw a neurologist. Patients who saw a neurologist were more likely to receive an MRI and an electrodiagnostic test. MRIs and electrodiagnostic tests are the main contributors to expenditures in the evaluation of peripheral neuropathy, and should be the focus of future efficiency efforts.

Medical societies, including the American Academy of Neurology (AAN), are working proactively to address health care inefficiencies through efforts to identify specific tests that are unnecessary or unwarranted in common clinical scenarios.1,2 Such efforts need to be informed regarding aggregate expenditures associated with individual tests so that the focus can be on high-impact tests.3 Identifying these high-impact tests in the evaluation of peripheral neuropathy is particularly important because it is a highly prevalent disease encountered by many provider types.4–7 However, the optimal...
diagnostic testing algorithm for this condition is unknown. While peripheral neuropathy comprises many different subtypes that may affect what constitutes appropriate testing, the vast majority of patients have a distal symmetric polyneuropathy. Recently, the AAN published a systematic review of diagnostic testing in distal symmetric polyneuropathy, and found evidence to support routine testing with serum protein electrophoresis (SPEP), B12, fasting glucose test, and glucose tolerance test (GTT). However, the strength of the evidence is modest (Class C), and the review does not address other commonly ordered tests for this condition, including MRIs of the neuroaxis and electrodiagnostic tests. More work is needed to establish what should constitute a clinically effective evaluation of peripheral neuropathy, and efficiency efforts should focus on the high-impact tests that drive expenditures.

Given the limited available evidence, it is not surprising that a survey found that physicians order many costly tests at the time of peripheral neuropathy diagnosis and also have substantial variation in evaluation patterns. Interestingly, high-cost, low-yield tests such as MRIs of the neuroaxis are frequently ordered (23.2%), whereas low-cost, high-yield tests like glucose tolerance tests are rarely ordered (1%). Furthermore, neurologists report that they would order more tests than internists when presented with the same clinical vignettes of distal symmetric polyneuropathy. Specifically, neurologists reported the increased utilization of laboratory and electrodiagnostic testing, but not MRIs of the brain or spinal cord, which internists indicated that they would order more often. However, the actual utilization of diagnostic testing and expenditures in patients with peripheral neuropathy who see a neurologist compared to those who see other provider types is unknown.

The purpose of this study was to determine the tests that have the largest effect on expenditures in patients with a new diagnosis of peripheral neuropathy within a nationally representative sample of patients aged 65 or over. In addition, we wanted to explore the differences in test utilization and expenditures by medical specialty to inform future physician intervention efforts aimed at improving this evaluation.

METHODS

Population

We used data from biennial interviews between 1998 and 2006 of the Health and Retirement Study (HRS) linked with Medicare Standard Analytic File data from 1997 to 2007, and the Dartmouth Hospital Referral Region (HRR) data (http://www.dartmouthatlas.org). These linked data provide rich demographic detail from the HRS, detailed health care utilization data from Medicare claims, and regional practice patterns from the Dartmouth HRRs. We identified individuals with incident peripheral neuropathy diagnosed between 1998 and 2007, as previously described. We defined an incident diagnosis in persons who had an International Classification of Diseases (ICD)-9 code of peripheral neuropathy in a Medicare claim and no previous neuropathy diagnosis during the preceding 30 months. We included all ICD-9 codes for peripheral neuropathy (354.5, 356.0–9, and 357.0–9). We included individuals who were at least 65 years old (30 months before diagnosis), were continuously enrolled in Medicare parts A and B fee-for-service from 30 months preceding the index diagnosis until the index date.
diagnosis through 6 months following the index diagnosis, and completed an HRS interview within 3 years prior to the diagnosis date.

**Standard protocol approvals, registrations, and patient consents**
The University of Michigan institutional review board approved this study.

**Demographics/health measures**
HRS data were used to obtain information on age, sex, race/ethnicity, education, and limitations in activities of daily living (ADLs), and to determine HRR region based on zip code. To identify patients with pain, we recorded the answer to the HRS question “Are you often troubled with pain?” during the HRS interview just prior to incident neuropathy diagnosis. The Medicare claims database provided the diabetes status of patients based on the following Chronic Condition Warehouse definition: 2 outpatient or Carrier claims with diabetes diagnostic codes (249.3, 250.3, 357.2, 362.01, 362.02, or 366.31) during the 24 months prior to the diagnostic period (time period from 6 months prior to 6 months after the neuropathy diagnosis), or at least one inpatient, skilled nursing, or home health claim with a diabetes diagnostic code. If and when a patient was evaluated by a neurologist was determined using Medicare claims to search for physician office visits with a neurology provider indicated as the specialty type within the diagnostic period. Using the Dartmouth HRR data, we calculated inpatient expenditures during the last 6 months of life, by HRR and year. A geographic expenditure indicator was calculated by separating patients into quartiles of HRR expenditures during the last 6 months of life in the year in which they were diagnosed with neuropathy. We also calculated a personal baseline expenditure indicator by summing all expenditures during the 12 months prior to the diagnostic period and categorizing patients by quartiles. Limitations in ADLs (bathing, eating, dressing, walking across a room, and getting in or out of bed) were used to determine the degree of disability. Moderate disability was defined as one limitation in an ADL, and severe disability was defined as 2 or more limitations in ADLs.

**Diagnostic tests**
We used Current Procedural Terminology codes to identify diagnostic tests in the Medicare claims. We selected tests based on their relevance to the diagnostic evaluation of peripheral neuropathy and included fasting glucose level, hemoglobin A1C (HA1C), GTT, SPEP, B12, antinuclear antibodies (ANA), erythrocyte sedimentation rate (ESR), thyroid-stimulating hormone (TSH), complete blood count (CBC), comprehensive metabolic panel, electrodiagnostic tests, and MRI studies (brain, cervical, thoracic, or lumbar sacral spine).

**Medicare expenditures**
We estimated Medicare payments (technical and professional components) by using the publicly available Centers for Medicare and Medicaid Services (CMS) 2012 national payment amounts for MRIs and electrodiagnostic tests (http://www.cms.gov/apps/physician-fee-schedule). For laboratory tests, we used the 2012 national limit payment amounts from the publicly available CMS clinical laboratory fee schedule (http://www.cms.gov/Medicare/Medicare-Fee-for-Service-Payment/ClinicalLabFeeSched). Test-specific expenditures were calculated by adding all payments from the diagnostic tests listed above. Total aggregate expenditures were calculated by adding all payments from the diagnostic tests for all patients.

**Statistical analysis**
We used t tests to compare normally distributed continuous variables for those who saw a neurologist vs those who did not (unpaired) and for comparing continuous variables for patients before and after their first neurology provider visit (paired). The χ² tests (unpaired) and McNemar tests (paired) were used to analyze categorical variables. Multivariable logistic regression models were developed to determine the association of neurology providers on the dependent variables electrodiagnostic and MRI utilization after adjusting for
demographics, health status including disability, a geographic expenditure indicator, and a personal baseline expenditure indicator. We calculated an overall concordance statistic (c statistic) in order to assess the predictive ability of the adjusted models. All analyses were performed with SAS 9.1 (Cary, NC).

RESULTS

Population

During the 10-year study period, an incident ICD-9 diagnosis of peripheral neuropathy was observed in 1,031 of the 8,783 (11.7%) patients. Demographic and other characteristics of the population are summarized in table 1. Mean age of the population was 77.6 years and 54.0% were female. Twelve percent were non-Hispanic black, 8.0% were Hispanic, 41.5% had diabetes, and 16.3% had diabetic complications. Thirty-six percent (n = 374) saw a neurology provider during the diagnostic period.

Test utilization

Patients who saw a neurologist received a mean (SD) of 11.1 (8.1) of the relevant tests compared to 7.2 (6.7) tests in those who did not see a neurologist (p < 0.0001). Laboratory tests endorsed by the AAN guideline (fasting glucose, B12, SPEP, GTT) were more commonly ordered in patients seen by a neurologist with the exception of fasting glucose, which had similar utilization between provider types (table 2). The 3 tests with the largest absolute difference in utilization between patients seen by neurologists and non-neurologists were nerve conduction studies (50% vs 17%, p < 0.0001), MRIs of at least one component of the neuroaxis (45% vs 13%, p < 0.0001), and EMG (37% vs 10%, p < 0.0001). Laboratory tests, including ANA, ESR, TSH, CBC, and comprehensive metabolic panels, were utilized more often among those who saw a neurologist with the exception of HA1C.

Table 1  Demographic and clinical variables in patients with neuropathy

<table>
<thead>
<tr>
<th>Measure</th>
<th>No. (%), unless otherwise specified (n = 1,031)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at index date, y, mean (SD)</td>
<td>77.6 (6.8)</td>
</tr>
<tr>
<td>Male</td>
<td>474 (46.0)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>82 (8.0)</td>
</tr>
<tr>
<td>Non-Hispanic Caucasian/other</td>
<td>822 (79.7)</td>
</tr>
<tr>
<td>Non-Hispanic African American</td>
<td>127 (12.3)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
</tr>
<tr>
<td>Less than high school graduation</td>
<td>338 (32.8)</td>
</tr>
<tr>
<td>High school</td>
<td>342 (33.2)</td>
</tr>
<tr>
<td>At least some college</td>
<td>351 (34.0)</td>
</tr>
<tr>
<td>Diabetes (30–6 months pre index date)</td>
<td>428 (41.5)</td>
</tr>
<tr>
<td>Diabetic complications (non-neurologic)</td>
<td>168 (16.3)</td>
</tr>
<tr>
<td>ADL limitations</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>719 (69.8)</td>
</tr>
<tr>
<td>1</td>
<td>164 (15.9)</td>
</tr>
<tr>
<td>2–5</td>
<td>147 (14.3)</td>
</tr>
</tbody>
</table>

Abbreviation: ADL = activities of daily living.
Total aggregate diagnostic expenditures in the neuropathy population were $497,544, with a mean of $483 per patient. MRIs of the neuroaxis accounted for 46% ($228,026) of total testing expenditures. Electrodiagnostic tests accounted for an additional 42% ($209,639). Laboratory tests, considered in aggregate, accounted only for 12% ($59,879). MRIs and electrodiagnostic tests were the dominant contributors to expenditures both in patients seen by neurologists (92%) and in patients not seen by neurologists (81%). Both mean test-specific expenditures and total aggregate expenditures were higher in those seen by a neurology provider compared with those not seen by a neurology provider ($849 vs $274, \ p < 0.0001; $317,526 vs $180,018, \ p < 0.0001) (table 3).

Of note, in the 374 patients with a neurology provider, a mean (SD) of 7.3 (5.8) tests were completed after the neurology visit, compared to 3.9 (4.9) before this visit, and most test-specific expenditures occurred after the neurology visit ($652 per patient vs $197).

**Predicting test utilization**

In an adjusted model, neurology provider (odds ratio \[OR\] = 5.30, 95% confidence interval [CI] 3.87–7.26), diabetes (OR = 0.63, 95% CI 0.46–0.87), age (OR = 0.96, 95% CI 0.93–0.98), and Hispanic ethnicity (OR = 3.62, 95% CI 2.01–6.51) were significantly associated with electrodiagnostic utilization (overall model \(c\) statistic = 0.764) (table 4). Similarly, neurology provider (OR = 5.00, 95% CI 3.63–6.89), higher education status (OR = 1.80, 95% CI 1.16–2.79), and age (OR = 0.96, 95% CI 0.93–0.98) were associated with MRI utilization (\(c\) statistic 0.759).

**Expenditures associated with tests**

Table 2 Test utilization by provider type

<table>
<thead>
<tr>
<th>Test</th>
<th>Neuropathy subjects without a neurology provider, n (%) [total = 657]</th>
<th>Neuropathy subjects with a neurology provider, n (%) [total = 374]</th>
<th>(p) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMG</td>
<td>65 (10)</td>
<td>139 (37)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nerve conduction study</td>
<td>111 (17)</td>
<td>186 (50)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Any MRI</td>
<td>87 (13)</td>
<td>168 (45)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Brain MRI</td>
<td>23 (4)</td>
<td>118 (32)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cervical spine MRI</td>
<td>16 (2)</td>
<td>41 (11)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Thoracic spine MRI</td>
<td>10 (2)</td>
<td>12 (3)</td>
<td>0.07</td>
</tr>
<tr>
<td>Lumbosacral spine MRI</td>
<td>52 (8)</td>
<td>65 (17)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ANA</td>
<td>41 (6)</td>
<td>73 (20)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ESR</td>
<td>134 (20)</td>
<td>159 (43)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SPEP</td>
<td>44 (7)</td>
<td>90 (24)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TSH</td>
<td>336 (51)</td>
<td>225 (60)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>GTT</td>
<td>2 (0)</td>
<td>7 (2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>B12</td>
<td>154 (23)</td>
<td>179 (48)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>146 (22)</td>
<td>89 (24)</td>
<td>0.56</td>
</tr>
<tr>
<td>CBC</td>
<td>457 (70)</td>
<td>289 (77)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CMP</td>
<td>333 (51)</td>
<td>212 (57)</td>
<td>0.06</td>
</tr>
<tr>
<td>HA1C</td>
<td>289 (44)</td>
<td>148 (40)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Abbreviations: ANA = antinuclear antibody; CBC = complete blood count; CMP = comprehensive metabolic panel; ESR = erythrocyte sedimentation rate; GTT = glucose tolerance test; HA1C = hemoglobin A1C; SPEP = serum protein electrophoresis; TSH = thyroid-stimulating hormone.
DISCUSSION

In this nationally representative sample of neuropathy patients aged ≥65 years, MRI studies were the single largest contributor to diagnostic testing expenditures. MRIs were performed in 25% of patients and accounted for 46% of total expenditures. Electrodiagnostic studies also contributed substantially to expenditures, being performed in 29% and accounting for 42% of expenditures. Laboratory tests, on the other hand, accounted for only a small proportion of total expenditures. Future efforts are needed to define what constitutes an appropriate and efficient evaluation of peripheral neuropathy. Specifically, a focus on the role of MRIs and electrodiagnostic tests should be a priority.

An additional finding of this study was the differences in the evaluation of neuropathy by medical specialty. Even though neurologists saw a minority of the patients, aggregate expenditures were significantly higher in patients seen by neurologists compared to those not seen by neurologists ($317,526 vs $180,018). Most of this difference was explained by higher utilization of MRI and electrodiagnostic tests. In addition, the dominant contributor to predicting MRI or electrodiagnostic utilization was being seen by a neurologist, even after adjusting for important confounders. Whether this increased utilization is appropriate cannot be determined with these data. Similarly, some tests may be more appropriate than others. For example, electrodiagnostic tests likely have more utility in the evaluation of peripheral neuropathy than MRIs, but future studies are needed to clearly define the role of both of these tests. Furthermore, patients who

<table>
<thead>
<tr>
<th>Test</th>
<th>Neuropathy subjects without a neurology provider, $, mean (SD) (total = 657)</th>
<th>Neuropathy subjects with a neurology provider, $, mean (SD) (total = 374)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMG</td>
<td>14 (47)</td>
<td>57 (84)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nerve conduction study</td>
<td>103 (313)</td>
<td>298 (445)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Brain MRI</td>
<td>22 (125)</td>
<td>233 (389)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cervical spine MRI</td>
<td>15 (101)</td>
<td>62 (186)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Thoracic spine MRI</td>
<td>10 (87)</td>
<td>22 (154)</td>
<td>0.17</td>
</tr>
<tr>
<td>Lumbosacral spine MRI</td>
<td>57 (226)</td>
<td>110 (263)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ANA</td>
<td>1 (4)</td>
<td>2 (7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ESR</td>
<td>1 (5)</td>
<td>2 (4)</td>
<td>0.08</td>
</tr>
<tr>
<td>SPEP</td>
<td>1 (7)</td>
<td>4 (13)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TSH</td>
<td>12 (20)</td>
<td>17 (27)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>GTT</td>
<td>0 (0)</td>
<td>0.1 (0.9)</td>
<td>0.32</td>
</tr>
<tr>
<td>B12</td>
<td>4 (11)</td>
<td>7 (13)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>2 (6)</td>
<td>2 (5)</td>
<td>0.96</td>
</tr>
<tr>
<td>CBC</td>
<td>14 (33)</td>
<td>16 (37)</td>
<td>0.41</td>
</tr>
<tr>
<td>CMP</td>
<td>7 (13)</td>
<td>10 (21)</td>
<td>0.04</td>
</tr>
<tr>
<td>HA1C</td>
<td>10 (18)</td>
<td>8 (17)</td>
<td>0.06</td>
</tr>
<tr>
<td>EDS total</td>
<td>117 (339)</td>
<td>355 (504)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MRI total</td>
<td>104 (325)</td>
<td>427 (624)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Laboratory total</td>
<td>53 (67)</td>
<td>67 (87)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total</td>
<td>274 (517)</td>
<td>849 (875)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Abbreviations: ANA = antinuclear antibody; CBC = complete blood count; CMP = comprehensive metabolic panel; EDS = electrodiagnostic studies (EMG and nerve conduction study); ESR = erythrocyte sedimentation rate; GTT = glucose tolerance test; HA1C = hemoglobin A1C; SPEP = serum protein electrophoresis; TSH = thyroid-stimulating hormone.
are seen by neurologists are different from those who are not seen by neurologists. Neurologists likely are referred patients with more severe neuropathy or rare neuropathy subtypes. Furthermore, we currently do not have evidence for or against the utilization of these tests. However, these data do suggest that neurologists are likely to be important targets for any physician interventions aimed at improving the efficiency of the evaluation of peripheral neuropathy. Neurologists may also be a more feasible target for interventions to optimize neuropathy evaluations because they are a much smaller group and have a more concentrated interest in neuropathy evaluation and management. Targeting neurologists is also important because their practice patterns are likely to diffuse into general medicine practices.

By comparison, non-neurologists had much lower utilization of MRIs and electrodiagnostic tests, but also had lower utilization of AAN-recommended laboratory tests for neuropathy.
Both vitamin $B_{12}$ and SPEP have been shown to have a substantial yield in neuropathy presentations and abnormal results influence further relevant management including specific treatments.

particularly vitamin $B_{12}$ levels and SPEP. Both vitamin $B_{12}$ and SPEP have been shown to have a substantial yield in neuropathy presentations and abnormal results influence further relevant management including specific treatments.\textsuperscript{8,11,12} This result highlights the fact that more testing does not equate with overutilization. Furthermore, the low utilization of AAN-recommended tests by all provider types emphasizes the opportunity we have to improve the care of many patients with neuropathy. In steps to optimize care, the initial step, especially for non-neurologists, may be a focus on increasing utilization of these tests, which would likely only lead to a minor increase in expenditures.

We identified one major difference when comparing the utilization results with our previous survey. Specifically, in our prior survey, neurologists indicated they would order fewer MRIs of the neuroaxis than internists (12.9\% vs 19.8\%) given the same distal symmetric polyneuropathy vignette, whereas in this study we found the opposite.\textsuperscript{5} Possible explanations include that peripheral neuropathy patients with a neurologist are more likely to have a separate indication for an MRI, the real-world practice of neurologists does not match their ideal intentions, the evaluation of neuropathy is different in Medicare vs non-Medicare populations, or neurology survey respondents were more likely to be lower MRI test utilizers. On the other hand, both studies demonstrated higher overall test utilization, including electrodiagnostic utilization, by neurologists. Similarly, both studies revealed higher utilization of the 4 AAN-recommended tests by neurologists. While our previous survey and current HRS-Medicare claims data reveal similar results, future work is necessary to address the reasons for the conflicting data on MRI utilization.

Potential limitations of our study include that cases were identified by ICD-9 codes, which may lead to misclassification bias. With this data, we are unable to determine the frequency of misclassification or how it might differ between provider types. The purpose of the current analysis was to describe utilization and expenditures in peripheral neuropathy. The study does not address whether the use or nonuse of a test in an individual patient was appropriate or not. We were also unable to investigate detailed information on why patients received specific tests. For example, patients who received MRIs may have had another indication for this test, such as additional features indicating the possible presence of brain or spinal cord disease. On the other hand, our previous work revealed substantially higher MRI utilization in this neuropathy group compared with propensity-matched controls. Moreover, we did not have information on neuropathy severity or subtype, both of which may influence testing and subsequent expenditures. However, we were able to include many patient characteristics into our predictive models, and the models had high predictive ability. We also were only able to study those 65 and older and enrolled in fee-for-service Medicare; therefore, how these results apply to a population that is younger and covered by other types of insurance is unknown.

MRIs and electrodiagnostic tests have the greatest effect on expenditures in the evaluation of peripheral neuropathy. These tests are performed more frequently in patients who are evaluated by a neurology provider. Therefore, MRIs and electrodiagnostic tests in patients seen by neurologists should be the main targets of cost-control and efficiency efforts in the evaluation of peripheral neuropathy.

Both vitamin $B_{12}$ and SPEP have been shown to have a substantial yield in neuropathy presentations and abnormal results influence further relevant management including specific treatments.
REFERENCES


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Peripheral Neuropathy in Adolescents and Young Adults With Type 1 and Type 2 Diabetes From the SEARCH for Diabetes in Youth Follow-Up Cohort

A pilot study

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For The SEARCH For Diabetes In Youth Study Group

OBJECTIVE.—To estimate the prevalence of and risk factors for diabetic peripheral neuropathy (DPN) in a pilot study among youth participating in the SEARCH for Diabetes in Youth study.

RESEARCH DESIGN AND METHODS.—DPN was assessed using the Michigan Neuropathy Screening Instrument (MNSI) (examination for foot abnormalities, distal vibration perception, and ankle reflexes). An MNSI exam (MNSIE) score $\geq$2 is diagnostic for DPN.

RESULTS.—The MNSIE was completed in 399 subjects, including 329 youth with type 1 diabetes (mean age 15.7 ± 4.3 years, duration 6.2 ± 0.9 years) and 70 with type 2 diabetes (mean age 21.6 ± 4.1 years, duration 7.6 ± 1.8 years). Glycated hemoglobin (A1C) was similar in both groups (8.8 ± 1.8% for type 1 vs. 8.5 ± 2.0% for type 2). The prevalence of DPN was significantly higher in youth with type 2 compared with those with type 1 diabetes (25.7 vs. 16.2%, P < 0.0001). In unadjusted analyses, diabetes type, older age, longer duration of diabetes, increased waist circumference, elevated blood pressure, lower HDL cholesterol, and presence of microalbuminuria (urinary albumin-to-creatinine ratio >30 mg/g) were associated with DPN. The association between diabetes type and DPN remained significant after adjustment for age and sex (odds ratio 2.29 [95% CI 1.05–5.02], P = 0.03).

CONCLUSIONS.—DPN prevalence among youth with type 2 diabetes approached rates reported in adult populations with diabetes. Our findings suggest not only that youth with diabetes are at risk for DPN but also that many already show measurable signs of DPN.

The incidence of both type 1 and type 2 diabetes in youth is increasing worldwide (1,2). Recent reports have projected that, if this trend continues, the prevalence of diabetes among the young in the U.S. could triple by the year 2050 (3). This could incur a significant burden on health care costs and on society, especially as these young people enter their peak working and earning capacity at the time when diabetes complications begin to occur. Diabetic peripheral neuropathy (DPN) is among the most distressing of all the chronic complications of diabetes and is a cause of significant disability and poor quality of life (4). Depending on the population and diagnostic criteria, the prevalence of DPN among adults with diabetes ranges from 30 to 70% (5–7). However, there are insufficient data on the prevalence and predictors of DPN among the pediatric population. Furthermore, early detection and good glycemic control have been proven to prevent or delay adverse outcomes associated with DPN (5,8,9). Near-normal control of blood glucose beginning as soon as possible after the onset of diabetes may delay the development of clinically significant nerve impairment (8,9). Therefore, children and adolescents with diabetes represent a critical target for primary prevention of this complication.

The American Diabetes Association (ADA) recommends screening for DPN in children and adolescents with type 2 diabetes at diagnosis and 5 years after diagnosis for those with type 1 diabetes, followed by annual evaluations thereafter, using simple clinical tests (10). Since subclinical signs of DPN may precede development of frank neuropathic symptoms, systematic, preemptive screening is required in order to identify DPN in its earliest stages.

There are various measures that can be used for the assessment of DPN. The Michigan Neuropathy Screening Instrument (MNSI) is a simple, sensitive, and...
specific tool for the screening of DPN (11). It was validated in large independent cohorts (12,13) and has been widely used in clinical trials and longitudinal cohort studies including the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) (13).

The aim of this pilot study was to provide preliminary estimates of the prevalence of and factors associated with DPN among children and adolescents with type 1 and type 2 diabetes.

**RESEARCH DESIGN AND METHODS**

**Study participants and data collection**

SEARCH for Diabetes in Youth is a population-based study of diabetes in young people of diverse racial and ethnic backgrounds in the U.S. (14). Study methods for SEARCH have previously been described (14). SEARCH is an observational longitudinal study of youth with diabetes diagnosed before the age of 20 years in the U.S. SEARCH participants are drawn from four geographically defined populations in Ohio, Washington, South Carolina, and Colorado; health plan enrollees in Hawaii and California; and Indian Health Service beneficiaries from four American Indian populations. Prior to protocol implementation, local institutional review board approval was obtained for each center. Participants 18 years and older and parents of youth under age 18 years provided written informed consent for participation; youth provided assent.

Once enrolled, the parent/guardian or study participants age 18 years and older were invited to complete a short survey that included questions about race and ethnicity, diabetes treatment, and other information. Participants who completed the initial survey were invited to a baseline study visit surveys were administered to obtain clinical and demographic information, as well as psychosocial burden. In addition, a physical examination was completed to measure systolic and diastolic blood pressure, height, weight, and waist circumference. A blood sample was collected by venipuncture. Youth whose diabetes was incident in 2002–2005. DPN assessment was conducted at the 60-month follow-up visit. In addition, a subset of youth with type 2 diabetes who were part of the 2001 prevalent cohort that participated in a baseline visit were also invited to participate in the DPN pilot study. The pilot study was approved by the institutional review board(s) at each study site. Diabetes type was categorized as type 1 or type 2 based on the health care provider diagnosis. Race/ethnicity was self-reported using the 2000 census questionnaire format, and five categories were created: non-Hispanic white, Hispanic (regardless of race), non-Hispanic black, Asian/Pacific Islander, and Native American. Current cigarette smoking was defined as having smoked cigarettes on ≥1 of the 30 days preceding the survey. Blood samples were obtained under conditions of metabolic stability after at least 8 h of fasting. Specimens were processed locally at the sites and shipped within 24 h to the central laboratory (Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington), where they were analyzed for measurement of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and glycated hemoglobin (A1C) as previously described (14). Urinary albumin levels were assessed on a random spot urine sample, and microalbuminuria was defined as urine albumin-to-creatinine ratio >30 mg albumin/g creatinine.

**Assessment of DPN**

DPN was assessed using the MNSI, a validated screening tool (11). SEARCH staff from each center were centrally trained and certified to perform the MNSI. The MNSI is a 15-item self-administered questionnaire (MNSIQ) and structured examination of the feet (MNSIE) scored for abnormalities of appearance (deformities, infection, and dry skin/callus), presence of ulcers, vibration perception threshold (VPT) at the distal great toe, and ankle reflexes. Threshold for DPN, established by prior validation studies performed among adults, is a score of >2 on the MNSIE (12,13).

**Statistical analyses**

Differences in the demographic, anthropometric, clinical, and metabolic parameters between adolescents and young adults with type 1 and type 2 diabetes were compared using Student t test for continuous variables and the χ² test for categorical variables. Factors associated with DPN were assessed independently using the cutoff score of 2 on the MNSIE to define DPN. Logistic regression analysis was conducted to estimate the odds of DPN associated with diabetes type, controlling for the variables found to be significant in the univariate analysis. The data were analyzed using SAS 9.2 (SAS Institute, Cary, NC). The level of significance was set at α = 0.05.

**RESULTS**—The characteristics of the study population are depicted in Table 1. A total of 399 youth (329 with type 1 and 70 with type 2 diabetes) participated in the pilot study. Youth with type 1 diabetes were younger (mean age 15.7 ± 4.3 years) and had a shorter duration of diabetes (mean duration 6.2 ± 0.9 years) compared with youth with type 2 diabetes (mean age 21.6 ± 4.1 years and mean duration 7.6 ± 1.8 years). Participants with type 2 diabetes had a higher BMI z score and waist circumference, were more likely to be smokers, and had higher blood pressure and lipid levels than youth with type 1 diabetes (all P < 0.001). A1C, however, did not significantly differ between the two groups (mean A1C 8.8 ± 1.8% [73 ± 2 mmol/mol] for type 1 diabetes and 8.5 ± 2.9% [72 ± 3 mmol/mol] for type 2 diabetes; P = 0.3) but was higher than that recommended by the ADA for this age-group (A1C ≤7.5%) (10). The prevalence of DPN (defined as the MNSIE score ≥2) was 8.2% among youth with type 1 diabetes and 25.7% among those with type 2 diabetes.

Unadjusted associations between demographic, anthropometric, and metabolic parameters and DPN are presented in Table 2. Youth with DPN were older and had a longer duration of diabetes, greater central obesity (increased waist circumference), higher blood pressure, an atherogenic lipid profile (low HDL cholesterol and marginally high triglycerides), and microalbuminuria. A1C, although above that recommended by ADA for optimal glycemic control among youth, was not significantly different between those with and without DPN (9.0% ± 2.0 or 75.0 ± 2.0 mmol/mol vs. 8.8% ± 2.1 or 72.0 ± 2.0 mmol/mol,
**Table 1—Characteristics of the study population by diabetes status: the SEARCH for Diabetes in Youth DPN pilot study**

<table>
<thead>
<tr>
<th>Participant characteristics</th>
<th>Type 1</th>
<th>Type 2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>329</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>15.7 (4.4)</td>
<td>21.6 (4.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>168 (51.0)</td>
<td>42 (60.0)</td>
<td>0.18</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>6.2 (0.9)</td>
<td>7.6 (1.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Race/ethnicity, n (%)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Non-Hispanic white</td>
<td>261 (79.3)</td>
<td>20 (28.5)</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic black</td>
<td>39 (11.8)</td>
<td>27 (38.5)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>27 (8.2)</td>
<td>17 (24.2)</td>
<td></td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>1 (0.3)</td>
<td>4 (5.7)</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>0 (0.0)</td>
<td>1 (1.4)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1 (0.3)</td>
<td>1 (1.4)</td>
<td></td>
</tr>
<tr>
<td>Income (USD), n (%)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&lt;25,000</td>
<td>34 (11.0)</td>
<td>14 (36.8)</td>
<td></td>
</tr>
<tr>
<td>25,000–49,000</td>
<td>57 (18.4)</td>
<td>6 (15.7)</td>
<td></td>
</tr>
<tr>
<td>50,000–74,000</td>
<td>52 (16.8)</td>
<td>1 (2.6)</td>
<td></td>
</tr>
<tr>
<td>≥75,000</td>
<td>127 (41.1)</td>
<td>2 (5.2)</td>
<td></td>
</tr>
<tr>
<td>DK/Ref</td>
<td>39 (12.6)</td>
<td>15 (39.4)</td>
<td></td>
</tr>
<tr>
<td>A1C</td>
<td></td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>mmol/mol</td>
<td>8.83 (1.8)</td>
<td>8.50 (2.9)</td>
<td></td>
</tr>
<tr>
<td>BMI z score</td>
<td>73 (2)</td>
<td>72 (3)</td>
<td></td>
</tr>
<tr>
<td>Insulin treated, n (%)</td>
<td>307 (99.3)</td>
<td>15 (51.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>40 (14.3)</td>
<td>14 (37.8)</td>
<td>0.0011</td>
</tr>
<tr>
<td>Positive MNSI examination, n (%)</td>
<td>27 (8.2)</td>
<td>18 (25.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>80.32 (15.7)</td>
<td>114.30 (30.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>62.59 (10.20)</td>
<td>74.10 (11.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>100.10 (13.0)</td>
<td>115.80 (14.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urine ACR (mg/g)*</td>
<td>2.47 (9.10)</td>
<td>18.09 (75.7)</td>
<td>0.0016</td>
</tr>
<tr>
<td>Microalbuminuria, n (%)</td>
<td>3 (1.1)</td>
<td>5 (7.9)</td>
<td>0.0080</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)*</td>
<td>91.10 (72.9)</td>
<td>177.1 (204.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>57.37 (14.5)</td>
<td>41.40 (10.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>95.22 (26.3)</td>
<td>107.80 (31.7)</td>
<td>0.0036</td>
</tr>
</tbody>
</table>

Data are means (SD) unless otherwise indicated. Microalbuminuria = albumin-to-creatinine ratio (ACR) >30 mg/g. DBP, diastolic blood pressure; DK/Ref, do not know/refused to answer; SBP, systolic blood pressure.

*Using log distribution for association tests.

P = 0.58). Although nearly 37% of youth with type 2 diabetes came from lower-income families with annual income <25,000 USD per annum (as opposed to 11% for type 1 diabetes), socioeconomic status was not significantly associated with DPN (P = 0.77).

In the unadjusted logistic regression models, the odds of having DPN was nearly four times higher among those with type 2 diabetes compared with youth with type 1 diabetes (odds ratio [OR] 3.8 [95% CI 1.9–7.5, P < 0.0001]). This association was attenuated, but remained significant, after adjustment for age and sex (OR 2.3 [95% CI 1.1–5.0], P = 0.03). However, this association was no longer significant (OR 2.1 [95% CI 0.3–15.9], P = 0.47) when additional covariates, significant in the univariate analyses (age, diabetes duration, waist circumference, blood pressure, HDL cholesterol, and microalbuminuria), were added to the model (Table 3).

**CONCLUSIONS**—In a pilot study of a multiethnic cohort of youth with diabetes, the prevalence of DPN was 8.2% among youth with type 1 diabetes and 25.7% among those with type 2 diabetes. Youth with DPN were more likely to have type 2 diabetes and an adverse cardiovascular risk profile (central obesity, higher blood pressure, and lower HDL) and microalbuminuria. The prevalence of DPN among type 1 diabetes youth in our pilot study is lower than that reported by Eppens et al. (15) among 1,433 Australian adolescents with type 1 diabetes assessed by thermal threshold testing and VPT (prevalence of DPN 27%; median age and duration 15.7 and 6.8 years, respectively). A much higher prevalence was also reported among Danish (62.5%) and Brazilian (46%) cohorts of type 1 diabetes youth (16,17) despite a younger age (mean age among Danish children 13.7 years and Brazilian cohort 12.9 years). The prevalence of DPN among youth with type 2 diabetes (26%) found in our study is comparable to that reported among the Australian cohort (21%) (15). The wide ranges in the prevalence estimates of DPN among the young cannot solely be attributed to the inherent racial/ethnic differences in this population but could potentially be due to the differing criteria and diagnostic tests used to define and characterize DPN. The Australian cohort used thermal and VPT to assess DPN, while the Danish cohort used VPT only.

Metabolic syndrome components such as central obesity, elevated blood pressure, and dyslipidemia have been implicated in the pathogenesis of DPN. In the EURODIAB study which prospectively followed 1,172 subjects with type 1 diabetes for 7.3 years (mean age 30 ± 8.8 years, A1C 8.0 ± 1.8%, and duration 12 ± 8 years) the authors reported that, apart from glycemic control, the incidence of DPN was significantly associated with potentially modifiable cardiovascular risk factors, including raised triglyceride levels, BMI, smoking, and hypertension (18). In the population-based Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA)/Cooperative Health Research in the Region of Augsburg (KORA) study, DPN assessed by MNSI was associated with increasing age and abdominal obesity (19). The Pittsburgh Epidemiology of Diabetes Complications Study (EDC) has established that low HDL cholesterol is associated with prevalent polyneuropathy (20) and that hypertension and smoking are predictors of incident polyneuropathy (21). These data thus support the relationship between DPN and cardiovascular risk factors (central obesity, lower HDL, and elevated blood pressure) found in our study, although in a much younger cohort.

In our study, the duration of diabetes was significantly longer among those with DPN, but A1C values did not differ significantly between the two groups, suggesting that a longer duration with its sustained impact on peripheral nerves is an important determinant of DPN.
Table 2—Differences in the demographic, anthropometric, and metabolic parameters between youth with and without DPN: the SEARCH for Diabetes in Youth DPN pilot study

<table>
<thead>
<tr>
<th>Variable</th>
<th>DPN* (MNSIE &gt; 2)</th>
<th>DPN* (MNSIE ≤ 2)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>17.91 (4.07)</td>
<td>16.11 (4.43)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23 (51.11)</td>
<td>187 (51.09)</td>
<td>0.87</td>
</tr>
<tr>
<td>Diabetes duration (years)*</td>
<td>7.0 (1.4)</td>
<td>6.3 (1.7)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Diabetes type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1, n (%)</td>
<td>27 (60.00)</td>
<td>302 (85.31)</td>
<td></td>
</tr>
<tr>
<td>Type 2, n (%)</td>
<td>18 (40.00)</td>
<td>52 (14.69)</td>
<td></td>
</tr>
<tr>
<td>Race/ethnicity, n (%)</td>
<td></td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td>Non-Hispanic white</td>
<td>28 (62.22)</td>
<td>253 (71.47)</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic black</td>
<td>10 (22.22)</td>
<td>56 (15.82)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>6 (13.33)</td>
<td>38 (10.57)</td>
<td></td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>1 (2.22)</td>
<td>4 (1.13)</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>0 (0.00)</td>
<td>1 (0.28)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>0 (0.00)</td>
<td>2 (0.56)</td>
<td></td>
</tr>
<tr>
<td>Income (USD), n (%)</td>
<td>6 (19.35)</td>
<td>42 (13.29)</td>
<td>0.67</td>
</tr>
<tr>
<td>&lt;25,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥25,000–49,000</td>
<td>6 (19.35)</td>
<td>37 (11.76)</td>
<td></td>
</tr>
<tr>
<td>≥50,000–75,000</td>
<td>4 (12.90)</td>
<td>49 (15.51)</td>
<td></td>
</tr>
<tr>
<td>≥75,000</td>
<td>9 (29.03)</td>
<td>120 (37.97)</td>
<td></td>
</tr>
<tr>
<td>DK/Ref</td>
<td>6 (19.35)</td>
<td>48 (15.19)</td>
<td></td>
</tr>
<tr>
<td>A1C %</td>
<td></td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>0.48 (1.50)</td>
<td>0.74 (0.90)</td>
<td>0.15</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>103.31 (42.21)</td>
<td>84.04 (18.21)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>110 (17.48)</td>
<td>101.90 (13.92)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>72.09 (12.66)</td>
<td>63.64 (10.71)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urine ACR (mg/g)*</td>
<td>9.76 (23.82)</td>
<td>4.98 (36.06)</td>
<td>0.0019</td>
</tr>
<tr>
<td>Microalbuminuria, n (%)</td>
<td>2 (2.22)</td>
<td>6 (1.13)</td>
<td>0.2339</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)*</td>
<td>116.84 (78.45)</td>
<td>107.96 (122.74)</td>
<td>0.07</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>48.28 (14.33)</td>
<td>54.82 (15.15)</td>
<td>0.01</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>96.08 (28.88)</td>
<td>98.07 (28.17)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Data are means (SD) unless otherwise indicated. Microalbuminuria = albumin-to-creatinine ratio (ACR) >30 mg/g, DBP, diastolic blood pressure; DK/Ref, do not know/refused to answer; SBP, systolic blood pressure.
*Using log distribution for association tests.

However, the A1C was above that recommended by ADA for optimal glycemic control among both youth with and youth without DPN. Cho et al. (22) reported an increase in the prevalence of DPN from 14 to 28% over 17 years among 819 Australian adolescents with type 1 diabetes aged 11–17 years at baseline, despite improvements in care and minor improvements in A1C (8.2–8.7%). The prospective Danish Study Group of Diabetes in Childhood also found no association between DPN (assessed by VPT) and glycemic control (23).

Nerve conduction studies are still regarded as the gold standard measure to assess DPN, but the discomfort associated with the procedure and significant investment in time, personnel training, and costs preclude their use in routine clinical practice. Assessment using the MNSI, however, is noninvasive and easy to use in large, multicenter cohorts and in adults was shown to be specific (95%) and sensitive (80%) for identifying the presence of DPN, with a positive predictive value of 97% and a negative predictive value of 74% (11,13).

The ADA recommends that health care providers perform an annual comprehensive foot examination for all patients with diabetes to identify neuropathy risk factors predictive of ulcers and amputations (24). This examination should include inspection, assessment of foot pulses, and testing for loss of protective sensations (10-g monofilament plus testing any one of the following: vibration using 128-Hz tuning fork, pinprick sensation, ankle reflex, or VPT). The MNSI incorporates most of these tests recommended by the ADA and is thus an ideal bedside screening tool for DPN.

Our study has limitations that must be taken into consideration. The results of this study should be interpreted cautiously, as the MNSI has not been validated in children and adolescents, although several of its components (vibration perception using 128-Hz tuning fork, pinprick sensation, and ankle reflexes) have been used among the pediatric population for DPN screening. The cross-sectional design of the study and the lack of information on prior A1C data could be a reason for the lack of association between DPN and A1C, as we could not include a weighted A1C over time into the model. The loss of the association between diabetes type and DPN with addition of covariates in the fully adjusted model could be due to power loss, given the small number of youth with DPN in the sample, or indicative of stronger associations between these covariates and DPN such that conditioning on them eliminates the observed association between DPN and diabetes type. More data are needed to further characterize the relationship between DPN and diabetes type in youth. While the data presented come from a pilot study conducted in 2009–2010, the SEARCH cohort study is currently collecting data on DPN in an estimated sample of 3,000 youth with type 1 and type 2 diabetes, which will have more power and allow a more comprehensive evaluation of prevalence of and risk factors for the development and progression of DPN.

In conclusion, our pilot study found evidence that the prevalence of DPN in adolescents with type 2 diabetes approaches rates reported in adults with diabetes. Several CVD risk factors such as central obesity, elevated blood pressure, dyslipidemia, and microalbuminuria, previously identified as predictors of DPN among adults with diabetes, emerged as independent predictors of DPN in this young cohort and likely accounted for the increased prevalence of DPN in youth with type 2 diabetes. Awareness and periodic assessment of this complication in its early subclinical stage might prevent the poor quality of life associated with DPN in the future by
allowing early application of suitable interventions. Further long-term study of DPN in youth is needed.

Acknowledgments—SEARCH for Diabetes in Youth is funded by the Centers for Disease Control and Prevention (PA no. 00097, DP-05-069 and DP-10-001) and supported by the National Institute of Diabetes and Digestive and Kidney Diseases. Site contract numbers are as follows: Kaiser Permanente Southern California (U48/CCU919219, U01 DP000246 and U18DP002714), University of Colorado Denver (U48/CCUB19241-3, U01 DP000247, and U18DP000247-06A1), Kuakini Medical Center (U58CCU919256 and U01 DP000245), Children’s Hospital Medical Center (Cincinnati) (U48/CCU519239, U01 DP000248, and U18DP002709), University of North Carolina at Chapel Hill (U48/CCU419249, U01 DP000254, and U18DP002708-01), University of Washington School of Medicine (U58CCU019235-4, U01 DP000244, and U18DP002710-01), and Wake Forest University School of Medicine (U48/CCU919219, U01 DP000250, and 200-2010-35171). The authors acknowledge the involvement of General Clinical Research Centers at the South Carolina Clinical & Translational Research Institute, at the Medical University of South Carolina (National Institutes of Health [NIH]/National Center for Research Resources [NCRR] grant UL1RR029882), Children’s Hospital and Regional Medical Center (grant M01RR00037), Colorado Pediatric General Clinical Research Center (grant M01 RR00069), the Barbara Davis Center at the University of Colorado at Denver (DERC, NIH P30 DK57516), the Institutional Clinical and Translational Science Award, NIH/NCCR at the University of Cincinnati (grant 1UL1RR026314-01), and the Children with Medical Handicaps program managed by the Ohio Department of Health.

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

The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention or the National Institute of Diabetes and Digestive and Kidney Diseases.

M.J. wrote the manuscript. A.L. analyzed data. C.L.M. contributed to the analysis plan and discussion and reviewed and edited the manuscript. R.A.B. and J.D. contributed to the discussion and reviewed and edited the manuscript. D.D. contributed to the analysis plan and discussion and reviewed and edited the manuscript. D.J.P., S.S., C.P., D.A.S., and B.L.R. contributed to the discussion and reviewed and edited the manuscript. R.F.-B. and E.L.F. contributed to the analysis plan and discussion and reviewed and edited the manuscript. E.L.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Parts of this study were presented in abstract form at the 72nd Scientific Sessions of the American Diabetes Association, Philadelphia, Pennsylvania, 8–12 June 2012. The SEARCH for Diabetes in Youth Study is indebted to the many youth, and their families and their health care providers, whose participation made this study possible. The authors thank Stacey A. Sakowski Jacoby, Deputy Managing Director, A. Alfred Taubman Medical Research Institute, University of Michigan, and Catherine Stables, postdoctoral fellow, Department of Neurology, University of Michigan, for critically reviewing this manuscript.

Table 3—Adjusted OR and 95% CI for factors associated with DPN from multiple logistic regression analysis: the SEARCH for Diabetes in Youth diabetic peripheral neuropathy pilot study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Adjusted OR</th>
<th>Lower</th>
<th>Upper</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 2 vs. type 1 diabetes</td>
<td>2.10</td>
<td>0.27</td>
<td>15.95</td>
<td>0.47</td>
</tr>
<tr>
<td>Age (years) (per 1 year)</td>
<td>0.94</td>
<td>0.79</td>
<td>1.11</td>
<td>0.48</td>
</tr>
<tr>
<td>Duration (years) (per 1 year)</td>
<td>0.80</td>
<td>0.45</td>
<td>1.41</td>
<td>0.44</td>
</tr>
<tr>
<td>Waist circumference (per 1 cm)</td>
<td>0.97</td>
<td>0.92</td>
<td>1.00</td>
<td>0.11</td>
</tr>
<tr>
<td>DBP (per 1 mmHg)</td>
<td>0.93</td>
<td>0.86</td>
<td>0.99</td>
<td>0.045</td>
</tr>
<tr>
<td>SPB (per 1 mmHg)</td>
<td>1.01</td>
<td>0.94</td>
<td>1.06</td>
<td>0.82</td>
</tr>
<tr>
<td>Urine ACR per 1 mg/g</td>
<td>1.01</td>
<td>0.96</td>
<td>1.06</td>
<td>0.60</td>
</tr>
<tr>
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Model adjusted for age, duration, waist circumference, diastolic blood pressure (DBP), systolic blood pressure (SBP), albumin-to-creatinine ratio (ACR), and HDL cholesterol.

References


Neuromuscular Disease and Nerve Injury
Two Dynamin-2 Genes Are Required for Normal Zebrafish Development

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Abstract

Dynamin-2 (DNM2) is a large GTPase involved in clathrin-mediated endocytosis and related trafficking pathways. Mutations in human DNM2 cause two distinct neuromuscular disorders: centronuclear myopathy and Charcot-Marie-Tooth disease. Zebrafish have been shown to be an excellent animal model for many neurologic disorders, and this system has the potential to inform our understanding of DNM2-related disease. Currently, little is known about the endogenous zebrafish orthologs to human DNM2. In this study, we characterize two zebrafish dynamin-2 genes, dnm2 and dnm2-like. Both orthologs are structurally similar to human DNM2 at the gene and protein levels. They are expressed throughout early development and in all adult tissues examined. Knockdown of dnm2 and dnm2-like gene products resulted in extensive morphological abnormalities during development, and expression of human DNM2 RNA rescued these phenotypes. Our findings suggest that dnm2 and dnm2-like are orthologs to human DNM2, and that they are required for normal zebrafish development.

Introduction

Dynamin are large GTPases involved in a wide range of cell and organelle fission events. The dynamin superfamily is made up of classical dynamins and dynamin-like proteins. Classical dynamins are critical components of clathrin-mediated endocytosis, where they contribute to the release of newly formed endosomes [1,2,3]. In addition to this well-characterized role in endocytosis, classical dynamins also participate in a variety of membrane trafficking functions including phagocytosis, caveolea internalization, and trans-Golgi transport [4,5,6]. In mammals, there are three classical dynamins: dynamin-1 (DNM1), dynamin-2 (DNM2), and dynamin-3 (DNM3). Of these three genetic isoforms, only DNM2 is ubiquitously expressed [7,8,9] and a requirement for DNM2 during development is evidenced by an embryonic lethal phenotype in Dnm2 knockout mice [10]. Furthermore, mutations in human DNM2 also cause two different neuromuscular disorders; Charcot-Marie-Tooth disease and centronuclear myopathy [11,12].

Currently, there is no published characterization of any classical dynamin in the zebrafish genome. Given the prominent role of DNM2 in cellular function and human disease, characterizing the endogenous zebrafish dynamin-2 is an important task. Several studies of zebrafish endocytosis have utilized putative markers or inhibitors of dynamin-2; however, none of these reports examined functional or structural similarity between human DNM2 and a zebrafish homolog [13,14,15]. Establishing this orthologous relationship will enable future studies of endocytosis and other dynamin-related pathways in the zebrafish.

In this study, we characterize two zebrafish dynamin-2 genes, dnm2 and dnm2-like. We demonstrate that dnm2 and dnm2-like are structurally similar to human DNM2 at both the gene and protein levels, and that these gene products are ubiquitously expressed in adult tissue. Using morpholino-mediated knockdown, we show that depletion of dnm2 and dnm2-like gene products causes morphological abnormalities in larval muscle. Overexpression of human DNM2 mRNA is able to rescue both dnm2 and dnm2-like phenotypes. Taken together, this evidence suggests that dnm2 and dnm2-like are structural and functional orthologs to human DNM2, and that they are required for normal embryonic development in the zebrafish.

Materials and Methods

Phylogenetic and Syntenic Analysis

Multiple species alignments and phylogenetic analyses were performed using Mega 5.1 software [16]. Phylogenies were created using the neighbor-joining method with 1000 bootstrap replicates. Syntenic genes were identified using NCBI and Ensembl databases, and orthology of these genes was confirmed using
reciprocal BLAST searches against the human and zebrafish genomes.

Animal Care and Ethics Statement

Zebrafish (AB strain) were bred and raised according to established protocols. Experiments were performed on zebrafish embryos and larvae between 1 and 2 days post fertilization (dpf). All animals were handled in strict accordance with good animal practice as defined by national and local animal welfare bodies, and all animal work was approved by the appropriate committee (University of Michigan UCUCA #09835).

Figure 1. Phylogenetic and syntenic analysis of dnm2 and dnm2-like. (A) Chromosomal locations of zebrafish homologues to human DNMI, DNM2 and DNM3. (B) Phylogenetic tree comparing dynamin-2 genes in multiple species. (C) Comparison of zebrafish classical dynamins with human classical dynamins. Percent identity was determined by BLASTP. The length of homologous overlap is in parenthesis (number of amino acids). (D) Syntenic organization of human DNM2 compared with zebrafish dnm2 and dnm2-like.

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RACE-PCR and RT-PCR

Rapid amplification of cDNA end (RACE) was performed to confirm the 3' sequence of zebrafish dnm2 using the 3'-RACE GeneRacer kit (Invitrogen) according to the manufacturer's protocol. To clone dnm2, total RNA was extracted from 2 dpf larvae using an RNaseasy kit (Qiagen). For expression studies, RNA was extracted from adult zebrafish and embryos at various developmental timepoints. For analysis of morpholino-mediated knockdown, RNA was extracted from morpholino-injected and control larvae at 2 dpf. cDNA was synthesized from RNA using the iScript cDNA synthesis kit (Bio-Rad). PCR was performed on a MyCycler thermocycler (Bio-Rad) using GoTaq Green 2X Master Mix (Promega) and the following primers: 5'-TCACCTGGGAGTAAAACACG-3' (f/2x forward), 5'-ACGAGCTCGATGGAACAGCGAAGGACTAT-3' (f/1x reverse), 5'-GGCCAAAAGGTGTAACCTGGAA-3' (dnm2 reverse), 5'-GGCTGTGCTCATTAACACACTCACC-3' ( dnm2-like reverse), 5'-TGTGGGACTTGGACAGGAGTCTCAAG-3' (dnm2-like reverse), 5'-ACACGGAGCAGAGAAACGTCTACA-3' (dnm2 reverse), and 5'-GGTGCAATGATGCTTTGGCATGA-3' (human DNM2 reverse).

RNA Synthesis

Wild-type human DNM2 plasmid was purchased from Invitrogen (ORF Gateway® Entry IOH53617). Expression vectors were generated by recombination of DNM2 with p5E-CMV/S6P, p3EpolyA, and pDestTol2pa2 cassettes from the Tol2kit v1.2, a kind gift of Dr. Chi-Bin Chien [17]. Gateway recombination reactions were performed using LR Clonase II Plus Enzyme Mix (Invitrogen). The DNM2 rescue plasmid was linearized with NotI and transcribed using the SP6 mMessage machine kit (Ambion).

Morpholino and RNA Injection of Zebrafish Embryos

For dnm2 and dnm2-like knockdown, the following custom splice-targeting morpholinos were designed and purchased, along with standard control morpholino, from Gene Tools: 5’-TGCGGTGCTCATTAAAAACACTCTACG-3’ (dnm2 MO), 5’-CAACCCACCTGCCTCACCAGGTCT-3’ (dnm2-like MO), and 5’-CCCTTTACCTCATTACATATA-3’ (GeneTools standard control). Fertilized eggs were collected after timed mating of adult zebrafish and injected at the 1-2-cell stage using a Nanoject II injector (Drummond Scientific). Embryos were injected with dnm2-like MO (0.1 mM) or dnm2 MO (0.3 mM) in a 4.6 nL volume. Injection of control morpholino (ctl MO; 0.3 mM) verifies that the described injections at this concentration do not confer morpholino-mediated toxicity, and the same morpholino concentrations were utilized in all experiments. For rescue experiments, embryos were coinjected with human DNM2 mRNA (50 ng/µl). Larvae were photographed using a Nikon AZ-100 microscope or a Leica MXIII Stereoscope.

Analysis of Motor Behavior

Spontaneous coiling was measured at 1 dpf by observing the number of coils in a 60 second period. Touch-evoked motor behaviors were measured in 3 dpf larvae by touching the tail with a pair of No. 5 forceps. Larvae that did not swim following three consecutive tail stimuli were recorded as “no response”.

Histopathologic Analysis

For semi-thin sections, zebrafish were fixed overnight in Karnovsky's fixative at 3 dpf and then processed for embedding in epon by the Microscopy and Imaging Laboratory core facility at the University of Michigan. Semi-thin sections were stained with toluidine blue and photographed using an Olympus BX43 microscope. Myofiber size was determined by measuring the length of two continuous myofibers spanning the first myosepta caudal to the yolk sac using Adobe Photoshop evaluation of photomicrographs from semi-thin sections. Electron microscopy was performed using a Phillips CM-100 transmission electron microscope as previously described [10].

In situ Hybridization

In situ hybridization against dnm2 was performed as described previously [18]. Probes were made by in vitro transcription with T7 or SP6 RNA polymerase (Promega), using templates generated by PCR. Probe template was generated by PCR using the following primers: 5’-ATTAGTGAGCAGCATATAGTCTGCA-GATGTGAGCAG-3’ (Forward, SP6), and 5’-TAA-TACGACTCATATAGGTTTCCAGG-TAAACGCTGCTC-3’ (Reverse, T7). PCR was performed on cDNA from 1 dpf wild-type (AB) embryos, and probe template sequence was verified by sequencing.

Statistical Analysis

Statistical analysis was performed on data using the GraphPad Prism 5 software package. Significance was determined using ANOVA or Fisher’s exact test.

Results

Structure and Organization of two Dynamin-2 Genes in Zebrafish

Using public databases (NCBI, ENSEMBL, ZFIN) and RACE-PCR, we identified two separate zebrafish genes, dnm2 and dnm2-like, which are highly related to human DNM2, on chromosomes 3 and 1 (Figure 1A; Genbank ID559534 and ID 406525; zfin zgc:114072 and zgc:77233). 3’ RACE-PCR on dnm2 identified an additional 3 exons not included in any databases. These exons shared sequence homology with the 3 final exons in human DNM2 and zebrafish dnm2-like. We additionally screened these databases for zebrafish genes with high sequence homology to other human classical dynamins. Comparison of the two putative zebrafish genes with human dynamins revealed that both dnm2 and dnm2-like share highest sequence homology with human DNM2 (Figure 1C). Phylogenetic analysis also grouped both genes into the DNM2 cluster (Figure 1B). Analysis of genes surrounding the human DNM2 revealed a conserved syntenic cluster including the dnm2 gene on zebrafish chromosome 3 (Figure 1D).

Both zebrafish proteins share all five major domains of human DNM2, including a GTPase domain, a GTPase effector domain (GED), a dynamin-specific middle domain, a pleckstrin homology (PH) domain, and a proline-rich domain (PRD). The two zebrafish dnm2 genes share similar intron-exon organization with human DNM2, although dnm2-like has substantially smaller introns than either other gene (Figure 2A). At the protein level, these domains all share close identity with the domains of human DNM2 (Figure 2B).

$dnm2$ and $dnm2$-like Genes are Widely Expressed in Adult and Embryonic Tissue

To determine the expression pattern of $dnm2$ and $dnm2$-like, we performed RT-PCR on adult zebrafish tissues and whole zebrafish larvae at several time points. Both $dnm2$ and $dnm2$-like mRNA was detected in all adult tissues examined (Figure 2C). Both genes products were also detected at the earliest stages of development,
indicating that both \(dnn2\) and \(dnn2\)-like are likely maternally deposited mRNAs (Figure 2D). Ubiquitous \(dnn2\) expression was additionally confirmed by in situ hybridization in 1 dpf embryos (Figure S1).

**Morpholino-mediated Knockdown of Zebrafish \(dnn2\) and \(dnn2\)-like Gene Expression**

To better clarify the roles of \(dnn2\) and \(dnn2\)-like, we used targeted morpholino oligonucleotides to knockdown expression of both genes during early development. Morpholinos were targeted to splice junctions in \(dnn2\) and \(dnn2\)-like pre-mRNAs (Figure 3A), and the resulting products were confirmed to be out of frame by sequencing the RT-PCR products (Figure 3B). A standard control morpholino was injected for comparison (Gene-Tools).

Both \(dnn2\) MO (0.3 mM) and \(dnn2\)-like MO (0.1 mM) injection resulted in pronounced but non-overlapping developmental phenotypes compared to ctl MO (0.3 mM) injection (Figure 3C). Knockdown of \(Dnm2\) caused a shorter body axis, small eyes, yolk and cardiac edema, shortened somites, and an upward tail.
curvature. Knockdown of Dnm2-like resulted in a thinned body axis, small eyes, and pigmentation defects. The severity and penetrance of morpholino phenotypes was consistent between injections (control n = 601, dnm2 n = 601, dnm2-like n = 587). At 2 dpf, both morpholino groups had a significant increase in abnormal morphology relative to control morpholino (Figure 3D; p < 0.0001, Fisher’s exact test); 93% of dnm2 morphants and 74% of dnm2-like morphants exhibited the described phenotypes, while only 4% of control embryos displayed any developmental abnormalities. To determine knockdown of dynamin-2 expression in dnm2 and dnm2-like morphants, isolated muscle fibers were stained with an antibody against dynamin-2. Cells from both dnm2

Figure 3. Morpholino-mediated knockdown of dnm2 and dnm2-like expression results in morphological changes. (A) Splice targeting morpholinos were designed against intron-exon boundaries within the dnm2 and dnm2-like genes. (B) Knockdown in morpholino injected embryos was verified using RT-PCR. Embryos were injected with a scrambled control morpholino (Ctl MO; 0.3 mM), dnm2 MO (0.3 mM), or dnm2-like MO (0.1 mM). Arrows indicate the alternative splice product induced by dnm2 MO and dnm2-like MO injection. dnm2 MO injection also resulted in an additional higher weight band due to activation of a cryptic splice site (*). (C) At 2 dpf, dnm2 MO-injected embryos exhibit shortened body length, upward curled tails, pericardial and yolk edema, and reduced head size when compared to control morpholino injected embryos. By contrast, embryos injected with dnm2-like MO have small muscle compartments, pigmentation defects, and mild tail curvature. (D) Percent of affected embryos at 2 dpf (ctl MO vs. dnm2 MO p < 0.0001, ctl MO vs. dnm2-like MO p < 0.0001; Fisher’s exact test). The total number of embryos is noted above each bar. doi:10.1371/journal.pone.0055888.g003
and dnm2-like morphants had reduced staining relative to control morphants (data not shown).

In order to further examine the effect of dynamin-2 depletion on embryonic and larval muscle, we assayed two motor behaviors during development. First, we looked at spontaneous contracting behavior in 1 dpf embryos. Spontaneous contracting is a highly stereotyped behavior detected in zebrafish embryos between approximately 17 and 26 hours post fertilization [19]. Control embryos contracted an average of 35.7 ± 1.5 times per minute and, similarly, dnm2-like morphants contracted 31.0 ± 1.6 times per minute (Figure 4A; control n = 119, dnm2-like n = 107; ns). By contrast, dnm2 morphants only contracted an average of 9.5 ± 1.2 times per minute (dnm2 n = 114; p < 0.001, ANOVA). Next, we examined touch-evoked behavior in 3 dpf larvae. At this stage of development, larvae typically respond to a tactile stimulus with a rapid escape response. However, 87.2% of dnm2 morphants failed to respond to a tail tap stimulus (Figure 4B-E, C, n = 203; Only 4.0% of control morphants and 20.3% of dnm2-like morphants did not respond to a tail tap stimulus (control n = 204; dnm2-like n = 197). Together, the reduced spontaneous contracting and diminished touch-evoked escape behaviors suggests that dnm2 morphants have a defect in motor function that is not shared by dnm2-like morphants.

Histopathological and Ultrastructural Abnormalities in dnm2 Morphant Muscle

In light of the observed motor defects in dnm2 morphants, we examined histological and ultrastructural features in muscle from 3 dpf larvae. Semi-thin sections were obtained from the trunks of 3 dpf larvae injected with control, dnm2, or dnm2-like morpholino (Figure 4D). While sections from dnm2 morphant muscle revealed striking fiber disorganization, as well as small somites and indistinct striations as compared with control muscle, sections from dnm2-like morphant muscle only revealed moderate effects on myofibers. Quantification of myofiber size indicated that fibers from dnm2 morphants were significantly and substantially smaller than those of control embryos (p < 0.009). Myofibers from dnm2-like morphants were also significantly smaller than fibers from larvae injected with control morpholino (p < 0.05; Figure 4E). The dnm2 morphant myofibers were, in addition, smaller than those from dnm2-like morphants; however, this difference did not reach statistical significance (p = 0.056 for direct comparison of dnm2 to dnm2-like). Similarly, electron microscopy of dnm2 morphant muscle revealed substantial disorganization with irregular membrane accumulations (Figure 4F, arrow) but only subtle changes in the dnm2-like morphants (data not shown). Of note, sarcomeric structures appeared normal in both groups, suggesting that dnm2 is not required for establishing basic myofibril organization.

Expression of Human DNM2 Rescues dnm2 and dnm2-like Knockdown

To rescue the dnm2 and dnm2-like morphant phenotypes, embryos were co-injected with human DNM2 capped mRNA and morpholino at the 1- to 2-cell stage (Figure 5). Expression of DNM2 did not cause any morphological abnormalities in control-injected embryos. At 2 dpf, the percent of normal-appearing embryos was significantly increased in both rescue conditions (control n = 796, dnm2 n = 840, dnm2-like n = 802). In dnm2 morphants, the percent of normal embryos increased from 8.2% to 67.1% (p < 0.0001, Fisher’s exact test). In dnm2-like morphants, the percent of normal embryos increased from 24.2% to 45.8% (p < 0.0001, Fisher’s exact test). Additional rescue experiments with human DNM1 and DNM3 reveal that, although all 3 classical dynamins can rescue the functional defects observed in dnm2 morphants to a similar extent, only DNM2 had a significant effect on the dnm2-like morphant behavior (data not shown). Together, these data support the contention that dnm2 and dnm2-like are functional orthologs of human DNM2.

Discussion

DNM2 plays an important role in endocytosis and several intracellular membrane trafficking pathways [20]. Given this prominent role in cellular function and the fact that mutations in DNM2 are associated with two disorders affecting nerve and muscle – Charcot-Marie-Tooth disease and centronuclear myopathy – understanding its specific role in nerve and muscle are critical to enhance our understanding of the role of DNM2 in these tissues in health and disease. In vitro and murine models of DNM2-related centronuclear myopathy have begun to shed light on how DNM2 contributes to muscle defects [20,21]; however, better models are needed to recapitulate disease characteristics and gain more meaningful insight into disease pathogenesis. Zebrafish are becoming an increasingly popular model for the study of muscle disorders; in addition to the many advantages of zebrafish as a model system, zebrafish muscle shares many histological features with mammalian muscle, their neuromuscular system is well-characterized, and various approaches facilitate the development of disease models. As a first step towards developing zebrafish models of DNM2-related neuromuscular disease, this manuscript describes the characterization of two zebrafish dynamin-2 orthologs, as well as the effects of altered gene expression on muscle histology and function.

In this study, we characterize two dynamin-2 genes in the zebrafish genome. The two genes are likely a product of the whole genome duplication that occurred in the ray fin fish lineage prior to the evolution of the teleost [22,23]. The syntenic organization of both genes supports this conclusion. dnm2 (zebrafish chromosome 5) shares close syntenic conservation with DNM2 (human chromosome 19), as it is directly flanked by homologs of the upstream and downstream neighbors of human DNM2 (TMED1 and QRT1). While dnm2-like (zebrafish chromosome 1) does not share this immediate syntenic block, the human homologs of at least four nearby genes are found within a 0.3 Mb distance of human DNM2 (TMED1, CDG37, OLFM2, COL5A3 and RDH8). Additionally, both zebrafish genes are found near chromosomal regions that have previously been reported to share homology with human chromosome 19 [24].

At both the gene and protein level, dnm2 and dnm2-like share structural similarity with human DNM2. All three genes have a
similar intron-exon organization, although dnm2-like has much smaller introns. Shrinkage of introns has been reported in several other teleost homologs to human genes [25,26,27]. At the protein level, the predicted amino acid sequences of Dnm2 and Dnm2-like share a high percent identity to human DNM2, as well as to each other. When we examined the DNA sequence of other human and zebrafish classical dynamins, phylogenetic analysis grouped dnm2 and dnm2-like with DNM2 rather than DNM1 or DNM3. Mammalian DNM2 is ubiquitously expressed in adult tissue [7,8,9]. In zebrafish, we found dnm2 and dnm2-like expression in every tissue we examined, which suggests these genes may also be ubiquitously expressed. Both genes were also expressed throughout early development. The early presence of these gene products...
makes it likely that dnm2 and dnm2-like mRNAs are maternally deposited. This contention is further supported by our observations following knockdown of either dnm2 or dnm2-like. Both morpholino reagents used in this study are splice-targeting morpholinos which only target unprocessed mRNA transcripts; therefore, expression of maternally deposited mRNAs will not be knocked down by the morpholino oligonucleotides. Since we detect dnm2 and dnm2-like mRNA at the one-cell stage, it is likely that both gene products are unaffected by morpholino knockdown during the first few hours of development. In spite of this, we see that both gene products are unaffected by morpholino knockdown dnm2 detected by the morpholino oligonucleotides. Since we morpholinos which only target unprocessed mRNA transcripts; functional abnormalities are present in zebrafish embryos following dnm2 and dnm2-like knockdown. Morphologically, dnm2 morphants exhibited a shortened body axis, upward tail curvature, small head size, and edema, while dnm2-like morphants displayed only mild tail curvature along with small muscle compartments and pigmentation defects. Further analyses of muscle histology revealed significant effects of both dnm2 and dnm2-like knockdown on myofiber length. The effects on fiber length in dnm2 morphants were greater than those observed in dnm2-like morphants, and dnm2 morphant embryos also exhibit irregular membrane structures upon EM. Similar histopathological changes in muscle have been previously described [29] and further support is provided by Durieux et al, who demonstrate decreased muscle size in transgenic mice heterozygous for mutant R465W-Dnm2, and Laporte et al, who describe histopathological features including centralized nuclei and fiber atrophy with adenosival overexpression of R465W-DNM2 in adult mouse muscle [29,30]. Interestingly, expression of R465W-DNM2 in this model was initiated in adult muscle, demonstrating that DNM2 plays an important role in muscle maintenance after myogenesis. Despite the occurrence of morphological effects in both dnm2 and dnm2-like morphants, however, behavioral characterization reveals a more varied effect on muscle function. dnm2 morphants exhibit a striking deficit in touch-evoked escape behavior, while dnm2-like morphants exhibit relatively mild phenotypes in this regard. Similar effects of mutant Dnm2 have been reported in heterozygous R465W-Dnm2 mice, which exhibit reductions in muscle force by 3 weeks of age [30]. Together, the current zebrafish data, along with insights gained from previously reported mutant Dnm2 mouse models, confirm a role for DNM2 in muscle structure and function.

In order to further support the hypothesis that dnm2 and dnm2-like share functional homology with human DNM2, we then examined the ability of human DNM2 to rescue the phenotype of both dnm2 and dnm2-like morphants. Human DNM2 expression can partially rescue the phenotypes resulting from knockdown of dnm2 and dnm2-like. This reduction in the fraction of abnormal embryos was greater for DNM2 rescue of the dnm2 morphants, although significant improvements were also seen for dnm2-like morphants injected with DNM2 RNA. Interestingly, additional rescue experiments in the zebrafish with human dynamins (data not shown) have also demonstrated that touch-evoked escape responses in the dnm2 morphants can be rescued by expression of DNM1, DNM2 or DNM3, while only DNM2 is able to convincingly rescue swimming behavior in dnm2-like morphants. This may suggest that dnm2-like is the more closely related DNM2 ortholog in zebrafish. However, previous studies have shown that the classical dynamins can co-oligomerize [31,32], and in vitro and knockout mouse studies both show that DNM1 and DNM3 can compensate for DNM2 loss [33]. Therefore, we are unable to conclusively distinguish dnm2 or dnm2-like as more closely resembling human DNM2, and maintain that both are likely orthologs of the human gene.

Together, our data support a functional connection between the dnm2 and dnm2-like orthologs in zebrafish; however, despite similar expression patterns and effects of dnm2 and dnm2-like knockdown on zebrafish muscle histology, the varying severity of these phenotypes along with the differential effects on functional assessments indicates that they play both overlapping and distinct roles in zebrafish muscle. On one hand, the observed functional differences could be due to differences in knockdown efficiency between the dnm2 and dnm2-like morphants. Alternatively, gene-specific functional differences could exist. Future gene-specific targeting and mutant DNM2 studies addressing the detailed mechanisms responsible for the observed histological and functional deficits in morphant zebrafish are warranted to comprehend the exact role these proteins are playing in muscle development and function. For example, activity-deficient DNM2 mutants could be employed to assess the contribution of enzymatic activity on endocytosis and muscle structure and function. Electrophysiological studies may also provide insight into the correlation of the observed morphological defects with functional outcomes. Finally, studies assessing potential disease-causing mechanisms may be required to understand the role of DNM2 in disease. Endocytosis and autophagy defects, altered oligomerization, abnormalities in muscle membrane structure development and maintenance, and effects at the neuromuscular junction are all important mechanisms [29,30,34,35] to consider and investigate to determine how DNM2 contributes to neuromuscular disorders.

Taken together, our findings show that dnm2 and dnm2-like are highly conserved orthologs to human DNM2 and are independently required for normal embryonic development in the zebrafish. It will be important to further examine these two genes in order to understand their specific cellular function in the zebrafish. The zebrafish provides an excellent system for examining aspects of membrane trafficking in vivo, and understanding the zebrafish dynamin-2 homologs will allow a more precise analysis of these pathways.

Supporting Information

Figure S1 Zebrafish dnm2 whole mount in situ hybridization. (A) Whole mount in situ of 1 dpf embryos reveals ubiquitous expression of dnm2; (B) Sense probe to dnm2 was used as a background control. (TIF)

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Author Contributions

Conceived and designed the experiments: EMG. Performed the experiments: EMG AED AT-G CB YH. Analyzed the data: EMG AED SAS JJD ELF. Wrote the paper: EMG SAS JJD ELF.
References

Neuromuscular junction abnormalities in DNM2-related centronuclear myopathy

Elizabeth M. Gibbs · Nigel F. Clarke · Kristy Rose · Emily C. Oates · Richard Webster · Eva L. Feldman · James J. Dowling

Abstract Dynamin-2-related centronuclear myopathy (DNM2-CNM) is a clinically heterogeneous muscle disorder characterized by muscle weakness and centralized nuclei on biopsy. There is little known about the muscle dysfunction underlying this disorder, and there are currently no treatments. In this study, we establish a novel zebrafish model for DNM2-CNM by transiently overexpressing a mutant version of DNM2 (DNM2-S619L) during development. We show that overexpression of DNM2-S619L leads to pathological changes in muscle and a severe motor phenotype. We further demonstrate that the muscle weakness seen in these animals can be significantly alleviated by treatment with an acetylcholinesterase inhibitor. Based on these results, we reviewed the clinical history of five patients with two different DNM2-CNM mutations (S619L and E368K) and found electrophysiological evidence of...
abnormal neuromuscular transmission in two of the individuals. All five patients showed improved muscle strength and motor function, and/or reduced fatigability following acetylcholinesterase inhibitor treatment. Together, our results suggest that deficits at the neuromuscular junction may play an important role in the pathogenesis of DNM2-CNM and that treatments targeting this dysfunction can provide an effective therapy for patients with this disorder.

Keywords Dynamic-2 · Myopathy · Neuromuscular junction · Acetylcholinesterase inhibitor

Introduction

Centronuclear myopathies (CNMs) are a clinically heterogeneous group of muscle disorders characterized by a high proportion of centralized nuclei on muscle biopsy. CNMs can have a wide spectrum of clinical presentations, ranging from severe infantile to mild adult-onset forms. Common features of the disease include generalized weakness, poor muscle tone, ptosis, and ophthalmoparesis. Mutations in four genes are known to cause CNM: myotubularin (MTM1), amphiphysin 2 (BIN1), dynamin 2 (DNM2), and the skeletal muscle ryanodine receptor (RYR1).

To date, 19 different mutations in DNM2 have been shown to cause autosomal dominant forms of CNM (Fig. 1a) [1]. While the relationship between mutation location and phenotype is not entirely clear, patients with severe early-onset DNM2-CNM often have mutations in the pleckstrin homology (PH) domain. The PH domain is responsible for localizing DNM2 to the plasma membrane during endocytosis, but this localization does not seem to be disrupted by disease-associated mutations in the PH domain [2, 3]. At the plasma membrane, DNM2 forms rings around budding vesicles, where it contributes to the release of newly formed endosomes. In addition to endocytic function, DNM2 has been implicated in caveolae internalization, trans-Golgi transport, and aspects of cytoskeletal regulation including lamellipodia extension, phagocytosis, cell motility, cell division, and centrosome cohesion [4–10].

Despite the well-characterized role of DNM2 in endocytosis, little is known about DNM2 function in muscle or the specific pathomechanisms that underlie DNM2-related CNM. The purpose of this study is to examine the structure and function of the neuromuscular junction (NMJ) in DNM2-CNM using a novel zebrafish model combined with clinical analysis of patients with DNM2-CNM. While NMJ defects have not typically been associated with CNM pathology, some patients with CNM exhibit symptoms consistent with congenital myasthenic syndromes, a group of disorders caused by defects in neuromuscular transmission. Case studies have reported NMJ abnormalities in patients with genetically uncharacterized CNM, including histopathologic features that resemble the post-synaptic changes seen in some myasthenic disorders [11–13]. Intriguingly, two recent studies describe patients with CNM who responded favorably to treatment with acetylcholinesterase inhibitors, a finding consistent with NMJ deficits [14, 15]. Despite this

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**Fig. 1** Overexpression of DNM2-S619L causes a muscle-specific phenotype. **a** CNM-causing mutations in human DNM2. The mutation used in this study, DNM2-S619L, is highlighted in yellow. **b** DNM2-WT and DNM2-S619L larvae at 2 dpf. Overexpression of DNM2-WT or DNM2-S619L does not cause any gross morphological changes during development. **c** RT-PCR of human DNM2 expression in developing zebrafish injected with high (150 pg/injection) or low (50 pg/injection) concentrations of DNM2-S619L RNA. All further studies were performed on embryos injected with 50 pg RNA.
mounting evidence that CNM can present with myasthenic features, there are no reports of NMJ dysfunction or acetylcholinesterase inhibitor treatment in patients with DNM2-CN.

There are currently no stable transgenic DNM2-CN animal models that recapitulate the major features of the disorder. Heterozygous knock-in mice carrying the R465W mutation display some histologic changes and modest muscle atrophy by 8 months, but exhibit no overt motor phenotype or centralized nuclei [16, 17]. By contrast, viral overexpression of DNM2-R465W in adult mouse muscle causes weak muscle and atrophy, and a significant increase in abnormally localized nuclei by 4 weeks postinjection [18]. Both of these mouse studies examined the DNM2-R465W mutation, a common CNM-causing mutation. Most patients with the DN2M-R463W mutation have a relatively mild disease course, with symptom onset ranging from late childhood to the fourth decade of life [19, 20]. In this study, we examine transient overexpression of DNM2-S619L, a mutation associated with severe neonatal weakness and hypotonia [21].

Zebrafish have been a successful model for many muscle disorders, and our lab has previously characterized a zebrafish model for myotubular myopathy, an X-linked form of CNM [22, 23]. In this study, we generate a novel model of DN2M-CN by transiently overexpressing DNM2-S619L in developing zebrafish larvae. These animals exhibit severe weakness and motor deficits. We demonstrate that NMJs are disorganized in these animals and that motor dysfunction can be rapidly alleviated by acetylcholinesterase inhibitor treatment. We also show electrophysiological evidence for NMJ deficits in two patients with DNM2-CN and report symptomatic improvement following acetylcholinesterase inhibitor treatment in five patients. Taken together, these results suggest that deficits in neuromuscular transmission are a significant component of DNM2-CN pathology and that therapeutics targeting the NMJ may provide effective treatment for this disorder.

Materials and methods

Animal care Zebrafish (AB strain) were bred and raised according to established protocols, under the guidelines of the University of Michigan Animal Care and Use protocols. Experiments were performed on zebrafish embryos and larvae between 1 and 3 days post fertilization (dpf). All animals were handled in strict accordance with good animal practice as defined by national and local animal welfare bodies. All animal work was approved by the University of Michigan Committee for the Use and Care of Animals (UCUCA protocol no. 09835).

RNA synthesis and injection of zebrafish embryos Wild-type human DNM2 plasmid was purchased from Invitrogen (ORF Gateway® Entry IOH53617). The S619L point mutation was introduced using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). Expression vectors were generated by recombination of DNM2 entry clones with p5E-CMV/SP6, p3E-polyA, and pDestTol2PA2 cassettes from the Tol2kit v1.2, a kind gift of Dr. Chi-Bin Chien [24]. Gateway recombination reactions were performed using LR Clonase II Plus Enzyme Mix (Invitrogen). DNM2 expression constructs were linearized with NotI and transcribed using the SP6 mMessage Machine kit (Ambion).

Fertilized eggs were collected after timed matings of adult zebrafish and injected at the one- to two-cell stage using a Nanoject II injector (Drummond Scientific). Embryos were injected with 50 or 150 pg of RNA (10.9 or 32.6 ng/μL in an injection volume of 4.6 nL) and raised in E2 embryo culture media [25].

Analysis of motor behavior Larvae were photographed using a Nikon AZ-100 microscope or a Leica MXIII stereoscope. For tail movement studies, larvae were embedded in 1 % low melting point agarose, and agarose surrounding the tail was gently cut away. Tail extension measurements were performed using ImageJ (NIH) and calculated as percent of body length. For acetylcholinesterase inhibitor studies, zebrafish larvae were bathed in 0.2 mg/mL edrophonium (Enlon®, Bioniche Pharmaceuticals) diluted in E2 media. Touch-evoked motor behaviors were elicited by touching the tail with a pair of no. 5 forceps. Speed measurements and video frame capture were performed using ImageJ (NIH).

Reverse transcription PCR RNA was isolated from embryos at 1, 2, or 3 dpf using the RNaseasy kit (Qiagen). cDNA was synthesized from RNA using the iScript cDNA Synthesis kit (Bio-Rad). PCR was performed on a MyCycler thermocycler (Bio-Rad) using GoTaq Green 2× Master Mix (Promega) and primers to zebrafish EF1-alpha or human DNM2. PCR primer sequences were as follows:

- ef1α forward: 5′-TCACCCCTGGAGTGAAACACGC-3′
- ef1α reverse: 5′-ACTTGCAAGGAAATTGAGCAG-3′
- Human DNM2 forward: 5′-ACACGGAGACAGA ACGTCTACA-3′
- Human DNM2 reverse: 5′-GGTGCGATGGTCTTG GCCATGA-3′

Histopathologic analysis For semi-thin sections, zebrafish were fixed in Karnovsky’s fixative at 3 dpf and then processed for embedding in Epon by the Microscopy and Imaging Laboratory core facility at the University of Michigan. Semi-thin sections were stained with toluidine blue and photographed using an Olympus BX43 microscope. Electron microscopy was performed using a Phillips CM-100 transmission electron microscope. For fluorescent
imaging, acetylcholine receptors were labeled with Alexa Fluor 594 conjugated alpha-bungarotoxin (Invitrogen, 1:100 in phosphate-buffered saline with Tween 20 (PBST)) and photographed using a Nikon Microphot FXA microscope. Total fluorescent intensity was measured using ImageJ (NIH). For motor neuron staining, labeling was performed as described previously, using 3-dpf larvae and a mouse monoclonal antibody against SV2 (1:100, Developmental Studies Hybridoma Bank) [26]. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on 3-dpf larvae as previously described [27], and cell death was evaluated by counting TUNEL-positive nuclei in the larval trunk.

**Myofiber cultures** Myofiber preparations from 3-dpf larvae were obtained as previously described [23]. Briefly, larvae were dissociated in 10 mM collagenase type I (Sigma) for 60–90 min at room temperature. Larvae were triturated approximately every 30 min. Dissociated preps were resuspended in CO2-independent media (Invitrogen), passed through a 70-mm filter (Falcon), and plated onto glass coverslips precoated with poly-L-lysine (Sigma). Culture media were changed after 2 h, after which cells were fixed for 15 min in 4 % paraformaldehyde. Cells were stained using alpha-bungarotoxin (Invitrogen, 1:100 in PBST).

**Statistical analysis** Statistical analysis was performed on data using the GraphPad Prism 5 software package. Where relevant, significance was determined using a one-way ANOVA, followed by Tukey’s multiple-comparison test. For maximum tail extension measurements, a paired Student’s t test was used to determine significance.

**Case studies** Case data were collected via institutional review board (IRB-approved protocols HUM00032624 and HUM00030880) at the University of Michigan.

**Results**

Motor behavior is impaired in DNM2-S619L-injected embryos

To examine the effect of DNM2-S619L overexpression in zebrafish, embryos were injected with DNM2-WT or DNM2-S619L capped mRNA at the one- to two-cell stage. Injections with either construct did not cause an obvious defect in general body morphology (Fig. 1b). Two RNA concentrations were tested, 50 pg per injection and 150 pg per injection. Reverse-transcriptase PCR was performed on RNA extracted from injected embryos at 1, 2, and 3 dpf. Although levels of injected DNM2 RNA decreased over time, RNA was still present at 3 dpf (Fig. 1c). Since injections at the lower concentration of RNA resulted in a pronounced motor phenotype, all measurements were performed on larvae injected with 50 pg of RNA. No significant increase in cell death was observed in the trunk of DNM2-WT or DNM2-S619L larvae as compared to uninjected larvae (data not shown).

At 2 dpf, larvae expressing DNM2-S619L had notably weaker swimming patterns and frequently exhibited a weak “flutter” tail movement. When lightly tapped, most DNM2-S619L larvae were unable to generate a substantial escape response (Fig. 2a and Supplementary video 1). To further quantitate this phenotype, we examined the escape response at 3 dpf. Thirty-five percent of DNM2-S619L larvae failed to respond to tactile stimulus, compared with 13 % of DNM2-WT larvae and 5 % of uninjected larvae (Fig. 2b; un injected, n=401; DNM2-WT, n=386; DNM2-S619L, n=398; data combined from ten trials). In addition to nonresponsive larvae, many DNM2-S619L larvae responded to touch with a light tail flutter or body coil, but did not swim away from the stimulus. Thirty-nine percent of DNM2-S619L larvae responded with a flutter or coil, compared with 12 % of DNM2-WT larvae and 1 % of uninjected larvae. Animals that were able to swim away from the stimulus (regardless of speed) were included in the “swim” category. Ninety-four percent of uninjected and 74 % of DNM2-WT larvae were able to swim away from the stimulus, while only 26 % of DNM2-S619L larvae responded by swimming.

Histopathological and ultrastructural abnormalities in DNM2-S619L zebrafish

To determine if there were structural abnormalities within skeletal muscle that were associated with the impaired motor function, semi-thin sections from the injected control, DNM2-WT, and DNM2-S619L embryos (2 dpf) were analyzed (n=3 per condition). As compared to control muscle, no overt histopathologic changes were observed in the DNM2-WT muscle. Conversely, abundant abnormalities were observed in the muscle from DNM2-S619L embryos. These changes included the presence of disorganized, amorphous cellular material in the perinuclear area (two black dots in Fig. 3a) and additional areas of swollen intracellular organelles (white arrow in Fig. 3b). Using the semi-thin sections, we also measured the cross-section area of DNM2-WT and DNM2-S619L muscle fibers. We found that DNM2-S619L fibers were 44 % smaller than WTs (n=10, p=0.0009). In addition, we further characterized larval muscle cell using electron microscopy (Fig. 3c). Large irregular membrane accumulations were seen between myonuclei and myofibrils in the DNM2-S619L larval muscle, which were not present in myonuclei from DNM2-WT larval muscle. Together, these findings at the light and
Fig. 2  DNM2-S619L larvae exhibit impaired motor function. a Video frames showing tail tap stimulus of a DNM2-WT larva (above) and a DNM2-S619L larva (below). b Response to tail tap stimulus at 3 dpf (uninjected, n=401 larvae; DNM2-WT, n=386; DNM2-S619L, n=398; response scores combined from ten different trials)

Fig. 3  Histopathological and ultrastructural abnormalities in DNM2-S619L zebrafish. a Semi-thin sections of DNM2-WT and DNM2-S619L muscle at 3 dpf. Note myonuclei (black arrow) with disorganized material in the perinuclear area (two black dots) in DNM2-S619L muscle. b Higher-magnification images of semi-thin sections. Vacuole-like structures (white arrow) are seen adjacent to myonuclei in DNM2-S619L larval muscle. c Comparison of perinuclear ultrastructure from DNM2-WT and DNM2-S619L larval muscle (N nuclei). Regions with swollen intracellular organelles are seen adjacent to the DNM2-S619L myonuclei. These regions likely represent dilated sarcoplasmic reticulum. Scale bar, 1 μm
electron microscopic levels are consistent with those reported for other models of CNM in zebrafish and are in keeping with the predicted features of acentronuclear myopathy [23].

To examine the organization of the developing NMJs, we used alpha-bungarotoxin to stain for acetylcholine receptor clusters (Fig. 4). In 3-dpf DNM2-S619L larvae, the clusters are weaker and more dispersed, although the general distribution appears normal and they localized with motor axon path. This attenuated staining was seen in 18 of 21 DNM2-S619L larvae and not observed in DNM2-WT larvae (n=19). No abnormalities in motor neuron organization were observed (Supplementary Fig. 1).

To quantify the intensity of alpha-bungarotoxin staining, total fluorescent intensity was measured in a restricted region of the trunk. The total fluorescent intensity was significantly reduced in the DNM2-S619L larvae (DNM2-WT, n=10; DNM2-S619L, n=11; p<0.0001, Student’s t test). To further examine the organization of acetylcholine receptor clusters in the DNM2-S619L muscle, we prepared dissociated myofiber preparations from DNM2-WT and DNM2-S619L larvae and stained the fibers with alpha-bungarotoxin. There was a significant decrease in the number of acetylcholine clusters per myofiber in the DNM2-S619L (5.3±0.3 clusters in DNM2-WT larvae, 3.0±0.2 clusters in DNM2-S619L larvae; p<0.0001, Student’s t test).

Acetylcholinesterase inhibitor treatment improves motor behavior in zebrafish

To further examine the role of the NMJ in DNM2-S619L larval swim defects, we examined the response of DNM2-S619L and DNM2-WT larvae to treatment with edrophonium, a short-acting acetylcholinesterase inhibitor. Short treatments with 0.2 mg/mL edrophonium (1–5 min) dramatically increased both spontaneous and evoked movement in DNM2-S619L larvae. Following treatment, DNM2-S619L larvae that had previously exhibited little or no movement were able to swim away from a tail tap stimulus (Fig. 5a and Supplementary video 2).

To quantify the improvement seen in acetylcholinesterase inhibitor-treated DNM2-S619L larvae, we measured the distance swum in approximately 182 ms following a tail tap stimulus (Fig. 5b, n=10 for all conditions). Only larvae that exhibited a motor response prior to treatment were included. In the measured time interval, uninjected larvae swam an average distance of 53.8±4.5 mm. Before treatment, DNM2-WT larvae swam 46.9±3.0 mm, and DNM2-S619L larvae swam 21.4±4.9 mm. The distance swum by the DNM2-S619L larvae was significantly smaller than the uninjected or DNM2-WT groups (p<0.001). Following acetylcholinesterase inhibitor treatment, the DNM2-S619L larvae swam significantly further than before treatment (41.5±3.8 mm, p<0.01). There was no significant difference.
between the distance swum by the treated DNM2-S619L group as compared to DNM2-WT larvae or to uninjected controls. Of note, neither DNM2-WT nor uninjected control larvae had a change in swim behavior following acetylcholinesterase inhibitor treatment.

To further examine the response to acetylcholinesterase inhibitor treatment, DNM2-S619L larvae were embedded in agarose with the tail and caudal portion of the fish free to move (Fig. 5c). All larvae exhibited weak and restricted tail beats during swim bouts \((n=6)\). Following 2 min of acetylcholinesterase inhibitor treatment, all larvae moved with increased tail curvature and stronger muscle contractions (Fig. 5c). The maximum tail extension of each larva was measured before and after treatment and was found to increase in all treated larvae (Fig. 5d). Prior to acetylcholinesterase inhibitor treatment, DNM2-S619L larvae had an average maximum tail extension of \(14.2\%\) of body length. Following treatment, the average maximum tail extension was \(51.8\%\) of body length \((p<0.0005)\).

Abnormal neuromuscular junction function and response to acetylcholinesterase inhibitor in five individuals with DNM2-related CNM

To understand the potential relevance of these findings in zebrafish to human DNM2-related CNM, five cases (two retrospective and three prospective) were analyzed.

Case 1 Case 1 is a 24-year-old male individual. He presented with weakness and respiratory failure shortly after birth, and he has been wheelchair- and ventilator-dependent for his entire life. In addition to severe extremity weakness, he also has ptosis, ophthalmoparesis, and lower facial weakness. Muscle biopsy performed in the first year of life was consistent with a diagnosis of centronuclear myopathy (significantly increased central nuclei, abnormal oxidative stain pattern, and type I fiber predominance and atrophy). Genetic testing was negative for mutations in \(MTM1\) but revealed a heterozygous mutation (S619L) in DNM2.
This individual had a relatively static course of illness until approximately 1 year ago. In the past year, he has experienced progressive fatigue as well as declining strength that is manifested by decreased volume of speech and diminished ability to raise his distal upper extremity against gravity. Clinical evaluation did not reveal any general medical condition to account for his decline, and his cardiopulmonary status (including nocturnal ventilatory status) was unchanged. Electrodiagnostic studies were performed, including 3-Hz repetitive nerve stimulation and single-fiber electromyography (SFEMG). A 6 to 12% decrement was observed with 2-Hz repetitive stimulation of the ulnar motor compound muscle action potential. SFEMG of the right extensor digitorum brevis revealed neuromuscular jitter (a measure of neuromuscular transmission synchrony between two motor units) in all motor unit pairs with a mean consecutive difference (MCD) from 88 to 300 μs (normal, 25 to 35 μs) with several motor unit pairs having >20% blocking, an indication of complete loss of neuromuscular synchrony.

Based on these findings, he was started on pyridostigmine therapy at a dose of 60 mg QID. Within 2 weeks of treatment, he reported improvement in several areas. Specifically, his speech was higher in volume and easier to understand, he had fewer complaints of fatigue, and, for the first time since early childhood, he was able to bring his hands to his mouth. He has been continued on this dose of pyridostigmine and has maintained these improvements in motor function. Of note, acetylcholine receptor antibody testing was negative.

**Case 2** Case 2 is a 28-year-old female who began to experience weakness at around 5 years of age. Her presenting symptoms included frequent falls, an abnormal gait, difficulties rising from a seated position, and impaired stair climbing. A diagnostic muscle biopsy performed at age 8 revealed changes consistent with centronuclear myopathy (excessive central nuclei, small type I fibers, and radial stranding with NADH staining). Genetic testing (performed at age 25) uncovered a heterozygous mutation (E368K) in DNM2. Basic clinical details of this patient have been reported previously [28].

She has had progressive decline in motor function, has additionally developed severe ptosis and ophthalmoparesis, and has, for the past 3 years, required a cane for ambulation and, for the past year, required a walker. She has also experienced worsening fatigue and exercise intolerance and, at her worst, was able to take only a few steps with a walker before needing to stop. Based on this increasing fatigue and declining motor function, she underwent an electrodiagnostic evaluation. Two-hertz repetitive stimulation of the ulnar motor and spinal accessory motor compound action potentials was normal with no decrement. SFEMG of the triceps revealed neuromuscular jitter with a MCD of 40 μs (normal, 25 to 35 μs). Of note, serum testing for acetylcholine receptor antibody was negative.

She was subsequently started on pyridostigmine therapy at a dose of 60 mg QID. She reported subjective improvement with medication, stating she had much more energy and improved ambulation with her walker. Her dose of medication was reduced to 30 mg QID due to excessive diarrhea, and with this dose, she continued to claim having increased exercise tolerance and diminished fatigue. However, due to sustained difficulty with medication side effects, she recently discontinued pyridostigmine.

**Case 3** Case 3 is a 9-year-old girl who presented in infancy with delayed motor milestones and feeding difficulties. She first walked at age 3.5 years. Nocturnal noninvasive ventilatory support was required from age 4 years, and she developed progressive scoliosis from age 5 years, managed with a spinal brace. Genetic testing revealed a de novo heterozygous mutation (E368K) in DNM2. Basic clinical details of this patient have been reported previously [28].

Current health concerns include marked dysphagia, poor weight gain, fatigue, and moderate generalized weakness. On examination at age 9 years, she weighed 19.5 kg (<1st percentile), her height was 120 cm (<1st percentile), and her head circumference was 48.4 cm (<1st percentile). There was marked generalized muscle wasting, a severe C-shaped scoliosis, markedly restricted neck movements, and mild elbow, knee, and ankle contractures. She walked only short distances inside the house with a marked pelvic tilt. She had hypophonic speech, marked generalized facial weakness, ptosis, poor eye closure, and limited mouth opening. There was moderate limitation of eye movements of up and downward gaze and mild limitation of horizontal gaze. Limb muscle strength was 3/5 for shoulder abduction and 3+ to 4/5 for other muscle groups (Medical Research Council scale).

Pyridostigmine was commenced at 10 mg three times daily (TID) and slowly increased to a maximum dose of 30 mg TID, limited by the onset gastrointestinal symptoms. At the highest dose, the family reported that she had more energy and was less fatigued in the afternoons; she walked more inside the house and was able to complete her homework more easily after school. She also appeared brighter, was more interactive, and had less ptosis. There was no subjective improvement in her dysphagia.

Strength testing while on pyridostigmine treatment revealed sustained improvement in five out of seven muscle groups (measured with a MicroFET handheld myometer; Supplementary Fig. 2). Prior to treatment, a 6-min walk test was not feasible; however, after 8 months of treatment, she walked 40 m with the assistance of a wheeled frame over 6 min. There was also a small improvement in the Motor Function Measure (MFM) scale from 60–61/95 at baseline.
to 62/95 at 3 months and 65/95 at 8 months. The greatest gain was in the axial and proximal motor function domain (Supplementary Fig. 2).

Repetitive nerve stimulation of the trapezius following 6 months of treatment showed no abnormal decrement. The same result (no decrement) was obtained following a brief cessation of treatment (48 h). Orbicularis oculi single-fiber EMG was attempted but not tolerated.

Cases 4 and 5: 6-year-old identical twin boys

Case 4 Case 4 presented with severe intrauterine growth retardation at 20 weeks gestation and premature birth at 34 weeks with a birth weight of 1,005 g. He required CPAP for the first week of life and nasogastric feeds for the first month. Motor delay and hypotonia were noted during infancy. He sat at 1 year of age and walked just before his second birthday. Bilateral ptosis and facial weakness were present by age 2. By age 4, he could jump and run but was slower and clumsier than his peers. He also has a history of hypospadias, unilateral strabismus, and epilepsy. A brain MRI at age 4 showed enlarged ventricular spaces and reduced white matter volume. A muscle biopsy performed at age 6 showed features typical of a centronuclear myopathy. Genetic testing identified a de novo heterozygous E368K DNM2 mutation.

At age 6, case 4 was ambulant, but tired after walking several hundred meters, fell frequently, and fatigued easily. Muscle strength was 4 to 4–5 in most muscle groups. Repetitive nerve stimulation (RNS) at 5, 10, and 20 Hz in the right abductor pollicis brevis at age 4, and at 5 and 20 Hz in the abductor digitii minimi at age 6, did not show a significant decrement. RNS of the trapezius and SFEMG were not tolerated. Based on clinical grounds, pyridostigmine treatment was initiated at 30 mg TID. With therapy, his parents noted increased exercise tolerance and reduced ptosis. MFMs improved modestly from 75–78/96 pre-treatment to 83/96 at 6 weeks of treatment and 80/96 after 9 weeks of treatment (Supplementary Fig. 2), mainly due to improvements in standing and with transfers. In addition, his 6-min walk distance improved from 325–350 m pre-treatment to 475 and 450 m after 6 and 9 weeks of treatment, respectively.

Case 5 Case 5 is case 4’s twin, and he shares the same de novo E368K mutation in DNM2. His birth weight was almost twice that of his twin (2,265 g). He required CPAP for only 3 days after birth and only a short period of tube feeding. Although his pattern of facial involvement and weakness is the same, he has always been larger and stronger than his twin, with better muscle bulk and more advanced motor skills. Feeding and weight gain have also been less of a concern. Of note, like his twin sibling, he also has a history of epilepsy.

At age 6, case 5 was ambulant but with significant fatigue. Repetitive nerve stimulation testing at ages 4 and 6 showed no abnormal decrement, and single-fiber EMG was not tolerated. Pyridostigmine therapy was initiated at 30 mg TID, and improvements were noted in both exercise tolerance and ptosis. MFMs scores changed little following treatment (Supplementary Fig. 2). However, the 6-min walk distance improved from 401–412 m pre-treatment to 500 m at 6 weeks and 450 m at 9 weeks of treatment.

Discussion

In this study, we demonstrate motor weakness and NMJ defects in a novel zebrafish model of DNM2-CNM. Larval fish overexpressing human DNM2-S619L have disorganized acetylcholine receptor patterning and severe motor deficits manifested in weak tail beats, slow swimming, and partial paralysis. We show that motor dysfunction in these animals can be rapidly alleviated by treatment with an acetylcholinesterase inhibitor. Additionally, we report clinical and electrophysiological findings suggestive of an NMJ deficit in five patients with DNM2-CNM. Each patient responded favorably to acetylcholinesterase inhibitor therapy, providing further evidence for a defect in neuromuscular transmission in DNM2-related CNM.

This study provides the first evidence of an NMJ defect in DNM2-related CNM. There are several previous reports of abnormal histologic findings in NMJs from patients with genetically unconfirmed CNM that support the relevance of our findings. At least three studies have described abnormal endplate elongation and irregular acetylcholinesterase staining [11, 13, 29], and one ultrastructural study reported post-synaptic junctional folds that were irregular and unelaborated [12]. Two recent studies provide clinical evidence for NMJ involvement in some CNM patients. Liewluck et al. described a patient with genetically uncharacterized CNM combined with clinical, histological, and electrophysiological features of myasthenia [14]. Similarly, Robb et al. reported three patients with genetically uncharacterized CNM and one patient with a mutation in MTM1 who exhibited fatigability and abnormal jitter on EMG [15]. All four patients responded favorably to acetylcholinesterase inhibitor treatment, and two patients showed substantial improvement with sustained therapy. In this same report, our lab demonstrated that a zebrafish model of myotubular myopathy has abnormal acetylcholine receptor patterning and that motor weakness in these animals can be partially alleviated by acetylcholinesterase inhibitor treatment. These case reports on myotubular myopathy and unconfirmed CNM thus corroborate our findings in DNM2-CNM.
Our data, when considered with these previous studies, support a growing body of evidence that NMJ defects are a clinical feature of many, if not all, subtypes of CNM. Neuromuscular transmission deficits have now been identified in two genetically defined subsets of CNM, those associated with mutations in MTM1 and DNM2. Together with previous reports of NMJ abnormalities in genetically uncharacterized patients, these findings underscore the importance of examining NMJ function in all CNM patient populations. While further study is needed to determine the prevalence of neuromuscular transmission deficits, it is possible that they are a common feature among all or most patients with CNM. Since this aspect of the disease is therapeutically tractable, this opens up new avenues for the treatment of CNM.

It is important to note the potential limitations of our study, particularly as they relate to the case presentations. The cases represent analyses of five individuals with DNM2 mutations, two of whom were analyzed only retrospectively. Each patient was treated with pyridostigmine based on the judgment of the clinician and his/her interpretation of neurodiagnostic evidence. While all five patients responded subjectively to pyridostigmine therapy, and objective measures of muscle function improved in three children tested, only the two adult cases had electrodiagnostic evidence of an NMJ defect. The evidence was strongest in case 1, while case 2 had only slightly increased jitter with single-fiber EMG. For the three pediatric cases, because they could not tolerate the procedure, it is obviously unknown whether changes would be observed on single-fiber EMG. The question remains therefore whether the positive response observed with pyridostigmine was due to specific amelioration of defective NMJ signaling or instead to nonpathomechanistic effects of the medication. Future prospective investigations are therefore needed to definitively establish a connection between DNM2-CNM, abnormalities in neuromuscular transmission, and response to therapy.

Our work suggests that DNM2 may directly participate in the establishment or regulation of the NMJ. Nothing is known about the function of DNM2 at the post-synaptic NMJ; however, several factors make it an excellent candidate to consider in the regulation of the post-synaptic NMJ. DNM2 is well characterized as a membrane-trafficking protein, and it plays a role in shaping and remodeling a variety of membrane structures in most cell types. In addition to functioning in membrane deformation, DNM2 associates with many components of the cytoskeleton, which is required for stabilization of NMJ post-synaptic components [30, 31]. At neuronal synapses, DNM2 has also been shown to interact with scaffolding proteins that shape the post-synaptic density during development [32]. In light of the evidence presented here, it will be important for future studies to examine the specific function of DNM2 at the NMJ.

In summary, we have shown that transient expression of DNM2-S619L in zebrafish is a novel model for DNM2-CNM. We have identified a new mechanism of disease based on this model, which is supported by clinical evidence from three patients with mutations in DNM2. These results suggest that NMJ deficits are a significant component of DNM2-CNM pathology and that acetylcholinesterase inhibitors may be an effective therapy for patients with this disorder. Our findings further show that zebrafish are an excellent model for DNM2-CNM and can provide important insight into the pathomechanisms of DNM2-CNM and related disorders.

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Conflict of interest The authors declare no conflict of interest in connection with the submitted material.

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Spontaneous Laryngeal Reinnervation Following Chronic Recurrent Laryngeal Nerve Injury

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Objectives/Hypothesis: To enhance understanding of spontaneous laryngeal muscle reinnervation following severe recurrent laryngeal nerve injury by testing the hypotheses that 1) nerve fibers responsible for thyroarytenoid muscle reinnervation can originate from multiple sources and 2) superior laryngeal nerve is a source of reinnervation.

Study Design: Prospective, controlled, animal model.

Methods: A combination of retrograde neuronal labeling techniques, immunohistochemistry, electromyography, and sequential observations of vocal fold mobility were employed in rat model of chronic recurrent laryngeal nerve injury. The current study details an initial set of experiments in sham surgical and denervated group animals and a subsequent set of experiments in a denervated group.

Results: At 3 months after recurrent laryngeal nerve resection, retrograde brainstem neuronal labeling identified cells in the characteristic superior laryngeal nerve cell body location as well as cells in a novel caudal location. Regrowth of neurons across the site of previous recurrent laryngeal nerve resection was seen in 87% of examined animals in the denervated group. Electromyographic data support innervation by both the superior and recurrent laryngeal nerves following chronic recurrent laryngeal nerve injury.

Conclusions: Following chronic recurrent laryngeal nerve injury in the rat, laryngeal innervation is demonstrated through the superior laryngeal nerve from cells both within and outside of the normal cluster of cells that supply the superior laryngeal nerve. The recurrent laryngeal nerve regenerates across a surgically created gap, but functional significance of regenerated nerve fibers is unclear.

Key Words: Laryngeal reinnervation, recurrent laryngeal nerve injury, vocal fold paralysis, superior laryngeal nerve, thyroarytenoid muscle, animal model.

INTRODUCTION

Normal laryngeal physiology requires complex interplay between multiple different components, including sensory input and highly coordinated motor function. Innervation of intrinsic laryngeal muscles is through the superior laryngeal (SLN) and recurrent laryngeal (RLN) branches of the vagus nerve. Motor innervation to all intrinsic muscles except for the cricothyroid muscle (CT) is thought to occur through the RLN, with the CT being supplied by the external branch of the SLN.1 Damage to laryngeal innervation leads to vocal fold paresis or paralysis, for which there currently is no clinical intervention that will reliably restore physiologic movement.

Spontaneous reinnervation of the larynx after RLN injury has been demonstrated in a variety of studies.2–7 Investigating the sources of spontaneous laryngeal reinnervation has been the subject of multiple studies in the past 3 decades.3,8–12 Review of the existing literature leads logically to three conclusions: 1) the larynx has a propensity for spontaneous reinnervation following RLN injury, 2) reinnervation is a multifactorial process, and 3) spontaneous reinnervation following transecting-type of injuries does not lead to physiologic movement, and reinnervation can be present in chronically immobile vocal folds. The broad long-term goal of our research is to develop techniques that deliver clinical improvement to laryngeal function following nerve injury. A clearer understanding of the processes involved with spontaneous reinnervation is integral to this goal.

Development of the experimental model utilized in the current study and data for control animals were previously published.13 In the previous study, methods for rat suspension microlaryngoscopy and endoscopic thyroarytenoid (TA) muscle injection with the retrograde tracer FluoroGold (FG; Fluorochrome, LLC, Denver, U.S.A.) were evaluated. The susceptibility of muscle fibers to reinnervation being dependent on the degree of injury, the authors examined the potential for reinnervation to occur after full-thickness transection of the RLN.
Colorado) were developed. Anatomic dissections for identification of RLN, SLN, nodose (NOD), superior cervical ganglia (SCG), and dorsal root ganglia (DRG) were refined. Histologic methods for mapping of neurons labeled with FG in brainstem and ganglia were tested. Collection of electromyography (EMG) data was initially pursued during model development, but available needles for insertion were felt at that time to be too traumatic to the TA muscle and therefore potentially a confounder for retrograde labeling experiments with muscle injection. Control retrograde brainstem mapping data were obtained from the TA muscle and for RLN and SLN neurons. Mapping to laryngeal sensory (NOD) and autonomic (SCG) ganglia was also performed.

Retrograde mapping data for the right TA muscle, SLN, RLN, and contamination controls are in the Supplemental Figure. The measurements for brainstem labeling in that figure are distance from the obex in millimeters (mm) on the x axis and number of cells on the y axis. Neurons of the RLN mapped consistently to a brainstem distribution with a bimodal peak in the NA and dorsal motor nucleus of the vagus nerve (N10) more caudal than the SLN peak with some overlap, consistent with published data. Injection of the TA muscle with FG yielded a mixed RLN and SLN brainstem distribution demonstrating that the TA is innervated by both nerves, contrary to classical teaching. If both nerves were cut before TA injection, no brainstem labeling occurred; these animals served as negative controls. Ganglia uptake in SCG and NOD was consistent with uptake by mixed motor, sensory, and autonomic fibers in both RLN and SLN, but the SLN had a much greater proportion of sensory and autonomic labeling than the RLN, as expected. No significant uptake was present in DRG for any rats, serving as a negative control for systemic uptake of FG.

The current study was undertaken after we had developed and published a model through previous studies. The aim of this study was to characterize the sources of spontaneous reinnervation in the rat TA muscle following long-term RLN injury. Hypotheses to be tested were 1) that the nerve fibers responsible for TA muscle reinnervation can originate from multiple sources and 2) that the SLN is involved in reinnervation of the TA muscle.

MATERIALS AND METHODS

Animals

Procedures were performed under the approval of the University of Michigan Committee on Use and Care of Animals. Initial experiments were carried out using 16 male Sprague-Dawley rats, age 80 days, divided equally into sham (S) and denervated (D) groups and treated according to the schema in Figure 1. The difference between the eight S and eight D group animals was only in the nature of the initial procedure as described in the following animal procedures section; the remaining treatment was the same for animals S1 through S8 and D1 through D8. Upon completion of these experiments and analyses of acquired data, further studies were specifically designed to explore in more detail and with a larger experimental group some of the most compelling initial results. For sake of clarity, this next set of experiments will be referred to in the following sections as the subsequent experiments. Twenty-one male Sprague-Dawley rats, age 80 days, were used for these subsequent experiments. Thirteen additional D group animals (D9–D21) were treated according to the schema in Figure 2, and eight other rats were used for development of EMG techniques and to acquire normal and acute denervation EMG data for comparison to experimental animals.

Animal Procedures

General anesthesia with spontaneous respiration and microlaryngoscopy was performed as previously described. In brief, intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) with inhaled 1.8% isoflurane were used to induce anesthesia for endoscopy to document vocal fold mobility. The larynx, trachea, and right RLN were then exposed under an operating microscope through a midline cervical incision. In those rats that were to undergo denervation, 5 mm of the right RLN was resected. Retrograde labeling procedures were performed according to Figures 1 and 2 using techniques previously published. Briefly, FG was injected into the right TA muscle of designated animals using endoscopic guidance. Transcervical application of FG to the proximal nerve stump of the RLN or SLN was performed in designated animals.
SUBSEQUENT EXPERIMENTS

**Denervation (D)** \(\text{n=13} \) Group

- Denervation and Initial Endoscopy
  - Endoscopy – 1 month
  - Endoscopy – 2 months
  - Endoscopy – 3 months

Rat #9-14: Inject FG RTA muscle

#15-21: Cut R SLN, apply FG to nerve end

Perfuse and harvest 6 days later

*Rats #9, 10, 11, 15, 16, 21: EMG prior to harvest

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**Transoral Laryngeal EMG**

Original previously published model development did not include TA muscle EMG because of concerns about muscle trauma from EMG needles that could affect retrograde labeling. After acquiring initial labeling data in the current studies, however, it was felt that transoral EMG should be pursued in the subsequent experiments to augment information about innervation status of examined muscles. A total of 14 rats underwent transoral laryngeal EMG: six rats from the subsequent experimental group (3 that underwent acute right SLN transection and FG application and 3 that underwent FG injection into the TA muscle), two EMG control rats that had undergone no prior procedures, three rats that underwent acute right RLN resection 7 days before EMG, and three rats that underwent acute right RLN and right SLN resection 7 days before EMG. Rats were anesthetized for microlaryngoscopy with spontaneous respiration as described. Using a 30-degree pediatric telescope for visualization, a 26-gauge concentric needle electrode (VIASYS NeuroCare, Madison, WI) was inserted into the TA muscle to measure spontaneous EMG during respiration (Fig. 3). This technique was adapted from Tessema et al.\(^{14}\) Spontaneous EMG was recorded for the bilateral TA muscles on all animals. EMG interpretation was performed by one author (K.E.K.), who was blinded to any prior denervation procedures.

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**RESULTS**

**Animal Procedures**

All 29 experimental animals and eight EMG (control and acute denervation) animals survived to the chosen endpoint, underwent designated procedures, and were successfully harvested. Eighteen endoscopic injections of FG into the right TA muscle were performed with three injections considered technically inadequate (animals S7, D5, and D12).

**Vocal Fold Motion Analysis**

All initial RLN resections correlated with immediate ipsilateral vocal fold paralysis (Table I). This includes all 21 D-group animals plus the six EMG acute RLN section animals. Four of 21 D-group rats developed partial movement, including rats D21 and D3. During the final FG procedures, rat D21 underwent transection of the SLN, and rat D3 underwent retransection of the proximal RLN. Retransection was performed to eliminate any contribution to reinnervation or movement through regenerated RLN fibers. Interestingly, the partial movement persisted in both of these rats. Three rats (D2, D8, and D20) developed nonpurposeful fibrillation.
of the vocal fold: D2 and D20 subsequently underwent SLN resection; fibrillations ceased in rat D2 after SLN section but remained in rat D20.

**Trachea With Associated RLN Histologic Section Analysis**

Immunohistochemical evidence of RLN regrowth across the resection gap by presence of neurofilament was evident in 13 of 15 (87%) D rats available for tracheal sectioning (Fig. 4). Visual comparison to the intact contralateral side showed regenerated axons were less robust and more disorganized, becoming more scattered as sections progressed distally. In addition, neurofilament was present in the distal nerve stumps, but the distal nerves were partially overtaken by fibrosis. RLN regrowth did not correlate with vocal fold motion.

**Retrograde Labeling**

Control data from preliminary studies have been previously published and are presented in the Supplemental Figure. In those studies and also in current initial experiments, all FG-positive neurons within the brainstem were mapped by their rostral-caudal position, but not to a specific nucleus. In the subsequent experiments, FG-positive neurons were mapped by rostral-caudal position and were also further characterized by location within the nucleus ambiguus (NA) or dorsal motor nucleus of the vagus (N10). N10 cells could be differentiated from NA cells by a more medial and ventral location and smaller size (Fig. 5). There were no FG-positive cells in the DRG of any animal, which served as a negative control.

**Initial Experiments: S Group (Fig. 6)**

**RLN and SLN mapping.** Results for the S-group animals essentially paralleled control data. RLN cell bodies (rat S3) map to a bimodal distribution in the brainstem, with the majority in a peak from the obex to 1 mm rostral to that point. Ganglia mapping through the RLN labeled to the ipsilateral and contralateral NOD. Cell bodies of the SLN (rat S4) map to a more rostral and narrow region of the brainstem, with a large rostral peak. Ganglia mapping through the SLN labeled much larger numbers of sensory and autonomic neurons.

**Right TA muscle injections.** Right TA injections demonstrated a pattern consistent with uptake primarily through the RLN with a lesser amount of SLN uptake (rats S4-S8). Ganglia mapping showed large uptake in the ipsilateral NOD, moderate amounts in the contralateral NOD and ipsilateral SCG, and small amounts in the contralateral SCG. When the TA muscle was injected after acute RLN (rat S1) or SLN (rat S2) transection, brainstem mapping showed a small rostral peak (S1) in the SLN distribution and a bimodal peak in the RLN

### TABLE I.

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<th>Group D</th>
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D = denervated; F = fibrillation; M = partial movement; NA = not applicable; + = full movement; – = paralysis.
distribution (S2), consistent with primarily RLN innervation to the TA with secondary SLN innervation.

**Initial Experiments: D Group (Fig. 7)**

**RLN and SLN mapping.** D-group animals yielded markedly different results from the S-group and control animals. Rat D3 underwent retranssection of the RLN proximal to the prior nerve resection followed by retrograde labeling through this stump. Retranssection was performed to eliminate any contribution through the RLN to reinnervation. This resulted in neurons mapping in reduced numbers to a region overlapping the more rostral area of prior

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Fig. 4. Histology of recurrent laryngeal nerves (RLNs) with associated trachea. White arrows indicate areas of immunohistochemical staining of neurofilament. (A) Intact left RLN with associated blood vessels. (B) Regrowth of disorganized bundle of neurofilaments across right RLN resection site. (C) Scar with smaller and more disorganized neurofilaments across right RLN resection site. (D) Distal nerve stump costained with 4',6-diamidino-2-phenylindole (blue) demonstrating decreased number of axons within a partially fibrotic nerve.

Fig. 5. FluoroGold-positive cells in nucleus ambiguus (A) and dorsal motor nucleus of the vagus (B) at the same magnification. Note the much smaller size of cells within the dorsal motor nucleus of the vagus compared to the nucleus ambiguus.
Fig. 6. Retrograde mapping of FluoroGold (FG)-positive cells in the brainstem (first column) and ganglia (second column) for group S. Measurements for brainstem are millimeters from the obex (x axis) and number of labeled cells (y axis). Blue lines are right-sided cells and red are left. Ganglia are superior cervical (SCG) and nodose (NOD) with number of labeled cells. L = left; R = right; RLN = recurrent laryngeal nerve; SLN = superior laryngeal nerve; TA = thyroarytenoid. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
RLN mapping, and to the SLN region at 0.7 to 2.6-mm rostral to the obex. There was also minimal mapping in the ipsilateral and contralateral NOD. Mapping of SLN neurons (rat D4) labeled cells in a new caudal region not identified in any other groups, as well as a large peak in the rostral brainstem partially overlapping with previously identified regions.
identified SLN neurons. Ganglia mapping showed similar findings to S-group and control animals in the ipsilateral NOD and SCG but much less in the contralateral SCG.

**Right TA muscle injections.** Right TA injections following RLN resection (rats D5-D8) showed a pattern similar to direct labeling through the SLN (rat D4), including cells within the novel caudal region, but reduced in numbers. Ganglia labeling showed a moderate to large amount in the ipsilateral NOD and a moderate amount in the contralateral NOD and both SCG. When the right TA muscle was injected after acute RLN transection (rat D1), there was continued labeling in the region of prior SLN or rostral RLN mapping, but with reduced numbers. Transection of the SLN before right TA injection (rat D2) essentially halted brainstem uptake with only a single labeled cell.

**Subsequent Experiments**

Analyses of initial experiments suggested that normal SLN neurons as well as neurons from a novel caudal location were involved with reinnervation of the TA muscle. In particular, paradigms used for animals D4 (labeling through the SLN) and D5-D8 (labeling following right TA muscle injection) were believed to warrant further specific investigation. These subsequent experiments were therefore undertaken to increase the sample size for labeling following TA muscle injection (D9-D14) or directly through the SLN (D15-D21).

**Right TA muscle injections.** Brainstem mapping showed a similar rostral-caudal pattern for D5 to D8 from the initial experiments having undergone the same procedure (Fig. 8). The majority of FG-positive cells were located in the ipsilateral NA, but two animals

![Fig. 8. Retrograde mapping of FluoroGold (FG)-positive cells in the brainstem (first column) and ganglia (second column) in rats from the subsequent experiments that underwent injection of FG into the right thyroarytenoid muscle. Measurements for brainstem are millimeters from the obex (x axis) and number of labeled cells (y axis). Vertical green lines indicate the most caudal point at which sections were available. Ganglia are superior cervical (SCG), nodose (NOD), and dorsal root ganglia (DRG) with number of labeled cells. L = left; LNA = left nucleus ambiguus; LN10 = left dorsal motor nucleus of the vagus; RNA = right nucleus ambiguus; RN10 = right dorsal motor nucleus of the vagus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]](image)
demonstrated a significant proportion of cells that mapped to the ipsilateral N10 (33% in rat D9 and 48% in rat D11). The rostral-caudal location of these N10 cells was similar to the main cluster of cells that mapped to the NA. The novel area of caudal cells that had been identified during the initial experiments in rats D4, D6, D7, and D8 was found only in rat D9, where a single cell from each nucleus was found at that caudal location. However, because of inadvertent tissue processing errors, the caudal extent of evaluation was truncated in several animals (indicated by the vertical green line in Fig. 8), and so it was not possible to assess this region in those animals. Ganglia mapping was similar to rats D5 through D8 in the initial group.

**SLN mapping.** Overall rostral-caudal position of FG-positive cells was similar to findings in rat D4, with a strong peak in the rostral brainstem (Fig. 9). The previously noted caudal cells were seen in four animals. All of these caudal cells mapped to the right N10. Because of tissue processing, the caudal extent of evaluation was again truncated in several animals (indicated by the vertical green line in Fig. 9). Ganglia mapping was similar to rat D4, with strong labeling to the ipsilateral NOD and very few cells mapping to the ipsilateral SCG.

![Fig. 9. Retrograde mapping of FluoroGold (FG)-positive cells in the brainstem (first column) and ganglia (second column) in rats from the subsequent experiments that underwent right superior laryngeal nerve transection and application of FG to the cut end. Measurements for brainstem are millimeters from the obex (x axis) and number of labeled cells (y axis). Vertical green lines indicate the most caudal point at which sections were available. Ganglia are superior cervical (SCG), nodose (NOD), and dorsal root ganglia (DRG) with number of labeled cells. L = left; R = right; RNA = right nucleus ambiguus; RN10 = right dorsal motor nucleus of the vagus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.](image-url)
Laryngeal EMG. EMG recordings from the right TA muscle of chronically denervated rats at 3 months after RLN resection demonstrated observable motor unit action potentials, but with decreased insertional activity compared to the left TA muscle in the same rats. In those rats that underwent chronic right RLN denervation followed by acute right SLN transection for the FG procedures, insertional activity was further decreased compared to those that underwent chronic right RLN denervation alone. Notably, rat D21 showed some recovery of right vocal fold movement on endoscopy and was also found to have nearly normal EMG signal in the right TA muscle at 3 months after RLN resection and 1 week after SLN transection.

Acutely denervated rats demonstrated fibrillation and positive sharp wave potentials in the ipsilateral TA muscle on laryngeal EMG 1 week after denervation. Transection of the RLN and ipsilateral SLN resulted in strong fibrillation and positive sharp wave potentials, but transection of the RLN alone yielded less consistent results, with fibrillation potentials noted in only one of three rats. Vocal fold motion assessment of all acutely denervated rats demonstrated right vocal fold immobility at the time of EMG recording. Control rats that had undergone no denervation procedures and the intact left TA muscle of the D-group rats consistently demonstrated normal motor unit action potentials as well as normal bilateral vocal fold movement. Figure 10 shows examples of EMG recordings from control and D rats.

DISCUSSION

It is clear from control results presented in a prior paper and current S-group animals that in the normal state, the rat TA muscle receives dual innervation from the RLN and SLN with the RLN predominating. Dual innervation is contrary to what is taught as textbook laryngeal neuroanatomy, but review of the literature finds this concept previously presented for humans and multiple other species.

Retrograde neural labeling directly through laryngeal nerves and following TA muscle injection provides evidence that the SLN is a source of spontaneous reinnervation to the TA muscle in the setting of chronic RLN injury. There is also evidence for central nervous system (CNS) plasticity in that both expected SLN neurons and a small number of neurons outside the normal location of cells projecting axons through the SLN are identified through retrograde labeling. In the subsequent set of experiments from the current study, these caudal cells were characterized both by linear distance from the obex and by their two-dimensional location in brainstem cross-section. Their location appears to be in N10, the parasympathetic nucleus of the vagus nerve. No S-group animals from the current study or control animals from the preliminary studies showed direct SLN labeling in this region.

The exact nature of the novel caudal-labeled cells in the D-group animals cannot be defined with our current data. Recently published evidence shows that multiple forebrain systems converge on lower motor neurons innervating the TA muscle. In addition, expression of nestin, a neural stem/progenitor cell-enriched marker, has been demonstrated in the NA following vagal nerve injury. This finding is an indication of rejuvenilization of NA neurons in response to peripheral nerve injury.
and a demonstration of CNS plasticity. These studies underscore the points that neural pathways involved with laryngeal innervation are complex and that we are truly just beginning to understand them in the normal and injured states.

Electromyographic data also support the SLN as a source of TA innervation after RLN injury. Rats that underwent chronic RLN resection followed by acute SLN transection as part of FG labeling procedures were found to have decreased insertional activity in the ipsilateral TA muscle compared to those rats that underwent chronic RLN resection alone. Together with the EMG data in acutely denervated rats, these findings suggest that the SLN is a source of innervation of the TA muscle in both the normal and denervated states.

Given prior evidence in the literature for regeneration of the RLN across experimentally created gaps, we examined this phenomenon histologically and found an 87% rate of regrowth. We were not able to determine definitively whether these axons form functional neuromuscular junctions in the TA or other laryngeal muscles from our current data. However, functional and EMG data from rat D21 showed continued vocal fold movement and observable motor unit action potentials in the setting of chronic RLN injury, even after acute transection of the ipsilateral SLN, suggesting that reinnervation includes sources other than the SLN.

One-third of D rats in the study developed some degree of right vocal fold mobility during the 3-month course of observation. Perhaps the most interesting findings were in those rats that developed movement and had subsequent nerve-sectioning procedures. Rat D2 underwent section of the SLN as a final procedure before FG injection at 3 months, with subsequent abatement of disorganized vocal fold movements that had been seen previously. However, rats D20 and D21 continued to have partial vocal fold movement, even after undergoing SLN section. Rat D3 had resectioning (to eliminate contributions from regenerated RLN fibers) of the RLN before application of FG to the nerve stump at 3 months, and the partial movement seen in that animal continued. These findings further support the conclusion that there are several sources of reinnervation in the setting of chronic RLN injury.

It would also be logical that with multiple possible reinnervation sources available following RLN injury, the specific pattern of reinnervation could vary significantly from case to case; that is, the relative contribution from each source would not be expected to be uniform. Defining all potential sources and if possible determining favorable ones would be valuable steps toward an ultimate goal of improving clinical outcomes after nerve injury. Strategies to augment favorable sources and/or restrict unfavorable ones could then be employed. McRae et al. have demonstrated in a rat model the ability to prevent laryngeal synkinesis following RLN injury through use of local neurotoxins.

Analyses of sensory (NOD) and autonomic (SCG) ganglia labeling provide additional insights about the makeup of neural components in the RLN and SLN and efferent and afferent innervation of the TA muscle. When considered together, control data from a prior study and S data from the current study indicate that the RLN carries a very limited amount of sensory and sympathetic fibers, with ipsilateral sensory (right NOD) being the greatest. In contrast to this, the SLN has a much greater sensory (NOD) component and also more autonomic (SCG) fibers, consistent with the known larger sensory function of the SLN. Notably, there is also bilateral labeling, and to our knowledge the anatomic pathways for this are not well defined.

**Limitations of the Current Study**

One limitation of this study is the small sample size for animals undergoing a particular intervention in the primary experiments. The labor-intensive nature of these experiments precluded a larger sample size for each intervention and labeling procedure in the initial studies. Therefore, the subsequent experiments were designed to corroborate the most interesting findings from these studies by increasing the number of D animals undergoing labeling through the SLN and directly from the TA muscle. We also note a potential limitation that the transport of FG through an injured/regenerated nerve may be unreliable. This makes it possible that all neurons reaching the TA through regenerated axons may not be identified through retrograde labeling. During the subsequent set of experiments in the current study, it became apparent that characterizing FG-positive cells by both linear distance from the obex and nuclear location in NA or N10 would be informative. Unfortunately, this differentiation of FG-positive cells into distinct nuclei was not performed in earlier animals; those cells were only described by their rostral-caudal location. Attempts to retrospectively analyze the brainstem slides were unsuccessful due to fading of fluorescent labeling. Another limitation was the inadvertent truncation of the brainstem specimens in several experimental animals, thus limiting evaluation of a caudal region of interest in four rats. This resulted from the inability to accurately identify the rostral-caudal position of the obex grossly during tissue harvest, because the precise location of the obex can only be determined during histologic review.

**Implications for Future Research**

Understanding the pathways through which the SLN contributes to laryngeal innervation in the setting of chronic RLN injury warrants further investigation. Previous work has suggested that intramuscular sprouting occurs in this setting, which may be the mechanism of TA muscle reinnervation. Determining the functionality of regenerated RLN fibers is also an important step in understanding the likely multidimensional process of reinnervation. Further studies are underway in both of these areas.

**CONCLUSION**

It is clear that, contrary to what has been taught traditionally in regard to anatomy, the rat TA muscle in...
its normal state receives dual innervation through the RLN and the SLN. In the setting of chronic RLN injury, there are changes in CNS organization of the SLN and reinnervation of widowed fibers in the TA through the SLN by cells that include typical SLN neurons as well as ones outside the normal cluster of medullary cells that supply the SLN. We also conclude that the RLN does have the ability to regenerate axons across a surgically created gap in a high percentage of cases, but the functionality of these regenerated axons is uncertain. The ultimate goal of this research remains the clinical improvement of laryngeal function following nerve injury. Further experimentation to understand the sources and processes involved with spontaneous laryngeal reinnervation is one key to achieving this goal.

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Summary

DNM2 is a ubiquitously expressed GTPase that regulates multiple subcellular processes. Mutations in DNM2 are a common cause of centronuclear myopathy, a severe disorder characterized by altered skeletal muscle structure and function. The precise mechanisms underlying disease-associated DNM2 mutations are unresolved. We examined the common DNM2-S619L mutation using both in vitro and in vivo approaches. Expression of DNM2-S619L in zebrafish led to accumulation of aberrant vesicular structures and to defective excitation-contraction coupling. Expression of DNM2-S619L in COS7 cells resulted in defective BIN1-dependent tubule formation. These data suggest that DNM2S619L may cause disease, in part, by interfering with membrane tubulation.

Introduction

Centronuclear myopathies are a clinically and genetically heterogeneous group of skeletal muscle disorders that share common muscle biopsy features. These features include myofiber hypotrophy, an increased number of centrally located myonuclei, and irregularities in oxidative stains. Recent evidence suggests that another unifying feature of centronuclear myopathies is abnormalities in triad structure (Al-Qusairi et al., 2009; Dowling et al., 2009; Nicot et al., 2007; Toussaint et al., 2011). The triad represents the intersection between the T-tubule and the terminal sarcoplasmic reticulum, and is a key component of the cellular apparatus that mediates excitation-contraction coupling. Changes in the structure of the triad have been observed in all genetically confirmed subtypes of centronuclear myopathy (Dowling et al., 2009; Nicot et al., 2007; Toussaint et al., 2011).

Mutations in dynamin-2 (DNM2) are the most common cause of autosomal dominant centronuclear myopathy (Hanisch et al., 2011; Jeub et al., 2008). They are also a rare cause of inherited peripheral neuropathy (Zuchner et al., 2005). DNM2 is a large GTPase that is a member of the dynamin family of membrane fission proteins. Numerous functions, including the regulation of endocytosis, cell migration, and cytokinesis, are associated with the normal function of DNM2 (Jones et al., 1998; McNiven et al., 2000; Thompson et al., 2004). The mechanism(s) via which dominant mutations in DNM2 cause myopathy, however, are incompletely understood. Data from cell culture models has been conflicting; for example, some studies have identified abnormalities in endocytosis while others have not (Bitoun et al., 2009; Koutsopoulos et al., 2011; Liu et al., 2011; Tanabe and Takei, 2009). Examination by immunohistochemistry of patient muscle biopsies revealed disorganization of the triad, and recent studies using viral expression in the mouse corroborated this observation (Cowling et al., 2011). However, the mechanism(s) by which DNM2 mutations impair triad formation is not known; furthermore, a functional effect on excitation-contraction coupling has yet to be documented.

In this study, we examine the S619L mutation in DNM2’s pleckstrin homology domain. The S619L mutation is one of the most common DNM2 mutations and is associated with a severe muscle phenotype (Bitou et al., 2007; Bohm et al., 2012). We determined the impact of DNM2-S619L expression in both the developing zebrafish and in a cell culture model of tubule formation (Bitou et al., 2007). We show that the mutation results in defective membrane tubulation, and propose that one mechanism of disease is the interference of BIN1-mediated tubule formation and subsequent impairment of excitation-contraction coupling.
Results and Discussion

Expression of DNM2-S619L in developing zebrafish disrupts triad structure.

To generate a model of centronuclear myopathy due to DNM2 mutation, we ubiquitously expressed either wild type or S619L mutation-containing human DNM2 RNA in the developing zebrafish starting at the 1-2 cell stage (Supplemental Figure 1). Expression of DNM2-S619L results in abnormalities in muscle structure (including abnormally located nuclei and perinuclear disorganization), impaired force generation, and altered motor function. The general phenotypic features of DNM2-S619L zebrafish are recently published (Gibbs et al., 2013). In this study we specifically focused on the triad because of the mounting data suggesting that triad defects are a critical aspect of centronuclear myopathy (Al-Qusairi et al., 2009; Dowling et al., 2009; Nicot et al., 2007; Toussaint et al., 2011). We performed ultrastructural evaluation of the triad structure in zebrafish with confirmed expression of either wild type DNM2 or DNM2-S619L.

Electron micrographs of skeletal muscle from 3 separate DNM2-WT or DNM2S619L zebrafish, obtained at 3 days post fertilization (dpf), are depicted in Figure 1. DNM2-WT muscle showed a normal appearance and distribution of T-tubule and terminal sarcoplasmic reticulum (Fig 1A, C). Conversely, muscle from DNM2-S619L larvae had extensive triad abnormalities (Fig 1B, D). While there were rare regions of normal triads, the majority did not resemble the native structure but were instead characterized by disorganized and swollen membrane structures. The most striking aspect of the triad defects was the abundant accumulation of large, irregular vesicular structures that correspond in location and by general appearance to the terminal sarcoplasmic reticulum (Fig 1B, D).

Figure 1: T-tubule and SR abnormalities in DNM2-S619L larval muscle. (A-D) Electron micrographs of longitudinal sections through zebrafish muscle at 3 dpf. (A, C) Muscle from DNM2-WT larvae shows the typical sarcomere striations of vertebrate striated muscle. (B, D) Muscle from DNM2-S619L larvae demonstrates extensive swelling and vacuolization in the region of the SR and T-tubules. Scale bars equal to 1 µm. (E-H) Confocal micrographs of isolated myofibers subjected to immunofluorescence analysis. (E) wild type (WT) myofibers showing the expected pattern of RyR1 staining. (F, G) RyR1 expression is irregular in DNM2-S619L myofibers, and is often found aggregated. (H) α-actinin staining was normal, indicating that other elements of the muscle structure in S619L myofibers are not disturbed.

Figure 2: Calcium activity in DNM2-S619L larvae muscle. (A) Embryos were co-injected with DNA for GCaMP (a genetically-encoded calcium indicator) and RNA for DNM2 (WT or S619L) for GCaMP fluorescent imaging in muscle during spontaneous muscle contractions at 24 hpf. (B) Representative trace of whole-embryo fluorescent intensity (deltaF/F) during spontaneous contractions. Although DNM2-S619L larvae displayed spontaneous contractions with normal timing, there was no substantial increase in GCaMP fluorescence. (C-D) Sequential images of GCaMP fluorescence from the larvae in panel B.
reticulum. Overall, these findings reflect substantial membrane abnormalities in the muscle of DNM2-S619L zebrafish.

To support our assertion that the abnormal structures seen by electron microscopy are, in fact, triads, we performed immunofluorescent analysis of triad markers on myofibers isolated from RNA injected embryos. Myofibers from uninjected and WT DNM2 injected embryos demonstrated the expected pattern of RyR1 (Figure 2E) and DHPR (data not shown) staining. Conversely, myofibers from DNM2-S619L zebrafish had widespread abnormalities in RyR1 and DHPR staining, including aggregation of staining and interruption of the normal confluent linear pattern (Figure 2F, G). Of note, alph-actinin staining (highlighting Z-bands) was normal (Figure 2H), suggesting that the changes in triad marker immunofluorescence was not due to non-specific dissolution of the entire myofiber structure. In all, these data corroborate the ultrastructural analysis and demonstrate severe disturbance of the triad structure with DNM2-S619L expression.

**Impaired excitation-contraction coupling in the muscle of DNM2-S619L larvae**

The abnormalities in triads in our zebrafish model correspond well with the immunohistological abnormalities previously reported in DNM2 patient biopsies and the ultrastructural changes described in the viral overexpression model of DNM2 associated CNM (Cowling et al., 2011; Toussaint et al., 2011). The potential impact of structural triad changes on triad function, however, has yet to be examined for any DNM2 model. In order to determine the functional effect(s) of DNM2-S619L expression on excitation-contraction coupling, we examined calcium transients in spontaneously contracting embryos using a genetically encoded calcium indicator, GCaMP3. Embryos co-injected with DNM2 RNA and GCaMP3 cDNA were screened for variegated GCaMP expression in skeletal muscle (Fig. 2A). Both DNM2-WT and DNM2-S619L larvae expressed GCaMP3 at a similar level. At approximately 24 hours post fertilization, embryos were embedded in agarose and fluorescence was measured during spontaneous muscle contractions. Both WT and S619L-DNM2 embryos displayed abundant spontaneous muscle contractions at this point. In DNM2-WT embryos, a pulse of fluorescence corresponding to SR-based calcium release was temporally linked to each contraction. In DNM2-S619L embryos, however, there was minimal or no visible increase in fluorescence during muscle contraction in 10 of 12 embryos examined (Fig 3C, D). In order to directly compare the fluorescence changes between embryos, we calculated the relative change in fluorescent recordings before vs. during a muscle contraction (ΔF/F). We observed a significant difference in this dynamic ratio of calcium transients in DNM2-WT as compared with DNM2-S619L embryos (Fig. 2B and Supplemental figure 2). This indicated that in addition to altering triad organization in zebrafish muscle, DNM2-S619L...
expression impairs stimulus-associated intracellular calcium release, a triad-mediated muscle function.

**DNM2-S619L expression alters formation of BIN1-induced tubules**

The above data reveal that DNM2-S619L expression can disrupt triad formation and function, but do not provide a potential mechanism via which it may do so. It has been previously established that wild type DNM2 can interact with BIN1 (also called amphiphysin-2), a protein that can sense and regulate membrane curvature (Takei et al., 1999). A muscle specific isoform of BIN1 (BIN1 isoform 8) has been shown to be required for T-tubule biogenesis, and recessive mutations in BIN1 cause an autosomal recessive form of centronuclear myopathy associated with triad defects (Lee et al., 2002; Nicot et al., 2007; Razzaq et al., 2001). Based on these known functions of BIN1, we hypothesized that DNM2-S619L mutations function to impair triad structure by interfering with BIN1-mediated tubulogenesis. To test this hypothesis, we used a well-established in vitro model of BIN1-dependent tubule formation (Nicot and Laporte, 2007; Nicot et al., 2007).

Expression of BIN1 isoform 8 (iso8) can induce the formation of tubular membrane structures when expressed in non-muscle cells, thus enabling an in vitro model of T-tubule biogenesis. To examine the impact of DNM2-S619L expression in this system, we co-expressed in COS7 cells GFP-tagged BIN1 iso8 and either wild type DNM-WT cDNA or DNM2-S619L cDNA tagged with mCherry. Consistent with previously published results, we found by confocal microscopy that co-expression of BIN1 iso8 and DNM2-WT resulted in extensive tubulation (Fig 3A). When DNM2S619L was co-expressed with BIN1 iso8, however, we observed predominantly the formation of short punctate structures instead of tubules (Fig 3B). To quantify these effects on membrane organization, tubulation in each cell was classified as short, long or intermediate. While DNM2-WT expressing cells exhibit a phenotype consisting of long tubules, only 19% of DNM2-S619L expressing cells have a similar phenotype (Fig. 3D; DNM2-WT n=68, DNM2-S619L n=73, cells from three independent experiments). Thus compared to WT, expression of DNM2-S619L results in a significant impairment in BIN1-dependent tubule formation.

**Summary**

We demonstrate that the common myopathy causing S619L mutation in DNM2 disrupts triad structure and function in vivo and BIN1-induced tubulation in vitro. These data represent the first direct evidence that a DNM2 mutation can interfere with tubulation and disrupt triadic function. Based on these novel findings, we hypothesize that muscle disease related to DNM2 mutations is caused by the production of dominantly active defective DNM2 proteins that interfere with the tubulation process. Disrupted tubulation, in turn, impairs triad formation and excitation-contraction, which results in abnormal muscle force generation and ultimately muscle weakness (Gibbs et al., 2013). Future experimentation, particularly with the testing of additional DNM2-associated CNM mutations, will be required to support this hypothesis. These data, however, provide an important step toward establishing the pathomechanisms related to DNM2 mutations.

**Methods**

**Animal care:** Zebrafish (AB strain) were bred and raised according to established protocols approved by the University of Michigan Animal Care and Use committee (UCUCA protocol #09835). **Plasmid construction and RNA synthesis:** Wild-type human DNM2 plasmid was purchased from Invitrogen (ORF Gateway® Entry IOH53617). The S619L point mutation was introduced using the QuikChange Lightning Site-directed Mutagenesis kit (Stratagene). Expression vectors were generated using the Gateway system and p5ECMV/SP6, p3E-polyA, and pDestTol2pA2 cassettes from the Tol2kit v1.2 (kind gift of Dr. Chi-Bin Chien). Gateway recombination reactions were performed using LR Clonase II Plus Enzyme Mix (Invitrogen). For RNA injection, plasmids were linearized with NotI and transcribed using the SP6 mMessage Machine kit (Ambion).

**RNA injection of zebrafish embryos:** Zebrafish embryos were injected as previously described (Dowling et al., 2009). Briefly, fertilized eggs were injected at the 1-2 cell stage using a Nanoject II injector (Drummond Scientific). Embryos were injected with DNM2 RNA (30 ng/µl) at 2.5 hpf in a 4.6 nL volume. **Muscle ultrastructure:** Zebrafish at 3 dpf were fixed overnight in Karnovsky's fixative and then processed for electron microscopy by the Microscopy and Imaging Laboratory (MIL) core facility at the University of Michigan. EM was performed using a Phillips CM-100 transmission electron microscope. **Myofiber immunofluorescence:** Myofibers were isolated from 3 dpf zebrafish embryos using our established protocol (Horstick et al., 2013). Fibers were fixed to coverslips for 10 minutes in 4% paraformaldehyde. Immunostaining was done as previously reporting using antibodies to α-actinin (Sigma), RyR1 (Developmental Hybridoma Bank), and DHPR (Abcam) (Dowling et al., 2009; Majczenko et al., 2012).

**Measurements of calcium transients:** The construct containing GCaMP under a muscle actin promoter was a kind gift of Dr. John Kuwada. Plasmid DNA was co-injected with DNM2 RNA at a concentration of 10ng/µL in 1-2 cell embryos. At 24 hours, embryos were
embedded and immobilized in 1% low melt agarose. Fluorescence intensity during 10 seconds of spontaneous muscle contractions was recorded using a Nikon AZ-100 microscope. For image processing, ImageJ was used to measure total fluorescence intensity in a region of the larval trunk over the recorded interval. Baseline fluorescent levels were used to calculate relative fluorescence (delta F/F).

**Ex vivo tubulation in COS cells:** Ex vivo tubulation was performed following the basic methods described in (Nicot et al., 2007). The BIN1 iso8-GFP construct was a kind gift of Dr. Pietro De Camilli. COS7 cells were maintained in DMEM + 10% FBS and cultured on 22 mm cover slips. Cells were transfected using the TransIT reagent (Mirus). For processing, cells were fixed with 4% paraformaldehyde for 15 min, washed in PBS, and then mounted on Superfrost plus slides with ProLong plus DAPI. Images were generated using an Olympus confocal microscope and the FluoView software package. Tubulation patterns were classified as previously described (Meunier et al., 2009; Nicot et al., 2007). Cells with “short” tubules exhibited punctate tubules with lengths no greater than 4 times the tubule width, while cells categorized as “long” displayed tubules that extended uninterrupted to the plasma membrane. Cells with tubules longer than 4 times the width that did not fully extend to the plasma membrane were classified as “intermediate”.

**Statistical analysis:** Statistical analysis was performed using the GraphPad Prism 5 software package. Significance was determined using Student’s t-test or Fisher’s exact test.

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**References**


**Translational Impact**

**Clinical issue:** Congenital myopathies are a heterogeneous group of childhood onset muscle diseases associated with significant morbidity and early mortality. To date, no therapies are available that modify the disease process for any congenital myopathy. Dominant mutations in DNM2 (dynamin-2) are associated with a common congenital myopathy subtype called autosomal dominant centronuclear myopathy. Much remains to be understood regarding the pathogenesis of this condition, with one key barrier being the relative lack of adequate models of the disease. The goal of this study was to further characterize zebrafish and cell culture models of DNM2-related myopathy and to use these models to better understand disease pathogenesis. *Results:* Using direct injection of both wild type and mutant DNM2 RNA into early stage zebrafish embryos, the skeletal muscle phenotype of the resulting animals was characterized. Larvae expressing mutant DNM2 (DNM2-S619L) had structural abnormalities in the triad, the skeletal muscle substructure that represents the intersection of the T-tubule and terminal sarcoplasmic reticulum. Functional studies revealed that contraction related calcium release was also defective in DNM2-S619L embryos. Taken with the ultrastructural data, these results indicate a defect in excitation-contraction coupling. This led to a hypothesis that *DNM2* mutation promoted defective tubulation. This hypothesis was tested using an *in vitro* tubulation assay. Exogenous expression of DNM2-S619L cDNA (but not WT DNM2) into Cos7 cells prevented the appearance of normal tubules, thus supporting the idea that *DNM2* mutation, at least in part, causes disease by preventing proper tubulation. *Implications and future directions:* The data in this short report demonstrate that the common DNM2-S619L mutation disturbs tubule formation, both *in vitro* and in a zebrafish model of the disease. In turn, this abnormal tubulation prevents normal excitation-contraction coupling, which likely leads to muscle weakness and impaired muscle force generation. This new knowledge of disease pathogenesis addresses an unanswered aspect of DNM2-related myopathy, and will serve as a springboard for rational therapy development for this devastating disorder of childhood.
GTPase dynamin 2, and genotype-phenotype correlation in autosomal dominant centronuclear myopathy. *Hum Mutat* 33, 949-59.


