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Amyotrophic Lateral Sclerosis
Novel Model to Assess Laryngeal Function, Innervation, and Reinnervation

Matthew O. Old, MD; Sang Su Oh; Eva Feldman, MD, PhD; Norman D. Hogikyan, MD

Objectives: Laryngeal paralysis remains an unsolved problem, and the behavior of the laryngeal muscles following injury to the native innervation appears to be a function of denervation and reinnervation. The aim of the present study was to develop a reliable, accurate, and multifaceted animal model for study of laryngeal function, innervation, and reinnervation.

Methods: A spontaneous-breathing anesthesia technique, suspension laryngoscopy, endoscopic evaluation of the rat larynx, and transoral injection of a retrograde neuronal tracer, hydroxystilbamidine (FluoroGold), were developed. We submitted 14 rats to the developed technique to map the brain stem projections of the superior laryngeal nerve and the recurrent laryngeal nerve and to determine the feasibility and accuracy of the endoscopic injection technique.

Results: This endoscopic technique provided full evaluation of the rat larynx. We performed transoral endoscopic injection of FluoroGold and transcervical application of the tracer to transected superior laryngeal and recurrent laryngeal nerves in 14 different rats and successfully created a neural projection map for the superior laryngeal nerve, the recurrent laryngeal nerve, and the cervical ganglia.

Conclusions: A reliable, accurate model for the characterization of laryngeal function, routes of innervation, and sources of spontaneous reinnervation following recurrent laryngeal nerve resection has been developed. This stable and reproducible model can serve as a dependable tool in future investigations of laryngeal nerve injury and recovery.

Key Words: laryngeal innervation, nucleus ambiguus, rat laryngeal anatomy, recurrent laryngeal nerve, reinnervation, superior laryngeal nerve.

INTRODUCTION

Normal laryngeal function requires complex coordinated interplay between multiple intrinsic and extrinsic muscles. The intrinsic muscles are innervated by branches of the vagus nerve, the superior laryngeal nerve (SLN), and the recurrent laryngeal nerve (RLN). Traditional anatomic teaching holds that the RLN serves all of the intrinsic muscles except for the cricothyroid muscle, which is innervated by the external branch of the SLN.1

Laryngeal paralysis remains a fundamentally unsolved clinical problem, because there are no interventions that can reliably restore physiological movement to a paralyzed vocal fold. Multiple treatment options are available, however, that can improve laryngeal function in the setting of paralysis. These include static geometric solutions that improve glottal closure in the setting of unilateral vocal fold paralysis, and reinnervation procedures that provide tone to vocal fold musculature.2 In cases of bilateral paralysis, reduction of glottal tissue can improve the airway at the expense of vocal quality, whereas tracheostomy can bypass laryngeal obstruction. Pacing procedures have also been described.3,4

Laryngeal musculature differs from most other skeletal muscle in that progressive muscular atrophy or fibrosis is not necessarily seen after clinical or experimental disruption of innervation. As stated by Crumley, studies "...have provided a sound basis for the presumption that laryngeal muscles generally regain various levels of innervation following RLN injury. Hence, it appears that aberrant and poorly functioning reinnervation, rather than denervation, is the most common laryngeal problem in patients following RLN injury."5(p369) Despite the immobility of the vocal folds following denervation injury, many electromyographic studies have demonstrated motor unit action potentials in the laryngeal musculature, rather than electrical silence.5-8 Both the abnormal motor unit recruitment in these

From the Departments of Otolaryngology–Head and Neck Surgery (Old, Hogikyan) and Neurology (Oh, Feldman), University of Michigan, Ann Arbor, Michigan. Supported by the Program for Neurology Research and Discovery. This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan.

Correspondence: Matthew O. Old, MD, Ohio State University, 456 West 10th Ave, Cramblett Hall, Suite 4A, Columbus, OH 43210.
muscles and the preservation of muscle morphology observed after RLN injury have been attributed to spontaneous reinnervation. In cases of neural injury in which the gross structure of the RLN is intact, the nucleus ambiguus is believed to be the source of the regenerated axons. In cases of severe injury of the nerve, misdirected growth of abductor and adductor fibers causes synkinesis, rather than purposeful movement. In cases of complete nerve transection, the source of the reinnervation is unclear, although there is evidence in the literature to support both regrowth of native motor nerve and an autonomic origin of reinnervation.

Hydman and Mattsson have reported studies of posterior cricoarytenoid muscle innervation in a rat model of RLN injury. Using retrograde tracing and electrophysiologic and immunohistochemical techniques, they demonstrated dual innervation of the normal posterior cricoarytenoid muscle by the RLN and the SLN. After chronic RLN injury, they also found that intact fibers of the SLN reinnervate the posterior cricoarytenoid muscle, most likely by intramuscular sprouting. Reinnervation studies were performed 6 weeks after RLN injury, and an open transcervical approach to the posterior cricoarytenoid muscle was used for retrograde tracer injections. Possible sensory or autonomic sources of reinnervation were not explored.

The majority of animal reinnervation models have relied upon open transcervical application of retrograde neuronal tracers, which requires significant dissection and disruption of tissue planes and potential sources of reinnervation. Tracheal transection, laryngofissure, and laryngeal windows have all been routinely used to perform laryngeal surgery in rodents because of the small laryngeal size. This increases the probability of morbidity and mortality, and typically requires intubation and ventilation during and after the procedures. Because the rat larynx is so small, intubation can cause mucosal trauma to the vocal folds. In addition, the endotracheal tube places pressure on the vocal fold, causing leakage of neural tracers after injection. These two factors are potential confounding variables in studies of denervation and reinnervation. The prolonged period of time necessary for neural regeneration makes any additional morbidity or mortality costly to a reinnervation study.

Ultimately, we seek to further elucidate the sources of spontaneous laryngeal reinnervation in the setting of RLN injury. Fundamental to this goal is development of a safe, reliable, and accurate animal model for observation and experimentation. Our present aims are to develop an animal model that 1) avoids intubation, significant cervical dissection, and open approaches to the larynx; 2) provides accurate assessment and visualization of vocal fold movement; and 3) is able to elucidate the source(s) of innervation and reinnervation by use of retrograde neural labeling techniques.

MATERIALS AND METHODS

The description of methods that follows includes the initial model feasibility and development procedures and the final methods used in the reported experimental animals.

Animals. The study consisted of 38 male Sprague-Dawley rats divided into two groups: model development and experimental. The model development group consisted of 24 rats that were used to develop the methods. The experimental group contained 14 animals studied by means of the final developed technique.

Anatomic Dissections. Preliminary anatomic dissections were performed in model development animals to define pertinent anatomy and determine the feasibility of techniques. Xiphoid-to-chin incisions were made, and the sternum was divided to expose the cervical and thoracic compartments. The anatomy of the rat neck and the RLN and SLN were dissected and analyzed. The superior cervical ganglion (SCG) was located on the medial surface of the carotid bifurcation. The course of the vagus nerves and nodose ganglia with their relation to the skull base was dissected. The anatomic relationships of pertinent nerves with respect to the trachea, thyroid cartilage, and cricoid cartilage were determined. Laryngofissure and tracheal transections were performed, and the laryngeal musculature was identified and analyzed microscopically.

Anesthesia. The depth of anesthesia was important for this procedure. Transoral endoscopic laryngeal surgery in general can be performed with or without intubation and mechanical ventilation. The success of the planned experiments, however, mandated the development of an anesthesia technique that would preclude intubation or mechanical ventilation. The small size of the rat larynx precludes accurate vocal fold injection or mobility observations if the rat is intubated. Furthermore, intubation trauma increases the risk of postoperative mortality or morbidity. Thus, a spontaneous-ventilation anesthesia technique was developed and refined to avoid the need for intubation and unnecessary manipulation of the larynx. This allowed for unimpeded access to the larynx and uneventful postoperative recovery in the operative suite we designed (Fig 1). Ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride...
(5 mg/kg) were given by intraperitoneal injection. After tail and paw reflexes were lost, 1.8% isoflurane was delivered via a fashioned nose cone until the respiratory rate slowed and became regular. The isoflurane concentration was reduced to 1.25% and could be easily adjusted as needed to maintain adequate depth of anesthesia and spontaneous respiration. This method allowed for suspension laryngoscopy, endoscopic analysis of vocal fold movement, and injection of the vocal folds.

**Laryngoscopy.** There are many commercially available laryngoscopes for small animals, but none that would provide adequate exposure of the rat larynx and suspension to perform transoral endoscopy and injection procedures. Transoral injection of retrograde neuronal tracers is a critical aspect of this investigation, and thus the development of a suspension laryngoscope for bimanual endoscopic surgery in the rat larynx was necessary. Metal was measured and shaped into an appropriate laryngoscope to allow endoscopy in young rats to adults more than 3 to 4 months of age without the need for multiple laryngoscopes of various sizes. A custom suspension device was integrated into the laryngoscope to allow bimanual surgery (Fig 2).

**Endoscopic Vocal Fold Evaluation.** The Animal Surgery Operating Rooms (ASOR Core) at the University of Michigan provided the endoscopic equipment for this portion of the procedure. Endoscopic procedures were recorded on compact disc or digital video disc. We used 0° and 30° Stryker pediatric telescopes (2.7 mm) for evaluation of the larynx. Larger telescopes did not provide enough room at the glottal level to manipulate instruments sufficiently (Fig 3).

With the suspension laryngoscope in position, the
telescope was used to depress the soft palate posteriorly and then advanced until the glottis was brought into view. The 30° telescope provided full visualization of the rat larynx, particularly during surgical procedures (Fig 4).

**Endoscopic Vocal Fold Injections.** Vocal fold injections were performed under suspension microlaryngoscopy by use of bimanual technique with the telescope in the nondominant left hand. The right hand held the suction, needle, or other instrument. A portion of the right hand rested on the laryngoscopy platform for stability. Dozens of injections were performed with different needles and modifications, followed by anatomic dissections to determine the placement and precision of the injection. A custom 2-inch (5-cm), 30-gauge Hamilton removable needle with a 45° bevel and point style 4 was designed and was found to be the most accurate. It was attached to a 10-μL Hamilton syringe. The removable needle hub design helped prevent extravasation of the retrograde tracer, hydroxystilbamidine (FluoroGold, Fluorochrome, LLC, Denver, Colorado), from the connection or leakage from the tip. A bend of about 30° was made approximately 3 to 4 mm from the distal end of the needle. This adjustment allowed for the proper trajectory into the vocal fold (Fig 5). The absence of this bend and a smaller bevel (less than 45°) resulted in placement of the needle tip through the vocal fold and injection of material into the subglottis or tracheal wall.

Dye volume studies were performed to determine the appropriate volume of injected solution. This was determined to be a maximal volume of 2 μL. Less than 0.5 μL of air was pulled into the distal tip of the syringe before insertion into the oral cavity, to prevent inadvertent application of FluoroGold to unintended areas. Once the proper position of the needle was confirmed, the injection was performed slowly. The FluoroGold could be visualized within the true vocal folds with the 30° endoscope. After the needle was removed, two small boluses of warm saline solution irrigation were applied with constant suctioning to cleanse the mucosal surfaces of any FluoroGold. Constant suctioning was necessary to prevent significant aspiration of the saline solution.

**Transcervical Procedures.** Dissections of the SLN and RLN were performed through a midline cervical incision. The large submandibular glands were identified, separated in the midline, and lateralized. The raphe of the infrathyroid strap muscles was identified and the muscles were retracted, allowing for dissec-
tion down to the anterior tracheal wall and larynx. The left RLN typically was visible on the anterolateral surface of the trachea. The right RLN was located in the tracheoesophageal groove.

Elevation of the thyroid gland medially and retraction of the infrahyoid strap muscles laterally allowed for identification of the SLN. It could be traced distally to its internal or external branches and proximally to its origin from the vagus nerve. Just proximal to this origin is a small bulge of the vagus nerve. This is the nodose ganglion, and its proximal portion frequently tracked superiorly into the skull base.

Application of FluoroGold to nerve stumps was performed to map out the nucleus ambiguus and projections of the SLN and RLN. The proximal end of the nerve stump was placed for 5 minutes in the cut end of an Eppendorf microtube filled with 5% solution of FluoroGold. The cavity was thoroughly irrigated, and the nerve stump was wrapped in subcutaneous fascia and fat harvested during the cervical approach. After the procedures were performed, the strap muscles were reaproximated and the skin was closed with staples. The FluoroGold was allowed to incubate for 6 days before perfusion and harvest.

Perfusion and Harvest. The rats were euthanized on postoperative day 6 with a lethal dose of pentobarbital sodium and intracardiac perfusion of 100 mL of lactated Ringer’s solution followed by 200 mL of 4% paraformaldehyde. Immediate microscopic dissection of the nerves, ganglia, trachea, and larynx was performed, and the specimens were kept in 4% paraformaldehyde overnight at 4°C. The specimens were then transferred to 15% sucrose in 1× phosphate-buffered saline solution and left for 24 hours. The specimens were then embedded in optimal cutting temperature medium and stored at –20°C.

Tissue Processing. Brain stem specimens were sectioned according to a predetermined method: 14 μm, 14 μm, and 20 μm serially. The 20-μm sections were used to count the cells positive for FluoroGold, and the precise rostral and caudal locations were recorded in relation to the obex. One set of the 14-μm sections was saved for future studies. All sections were placed on glass slides. The ganglia were sectioned at 14 μm and placed on glass slides. All slides were stored at –20°C until further processing.

Immunofluorescence. The slides were dried for 10 minutes at 55°C and then rehydrated in phosphate-buffered saline solution for 35 minutes. Extra phosphate-buffered saline solution was wicked away, and 1 or 2 drops of Invitrogen’s ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) was applied along with glass coverslips. DAPI is a nuclear and chromosome stain that emits blue fluorescence upon binding to DNA. The slides were cured overnight in the dark at room temperature, and then the edges were sealed with nail polish. The slides were then stored at –20°C until the cells were counted.

The slides were visualized with a wide-band ultraviolet filter that also excited DAPI (excitation at 372 nm; Fig 6). Motor neuron cell bodies with a clearly visible nucleolus were counted and recorded according to side (left, right) and location in relation to the obex. The nucleus ambiguus is located approximately 2 to 3 mm rostral and caudal to the obex. Every fifth section from the ganglia was counted and summed. Data were collected on a spreadsheet, and a profile of FluoroGold-positive cells according to location was created that included motor (nucleus ambiguus), autonomic (SCG), sensory (nodose), and negative control (dorsal root ganglion) groups.

RESULTS

Rat Anatomy. Anatomic dissections and endoscopic analysis of the larynx with the designed suspension system elucidated the peculiar anatomy of the rat larynx (Figs 3 and 4). The ratio of the length of the musculomembranous vocal fold to that of the vocal process is significantly lower in rats than in humans. The arytenoid cartilage and vocal processes dominate the glottal opening and may deceive an investigator into thinking that these are the entire true vocal folds. Coupled with anatomic dissection, endoscopic analysis of the larynx with a 30° endo-
scope reveals the musculomembranous true vocal fold location more anteriorly than expected. A majority of published pictures of the rat larynx consist of pictures of the vocal processes similar to our Fig 3.16-19 The use of the suspension laryngoscope and 30° endoscope provided adequate visualization of the entire rat larynx.

Laryngoscopy, Injections, and Transcervical Procedures. All 14 of the experimental rats survived the anesthesia technique, endoscopy, transoral injections, and transcervical operations. The brain stem, trachea, larynx, right and left RLNs, right and left nodose ganglia, and right and left SCGs were harvested and cryopreserved successfully in all 14 animals.

Mapping of RLN, SLN, and Ganglia. Mapping of the RLN and SLN to the nucleus ambiguus and SCGs revealed consistent results among the rats tested (Fig 7). The RLN primary cell bodies reside in a broad distribution in the nucleus ambiguus, ranging from –0.7 mm caudal to 2.6 mm rostral to the obex (Fig 7D). The SLN pattern had a narrower distribution in the nucleus ambiguus, with a large peak more rostral than the pattern of the RLN (Fig 7B). The peak overlapped with the RLN distribution.

Endoscopic Injection Technique. Sectioning of the RLN or SLN (Fig 7A,C) before injecting FluoroGold into the right thyroarytenoid muscle revealed a pattern similar to the pure SLN and RLN distributions seen after soaking the proximal nerve.
stumps in FluoroGold (Fig 7B,D). Injection of the right TA muscle in 4 of the experimental rats demonstrated a mixed RLN and SLN pattern (Fig 7E), as though the two distributions were superimposed on each other. This finding corroborates the accuracy of the endoscopic method and demonstrates that this method appropriately incorporates the innervation of the TA muscle.

**Contamination and Negative Controls.** Negative contamination controls were performed in 2 rats (Fig 7F). The RLN and SLN were cut before right TA muscle injection. No uptake in the nucleus ambiguous was noted.

**Ganglia Distribution Patterns.** In the RLN projections (Fig 7C), a minimal amount of FluoroGold-positive cells was detected in the ipsilateral nodose ganglion. A small amount was detected in the ipsilateral SCG, consistent with a mixed distribution of motor, sensory, and autonomic fibers in the RLN bundle. The large amount of uptake in the contralateral nodose ganglion seen in the RLN projections is likely due to cross-sensory innervation of the larynx as represented by the uptake pattern in the contamination control (Fig 7F) and the injection of the TA muscle after the ipsilateral SLN was sectioned (Fig 7C). In corroboration of this, Fig 7D demonstrates no uptake in the contralateral nodose ganglion, as this is from pure uptake in an ipsilateral nerve with application of FluoroGold to the nerve stump. The SCGs had a similar but less prominent pattern.

The SLN pattern (Fig 7A,B) demonstrated increased numbers of positive cells detected in the ipsilateral nodose ganglion and SCG and in the contralateral nodose ganglion and SCG. This finding is consistent with the predominant sensory and autonomic nature of the SLN. As a negative control for systemic FluoroGold distribution, the dorsal root ganglia were harvested and evaluated for FluoroGold-positive cells in all rats. No significant uptake was noted in the dorsal root ganglia.

**DISCUSSION**

The anterior-posterior anatomic position of the rat larynx is similar to that in humans, but the components are different in size. The arytenoid cartilages and vocal processes dominate the rat laryngeal introitus, and with the aid of a 30° endoscope and suspension microlaryngoscopy, full visualization of this anatomy is possible. A majority of studies have provided pictures of the rat arytenoid cartilages and vocal processes, but not the true vocal folds. The ratio of the length of the musculomembranous vocal folds to that of the vocal processes is quite different in humans, because the musculomembranous portions dominate the human glottal opening, in contrast to what is found in the rat larynx. Despite this difference, anatomic and muscle-staining studies support the use of the rat larynx for modeling human diseases.

The RLN has a mixed composition of motor, sensory, and autonomic fibers, but is predominantly motor, as demonstrated previously. These projections are to the nucleus ambiguous, the nodose ganglia, and the SCGs, respectively. Interestingly, the sensory and autonomic projections to the true vocal folds are bilateral.

Severe injury to the RLN results in an immobile but innervated vocal fold. The source of reinnervation is unclear, but the current leading hypothesis is misdirected motor fiber regrowth leading to synkinesis. Different studies have investigated this concept, but none have looked at all of the potential sources of reinnervation in a single study. This model is designed to account for all of the potential reinnervation sources, and will serve future investigations of laryngeal reinnervation.

One limitation of this study is the lack of electromyographic data. Previous studies have used electromyography to establish physiological evidence that the denervated vocal fold has been reinnervated. Our approach does provide an excellent functional measure, however, via serial video analysis of vocal fold movement and position. Another possible limitation of our approach is the way in which FluoroGold is transported to neuronal cell bodies. FluoroGold uptake requires retrograde transport along axons to the cell body, and it is possible, but not known, that this transport is less efficient in repaired axons. Hydman et al demonstrated that primary neuronal cell bodies in the nucleus ambiguous survive after complete axonal injury. Application of FluoroGold proximal to the injured site resulted in good uptake. Application was not performed distally in their study, however. We chose to apply neural tracer to the muscle to demonstrate all potential reinnervation sources. As controls, we applied FluoroGold to nerve stumps in an approach similar to that of Hydman et al. Limiting application of tracers to the RLN cannot address sources of reinnervation other than the injected RLN; thus, injection of...
tracers directly into the muscle allows for detection of cross-reinnervation.

**CONCLUSIONS**

We have developed an accurate, minimally injurious, and reproducible method to study sources of laryngeal innervation and to help elucidate the cause of spontaneous laryngeal reinnervation. This method combines spontaneous-ventilation anesthesia, suspension laryngoscopy, analysis of vocal fold movement, and injection of the unilateral TA muscle and will serve as a powerful tool and model in future animal laryngological studies.

Studies are ongoing to determine the extent and source of spontaneous laryngeal muscle reinnervation following RLN resection. In addition to FluoroGold profiles of the brain stem, SCGs, and no-dose ganglia, the regrowth of new axons at the site of RLN resection will be evaluated. Such studies are designed to investigate the sources of reinnervation and to help determine the pattern and causes of the problem of an immobile but reinnervated vocal fold.

**REFERENCES**


Computational methods for identifying a layered allosteric regulatory mechanism for ALS-causing mutations of Cu-Zn superoxide dismutase 1

Adam D. Schuyler,1* Heather A. Carlson,2 and Eva L. Feldman3

1 Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut
2 Department of Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan
3 Department of Neurology, University of Michigan, Ann Arbor, Michigan

INTRODUCTION

ALS and SOD1

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease.1 Approximately 5–10% of ALS cases are due to genetically linked mutations and are termed familial ALS (fALS), while the remaining cases are of unknown cause(s), but present identical symptoms,2 suggesting a similar underlying mechanism. The most common fALS type is caused by any of the more than 100 known mutations of the enzyme Cu-Zn superoxide dismutase 1 (SOD1).2 All of these mutations maintain the enzyme’s natural antioxidant activity, suggesting that a new, toxic, function is introduced by mutation. This function, let alone its mechanism of action, is unknown.

There have been numerous studies of proposed disease mechanisms and treatments.3–8 Toxic aggregation of dissociated monomers has been implicated as a driving force in disease progression. Ray et al.6 demonstrate that small molecule docking at the dimer interface stabilizes several fALS mutants by resisting aggregation and unfolding. However, Rodriguez et al.9 identify several SOD1 mutations that are more stable than the wild type (WT). There is no single property (e.g., dimer stability, net charge, metallation) that correlates mutation type with disease progression. It is thus assumed that ALS results from multiple contributory mechanisms.7

The SOD1 mutations that cause ALS are unrelated, ranging widely in their chemical nature and spatial distribution within the structure. As further evidence of their diversity, patient survival times range from 1 to 17+ years, depending upon the mutation. These factors lead to our:

ABSTRACT

The most prominent form of familial amyotrophic lateral sclerosis (fALS, Lou Gehrig’s Disease) is caused by mutations of Cu-Zn superoxide dismutase 1 (SOD1). SOD1 maintains antioxidant activity under fALS causing mutations, suggesting that the mutations introduce a new, toxic, function. There are 100+ such known mutations that are chemically diverse and spatially distributed across the structure. The common phenotype leads us to propose an allosteric regulatory mechanism hypothesis: SOD1 mutants alter the correlated dynamics of the structure and differentially signal across an inherent allosteric network, thereby driving the disease mechanism at varying rates of efficiency. Two recently developed computational methods for identifying allosteric control sites are applied to the wild type crystal structure, 4 fALS mutant crystal structures, 20 computationally generated fALS mutants and 1 computationally generated non-fALS mutant. The ensemble of mutant structures is used to generate an ensemble of dynamics, from which two allosteric control networks are identified. One network is connected to the catalytic site and thus may be involved in the natural antioxidant function. The second allosteric control network has a locus bordering the dimer interface and thus may serve as a mechanism to modulate dimer stability. Though the toxic function of mutated SOD1 is unknown and likely due to several contributing factors, this study explains how diverse mutations give rise to a common function. This new paradigm for allosteric controlled function has broad implications across allosteric systems and may lead to the identification of the key chemical activity of SOD1-linked ALS.
**Allosteric mechanism hypothesis**

SOD1 mutants alter the correlated dynamics of the structure and differentially signal across an inherent allosteric network, thereby driving the disease mechanism at varying rates of efficiency.

The methods discussed thus far have one thing in common: they are external to SOD1 and observational, in that they identify products of the disease, but not causal components of the mechanism. To best understand these contributory factors, and their potentially compounding interactions, atomic level descriptions of their respective modes of action are essential. In this study, we utilize computational methods to elucidate a group of atomic level interactions that support an allosterically modulated mechanism. Such a mechanism may not be universally accessible to all SOD1 mutant forms, but deriving the mechanism across a set of SOD1 mutants allows for the cohesive allosteric signal to stand out from the noise.

**Allosterity**

Allosterity is most generally described as an event at one structure location triggering a response at another (e.g., ligand binding at one location triggering a structure rearrangement that modulates the binding affinity at a secondary site). Allosterity has been recognized as a regulator of protein stability and a carrier of structure entropy. Gunasekaran et al. even hypothesize that all dynamic proteins have the potential to be controlled allosterically.

The cause and effect of allosteric communication may be readily observed, but the signal transmission mechanism is frequently not well understood. A variety of techniques have been employed, including solution NMR, molecular dynamics, Markov models and network analysis metrics. The current study of SOD1 utilizes the “static” and “dynamic” allosteric site prediction methods recently developed by the authors. Both methods were validated against the well studied dihydrofolate reductase and generated allosteric control site predictions with significance values of $P < 0.005$.

**Ensemble representations**

The ensemble representation of conformation space and structure dynamics has advanced many modeling approaches, with significant improvement coming in two related areas. First, drug design has evolved from the “lock and key” and “induced fit” paradigms to a notion of pre-existing conformation ensembles. The structure dynamics inherently captured by conformation ensembles greatly improve binding models and have led to better drug design methods. Second, transition state modeling reveals intermediate structures that serve as way-points along possible transition pathways. The intermediates display structure dynamics that are not locally accessible to the stable endpoints, but may be most relevant to the biological function.

The drug design and transition state modeling methods are illustrative examples of how ensemble representations more accurately describe structure dynamics as they pertain to molecular binding interactions. The ensembles in these methods are of the traditional sense: samples in conformation space around a single structure. In the current SOD1 analysis, we take a different approach, but with a similar motivation. The dynamics of SOD1 are accessed with a mutation ensemble. Rather than sampling conformations around a single structure, we are sampling the dynamics across a family of related structures. The cumulative result is an ensemble of dynamics, distributed over an ensemble of structure variations.

Our use of mutation ensemble derived dynamics is consistent with other approaches in the literature, particularly with respect to normal mode analysis (NMA) based techniques. Van Wynsbergh and Cui establish that correlated dynamics are most accurately identified from an ensemble of normal modes, not just from analyzing individual mode shapes. Petrone and Pande use NMA to study allosteric structure transitions and conclude that localized residue motions are often observed, thereby necessitating a large set of NMA modes to capture the motions. Zheng et al. observe the conservation of low-frequency normal modes that relate to allosteric transitions. This conservation is quantified as a robustness to sequence variation, a result which strongly supports the current SOD1 approach.

**METHODS**

The mutation ensemble of SOD1 is assembled from crystal structures available in the protein data bank (PDB) and from computationally generated structures produced by the mutagenesis tool in PyMOL (version 1.0,33). These procedures are given in the following sections. The SOD1 structures included in this study are listed in Table I, along with sets names that are used during the analysis to reference subgroups of structures. The dimer is shown in Figure 1, with the mutation sites highlighted and key structure locations labeled.

The allosteric mechanism hypothesis is tested with the application of the “static” and “dynamic” allosteric control site prediction methods. The static method predicts control sites with a geometric analysis of a single conformation and reveals the structural basis for an inherent allosteric network. The dynamic method compares the harmonic motions (i.e., normal modes) between a WT and mutant; atoms that become substantially more/less involved in correlated motion are identified as allosteric control sites. These allosteric prediction methods are briefly discussed in the following sections; complete details are available in Ref. 18.
Structure preparation

Crystal structures

The SOD1 WT is available in the PDB as a crystal structure of excellent resolution. Only four of the 100+ \(f\)ALS causing SOD1 mutations are single residue mutations with complete crystal structures of similar resolution. Multiple mutations may introduce complicating secondary effects and are excluded from this analysis.

Each crystal structure contains multiple copies of the SOD1 dimer. The dimer selected for analysis is the one most central to the ensemble and is determined as follows:

1. Any dimers with missing atoms are excluded.
2. A set of RMSD values is computed for each dimer by optimally aligning it to every other dimer.
3. The average RMSD is computed for each dimer’s set of RMSD values.
4. The dimer with the minimum value is selected.

### Table 1

<table>
<thead>
<tr>
<th>SOD1 Structures</th>
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<tbody>
<tr>
<td>X-ray structures from PDB</td>
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Computationally derived from WT

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The crystal structures are listed in the top panel along with their PDB tags, crystal resolution (Å), primary literature reference, ordered pairing of chain identifiers defining the dimer and RMSD from WT (Å, taken between all common atoms). The bottom panel lists the computationally derived mutant structures, which are based on the WT crystal structure and produced with the mutagenesis wizard in PyMOL. The “set names” categorize the structures into 4 groups based on their link to ALS and their method of derivation.

### Figure 1

WT SOD1 and Mutation Sites. SOD1 contains 153 residues and is biologically active in a homodimer form shown here, with each monomer containing a copper and zinc binding site. The bulk of the structure is a compact β-barrel and backs the active site. The residues associated with the mutations listed in Table I, are shown with balls and sticks. Mutations with crystal structures are colored red and those that are computationally generated are colored yellow. The copper and zinc atoms are shown as dotted spheres (Image prepared with PyMOL).
the highest quality set of structures the only mutations considered are those whose heavy atom topologies are equivalent to, or a subset of, the WT configuration. These permissible mutations come in two forms. A “swap” is the substitution of one atom type for another (e.g., in Asp → Leu, both δ-oxygens become carbons). A “clip” is the truncation of a WT residue into a smaller residue type (e.g., in Ile → Val, the δ-carbon is removed). This approach is applied to all applicable mutant types and produces 20 additional fALS structures: 12 “clip” and 8 “clip” + “swap.”

The NCBI Single Nucleotide Polymorphism Database lists three missense entries for SOD1. Two of the entries are known fALS mutations: D90A (SNP tag rs80265967) and N86S (SNP tag rs11556620). The remaining entry is presumed to be a non-fALS mutant: T39I (SNP tag rs1804450). This mutation is not of the simple “clip” and “swap” variety and requires complete side chain replacement. Given the rarity of identifying a non-ALS mutant, it is included in the analysis and serves as a powerful negative control.

**Analysis techniques**

**Static method**

Allosteric communication is the flow of a signal across a molecular network. Any model must therefore define the molecular network and derive a function for quantifying signal transmission.

The static method defines its network such that all atom pairs within a cutoff distance are considered linked. This concept is expressed as the adjacency matrix \( A \) where entry \( A_{ij} = 1 \) if, and only if, the distance between atoms \( i \) and \( j \) is not more than the cutoff distance. Self contact is not allowed (\( A_{ii} = 0 \)).

A standard metric in graph theory is degree, which is the number of connections that radiate from a node in a network. This quantity is the sum of values down the corresponding column (or row) of \( A \). The static method generalizes this local description of contact density to a network-wide description by considering paths in the network that connect pairs of atoms. All such paths of a given length \( s \) are obtained by computing \( A^s \); these values quantify the relative connectedness of each atom to the entire structure and serve as the basis for quantifying signal transmission in the static model.

Consider a structure location which is highly connected to the surrounding structure (i.e., high values down the corresponding column of \( A \)). Any allosteric signal that flows out of this node is distributed across its dense network of connected paths and diffuses. In contrast, a network location with relatively few radiating paths, transfers a greater magnitude of its allosteric signal down each path. This is the inverse contact path model (iCPM) and it probes the network flow of an allosteric signal. Residues are identified as control sites based on how much their iCPM values deviate from the mean of all residue iCPM values.

**Dynamic method**

The dynamic method assigns the known functional differences between two structures to the differences observed in their accessible motions. This comparison is made on the foundation of elastic network based NMA. Normal modes, which in this case are obtained with the cluster-based cNMA approach, provide two critical features. First, a structure’s complete set of normal modes defines a coordinate system for the motion space. That is, any structure motion can be represented as some linear combination of the normal modes. Second, through statistical mechanics, each mode’s eigenvalue is used to assign a relative significance, which indicates the importance of the mode to the overall fluctuations of the structure. As a consequence of these properties, the normal modes of one structure, along with their relative significance values, are decomposed over the normal modes of a modified structure. This weighted mapping reveals the extent to which each mode of one structure becomes more significant or less significant when mapped onto the motion space of the second structure.

Given two structures, a WT and a mutant, the dynamic method partitions the normal modes of the WT into two sets: \( \text{WT}^+ \) = {WT modes that become relatively more significant when mapped onto the mutant} and \( \text{WT}^- \) = {WT modes that become relatively less significant when mapped onto the mutant}. Similarly, the mutant modes are partitioned into two sets: \( \text{M}^+ \) = {mutant modes that become relatively more significant when mapped onto the WT} and \( \text{M}^- \) = {mutant modes that become relatively less significant when mapped onto the WT}. For each of these 4 sets, the dynamic method quantifies how involved each atom becomes in positively correlated motions and negative correlated motions. The residues with the largest magnitudes of correlated dynamics are identified as control sites on the allosteric network.

**Control site identification**

The static method generates iCPM data and the dynamic method generates eight channels of correlated dynamics data. Each of these nine raw data sets is the basis for predicting allosteric control sites. Let a candidate raw data set be given by the vector \( x \), where each entry in the vector corresponds to a residue in the structure. The control sites are identified according to the following procedure (as established in Ref. 18):
1. Compute the following ratio for each α-carbon: $\mu_i = (x_i - \langle x \rangle) / \langle x \rangle$, where $\langle x \rangle$ is the mean value of $x$.

2. Identify all residues with $\mu$ values at least as big as the threshold cutoff, $\tau$.

3. Group the residues into runs of consecutive indices; each group is the top of a peak in the data.

4. Pick the residue with the largest $\mu$ value within each grouping.

This procedure is parameterized by the $\tau$ value, which defines the threshold relative to the mean. For example, a $\tau$ value of 0.25 corresponds to data that is 25% above the mean. As established in Ref. 18 the threshold cutoff for iCPM data is $\tau = 1$ and the threshold cutoff for correlated dynamics data is $\tau = 0.45$. The iCPM and correlated dynamics data are fundamentally different quantities and are not scaled on the same interval.
the same range; accordingly, they are subject to different \( \tau \) values.

**RESULTS**

**Static method**

The static method is applied to all structures listed in Table I. Figure 2 shows a dual plot series for the WT. The adjacency matrix (\( s = 1 \) plot) morphs into a steady state representation of network connections (\( s = 20 \) plot). The normalized degree metric shown in the bottom panel of each dual plot is the basis for computing the iCPM data. The iCPM data are computed for all of the structures in the SOD1 ensemble at a path length of \( s = 20 \), and, as expected, the profiles are nearly identical: the average alignment between the WT profile and each of the other 25 structure profiles is \( \geq 0.99 \). Accordingly, only the WT iCPM data are presented in Figure 3.

Residues are identified as control sites when their iCPM values are 100% larger than the mean (i.e., \( \tau = 1 \), as established in Ref. 18) This yields five predicted control sites in each monomer. By visual inspection, the iCPM data appears to have a secondary set of seven control sites which do not pass the \( \tau \) threshold, but do clearly stand out relative to the background data. Both tiers of predicted control sites are highlighted in Figure 3. The complete profile is color mapped onto the WT structure in Figure 4, to give a three-dimensional view of the control sites and their relative positions. The functional significance of this network is addressed in the Discussion.

**Dynamic method**

The dynamic method is applied in several stages to build up a comprehensive representation of the allosteric activity. The first stage of the analysis applies the dynamic method to each of the four yALS-xray structures paired against the nALS-xray structure. This analysis establishes a comparative baseline by (i) utilizing just crystal structures to avoid introducing any potential error from computationally modeled structures and (ii) enhancing the signal-to-noise ratio by averaging over several structures. The data are shown in Figure 5, where each residue that passes the threshold cutoff (\( \tau = 0.45 \), as established in Ref. 18) is identified as a control site and highlighted in yellow. As with the static analysis, there is a prominent second tier of control sites that fall below the \( \tau \) threshold; these control sites are labeled and highlighted in red.

The second stage of the analysis introduces computationally generated structures: the dynamic analysis is performed on the 20 yALS-comp structures paired against the nALS-xray structure. The averaged data profile (not
shown) is nearly identical to that shown in Figure 5. Each of the four components (WT\textsuperscript{−}, WT\textsuperscript{+}, M\textsuperscript{−}, and M\textsuperscript{+}) contains data for positive and negative correlations, giving 8 total data sets. Comparing each of the data sets from \{yALS-xray vs. nALS-xray\} with the corresponding data set from \{yALS-comp vs. nALS-xray\} produces an average alignment value >0.99. This demonstrates that computationally generated structures are virtually indistinguishable from the crystal structures under the dynamic analysis technique. The consensus control site identifications made in \{yALS-xray vs. nALS-xray\} are also further corroborated.

The third stage of the dynamic analysis introduces a negative control by comparing the nALS-comp structure (T39I mutant) against the average profile taken across all 24 yALS structures (i.e., the 4 yALS-xray and the 20 yALS-comp). The average alignment value of this negative control with the consensus of the 24 yALS structures is 0.95. To isolate the functional significance of this difference, the negative control is subtracted from the consensus profile. The WT\textsuperscript{−} and M\textsuperscript{+} components are nearly identical (normalized alignment >0.99) and are thus averaged to produce a single allosteric channel. Similarly, the WT\textsuperscript{+} and M\textsuperscript{−} components are also nearly identical (normalized alignment >0.99) and are averaged to produce a single allosteric channel. This two channel allosteric profile (Fig. 6) gives a cleaner representation of the control site locations than the original analysis (Fig. 5).

Each of the channels in Figure 6, is color mapped onto the WT structure in Figure 7, to give a three-dimensional view of the control sites and their relative positions. The functional significance of these two networks is addressed in the Discussion.

Figure 6, shows the WT\textsuperscript{−}/M\textsuperscript{+} and WT\textsuperscript{+}/M\textsuperscript{−} channels as combined, but Figure 5 shows all channels explicitly. It should be noted that the data in Figure 5 is also suitable for a combined plot, but since it is the first plot showing data from the dynamic method, all channels are shown for completeness. The combined plot is used to highlight the connection between the two channels of Figure 6 and the two columns in Figure 7.

**DISCUSSION**

The static method evaluates network flow and establishes the existence of an inherent allosteric framework in SOD1, which is observed across all 26 structures. The iCPM data in Figure 3, are not exactly mirrored between the monomers, but the predicted control sites at the \(\tau = 1\) level are exactly the same between monomers. The second tier control sites are also the same. The matching control sites, despite the structural variation between the two monomers, indicates the robust nature of the method and supports the allosteric mechanism hypothesis.
Figure 5
Dynamic Method: γALS-xray. The average values of the dynamic method over all 4 γALS-xray structures. The control sites that pass the $\tau = 0.45$ cutoff are highlighted in yellow. The second tier control sites are highlighted in red. The residue indices on the horizontal axis are across the dimer, while the residue labels in the plot are given within each monomer. The dashed lines partition the monomers.

Figure 6
Dynamic Method: γALS masked by T39I. The averaged allosteric profiles from all 24 γALS structures, masked by the T39I negative control. The WT− and M+ components are averaged to produce the top channel and the WT+ and M− components are averaged to produce the bottom channel. Residues that pass the $\tau = 1$ cutoff are labeled and highlighted in yellow.
Color mapping the iCPM data of Figure 3, onto the structure in Figure 4, reveals critical insight into the underlying allosteric network. The groove in the top and the cavity in bottom of the structure are both lined with allosteric residues and are connected by a path of allosteric residues that run along β-strand 6 (residues 95–101).
The groove and cavity are adjacent to the dimer interface and suggest that the allosteric activity is a regulatory mechanism for modulating dimer stability. This is consistent with the leading hypothesis in the literature involving toxic aggregation of dissociated monomers.

The multistage dynamic analysis is complex, but produces several key pieces of evidence that add to the understanding of allosteric regulation in SOD1. The T39I non-ALS mutant is used as a negative control to isolate the functionally significant allosteric activity (Fig. 6). This analysis reveals that SOD1 has the capacity to operate a pair of allosteric networks, which are shown in Figure 7. Of particular significance, these networks are disjoint, which is a critical feature consistent with the observation that antioxidant and toxic functions simultaneously operate in fALS SOD1 mutants.

The WT−/M+ channel (illustrated in left column of Fig. 7) corresponds to allosteric activity that is more prominent in the WT than the yALS mutants. This channel shows control sites that form a cross-sectional plane directly through the active site. The central axis of this plane is defined by the pathway of adjacent residues: Pro11, Asp13, Arg14, Val119, His46, Phe45, Leu84. The WT−/M+ channel may regulate catalytic site activity and control the normal antioxidant function of SOD1.

The WT+/M− channel (illustrated in right column of Fig. 7) corresponds to allosteric activity that is more prominent in the yALS mutants than the WT. This channel shows a tunnel of control sites from the solvent exposed α-helix fragment and surrounding residues to β-strand 1 and its surrounding (buried) residues on the dimer interface. The WT+/M− channel may regulate dimer interface dynamics and control dimer stability. Ray et al.5 approached dimer stability by targeting ligand docking at the bottom cavity, which is part of the dimer interface. The WT+/M− channel indicates that dimer interface dynamics can also be controlled via the allosteric linkage to the solvent exposed locus of residues surrounding Gly130.

The static and dynamic methods have a common goal, but are based on fundamentally different principles. There are observations to be made in comparing the results of each method.

First, the control sites of the static method (Fig. 3) appear similar to the WT−/M+ channel of the dynamic method (Fig. 7, left column), but yet the control sites of the static method are attributed to regulating dimer stability, while the control sites of the WT−/M+ channel of the dynamic method are attributed to antioxidant function. The critical difference is that the WT−/M+ channel of the dynamic method shows a path of allosteric residues running through the active site, while the static method shows no allosteric activity in this area.

Second, the control sites of the static method and the control sites of the WT+/M− channel of the dynamic method (Fig. 7, right column) do not appear similar, but yet both are attributed to regulating dimer stability. The static method identifies allosterically active residues along the top groove and bottom cavity, while the WT+/M− channel of the dynamic method identifies internal activity at the dimer interface. It is possible that these dimer destabilizing networks may be dynamically linked. However, these networks are identified by vastly different methods and elucidating such a linkage is nontrivial. The other possibility is that two modes of dimer destabilization may be active. This is not surprising, given the spatial distribution of the known fALS mutants and the diversity of observed biophysical properties.

Third, the data of the dynamic method naturally falls into two groups: WT−/M+ and WT+/M−. It is rather remarkable that these groups identify disjoint allosteric networks and that each network is coherent and recognizable as a possible mechanism behind each of the two biological functions of SOD1. In contrast, the static method reveals only a single allosteric network. The static method is incredibly simplistic in design and is almost certainly insensitive to single residue mutations. It is therefore interesting to note that the static method identifies a potential dimer stabilization network, even in the WT structure. This indicates that while the fALS mutations may be required to activate the allosteric network, the SOD1 structure appears to inherently support such activity. This may explain why so many mutations are capable of triggering ALS.

In conclusion, the static and dynamic methods yield compatible and highly detailed descriptions of allosteric activity across the SOD1 structure ensemble. The following characterization is made in support of the allosteric mechanism hypothesis: Dual allosteric networks are independently involved in (1) the normal antioxidant activity of SOD1 and (2) the modulation of dimer stability. Controlling multiple biological functions (or even multiple components of a single function) via layered allosteric networks may be the unifying mechanism underlying ALS and may prove to be a feature of allosteric activity, in general.

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REFERENCES


The Association of Exposure to Lead, Mercury, and Selenium and the Development of Amyotrophic Lateral Sclerosis and the Epigenetic Implications

Brian Callaghan    Daniel Feldman    Kirsten Gruis    Eva Feldman
University of Michigan, Ann Arbor, Mich., USA

Key Words
Amyotrophic lateral sclerosis · Lead exposure · Environmental risk factors

Abstract
Metal exposures are an intriguing potential culprit in the cause of sporadic amyotrophic lateral sclerosis (ALS). For one, there are numerous case reports linking different metals to an ALS phenotype. Furthermore, some investigators have demonstrated higher levels of certain metals in the blood, bone, cerebrospinal fluid, urine, or spinal cords of patients with ALS compared to controls. There are also many case-control studies looking at the possible association of certain metals with the development of ALS. We have reviewed the relevant literature regarding metal exposures and the risk of developing ALS. We found that many different metals have been implicated as having a role in ALS, but there is more literature investigating the role of lead than any other metal. Despite many studies, the role, if any, of this metal in the pathogenesis of ALS remains unclear. Similarly, other metals either have inconclusive, conflicting, or insufficient results in order to make a definitive conclusion. One explanation for these findings is that metal exposures alone are insufficient for the development of ALS. Perhaps an interaction between the metal exposure and an individual’s genetic makeup is required to produce epigenetic changes that ultimately lead to ALS.

Amyotrophic Lateral Sclerosis (Background)

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neurodegenerative disease that occurs in sporadic (90%) and familial forms (10%) [1]. The cause of sporadic ALS remains unknown, and only a small percentage of familial cases have an identified genetic abnormality [1]. ALS is characterized by motor neuron degeneration which leads to progressive muscle weakness, swallowing difficulties, and respiratory failure. An aggressive disease, ALS is usually fatal within 3–5 years after the onset of symptoms [1]. The devastating nature of this condition combined with an incidence of approximately 2 per 100,000, makes ALS a pressing target for investigation [2].
Metals and Trace Elements

Abnormal exposure to metal ions may be a risk factor for ALS. Overexposure to any of several metal ions, including lead, mercury, selenium, cadmium, manganese, arsenic, copper, aluminum, and zinc, is known to be neurotoxic [3]. Specifically, motor neurons appear to be susceptible to metal ion toxicity. Lead toxicity, for example, is known to mimic motor neuron disease [4]. Motor symptoms are also associated with selenium toxicity [3]. Similarly, iron supplementation decreases motor activity in animals [5]. Furthermore, oxidative stress is hypothesized to play a role in the pathogenesis of ALS which may be one mechanism by which metals cause neurotoxicity. The role of oxidative stress is supported by evidence of oxidative damage to proteins, lipids, and DNA in post-mortem tissue samples of both familial and sporadic forms of the disease [6, 7]. Additionally, evidence of oxidative stress is present in the sera and spinal fluid of living ALS patients [7]. Metal ions are suitable mediators of oxidative insults due to their propensity to catalyze oxidation and reduction reactions. Several transition metals are known to cause neurotoxic effects by generating reactive oxygen species. Examples include lead, mercury, manganese, cadmium, and copper. We will focus on the three metals with the most literature to support their role in the development of ALS, namely lead, mercury, and selenium.

Lead

Lead exposure is among the most studied environmental risk factors in ALS. The association between the two dates back to 1850, when Aran first described motor neuron disease in the context of lead exposure. Three of his 11 patients with progressive muscular atrophy had been exposed to lead, 2 of them having suffered from outright lead poisoning [8]. In 1907, Wilson reported 4 additional cases of ALS associated with chronic lead poisoning [9]. There are also modern case reports that describe ALS in the setting of acute lead exposure [10]. Since the second half of the twentieth century, these associations have been examined in much greater detail.

Several early studies investigated lead levels within different body compartments of ALS patients compared to controls. Such studies focused on the abnormal turnover, tissue distribution, detoxification, and excretion of lead. Abnormal lead levels were thought to result in subsequent lead uptake by neurons, eventually manifesting in toxic neurodegeneration [11]. Conradi et al. [9] reported increased levels of lead in the cerebrospinal fluid of ALS patients and confirmed this finding again in 1980. Meanwhile, Kurlander and Patten [12] measured elevated levels of lead in spinal ventral horn tissue. These values directly correlated with the duration of the illness. Whereas Conradi et al. [9] found significantly elevated plasma lead levels in ALS patients, whole blood lead levels did not appear to differ between cases and controls in an earlier study [9]. In other pathologic states related to lead toxicity, such as acute lead intoxication, the hypothesis is that plasma lead levels are more meaningful than levels found within whole blood [13]. In contrast to many of these early findings, a small study consisting of only 9 patients found no association between ALS and lead levels in whole blood, plasma, erythrocytes, or CSF [14]. However, one must consider the possibility of drawing erroneous conclusions from such a small sample of ALS subjects. There is also conflicting evidence as to whether elevated levels of lead in skeletal muscle are present in ALS patients [11, 15]. Lastly, the administration of a metal chelating agent to ALS patients did not lead to detectible mobilization of lead stores [16]. Admittedly, however, the amount of lead excreted in the urine may not accurately depict the amount of lead that has accumulated in neurons.

More recently, investigators began to consider lead levels as surrogates for prior lead exposure, which is now viewed as a potential risk factor for ALS in susceptible patients. Consistent with previous data, Vinceti et al. [17] found no abnormalities in whole blood lead levels during early stages of the disease. However, they noted a positive correlation between whole blood lead levels and the progression of the disease, measured in this case by worsening functional impairment. Thus, elevated whole blood levels appear in later stages of the disease, similar to observations made by Kurlander and Patten [12] with respect to lead levels in the spinal cord. Since blood levels rise only after the onset of clinical symptoms, this abnormality likely reflects changes associated with disease progression rather than continued accumulation of lead in response to exposure. Interestingly, a more recent study found ALS patients to have elevated whole blood lead levels with a dose-response relationship between lead levels and risk of ALS [18]. Additionally, they found weaker, less consistent correlation between ALS and elevated bone lead levels. Because patients in the study were not stratified based on the stage of their disease, one might argue that, in light of the findings of Vinceti et al. [17] and Kurlander and Patten [12], this patient population might have been biased towards end stage patients, who would be more likely to have elevated lead levels. However, after
excluding all patients who were diagnosed more than 1 year prior to enrolling, their findings were unchanged. Furthermore, neither blood nor bone levels correlated with the time since diagnosis. It is also worth noting that several patients who met this study’s inclusion criteria elected not to participate because they were too sick or because travel was too difficult, implying that end-stage patients self-selected out of the case population. One potential limitation is the low participation rate for the controls in this study, which brings up the possibility of selection bias.

Clearly, these findings are insufficient to make any convincing arguments regarding the role of lead as a risk factor in the pathogenesis of ALS. In response, a number of additional case-control studies have compared self-reported histories of lead exposure between ALS patients and controls. Overall, we have identified 10 case-control studies investigating the possible pathogenic role of lead exposure [18–27]. These studies are summarized in Table 1. Conclusions clearly vary from study to study. Several factors influence the findings in these studies. One factor is the number of cases within each study, which varied from 25 to 518. Thus, smaller studies may be inadequately powered to detect an association between exposure and disease. Another factor is the inherent recall bias associated with case-control studies. This point is emphasized in McGuire et al. [20], which show that self-reported exposure to lead is exaggerated in comparison to the level of exposure determined by a panel of 4 industrial hygienists based on self-reported job descriptions. Such overestimations of past exposures may lead one to erroneously identify associations when, in fact, no such association exists. Overall, most of the studies showed an association between lead exposure and risk of ALS, but a few did not, including the study that assessed the largest sample size.

An epidemiologic investigation conducted in Jefferson County, Missouri, reveals a small but statistically significant standardized prevalence ratio of ALS (6.4, p = 0.0437) within proximity of a lead smelter known to be responsible for local atmospheric and soil contamination [28]. Recently, Kamel et al. [29] were able to follow up on 91% of the cases included in their previous case-control trial that confirmed an increased risk of ALS associated with a history of lead exposure [18]. Unexpectedly, they now note that lead exposure appears to be directly correlated with disease survival among the ALS cases. Though unintuitive, this finding is consistent with some previous reports. For example, one study showed a greater 5-year survival rate among ALS cases with a history of high lead exposure compared to cases with no history of lead exposure [8]. Kamel et al. [29] postulate that, together, the evidence implies that lead exposure promotes disease onset, but delays disease progression. They argue that the possibility that lead exposure is simply a marker associated with a different factor is unlikely since the findings were unchanged after adjusting for age, sex, smoking, education, BMI, physical activity, bulbar onset of symptoms, diagnostic delay, family history of ALS, and respiratory dysfunction. However, the mechanism by which lead could slow disease progression is unclear and makes definitive conclusions from this study difficult. Although there is evidence to suggest that lead exposure may be associated with ALS, and may even affect disease progression, the specific role of lead in the pathogenesis of ALS remains poorly defined and deserves further study.

One potential explanation for these conflicting results is that lead exposure is only part of the equation. Recent evidence has shown that lead exposure can cause epigenetic changes, which are DNA modifications that do not cause a change in the DNA sequence [30]. In this case, these changes are modifications in the methylation patterns that can subsequently lead to alterations in the transcription and subsequent translation rates of genes and proteins throughout the genome. Pilsner et al. [30] showed that maternal lead exposure as determined by patella lead levels was inversely correlated with methylation levels of her child’s DNA in cord blood. These results indicate that even maternal lead exposure can alter the epigenome of her offspring, which may have implications for subsequent disease susceptibility. Given the fact that lead can cause significant epigenetic changes, perhaps the reason that so many studies have been unrevealing is that we are missing the essential interaction of lead with the epigenome.

Further evidence that epigenetic changes are involved in the pathogenesis of ALS comes from studies of genes known to cause familial ALS. Two of these genes, FUS and ELP3, encode proteins that can cause alterations in histone acetylation [31]. Histones are an essential component of the organization of DNA within the cell. DNA wraps around an octamer of histones to form a nucleosome, which is the fundamental building block of the chromosome. However, histones are not merely a scaffold for the organization of our genetic material, but also undergo modifications that can have a profound influence on the transcriptional activity of nearby genes [31]. The best studied modifications are acetylation by histone acetyltransferases (HAT) and deacetylation by histone deacetylases. In regard to ALS, FUS interacts with the CREB binding protein decreasing this protein’s HAT ac-
Table 1. Case control studies investigating the association between lead exposure and the risk of developing ALS

<table>
<thead>
<tr>
<th>Reference</th>
<th>Location</th>
<th>Time period</th>
<th>ALS cases</th>
<th>Conclusions</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Felmus et al. [22]</td>
<td>Methodist Hospital in Houston, Tex.</td>
<td>Not specified</td>
<td>25</td>
<td>Self-reported lead exposure is a risk factor for ALS in predisposed individuals.</td>
<td>Small number of cases.</td>
</tr>
<tr>
<td>Pierce-Ruhland and Patten [23]</td>
<td>Houston, Tex.</td>
<td>Not specified</td>
<td>80</td>
<td>In genetically susceptible individuals, lead exposure is a possible antecedent event in ALS pathogenesis.</td>
<td>Exposure to lead alone was not significantly associated with increased risk. Only in conjunction with past mercury exposure was there a statistically significant association.</td>
</tr>
<tr>
<td>Gresham et al. [19]</td>
<td>San Diego, Calif.</td>
<td>1985</td>
<td>66 males</td>
<td>No association between self-reported occupational exposure and risk of ALS in males.</td>
<td>11.6% of cases had a family history of ALS. 39% of cases had traveled to the Marina islands. Possible overmatching of controls.</td>
</tr>
<tr>
<td>Deapen and Henderson [24]</td>
<td>United States (nationwide)</td>
<td>1977–1979</td>
<td>518</td>
<td>No relationship exists between self-reported lead exposure and the risk of ALS.</td>
<td>Study was not designed to investigate lifetime lead exposure in detail.</td>
</tr>
<tr>
<td>Armon et al. [21]</td>
<td>Mayo Clinic in Rochester, Minn.</td>
<td>Not specified</td>
<td>47 males</td>
<td>Among men, there is an association between ALS and self-reported exposure to lead vapor, although true etiology appears to be multifactorial in nature.</td>
<td>Small number of cases.</td>
</tr>
<tr>
<td>Gunnarsson et al. [25]</td>
<td>Southern Sweden</td>
<td>1990</td>
<td>92</td>
<td>A non-significant increased risk for ALS is associated with self-reported occupational lead exposure.</td>
<td>Possibility of confounding variables: lead exposure highly correlated to solvent exposure and the occupation of welding.</td>
</tr>
<tr>
<td>Chancellor et al. [26]</td>
<td>Scotland</td>
<td>Disease diagnosed 1990–1991</td>
<td>103</td>
<td>Occupational lead exposure is a risk factor for ALS.</td>
<td>Selection and recall bias. The patients included in the study had a trend towards longer survival.</td>
</tr>
<tr>
<td>Kamel et al. [18]</td>
<td>New England</td>
<td>1993–1996</td>
<td>109</td>
<td>An increased risk of ALS is associated with self-reported occupational lead exposure in a dose-dependent pattern. No increased risk of ALS associated with residential or recreational exposure.</td>
<td>Possibility of recall bias.</td>
</tr>
<tr>
<td>Qureshi et al. [27]</td>
<td>Massachusetts General Hospital, Boston, Mass.</td>
<td>1998–2002</td>
<td>95</td>
<td>A significant association exists between self-reported lead exposure and risk for ALS.</td>
<td>Failed to demonstrate smoking as risk factor contrary to most of the literature on this subject.</td>
</tr>
</tbody>
</table>
tivity, thus downregulating CREB target genes that may be involved in the pathogenesis of ALS [31]. Similarly, ELP3 is able to alter the expression of HSP70 through its HAT activity [32]. Interestingly, overexpression of HSP70 has been shown to decrease the amount of insoluble SOD1 in a SOD1 mouse model of familial ALS as well as prevent neuronal cell death in a mammalian cell model [33, 34]. These findings are the first glimpse that epigenetic changes are involved in the pathogenesis of ALS and open up a whole new avenue for potential experiments and perhaps future treatments of this devastating condition.

Mercury

There is a large amount of evidence suggesting that mercury is toxic to motor neurons. In animals, mercury accumulates in motor neurons following its injection into blood [35]. In humans, Pamphlett and Waley [36] report the preferential uptake of mercury by cortical motor neurons over other cerebral neurons following a suicide attempt involving intravenous injection of mercury. Arvidson [37] theorizes that mercury enters the CNS from peripheral exposure through uptake at neuromuscular junctions, which is followed by retrograde axonal transport. Mercury may mediate its neurotoxicity through one or more of several theorized mechanisms. These include inhibition of the activity of superoxide dismutase, damage to microtubules or other cytoskeletal components, and impairment of axonal transport [38].

A case report from 1996 [39] describes the onset of ALS in a 38-year-old woman 3.5 years after accidental injection of mercury into her hand. Importantly, mercury (and lead) concentrations in her blood, urine, and hair were not elevated at the time of diagnosis. Regardless of whether or not mercury exposure was responsible for the development of ALS in this patient, this finding is significant in that it shows that detection of body levels of a particular toxin via these methods may not account for previous exposure. Also of note, despite the fact that urinary mercury excretion increased dramatically after chelation treatment with dimercaptopropanosilic acid (DMSA), symptoms failed to improve, and actually worsened. Pralong et al. [40] report a more recent case of ALS in an 81-year-old woman following chronic exposure to mercury vapor from a tank used for pressure therapy to treat lymphadenopathy in her left arm. The patient had only slightly elevated mercury levels in her blood, but enormous levels were detected in her urine, even though her last exposure had taken place 5 months before. Despite the fact that urinary levels of mercury decreased signifi-

stantly in response to 4 weeks of DMSA therapy, her symptoms, like those of the previous case, continued to worsen. This failure to clinically respond to chelation therapy is consistent with findings from animal studies that show that DMSA is unable to decrease the brain burden of mercury [41]. Paradoxically, DMSA might appropriately increase the exposure of motor neurons to mercury as a result of redistribution of mobilized ions [41]. Other case reports describe similar examples of the onset of ALS preceded by mercury exposure [42, 43]. In the cases described by Adams et al. [42] and Barber [43], symptoms resolved once the exposure was removed.

A study that investigated urinary excretion of mercury and lead after administration of a chelating agent found no difference between ALS patients and controls in the amount of background excretion of mercury or excretion 48 h after chelation therapy [16]. Similar results were reported with respect to lead with the exception of background urinary lead excretion in males, which was significantly elevated in male ALS patients compared to controls. This is attributed to the combination of the fact that ALS males were significantly more likely to have possible occupational lead exposure than control males with no such difference among females. The fact that urinary mercury excretion increased only slightly after chelation is inconsistent with the above case reports, in which patients had known histories of significant mercury exposure [39]. The authors conclude, however, that the amount of mercury available for excretion may not represent the amount which has accumulated in motor neurons. Alternatively, the neurotoxic amount of mercury accumulated in neurons may indeed be mobilized by chelation, but is too small to be detected in the urine.

Most case-control trials that studied mercury as a risk factor for ALS investigated exposure to several other neurotoxic agents at the same time. Some of these trials were mentioned above as they pertained to lead. In short, mercury was not associated with an increased risk of ALS in two studies [19, 20] but was associated with an elevated risk in a third [23]. A cohort study amongst 83 Japanese mercury miners poisoned by or exposed to mercury vapor failed to identify any cases of ALS in 18 years of follow-up. No cases were reported among the 65 controls either. Overall, there is no conclusive evidence to suggest increased mercury exposure is associated with ALS but the literature on this subject is limited.

Selenium

As previously mentioned, selenium is neurotoxic in humans [3]. Suspicion regarding a link between selenium...
exposure and ALS was described in a 1977 report of 4 sporadic cases occurring among unrelated farmers living within a 15-km radius of each other in a selenium-rich region of South Dakota [44]. These reports are consistent with those of ALS-like syndromes associated with nearby livestock also exposed to toxic levels of selenium [45]. Not only did these animals show signs of progressive motor neuron disease culminating in respiratory failure, but degeneration of the ventral horns of the spinal cord was also present upon postmortem examination of some of the swine, further indicating motor neuron degeneration.

Vinceti et al. [46] studied a population that was also exposed to high levels of environmental selenium in Rivalta, Italy, where the municipal tap water contained elevated levels of selenium between 1972 and 1988. A cohort of residence living in Rivalta between 1980 and 1985 were found to have a standardized incidence ratio of 4.22 (95% CI: 1.15–10.8) compared to the unexposed population of the municipality. Moreover, a cohort of residence living in Rivalta since 1974 was found to have a higher standardized incidence ratio of 8.90 (95% CI: 2.43–22.79). Comparing these findings, this study demonstrates a higher incidence ratio of ALS in those Rivalta residents with a longer exposure to tap water with elevated selenium.

Several case-control trials examine the role of selenium exposure and diagnosis of ALS. Many of these trials use selenium levels in various body compartments as markers of selenium exposure. Early work from Nagata et al. [47] reveals a statistically significant elevation in erythrocyte selenium levels of ALS patients when compared to controls. However, more recent findings challenge these data. For example, Moriwaka et al. [48] found an inverse relationship between disease progression and both serum and erythrocyte selenium levels. Vinceti et al. [17] report a similar pattern with serum selenium levels. When they restricted their analysis to ALS patients with only a limited degree of disability, they found no difference in serum selenium levels compared to matched controls. This suggests that as the disease progresses, selenium levels in the blood fall. This relationship is believed to reflect the poor nutritional status associated with advanced disease. Most recently, Pamphlett et al. [49] observed no difference in whole blood, plasma, and erythrocyte selenium levels between ALS patients and controls, further confirming previous findings. Together, these data suggest that acute exposure to selenium does not necessarily precede disease onset, and that selenium levels change throughout the course of the disease. In order to study the influence of chronic selenium exposure on the risk of ALS, Bergomi et al. [50] used toenail metal concentrations, which reflects selenium exposure roughly over the prior 12–15 months. This study also failed to uncover an association between selenium exposure and risk of ALS. Their findings do, however, offer further support for the inverse relationship between selenium levels and disease progression.

Neurologic tissue has also been studied for associations between ALS and selenium. Mitchell et al. [51] report a significant elevation of selenium within the cervical cords (as well as livers and bones) of motor neuron disease patients. However, a similar trend is absent in the thoracic and lumbar spines. Kurlander and Patten [12] found elevated selenium in the anterior horn cells of only one of 7 motor neuron disease patients, but in 3 of 12 controls. In a larger study, Ince et al. [52] report significantly elevated levels of selenium in the lumbar spine of ALS patients, where there is also significantly elevated selenium-dependent glutathione peroxidase activity. Increased lumbar spine selenium levels appear to be independent of duration of disease, disease severity, and L4 segment lower motor neuron count, suggesting that its accumulation is unlikely to be a consequence of disease progression, but rather an earlier event in the course of the disease [53].

Ince et al. [52] caution, however, that the elevated selenium levels may be secondary to increased glutathione peroxidase enzymatic activity in response to oxidative stress from another source. Given all of the studies, there is very little data to suggest that selenium has a major role in the pathogenesis of ALS.

**Epigenetic Changes**

For many years, the dogma in biology was that DNA sequence-specific transcription factors were the only regulatory elements dictating gene expression. In the last decade, the field of epigenomics has emerged, revealing that DNA modifications, including DNA-bound histones, DNA methylation, and chromatin remodeling also provide levels of gene regulation and alter gene expression [31]. Epigenetic factors are probably much more suited than genetic factors to explain disease onset and progression in ALS, since aberrant epigenetic patterns may be acquired throughout life. One hypothesis is that environmental life exposures result in a failure to maintain epigenetic homeostasis in the nervous system which in turn leads to aberrant gene expression, contributing to nervous system dysfunction and in some cases the development of ALS. Metals are one of the most likely culprits to be a key
exposure risk factor in the development of ALS given their well-documented potential for neurotoxicity and involvement in oxidative mechanisms of injury. However, to date the epidemiologic literature supporting the role for metals in ALS pathogenesis has been disappointing. One potential reason for this discrepancy is the failure to understand the importance of the epigenomic background of patients and its interaction with exposures. Future studies are needed to investigate this relationship.

**Conclusions**

The role of metal exposures in the pathogenesis of sporadic ALS is unclear. There have been many studies investigating the role of metals such as lead, mercury, selenium, and cadmium. Unfortunately, many of the studies have had contradictory or indefinite findings. However, given the potential neurotoxicity of metals and their ubiquitous nature, further large-scale studies with rigorous epidemiologic designs are needed to reach more definitive conclusions. Furthermore, the interaction of lead and other metals with the epigenome of patients needs to be investigated to determine if epigenetic changes are the missing link between exposure and disease. Establishing or excluding the role of metals in the development or progression of ALS could lead to new insights into the cause of this devastating condition and potentially long overdue therapeutics. Moreover, the field of epigenetics has the potential to cause a major change in our current understanding of the pathogenesis of ALS and therefore new therapeutic targets.

**References**


ALS and Stem Cells
NEUROLOGICAL PROGRESS

Stem Cell Technology for Neurodegenerative Diseases

J. Simon Lunn, PhD, Stacey A. Sakowski, PhD, Junguk Hur, PhD, and Eva L. Feldman, MD, PhD

Over the past 20 years, stem cell technologies have become an increasingly attractive option to investigate and treat neurodegenerative diseases. In the current review, we discuss the process of extending basic stem cell research into translational therapies for patients suffering from neurodegenerative diseases. We begin with a discussion of the burden of these diseases on society, emphasizing the need for increased attention toward advancing stem cell therapies. We then explain the various types of stem cells utilized in neurodegenerative disease research, and outline important issues to consider in the transition of stem cell therapy from bench to bedside. Finally, we detail the current progress regarding the applications of stem cell therapies to specific neurodegenerative diseases, focusing on Parkinson disease, Huntington disease, Alzheimer disease, amyotrophic lateral sclerosis, and spinal muscular atrophy. With a greater understanding of the capacity of stem cell technologies, there is growing public hope that stem cell therapies will continue to progress into realistic and efficacious treatments for neurodegenerative diseases.

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Neurodegenerative diseases are characterized by the loss of neurons in the brain or spinal cord. Acute neurodegeneration may result from a temporally discrete insult, such as stroke or trauma, leading to a localized loss of neurons at the site of injury. Chronic neurodegeneration may develop over a long period of time and results in the loss of a particular neuronal subtype or generalized loss of neuronal populations. In the brain, Alzheimer disease (AD) and Huntington disease (HD) result in widespread loss of neurons, whereas Parkinson disease (PD) involves the specific and localized loss of dopaminergic (DA) neurons in the substantia nigra. In the brainstem and spinal cord, amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) involve the degeneration and loss of motor neurons (MNs). Although these conditions all exhibit unique neuronal pathologies, the exact mechanisms for neuronal loss are complex, making the identification of efficacious treatments elusive.

The lack of effective therapies for these neurological diseases creates an enormous burden on society. In the USA, approximately 7 million people are living with AD, HD, PD, ALS, or SMA (Fig 1A; http://www.alz.org/, http://www.apdaparkinson.org/, http://www.alsa.org/, http://www.hdsa.org/). Projected research spending by the National Institutes of Health (NIH) in 2011 for these 5 diseases totals $768 million, and spending allocation is proportional to the number of individuals affected by each disease (see Fig 1A; http://report.nih.gov/rcdc/categories/). In the USA, an estimated 5.3 million individuals suffered from AD in 2010, making it by far the most prevalent neurodegenerative disorder, carrying an estimated health care cost of $172 billion (http://www.alz.org/). PD affects up to an estimated 1.5 million people in the USA, with approximately 50,000 new cases diagnosed each year, and the estimated costs of PD stood at $11 billion per 500,000 affected Americans in 2009.1 Furthermore, HD and ALS each affect 30,000 Americans, and SMA affects 25,000 Americans.

Despite the destructive nature of these diseases, the number of affected individuals, and health care costs surpassing billions of dollars, there is a stunning lack of treatment options. Recently, cellular therapies have earned increased attention as potentially feasible novel therapies. Analysis of published scientific articles demonstrates that <2% of all papers per disease field examine the application of stem cells (see Fig 1B). However, it is

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Address correspondence to Dr Feldman, Department of Neurology, University of Michigan, 109 Zina Pitcher Place, 5017 BSRB, Ann Arbor, MI 48109-2000. E-mail: efeldman@umich.edu

From the Department of Neurology, University of Michigan, Ann Arbor, MI.

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likely that these figures will increase as the field of cellular therapy advances. In the past 5 years this percentage rose for each neurodegenerative disease analyzed (see Fig 1B), and NIH spending on stem cell research doubled between 2006 and 2011 (see Fig 1C; http://report.nih.gov/rcdc/categories/). Although stem cell therapies in the USA are methodically examined via cautiously designed trials and basic laboratory science, the lack of available and effective treatments has prompted people suffering with neurologic diseases to turn elsewhere for "stem cell treatments." Reports of "stem cell treatments" from clinics in China and India are followed by glowing patient testimonials; however, these treatments are not the result of rigorous trials investigating safety and efficacy. Analysis of 7 spinal cord injury patients receiving treatment at a clinic in China included uncertainties concerning the type of cells utilized, appropriate delivery for the level of injury, and ultimate presence of any clinical benefit.2 Since this analysis, improvements in cell quality, cell identification, delivery accuracy, and delivery targeting and reduced postoperative complications may have been made but have clearly not been documented.

Neurodegenerative diseases create a tremendous burden on society, and despite decades of research, effective treatments do not exist. Cellular therapies are attractive options, and the application of stem cell research to neurodegenerative diseases is rapidly expanding. In the current review, we detail the current state of stem cell research for neurodegenerative diseases, beginning with a brief introduction to the various stem cell technologies available. We also describe the rationale and remaining hurdles associated with transitioning stem cell therapies from bench to bedside. Finally, we discuss the current data and progress for translational stem cell therapies for AD, PD, HD, ALS, and SMA.

Stem Cell Technology

Stem cells have the capacity to proliferate and differentiate into multiple cellular lineages. There are different classifications of stem cells that reflect the range of possible cell types they can produce and the ways in which the stem cells are derived. These include embryonic stem (ES) cells, progenitor cells, mesenchymal stem cells (MSCs), and induced pluripotent stem (iPS) cells (Fig 2). To appreciate the potential applications of stem cell technology to neurodegenerative diseases, it is important to understand the characteristics of the various stem cell types available and the potential impact of cellular therapies on disease mechanisms.

Stem Cell Classifications

Each stem cell type possesses certain qualities and advantages, and the rationale for utilizing each depends on the desired applications and outcomes. ES cells are derived
from the inner cell mass of a developing blastocyst and are pluripotent, possessing the capacity to give rise to all 3 germ layers. Progenitor cells, which are derived from more developed fetal or adult tissues, are multipotent, meaning they give rise to more restricted lineages than ES cells. These potential lineages are usually determined by the germ layer of origin. For example, neural progenitor cells (NPCs), or neural stem cells (NSCs), are capable of differentiating to cell types within a neural lineage. NPCs may be derived directly from fetal or adult neural tissue, or by directed differentiation of ES cells via cell culture manipulation. Along with this limited differentiation potential, NPCs also appear to have a more restricted self-renewal potential; although the self-renewal state may be maintained in culture, cells stop proliferating and start to differentiate when transplanted in vivo.

MSCs are an alternative source of multipotent self-renewing cells and are derived from adult bone marrow. Naturally, they differentiate to produce osteoblasts, chondrocytes, and adipocytes; however, there is evidence that they can transdifferentiate to a neural lineage. MSCs provide an accessible alternative to ES cells and potentially circumvent the need for immunosuppression in cellular therapies because they are derived from an autologous source. Using autologous cells, however, may be less desirable when dealing with genetic diseases because the cells may possess the same genetic predisposition to disease. For example, MSCs derived from ALS patients exhibit diminished growth and differentiation capacity; however, these issues may be circumvented by enhancing cell culture techniques and establishing optimal cell passage numbers.

More recently, the development of iPS cells has provided an additional source of autologous stem cells for modeling and treating diseases. iPS cells are generated from somatic tissue such as fibroblasts and are reprogrammed into ES-like cells by the addition of select transcription factors. The original approach utilized Oct 3/4, Klf, Sox2, and c-Myc, and multiple research groups have now accomplished successful reprogramming of fibroblasts using various combinations of factors delivered by vector-, virus-, protein-, or RNA-mediated approaches. Although many neurological disorders rely on complex genetic rodent models or chemical treatments that may not fully represent human neurodegenerative diseases, these cells afford options for disease modeling and provide novel sources for autologous cellular therapies. It should be noted that residual alterations from the genetic reprogramming required to induce pluripotency are possible; therefore, careful characterization of patient iPS lines must be performed. With the continued advancement of iPS technology, however, directed differentiation of patient iPS cells...
may be utilized to model human disease processes for mechanistic and therapeutic discovery.

**Cellular Therapy Strategies and Applications**
Cellular therapies utilize cell or tissue grafts to treat diseases or injury (see Fig 2). Treatment objectives of stem cell therapies typically center on cellular replacement or providing environmental enrichment. Cellular replacement for neurodegenerative diseases involves the derivation of specific neuronal subtypes lost in disease and subsequent grafting into affected areas of the nervous system. The newly transplanted neurons may then integrate, synapse, and recapitulate a neural network similar to that lost in disease. Alternatively, stem cells may provide environmental enrichment to support host neurons by producing neurotrophic factors, scavenging toxic factors, or creating auxiliary neural networks around affected areas. Many strategies for environmental enrichment utilize stem cells to provide de novo synthesis and delivery of neuroprotective growth factors at the site of disease. Growth factors such as glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-I), and vascular endothelial growth factor (VEGF) are protective in neurodegenerative disease models and provide in situ support at the main foci of disease. The appropriate objective of cellular therapy for each neurodegenerative disease must be based on the specific neuronal pathology of each disorder. Whereas cellular replacement may be effective in diseases like PD where a specific neuronal subpopulation is lost, ALS is most likely to benefit from cellular therapies that enrich the local spinal cord environment to support the remaining MNs. Factors such as how well grafted neurons integrate and migrate within the host tissue, and the distances that axons must extend to reach their targets, must be considered when determining the potential efficacy of cellular therapies for neurodegenerative diseases.

**Cellular Therapy for Neurodegenerative Diseases**
Selecting the appropriate stem cell type and understanding the desired mechanism of support is only 1 step in developing and translating cellular therapies to patients. The course from bench to bedside is long and complex; and although each disease and cellular therapy is unique, certain universal issues must be considered for a safe transition to patient therapies.22–24 The Table describes some of these issues that are pertinent to the development of any clinical trial, and describes some of the issues that arise along these lines for stem cell therapies. This transition from bench to bedside may take well over a decade of in vitro, in vivo, and large animal studies (Fig 3). As we acknowledge and overcome these issues, however, advances in the field of translational stem cell therapy will continue to gain momentum. Next, we will discuss the potential for therapeutic success, supported approaches, and current progress in translating stem cells from bench to bedside for specific neurodegenerative diseases.

**AD**
AD, the most frequent form of dementia, is characterized by memory loss and cognitive decline.25 As the disease progresses, there is a widespread loss of neurons and synaptic contacts throughout the cortex, hippocampus, amygdala, and basal forebrain.26 Although the exact pathology of AD remains unclear, pathologic hallmarks include Aβ plaques and neurofibrillary tangles.26,27 There is an increased risk of developing AD with age, and the majority of AD cases are late onset, developing after 65 years of age.28,29 With an increasingly aging population, the burden from AD is anticipated to rise.

Current treatment options for AD are centered on regulating neurotransmitter activity. Enhancing cholinergic function improves AD behavioral and cognitive defects.30 Targeting the cholinergic system using stem cell therapies may provide environmental enrichment. Neurogenesis in the hippocampus decreases as we age and is exacerbated in AD;31,32 therefore, cellular therapies that enhance neurogenesis or replace lost neurons may also delay the progression of AD. Enhancing BDNF levels, which are decreased with age and in AD, promotes neurogenesis and protects neuronal function.33 Rodent AD models receiving NPC grafts demonstrate increased hippocampal synaptic density and increased cognitive function associated with local production of BDNF.19 Similarly, BDNF upregulation along with NPC transplants also improves cell incorporation and functional outcomes in an AD rat model.21 Nerve growth factor (NGF) production is another mechanism of cellular therapy efficacy. Genetically engineered patient fibroblasts that produce NGF are currently being examined in a phase I trial for AD.34,35 Integration of NGF fibroblasts into a major cholinergic center of the basal forebrain provided some benefit to AD patients.34 The Danish company NeGene (http://nsgene.dk/) is currently developing an NGF-releasing therapy using encapsulated epithelial cells. Combining engineered growth factor overexpression with the benefits of NPC integration into neural networks may provide an enhanced approach to treating AD. Furthermore, given the widespread neuronal loss involved in AD pathogenesis, targeting multiple systems simultaneously may be advantageous.
PD

PD results from the progressive loss of DA neurons in the substantia nigra. Patients suffer from severe motor deficits manifesting as tremors, muscle rigidity, and unstable gait and posture. Current treatment options include deep brain stimulation or therapies that aim to increase dopamine levels by providing a dopamine precursor, L-dopa, or providing dopamine agonists. These treatments are effective early in disease to alleviate symptoms, but long-term efficacy is uncertain; they do not correct the deficit, have long-term side-effects, and become increasingly ineffective with PD progression.

Cellular approaches for PD, on the other hand, focus on the replacement of lost DA neurons. Initial cellular therapies for PD utilized fetal ventral midbrain tissue as a source of DA neurons. Clinical trials have had varying degrees of success, but they supported cellular therapies for a potential functional benefit in PD. Potential limitations of utilizing fetal tissue, however, include ethical concerns, and the ability to obtain adequate amounts of tissue for treatment. Alternatively, ES cells offer sources for large-scale production of neurons that acquire a midbrain DA phenotype. Grafting both ES- and MSC-derived DA neurons into rodent PD models results in functional recovery. The ability to produce patient-specific DA neurons has recently been demonstrated using iPS cells.

Transplantation of these cells into a rodent PD model improved functional deficits and demonstrated cell integration in the host tissue. These reports are among the first to demonstrate a therapeutic use for iPS cells in a neurodegenerative disease.

Although studies maintain cellular replacement as a viable approach for treating PD, environmental enrichment may also support existing DA neurons and slow or prevent further degeneration. Growth factor therapy through direct delivery or viral-based systems protects against neuronal decay in PD. MSCs and NPCs engineered to produce growth factors such as BDNF, VEGF, GDNF, and IGF-I provide prolonged local growth factor production in situ. Transplantation of growth factor-producing MSCs and NPCs protects DA neurons and promotes functional recovery in rodent

### TABLE: Common Considerations When Translating Stem Cell Therapies to Neurodegenerative Disease Patients

<table>
<thead>
<tr>
<th>Inclusion/exclusion criteria</th>
<th>Enrolling late stage patients may prevent loss of quality of life</th>
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<tbody>
<tr>
<td></td>
<td>Late stage patients may mask any positive effects due to the intervention occurring too late in the disease course</td>
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<tr>
<td>Realistic expectation</td>
<td>Informed consent forms must clearly illuminate the goals of the study</td>
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<td></td>
<td>Safety trials vs efficacy trials</td>
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<td></td>
<td>Expectations of therapeutic effects based on disease state at intervention</td>
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<tr>
<td>Controlled study</td>
<td>Ideal study is a double-blind placebo study</td>
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<td></td>
<td>Late stage patients may mask any positive effects not observed due to the intervention occurring too late in disease</td>
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<td></td>
<td>Original PD studies offered control arm treatment after a 1-year follow-up, which confuses interpretation of efficacy</td>
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<tr>
<td>Immunosuppression</td>
<td>Although the brain remains an immunologically privileged site due to the blood–brain barrier, there is evidence that this barrier can be compromised in disease</td>
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<td></td>
<td>Studies of cell graft survival demonstrate that immunosuppression increases the survival of graft tissue</td>
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<tr>
<td>Potential side effects</td>
<td>Prevent/minimize potential side effects (ie, meningitis, fever)</td>
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<td></td>
<td>Avoid exacerbation of disease and tumor formation</td>
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<td></td>
<td>Risk vs quality of life</td>
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<tr>
<td>Safety of cellular therapy administration</td>
<td>Consider CNS accessibility and safety of delivery methods</td>
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<tr>
<td></td>
<td>Pros/cons of systemic delivery, lumbar puncture, or stereotactic injection are important</td>
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CNS = central nervous system; PD = Parkinson disease.
models of PD.\textsuperscript{18,48–54} Taken together, the combination of cellular replacement and environmental enrichment may improve the efficacy of cellular therapies for PD.

**HD**

HD is an autosomal dominant polyglutamine disease caused by the accumulation of CAG repeats in the huntingtin gene.\textsuperscript{55} Onset typically occurs in the 4th to 5th decade of life, with a disease course of approximately 20 to 30 years.\textsuperscript{56} HD manifests with involuntary motor activity, dementia, personality changes, and cognitive impairment associated with the progressive loss of medium spiny neurons (MSNs).\textsuperscript{57} Loss of these GABAergic neurons in the striatum is also accompanied by degeneration in the cortex, brainstem, and hippocampus.\textsuperscript{57} Despite the known genetic basis for HD, insight into disease mechanisms and identification of effective therapies remain elusive.

Cellular therapies have provided some of the only positive treatment outcomes for HD. Initial therapies utilized fetal-derived tissue,\textsuperscript{58} and grafting using the whole ganglionic eminence offered an optimal source of MSNs for HD.\textsuperscript{59} The transplantation of neural cells and striatal grafts into rodent HD models demonstrated that MSNs integrate and form circuitry in the host.\textsuperscript{60,61} Translation of fetal tissue grafting into HD patients prompted slight transient improvements and a period of stabilization prior to the inherent decline.\textsuperscript{62} Key issues still remain, based on the ethical implications of utilizing fetal tissues and the dangers associated with cellular therapies such as graft overgrowth and the presence of non-neuronal cells within grafts.\textsuperscript{62} Overall, the relative safety of the technique has been demonstrated in trials for both HD and PD.\textsuperscript{22,23,58,59}

Stem cells also have the potential to restore functional loss of MSNs in HD. Striatal injections of NPCs into HD rodents demonstrated incorporation as well as migration to secondary sites associated with the disease.\textsuperscript{63} The resulting functional improvements confirmed that isolated cell types provide similar functional benefits to those observed with fetal tissue, although mechanisms of cellular therapy protection were not examined. To address the role of environmental enrichment in cellular therapy for HD, NPCs engineered to overexpress GDNF were transplanted into HD rodents. Whereas unmodified NSCs provided no neuroprotective effects, NPCs expressing GDNF protected neurons and promoted functional recovery.\textsuperscript{18,20} This study validates that environmental enrichment and protection of endogenous neurons may lead to functional recovery.

**ALS**

ALS is an adult onset disorder involving the degeneration and loss of MNs. Patients present with loss of coordination and muscle strength with transition to complete loss of muscle control. Death typically results from respiratory failure within 2 to 5 years of diagnosis. Multiple cell types and mechanisms are likely involved in ALS pathogenesis,\textsuperscript{64} which makes the development of conventional drug therapies difficult. Cellular therapies for ALS provide both an integrating neural component and environmental enrichment to support and protect MNs from degeneration.\textsuperscript{16,17} Assessment of several stem cell types, including NPCs and MSCs, in ALS rodent models demonstrates that systemic and direct intraspinal injection ameliorates disease progression,\textsuperscript{5,65–71} suggesting that intervention prior to the irreversible loss of critical MN numbers may improve outcomes. Because it is crucial to protect the remaining MNs in ALS, the ability of stem cells to provide environmental enrichment via GDNF, VEGF, and IGF-I expression has also been examined, as
these growth factors all confer neuroprotection to MNs.\textsuperscript{17} MN axonal degeneration precedes symptom onset and loss of MNs in ALS; therefore, providing distal support to MNs at neuromuscular junctions may also prevent neurodegeneration. Distal production of GDNF in muscle protects neuromuscular junctions and promotes MN protection, likely by retrograde transport.\textsuperscript{71} Overall, the literature supports targeting cellular therapies to maintain MNs in the spinal cord and provide environmental enrichment to MNs and neuromuscular junctions.\textsuperscript{72}

These supporting studies have created the foundation for the first phase I trial for ALS using fetal spinal cord-derived NPCs (http://neuralstem.com/). The cells utilized in the trial integrate safely into the spinal cord, synapse, and interact with host MNs, and also provide a source of growth factors upon direct spinal cord injection in rodents.\textsuperscript{5,73} Delivery optimization and safety was further established in minipigs.\textsuperscript{74,75} NPCs are being delivered to nonambulatory, and ultimately ambulatory, ALS patients through direct lumbar and cervical intraspinal injections to demonstrate the safety of the procedure and lack of toxicity from the cellular therapy. Because cellular therapies for ALS are designed to provide support and enrichment to existing MNs, it is likely that treatment efficacy in future trials will be best examined in earlier stage patients.

**SMA**

SMA involves the selective loss of MNs and presents with a broad range of onset and severity. SMA type I is the leading genetic cause of infantile mortality\textsuperscript{76} and is characterized by early onset severe muscle weakness and fatality within 2 years. SMA is caused by a mutation or loss of the \textit{SMN1} gene,\textsuperscript{77} and the resulting decrease in SMN protein levels contributes to MN loss. In humans, low levels of SMN protein may be produced by alternative splicing variants encoded by the \textit{SMN2} gene. Current pharmaceutical developments and gene therapy treatments focus on regulating \textit{SMN2} to treat SMA. Cellular therapies, however, have been examined in mouse models of SMA, where grafting of ES cell-derived NPCs protected MNs from degeneration and improved survival.\textsuperscript{78,79} It is possible that for SMA, transient rescue of the developmental loss of SMN may be sufficient to confer efficacy, which may not be the case for other neurodegenerative diseases where long-term degeneration of the transplanted cells is a valid concern.

**Future Challenges**

Neurodegenerative diseases create a tremendous societal burden due to their devastating nature, cost, and lack of effective therapies. Cellular therapies offer great promise for the treatment of these diseases, and research progress to date supports the utilization of stem cells to offer cellular replacement and/or provide environmental enrichment to attenuate neurodegeneration. In diseases where specific subpopulations of cells or widespread neuronal loss are present, cellular replacement may reproduce or stabilize neuronal networks. In addition, environmental enrichment may provide neurotrophic support to remaining cells or prevent the production or accumulation of toxic factors that harm neurons. In many cases, cellular therapies provide beneficial effects through both mechanisms.

Many questions still remain unanswered, and certain issues must be addressed as we continue the translation of cellular therapies from the bench to the bedside (see Table). The pathophysiology of each neurodegenerative disease discussed in this review is unique, and thus requires careful attention to the following topics. Which type of cells offers the best approach to treat this disease? What do we expect the stem cells to do, and what outcomes are predicted? How do we anticipate patients early and later in the disease course will respond to treatments? As we begin to design clinical studies that take into account these questions and learn lessons from the trials currently underway, we are poised to maximize the potential of cellular therapies to provide much-needed treatments for neurodegenerative diseases.

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**Potential Conflicts of Interest**


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Optimization of Immunosuppressive Therapy for Spinal Grafting of Human Spinal Stem Cells in a Rat Model of ALS

Michael P. Hefferan,* Karl Johe,† Eva L. Feldman,‡ J. Simon Lunn,‡ and Martin Marsala*

*Neuroregeneration Laboratory, Department of Anesthesiology, University of California-San Diego, La Jolla, CA, USA
†Neuralstem Inc., Gaithersburg, MD, USA
‡Department of Neurology, University of Michigan, Ann Arbor, MI, USA

Previous rodent studies employing monotherapy or combined immunosuppressive regimens have demonstrated a variable degree of spinal xenograft survival in several spinal neurodegenerative models including spinal ischemia, trauma, or amyotrophic lateral sclerosis (ALS). Accordingly, the characterization of optimal immunosuppressive protocols for the specific neurodegenerative model is critical to ensure reliable assessment of potential long-term therapeutic effects associated with cell replacement. In the present study we characterized the survival of human spinal stem cells when grafted into the lumbar spinal cords of a rodent model of ALS, SOD1 (G93A) male and female rats (60–67 days old). Four different immunosuppressive protocols were studied: i) FK506 (q12h); ii) FK506 (qd) + mycophenolate (PO; q12h, up to 7 days postop); iii) FK506 (qd) + mycophenolate (IP; q12h, up to 7 days postop); and iv) FK506 (qd) + mycophenolate (IP; qd, up to 7 days postop). Three weeks after cell grafting the number of surviving human cells was then systematically assessed. The highest density of grafted cells was seen in animals treated with FK506 (qd) and mycophenolate (IP; qd; an average 915 ± 95 grafted cells per spinal cord section). The majority of hNUMA-positive cells colocalized with doublecortin (DCX) immunoreactivity. DCX-positive neurons showed extensive axodendritic sprouting toward surrounding host neurons. In addition, migrating grafted cells were identified up to 500 µm from the graft. In animals treated with FK506 (q12h), FK506 (qd) + mycophenolate (PO; q12h) or FK506 (qd) + mycophenolate (IP; q12h), 11.8 ± 3.4%, 61.2 ± 7.8%, and 99.4 ± 8.9% [expressed as percent of the FK506 (qd) and mycophenolate (IP; qd)] cell survival was seen, respectively. In contrast to animals treated with a combination of FK506 + mycophenolate, robust CD4/8 immunoreactivity was identified in the vicinity of the injection tract in animals treated with FK506 only. These data suggest that a combined, systemically delivered immunosuppression regimen including FK506 and mycophenolate can significantly improve survival of human spinal stem cells after intraspinal transplantation in SOD1 (G93A) rats.

Key words: Amyotrophic lateral sclerosis (ALS); Superoxide dismutase 1 (SOD1); Human spinal stem cells; FK506; Mycophenolate

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a lethal neuro-muscular disease characterized by progressive degeneration of upper and lower motor neurons and corresponding motor and respiratory dysfunction. Approximately 10% of patients have an inherited form of the disease, and of these 20% carry a mutation in the superoxide dismutase 1 (SOD1) gene. Creation of the G93A SOD1 transgenic mouse and rat has provided experimental rodent models of ALS that mimic human disease with comparable progressive motor dysfunction and respiratory failure (17,23).

Despite the discovery of several putative mechanisms underlying the onset and progression of ALS, there are no therapeutic strategies that successfully modify disease progression or outcome (33). Recently, cell-based therapies have emerged as potential treatments for several neurological disorders including ALS (18,28). For example, Xu et al. (34) reported that spinal grafting of human spinal stem cells (hSSCs) to the lumbar spinal cord of G93A SOD1 rats delayed the onset and progression of disease, while also extending the life span by more than 10 days. Glial-derived neurotrophic factor (GDNF)-producing human cortical neural precursors spinaly grafted to G93A rats significantly improved
motoneuron survival (30). However, cell graft survival appears to be inconsistent in the G93A SOD1 spinal cord and seems to greatly depend on the immunosuppression regimen. Yan et al. (35) reported that FK506 monotherapy after hSSCs grafting to the spinal cord of G93A SOD1 mice was inferior compared to combination therapy with rapamycin. Similarly, Suzuki et al. (30) and Klein et al. (19) both reported poor graft survival with cyclosporin A immunosuppression after spinal grafting of human neural progenitors in the G93A rat. Other studies, while reporting positive functional outcomes after spinal cell grafting to rodent ALS spinal cord, did not provide systematic data on cell survival (9,10,34).

In contrast to rodent G93A SOD1 models, spinal grafting of human neuronal or neural precursors in models of spinal trauma or spinal ischemia show much more consistent survival and maturation using similar immunosuppressive protocols (4,5,24,31). The nature of this differential response to identical immunosuppressive therapies is not clear but may reflect several possible mechanisms including distinct types of acute and chronic inflammatory responses. Experimental and clinical data demonstrate a complexity of pathological and resulting inflammatory changes in ALS rodent models and human patients [see review (25)]. Thus, it is possible that the increasingly hostile environment (e.g., inflammatory milieu resulting from cellular degeneration and vascular leakage) present in the spinal cords of rodent ALS models might attenuate graft survival and that a more aggressive immunosuppressive therapy than routinely required in other spinal cord injury models would be needed to achieve consistent graft survival. Accordingly, the current study was designed to optimize an immunosuppressive protocol for the spinal transplantation of hSSCs to presymptomatic G93A SOD1 rats.

MATERIALS AND METHODS

Spinal Cord Implantation of hSSCs

All animal studies were approved by the UCSD institutional animal care and use committee. SOD1 (G93A) ALS male and female rats (UCSD colony, Dr. D. W. Cleveland; 60–67 days old) were anesthetized with isoflurane (1.5–2% maintenance; in room air), placed into a spinal unit apparatus (Stoelting, Wood Dale, IL, USA), and a partial Th12–L1 laminectomy was performed using a dental drill (exposing the dorsal surface of L2–L5 segments). Using a glass capillary (tip diameter 80–100 µm) connected to a microinjector (Kopf Instruments, Tujunga, CA, USA), rats were injected with 0.5 µl (10,000 cells per injection) of the hSSCs in hibernation buffer. The duration of each injection was 60 s followed by a 30-s pause before capillary withdrawal. The center of the injection was targeted into the base of the ventral horn (distance from the dorsal surface of the spinal cord at L3 level: 1.1–1.2 mm) (16). Five injections (~800 µm rostrocaudally apart) were made on each side of the lumbar spinal cord. After injections, the incision was cleaned with penicillin-streptomycin solution and sutured in two layers.

Derivation of the Spinal hSSCs

Human SSCs were prepared from the cervical–upper thoracic region of spinal cord tissue obtained from a single 8-week human fetus after an elective abortion. The fetal tissue was donated by the mother in a manner fully compliant with the guidelines of NIH and FDA and approved by an outside independent review board. The spinal cord tissue was removed of meninges and dorsal root ganglia and dissociated into a single cell suspension by mechanical trituration in serum-free, modified N2 media. The modified N2 media was composed of: 100 mg/L human plasma apo-transferrin, 25 mg/L recombinant human insulin, 1.56 g/L glucose, 20 nM progesterone, 100 µM putrescine, and 30 nM sodium selenite in DMEM/F12. For growth of the hSSCs, 10 ng/ml basic fibroblast growth factor (bFGF) as the sole mitogen was added to the modified N2 media (growth media). The initial culture was serially expanded as a monolayer culture in precoated flasks (T-175) or plates (15). Briefly, the precoated vessels were prepared by incubating them for 1 h at room temperature with 100 µg/ml poly-D-lysine in 10 mM HEPES buffer at 0.165 ml/cm². The vessels were washed three times with water and allowed to completely dry aseptically in the hood. They were then further incubated with 100 µg/ml fibronectin/PBS for 5 min or alternatively 25 µg/ml fibronectin/PBS for 1 h. The fibronectin solution was aspirated and the vessels were used immediately without drying. Approximately 6.1 × 10⁶ total cells were obtained upon the initial dissociation of the spinal cord tissue. All of the cells were plated onto one 150-mm plate in 20 ml of the growth media.

The growth medium was changed every other day and in the alternate days, 10 ng/ml of bFGF was directly added to the culture. The first passage was conducted 16 days after plating. At this point, the culture was composed mostly of postmitotic neurons and mitotic hSSCs. The mitotic cells were harvested by brief treatment with trypsin (0.05% in 0.53 mM EDTA). Trypsin was stopped by addition of soybean trypsin inhibitor to 0.05% final concentration. The cell suspension was triturated with a pipette to obtain a single cell suspension and centrifuged at 1400 rpm for 5 min. The cell pellet was resuspended in growth media and the cells were replated in new precoated plates at 1.2 × 10⁶ cells in 20 ml of growth media per 150-mm plate. The cells were harvested at approximately 75% confluence, which occurred in 5–6 days.
This process was repeated for 20 passages. At various passages, the cells were frozen in the growth medium plus 10% dimethyl sulfoxide (DMSO) at $5 \times 10^6$–$10 \times 10^6$ cells/ml using a programmable freezer. The frozen cells were stored in liquid nitrogen. Upon thawing, the overall viability and recovery was typically 80–95%. The resulting cell line, which was produced by epigenetic means only, using bFGF as the sole mitogen, was named “566RSC.” A cell bank of passage 16 cells was prepared and used for this study.

**Preparation of hSSCs for Implantation**

One day prior to each surgery day, one cryopreserved vial of the previously prepared passage 16 cell bank was thawed, washed, concentrated in a hibernation buffer, and shipped from the cell preparation site (Neuralstem, Inc., Rockville, MD, USA) to the surgery site (UCSD, San Diego, CA, USA) at 2–8°C by overnight delivery. Upon receipt the following day, the cells were used directly for implantation without further manipulation. Before and after implantation the viability of cells was measured with trypan blue (0.4%; Sigma). On average a 75–85% viability rate was recorded.

**Experimental Groups: Immunosuppression Protocols**

Before cell grafting animals were randomly divided into four experimental groups and received immunosuppressive treatment with Prograf (FK506; Astellas Pharma, Deerfield, IL, USA) alone or in temporary combination with Cellcept (mycophenolate mofetil; Roche Pharmaceuticals, Nutley, NJ, USA) as described in Table 1. These groups allowed us to examine the effect of adding oral or intraperitoneal mycophenolate to the FK506 regimen, and for practical reasons, whether once-a-day treatment would be effective.

**Immunohistochemistry**

Three weeks after cell grafting, rats were deeply anesthetized with pentobarbital and phenytoin and transcardially perfused with 200 ml of heparinized saline followed by 250 ml of 4% paraformaldehyde in PBS. The spinal cords were dissected and postfixed in 4% formaldehyde in PBS overnight at 4°C and then cryoprotected in 30% sucrose PBS until transverse sections (30 µm thick) were cut on a cryostat and stored in PBS. Sections were immunostained overnight at 4°C with primary human-specific (h) or nonspecific antibodies made in PBS with 0.2% Triton X-100: mouse anti-nuclear matrix protein/h-nuc (hNUMA; 1:100; Millipore, Temecula, CA, USA); goat anti-doublecortin (DCX; 1:1000; Millipore). Mouse anti-CD4 and anti-CD8 antibodies (1:500; AbD Serotec, Raleigh, NC, USA) were used for identification of T-lymphocyte infiltration. After incubation with primary antibodies, sections were washed three times in PBS and incubated with fluorescent-conjugated secondary donkey anti-mouse, or donkey anti-goat antibodies (Alexa 488, 546; 1:250; Invitrogen Corp., Carlsbad, CA, USA) and DAPI for general nuclear staining. Sections were then mounted on slides, dried at room temperature, and covered with Prolong anti-fade kit (Invitrogen Corp., Carlsbad, CA, USA).

**Quantification of Grafted Cells**

Fluorescent images were captured using a Leica DMLB microscope with a Zeiss AxioCam MRm monochrome camera. For quantification, four sections spanning two injection sites were taken from each animal and stained with hNUMA antibody. Quantification was performed using Image-Pro Plus (v.6.2.0.424; Media Cybernetics Inc., Bethesda, MD, USA), counting only hNUMA-positive nuclei and eliminating cells with less than 30% of the maximum staining intensity. Sections that included an obvious injection tract were excluded from analysis because the tract scar introduced significant artifact in the automated quantification. Sections within two to three sections (60–90 µm) were used. Any postprocessing was done with Adobe CS3 (Adobe Systems, Inc., San Jose, CA, USA) with equal changes to any images being compared.

**Statistical Analysis**

Multiple comparisons between individual grafting groups were performed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls
test. All results are shown as mean ± SEM. A value of $p < 0.05$ was considered to be statistically significant.

**RESULTS**

**Tolerability of Immunosuppressive Treatment**

Preliminary experiments were performed to determine tolerable drug doses for both FK506 and mycophenolate in naive animals. We initially tested four mycophenolate doses: 30, 50, 70, and 100 mg/kg/day. After 2 days of treatment, doses of 50 mg/kg or higher resulted in severe diarrhea in 100% of animals with dramatic weight loss (up to 10% per day) and development of lethargic behavior. A dose of 30 mg/kg/day resulted in mild diarrhea in all animals by 3–5 days of treatment but only mild weight loss (<5% per day) and negligible lethargic behavior. FK506 doses of 1 and 3 mg/kg/day were initially tested in naive animals. Using 3 mg/kg/day resulted in continued body weight decline until treatment was halted.

Using data from the dose tolerance study, it was determined the most suitable drug doses were 30 mg/kg/day mycophenolate and 3 mg/kg FK506. The FK506 dose was lowered to 1 mg/kg/day at 14 days postop due to animal health issues noted in earlier studies using higher doses of FK506 in long-term treatment. At the doses described here, the animals tolerated each immunosuppression regimen well, with the worse side effects being mild diarrhea, which subsided once the mycophenolate was stopped (7 days postop). Long-term FK506 treatment appeared to have no side effects (at 1 mg/kg/day).

**Survival of Grafted Cells in all Experimental Groups**

In the first group (group A) treated with FK506 monotherapy, only sporadic survival of hSSCs was noted in one animal (from five total); detection in other animals was unsuccessful (Fig. 1). In that one animal, the identified hNUMA-immunoreactive (IR) cells appeared near the injection tract, typically within the graft core (Fig. 2A–D). In sharp contrast, animals treated with FK506 monotherapy lacked DCX and hNUMA staining in the injection tracts and putative graft core (Fig. 2E–H). In the one animal (FK506 only) that had sparse hNUMA staining in the injection tract, weak and disorganized DCX-IR structures were sometimes found in the injection sites (Fig. 2I–L).

**CD4/8 immunohistochemistry was used to assess T-lymphocyte infiltration into the spinal cord after cell transplantation.** In group A (FK506 only) injection tracts that were typically void of hNUMA-IR contained large populations of CD4/8-IR cells and no DCX staining (Fig. 3A–C). In sharp contrast, no or minimal CD4/8-IR cells were noted in the other three experimental groups with combined immunosuppression (Fig. 3D). In those latter three groups the injection tracts frequently void of CD4/8-IR consistently were strongly reactive for DCX (Fig. 3D–F).

**DISCUSSION**

Cell-based therapies are emerging as possible treatment options for numerous neurological disorders including spinal cord injury, spinal ischemia, stroke, Parkinson’s disease, and ALS. However, similar to organ transplantation, graft rejection by the host immune system remains an obstacle in achieving long-term survival and maturation of grafted cells. Given the time frame required for the maturation of grafted human neural precursors to fully functional neurons with well-developed synaptic networks (2–3 months), optimal immunosuppressive protocols are required for each disease process to ensure a reliable and accurate assessment of the potential beneficial effects expected after spinal cell grafting.

Our current study was designed to optimize an immunosuppression protocol for spinal grafting of hSSCs to...
the lumbar spinal cord of a rat model of ALS. We used two drugs, as monotherapy or in combination: FK506 (tacrolimus) and mycophenolate. FK506 is a macrolide and acts by binding to the immunophilin FKBP12 (FK506 binding protein); this new complex interacts with and inhibits calcineurin, thus inhibiting both T-lymphocyte signal transduction and IL-2 transcription (22). Mycophenolic acid (active form of mycophenolate mofetil after hepatic metabolism) inhibits inosine monophosphate dehydrogenase, the rate-limiting enzyme for the synthesis of guanine monophosphate in the de novo pathway of purine synthesis used in B- and T-lymphocyte proliferation. In the current study, we found extensive CD4/8 staining near the injection sites in animals that received FK506 only and concurrently no grafted cells were noted at 3 weeks after grafting. While the complexity of xenograft rejection by a host has yet to be fully understood, T lymphocytes appear to play a key role [see review (14)]. The importance of T cells in rejection of neural grafts is exemplified by the long-term survival of transplanted human cells in athymic rats (13,36). The strong CD4/8 staining and lack of hNUMA-IR cells at injection sites in animals treated with FK506 monotherapy suggests that despite the actions of FK506, it is insufficient as a single immunosuppressive agent in ALS rats at the dosing regimen employed in the present study. Addition of mycophenolate dramatically improved hSCC graft survival and concurrently decreased detectable CD4/8-IR. No significant difference in graft survival was noted when the drugs were administered once a day or divided twice daily.

It is interesting that while FK506 has been successfully used as monotherapy for spinal transplantation of human cells in our previous spinal ischemia studies (4,24), it failed in the present and earlier studies involving G93A SOD1 animal models of ALS (35). While not assessed directly in the current study, a possible determining factor of the effectiveness of a given immunosuppressive treatment is the degree and duration of inflammation in the vicinity of grafted cells. In G93A...
SOD1 models of ALS, there is a progressive inflammatory response characterized by the presence of activated microglia and macrophages. Indeed, active inflammation is present from the presymptomatic to the end stages of the disease process (12). Additionally, graft survival is likely affected by infiltration of peripheral immune cells through the compromised blood–brain barrier in ALS-diseased rodents. Recent studies have demonstrated progressive disruption of the blood–brain barrier with vascular leakage and extracellular edema in G93A SOD1 mice (7,8). Zhong et al. (37) also reported progressive blood–spinal cord barrier disruption in SOD1 mice, resulting in microhemorrhages, release of neurotoxic hemoglobin-derived products, and reduced microcircula-

Figure 2. Immunofluorescence staining of human grafted cells (hNUMA green) and doublecortin (DCX red). In FK506-treated rats, hNUMA and DCX were either absent completely (A–D) or only sparsely found (E–H) within the injection site. Large grafts of human origin were easily detected in animals treated with FK506 and mycophenolate (I). A majority of hNUMA-IR nuclei were found within DCX-positive structures, indicating a neuronal phenotype (J–L, and inset). Dashed line indicates an injection tract. Scale bar: 100 µm, and 25 µm for the inset (L).
tion. Similarly, infiltration of several vascular high molecular weight components (such as albumin, IgG, and C3 complement) into the cerebrospinal fluid in ALS patients was reported (1,20).

In contrast to ALS where spinal inflammation continues, likely worsening, until animal end stage, the traumatic-injured spinal cord is typically characterized by an initial acute inflammatory phase (hours to days) followed by a progressive loss of most inflammatory markers from the core of the injury over the following days to months (6). For example, there is at least partial restoration of blood–spinal cord barrier functionality 14–28 days after spinal trauma, as evidenced by a lack of extravasation of large and low molecular weight tracers (26). Interleukin-1β mRNA peaked by 12 h, and returned to levels found in sham-operated mice by 28 days

![Figure 3](image) **Figure 3.** Immunohistochemical staining of T cells (CD4/8) and doublecortin (DCX). In FK506 monotherapy-treated animals, strong CD4/8 staining was noted in the spinal cord near and around all identified injection tracts and these sites were negative for DCX-IR (A–C). However, with combined immunosuppression (FK506 + mycophenolate) extensive DCX-IR was noted throughout the injection sites with elaborate DCX-positive fibers extending from the graft site (E) but were virtually absent of CD4/8-IR (D). Dashed line indicates an injection tract. Scale bar: 100 µm.
after spinal cord contusion injury (27). Thus, it is possible that a differing time course and degree of spinal inflammation noted between mechanical spinal cord injury and experimental/clinical ALS influences cell graft survival and the efficacy of a given immunosuppressive regimen used to prevent cell graft rejection.

Additionally, it is possible that the plasma levels for both drugs have different plasma/clearance profiles and can directly contribute to the failure of the FK506 as monotherapy even if administered twice daily. Previous kinetic studies in rats show that an IV bolus of FK506 (1 mg/kg, bid) resulted in trough concentrations below 10 ng/ml by 6–12 h after injection (29). Therapeutic doses of FK506 have been reported to be 10–20 ng/ml, while doses of 20 ng/ml and higher are associated with neurotoxicity in rats and humans (2,29). Thus, it is possible that FK506 was not effective because the targeted plasma level was only transiently achieved after each injection. Mycophenolate has a significantly longer half-life than FK506, 16–18 h compared to 11 h, respectively (21,32); thus, it is possible that animals receiving mycophenolate benefited mostly from its longer half-life rather than its mechanism of action.

In animals that received combined immunosuppression, sizable human cell grafts were identified that were also intensely immunoreactive for DCX. DCX is a microtubule-associated protein and with few exceptions is exclusively expressed by immature neurons from about embryonic day 10 to about 2–3 weeks after mitosis is complete (3,11). This would suggest that the majority of grafted cells developed a neuronal phenotype by 3 weeks after grafting.

Combined immunosuppression also nearly completely prevented CD4/8 staining in grafted spinal cords. Given that FK506 and mycophenolate have relatively different mechanisms of action, and that the latter can also affect B cell activity, it is likely that these actions complement each other and result in a synergistic interaction. Our results are supported by a previous study by Yan and colleagues (35) that used the same cell line as the current report, and demonstrated that combination therapy of FK506 and rapamycin significantly improved cell graft survival and animal behavior in the G93A mouse model of ALS. Inconsistent cell graft survival using immunosuppression monotherapy has been reported previously. Cyclosporine monotherapy was used by Suzuki et al. after transplanting human-derived cortical neural precursors to the spinal cord of G93A rats and grafts were found in only 3/6 animals at 2 weeks postgrafting and 4/6 animals by 6 weeks. In a similar study the same group described random variability of cell survival while grafts were identified in only 8 of 14 rats (19). In several other cell replacement studies that employ rodent ALS models and report positive results in functional outcome (e.g., improved survival and neurological outcome) no systematic data on the graft survival in individual animals and correlative behavior were provided (9,10,34).

Overall, we demonstrated that the immunosuppression provided by FK506 alone is insufficient to maintain a low population of active T cells after cell transplantation in the G93A ALS rat model. Addition of mycophenolate seemed to supplement the inhibition of T-cell proliferation and leads to a robust graft survival when analyzed at 3 weeks after grafting.

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REFERENCES


8 HEFFERAN ET AL.
Stem cell technology for the study and treatment of motor neuron diseases

Amyotrophic lateral sclerosis and spinal muscular atrophy are devastating neurodegenerative diseases that lead to the specific loss of motor neurons. Recently, stem cell technologies have been developed for the investigation and treatment of both diseases. Here we discuss the different stem cells currently being studied for mechanistic discovery and therapeutic development, including embryonic, adult and induced pluripotent stem cells. We also present supporting evidence for the utilization of stem cell technology in the treatment of amyotrophic lateral sclerosis and spinal muscular atrophy, and describe key issues that must be considered for the transition of stem cell therapies for motor neuron diseases from bench to bedside. Finally, we discuss the first-in-human Phase I trial currently underway examining the safety and feasibility of intraspinal stem cell injections in amyotrophic lateral sclerosis patients as a foundation for translating stem cell therapies for various neurological diseases.

Stem cells: the right tool for the right job

‘Stem cell’ is a term commonly used to describe several distinct cell populations that share specific cellular characteristics. The basic tenets of a stem cell are the ability to self-renew and differentiate into multiple cell types, and there are a range of cell lineages including embryonic stem (ES) cells [1,2], neural progenitor cells (NPCs) [3,4], adult neural stem cells [5], and adult non-neural mesenchymal stem cells (MSCs) [6,7] that differ in their source of derivation and differentiation potential (Figure 1). Pluripotent cells such as ES cells have unrestricted ability to differentiate into cells from all three germ layers, whereas the differentiation of NPCs and MSCs are inherently limited to their respective lineage. While the pluripotency of ES cells and the neuronal differentiation potential of NPCs and neural MSCs are appealing characteristics for the treatment of motor neuron diseases, adult non-neural MSCs are more abundant and more readily isolated than any of the other adult stem cells. These cells provide an option for the development of autologous cellular therapies to circumvent the immunoreactive issues of tissue grafting, but development for the treatment of neurological disorders relies in part on the ability to differentiate across lineages, from mesenchymal to neural. Many papers have described the neuronal differentiation of MSCs to varying degrees [8,9]; however, the generation of MSCs or any other stem cell from a patient with a degenerative disease carries the risk of the resultant population being compromised in some way.

When faced with the question of which stem cells are correct for the treatment of disease, it is likely that there is no right answer. Better questions are what do these particular stem cells offer for a particular disease, and what is the stem cell expected to do? There are multiple ways a stem cell can interact with its environment in the context of motor neuron diseases. Stem cells can offer cellular replacement, augment a cellular population, or provide an enriched extracellular microenvironment [10]. In motor neuron diseases it is unlikely that direct...
replacement of the motor neuron populations will be a viable option. While there are established protocols to purify and enrich stem cells for motor neuron differentiation, there is no evidence that cellular replacement would lead to target muscle innervation [11–15]. In a disease like ALS where the non-neuronal component of disease is perpetuated by glial dysfunction, addition of ‘fresh’ glia to augment the resident glial population may alleviate the stress on the endogenous population [16]. Trophic support by grafted cells through the production of growth factors may also provide first aid to diseased motor neurons [10,17]. Many growth factors have very potent neuroprotective properties and may enrich the microenvironment of the spinal cord [18]. Each individual stem cell line will have a unique signature with respect to its secretion profile and its terminal differentiation capabilities. The strength of stem cell therapy is that the potentially beneficial effects are not mutually exclusive, and both cellular and trophic interventions are afforded by a single treatment.

Exploration of motor neuron disease mechanisms

- Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is an adult-onset, rapidly progressing neurodegenerative disease characterized by the selective loss of both upper and lower motor neurons. ALS has an estimated incidence of 3–5 cases per 100,000 in the USA [18], with approximately 10% of patients affected by familial ALS (fALS). Autosomal dominant fALS is clinically and pathologically indistinguishable from sporadic ALS and both forms are lethal within 3–5 years of onset. Typically, ALS diagnosis occurs very late in the disease course after symptoms present, at a point when a large number of motor neurons are already lost. In 1993, mutations were reported in cytosolic Cu²⁺/Zn²⁺ superoxide dismutase (SOD1) in several fALS families [19] and currently more than 150 missense mutations in SOD1 have been identified [20,21]. Despite over a decade of study, however, the etiology of the toxic gain-of-function associated with mutant SOD1 has remained elusive. Studies have described a plethora of mechanisms including mitochondrial dysfunction, protein transport defects, aggregation, reactive oxygen species, excitotoxic stress, glial and astrocyte dysfunction, and many others [22]. More recently, mutations discovered in the DNA/RNA binding proteins TDP-43 and FUS/TLS have also added a new dimension to the genetic component of ALS and proposed potential mechanisms [23–26]. The growing consensus, however, is that the complex ALS disease phenotype is likely due to contributions from multiple mechanisms.

Traditionally, mutations associated with fALS have been utilized in both rats and mice to develop models for ALS. These rodent models display the same loss of motor neurons observed in human forms of ALS [27,28]. The SOD1-G93A mouse shows disease onset at 180 days, progressive motor defects and muscle weakness, and endstage at approximately 240 days [28]. The SOD1-G93A rat presents a very aggressive disease course, with disease onset around 115 days followed by rapid progression to endstage within 11 days, with associated symptomatic deficits in muscle strength throughout the disease course and motor neuron loss [27]. Finally, TDP-43 mutant mice develop progressive motor neuron, weight and muscle loss and provide an additional model to study ALS pathogenesis [29].

Research over the last decade has produced a single US FDA-approved drug for ALS, riluzole [30]. Since that time, several novel therapeutic approaches with a strong potential to treat ALS have also been investigated. Silencing of mutant SOD1 using a lentivirus-delivered short hairpin to SOD1 delays disease onset and disease progression when given prior to symptom onset in SOD1-G93A mice [31]. The β-lactam antibiotic...
celfriaxone delays motor neuron loss, improves muscle strength and increases survival in the SOD1-G93A mouse, either through increased glutamate transporter expression [32] or by acting as a metal ion chelator [33]. Pioglitazone, an anti-inflammatory/antidiabetic agent, also improves muscle strength and motor neuron survival in SOD1-G93A mice [34]. Both manganese porphyrin and pyruvate treatments have shown beneficial effects on disease progression of the SOD1-G93A mouse [35,36]. Finally, growth factors have been increasingly described for their potential as therapeutic agents. Changes in cerebrospinal fluid levels of growth factors such as IGF-I, VEGF and others have lead to the hypothesis that upregulating and maintaining growth factor levels may provide neuroprotection for motor neurons [37,38]. Indeed, growth factor treatment demonstrates neuroprotective properties on motor neuron survival in vitro and in vivo [39–43]; however, translation to larger animals and to humans has been slow and not yielded the expected outcomes [44–48]. These results could potentially be related to the limited accessibility of such treatments to the motor neurons residing within the spinal cord [43].

- Spinal muscular atrophy

Spinal muscular atrophy is an inherited autosomal disease that presents clinically with a broad range of onset and severity associated with the selective loss of motor neurons within the spinal cord and muscle weakness. Currently there is no effective treatment available for SMA and care options are based either around palliative care, respiratory protocols using Bipap machines, or a tracheotomy to ventilate the patient to assist in breathing. Clinically, there are four distinct forms of SMA [49]. SMA type I is the leading genetic cause of infantile mortality, and is the most severe and common form of SMA with an incidence of 1 in 6000. SMA type I is typically diagnosed within the first 6 months of life and has a poor prognosis, commonly associated with respiratory failure and death within 2 years. Infants present with proximal weakness, poor muscle tone and the inability to support themselves or hold their head up. SMA type II is less severe than type I with a slightly later onset and longer life expectancy, while SMA types III and IV both present with slow, mild muscle weakness and patients exhibit a normal lifespan. Because of the severity of SMA type I and its presentation in such young infants, it is a key disease to target for the development of stem cell therapies.

Over 90% of SMA cases are caused by a homozygous deletion of the survival motor neuron (SMN)1 gene. Despite the fact that most cases of SMA are associated with the lack of a functional SMN1 gene, the lost function of SMN1 that causes disease still remains in debate. In humans, a second copy of SMN exists, SMN2 [50]. SMN2 is identical to SMN1 with the exception of a single nucleotide mutation that prompts alternative splicing of the transcript, resulting in a nonfunctional truncated protein in which exon 7 is deleted. A small proportion of SMN2-derived transcripts do not get alternatively spliced, however, leading to a very small amount of functional full length protein that can partially compensate for the loss of SMN1 [51,52]. Thus, modulating the proportion of SMN2 that gives rise to full-length protein as a means to treat SMA is the subject of multiple studies [51].

Original attempts to create animal models of SMA relied on a knockout approach; however, other animals do not have the same SMN duplication as humans and knockout of SMN results in embryonic lethality [53]. On the other hand, Smn+/− mice that exhibit approximately a 50% reduction in SMN protein levels in the spinal cord resemble the less severe SMA type III with a slow disease progression and later-onset motor neuron loss [54]. Models based on the introduction of human SMN2 on a SMN knockout background (SMN2tg/Smn−/−), however, have been developed that mimic the genetic basis of human SMA type I and circumvent the embryonic lethality of traditional SMN knockout models [55]. The severity of these models is dependent on the dosage of SMN2; mice expressing 1–2 copies survive to postnatal day 5, whereas more than six copies abrogate the SMA phenotype in the mice [56,57]. These models further validate the feasibility of targeting SMN2 expression levels for SMA therapeutic development.

- Induced pluripotent stem cells: the future of disease modeling & therapeutic development

Patient-specific induced pluripotent stem (iPS) cells provide ideal new models for the study of diseases such as SMA and ALS, as they essentially link cell behavior to donor disease phenotypes. iPS cells, which are developed by reprogramming somatic cells back to a pluripotent state, offer numerous advantages for studying disease mechanisms and discovering and developing novel therapies [58–61]. For diseases
like ALS where the majority of cases have no known genetic etiology, iPS cells provide a means to develop models of both familial and sporadic disease. Patient-specific iPS cell lines can then be used to examine disease mechanisms, for drug discovery, or as a means to provide cells for cellular replacement therapy. To generate iPS cells, fibroblasts are isolated after a skin punch biopsy (Figure 1). After fibroblast expansion, multiple methods exist to generate iPS lines. The original reported methodology involves retroviral delivery of a cocktail of four transcription factors known as the Yamanaka factors, consisting of oct3/4, Sox2, c-Myc and Klf4, which are sufficient to change the phenotype of the fibroblasts, reprogramming them back to a stem cell state [62]. iPS cells can then be subsequently differentiated into neurons to generate a new human model of disease. Since the first reports of iPS cells, many groups have worked to improve the technology using different combinations of up to six factors [60, 62–69]. Various viral delivery mechanisms, or alternatively direct protein treatments, have also been examined to circumvent some concerns with genomic incorporation and silencing of the factors, and methods utilizing nonintegrating protocols now exist [60, 62–70]. Some concerns still remain regarding the appropriate protocols for reprogramming factor combinations and method of gene transfer; however, iPS technology is progressing rapidly for the modeling of human diseases and drug discovery.

Induced pluripotent stem cell lines have recently been established from patients with multiple neurodegenerative diseases including Huntington’s disease [71], Parkinson’s disease [72] and Freidreich ataxia [73], as well as from ALS [74] and SMA [75] patients. Dimos et al. described the first iPS cells generated from an 82-year-old ALS patient in 2008 [74]. The patient demonstrated a rare, slowly progressing disease with clear clinical symptoms. This study demonstrated that neither age nor disease state perturbs the ability to generate iPS cells. They further showed that iPS cells could generate motor neurons, although additional assessment of the motor neurons for ALS disease characteristics was not presented. The first iPS cells from a patient with SMA was described a year later when iPS cells were derived from a patient with SMA type I, the rapidly progressing infantile form of SMA [75]. This study revealed the power of SMA iPS cells as a novel disease model, demonstrating multiple SMA characteristics including lowered SMN1 transcript levels and selective reduction of the protein in motor neurons. In addition, these SMA iPS cells reacted to drug treatments that increased SMN levels, thus validating the iPS platform for drug discovery in motor neuron disease. Based on these findings using SMA iPS cell lines, the field is poised for novel drug discovery efforts to identify much-needed treatments for both ALS and SMA.

While iPS cells are an exciting development for disease modeling and potential autologous treatments, there are cautionary notes that need to be considered for such a new technology. The true equivalence of iPS to ES cells is still under investigation, and while genomic analysis has demonstrated highly similar profiles for certain lines [76], some lines appear to only be partially reprogrammed. This is of high importance given that many lines are described only for their morphological appearance, with minimal description of their capability to differentiate into germ layers. There are also differences in the efficiency and variability of neuronal differentiation between lines, making line selection an important consideration for modeling and therapeutic studies [77]. Other potential issues such as genetic stability, point mutations, Yamanaka factor insertion sites, incomplete transgene silencing and global epigenetic modifications highlight the need for full characterization of individual cell lines for further advancement of the field [67, 78–82].

**Stem cell efficacy for motor neuron diseases**

- **Stem cell efficacy for ALS**

Recently, several studies have addressed the potential of stem cells to modulate ALS disease progression (Table 1). Endogenous NPCs, as well as direct spinal injection of MSCs and NPCs, have been examined for their potential to provide cellular protection, growth factor delivery and cellular augmentation. MSCs provide an easily accessible source for autologous cellular replacement therapies in ALS. Systemic delivery and intrathecal injection of MSCs in rodent models of ALS demonstrate integration of a small proportion of cells into the spinal cord [83–86]. Characterization of these MSCs indicate that they are capable of differentiating into both neurons and astrocytes [83, 84, 87]. Furthermore, MSC transplantation in SOD1-G93A mice delays disease progression and motor neuron loss, and also improves lifespan [16, 83, 84, 86–88]. NPCs have also been extensively studied for efficacy in ALS. During the progression of ALS, the endogenous population
Table 1. Stem cell technology utilization in motor neuron disease.

<table>
<thead>
<tr>
<th>Species Type</th>
<th>Genetic Modification</th>
<th>Model</th>
<th>Delivery; treatment age</th>
<th>Disease onset</th>
<th>Survival increase</th>
<th>Outcome</th>
<th>Additional notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES</td>
<td>MN differentiated</td>
<td>Rat</td>
<td>SOD1-G93A</td>
<td>Direct spinal implant; 10 weeks</td>
<td>None</td>
<td>None</td>
<td>Loss of grafted cells by end stage</td>
</tr>
<tr>
<td>BM</td>
<td>N/A</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Intraperitoneal injection; 4 weeks</td>
<td>Delay</td>
<td>13 days</td>
<td>MN protection; differentiation into neurons and contribution to non-neuronal tissue</td>
</tr>
<tr>
<td>BM</td>
<td>GFP tag</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Intra-bone marrow transplantation; 12 weeks</td>
<td>None</td>
<td>~10 days</td>
<td>No MN protection; GFP-positive cells in spinal cord; differentiation into glia; no neuronal differentiation</td>
</tr>
<tr>
<td>BM</td>
<td>Lin-c-kit + specific cells</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Intravenous injection; 70 days</td>
<td>4-day delay</td>
<td>17 days</td>
<td>MN protection, slowed progression; neuronal differentiation</td>
</tr>
<tr>
<td>NPC</td>
<td>Le + CX + specific cells</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Direct spinal implantation; 70 days</td>
<td>Delay</td>
<td>23 days</td>
<td>MN protection; neuronal differentiation</td>
</tr>
<tr>
<td>Primary NPC</td>
<td>GFP</td>
<td>Rat</td>
<td>SOD1-G93A</td>
<td>Intravenous injection; 14, 26 weeks</td>
<td>N/A</td>
<td>N/A</td>
<td>NPC migration to the CNS and differentiation</td>
</tr>
<tr>
<td>GRP</td>
<td>N/A</td>
<td>Rat</td>
<td>SOD1-G93A</td>
<td>Direct spinal implantation; 90 days</td>
<td>None</td>
<td>17 days</td>
<td>MN protection; astrocyte differentiation; decreased microgliosis</td>
</tr>
<tr>
<td>UBC</td>
<td>N/A</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Intravenous injection; 7–8 weeks</td>
<td>Delay</td>
<td>Various</td>
<td>Dose-dependent changes in survival; cytokine regulation</td>
</tr>
<tr>
<td>UBC</td>
<td>N/A</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Intravenous injection; 66 days</td>
<td>Delay</td>
<td>2–3 weeks</td>
<td>Migration to the CNS; neuronal differentiation</td>
</tr>
<tr>
<td>UBC</td>
<td>VEGF, FGF2</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Retro-orbital injection; 24–28 weeks</td>
<td>N/A</td>
<td>N/A</td>
<td>Migration to the CNS; differentiation to microglia and astrocytes</td>
</tr>
<tr>
<td>MSC</td>
<td>GDNF producing</td>
<td>Rat</td>
<td>SOD1-G93A</td>
<td>Intramuscular transplantation; 80 days</td>
<td>None</td>
<td>28 days</td>
<td>Protection of MN in the spinal cord and NMJ integrity</td>
</tr>
<tr>
<td>MSC</td>
<td>Derived from ALS patients</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Injection into cisterna magna; 60 days</td>
<td>None</td>
<td>7 days</td>
<td>Dose-dependent effect on survival; slowed progression</td>
</tr>
<tr>
<td>NPC</td>
<td>VEGF producing</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Intrathecal injection; 70 days</td>
<td>Delay</td>
<td>12 days</td>
<td>Regulation of apoptosis pathway</td>
</tr>
<tr>
<td>NPC</td>
<td>BDNF, IGF-I, VEGF, NT-3, GDNF</td>
<td>Mouse</td>
<td>SOD1-G93A</td>
<td>Injection into cisterna magna; 75 days</td>
<td>None</td>
<td>None</td>
<td>Astrocyte differentiation and MN protection with production of GDNF or IGF-I</td>
</tr>
<tr>
<td>NPC</td>
<td>N/A</td>
<td>Rat</td>
<td>SOD1-G93A</td>
<td>Direct spinal implantation; 62 days</td>
<td>Delay</td>
<td>10 days</td>
<td>Neuronal differentiation, MN protection, delayed progression, growth factor production</td>
</tr>
<tr>
<td>NPC</td>
<td>N/A</td>
<td>Rat</td>
<td>SOD1-G93A</td>
<td>Direct spinal implantation; 56 days</td>
<td>N/A</td>
<td>N/A</td>
<td>Integration and formation of synaptic contacts between graft and host</td>
</tr>
<tr>
<td>NPC</td>
<td>GDNF producing</td>
<td>Rat</td>
<td>SOD1-G93A</td>
<td>Direct spinal implantation; 90/100 days</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>NPC</td>
<td>GDNF producing</td>
<td>Rat</td>
<td>SOD1-G93A</td>
<td>Direct spinal implantation; 70 days</td>
<td>None</td>
<td>None</td>
<td>MN protection</td>
</tr>
</tbody>
</table>

ALS: Amyotrophic lateral sclerosis; BDNF: Bone-derived neurotrophic factor; BM: Bone marrow; GDNF: Glial-derived neurotrophic factor; GFP: Green fluorescent protein; GRP: Glial restricted precursor; ES: Embryonic stem; MN: Motor neuron; MSC: Mesenchymal stem cell; N/A: Not applicable; NMJ: Neuromuscular junction; NPC: Neural progenitor cell; NT: Neurotrophin; UBC: Umbilical cord cell.
of NPCs residing beside the central canal exit quiescence, proliferate, and migrate to the ventral horn of the spinal cord [89]. While this event provides support and cells to the diseased spinal cord, it is likely that there are insufficient cells to mount a viable defense against disease progression. Direct implantation of exogenous stem cells into the diseased spinal cord, however, is likely to have the greatest impact on disease. Studies have demonstrated the ability of astrocytes derived from human NPCs to protect motor neurons from degeneration in SOD1-G93A rats [90], and neurons derived from human NPCs to prevent motor neuron degradation and prolong survival [4,10]. NPCs grafted directly into the ventral horn of the spinal cord of SOD1-G93A rats delayed disease onset by 7 days and prolonged the lifespan by 11 days [4]. The NPCs produced detectable levels of the growth factors glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) within the cerebrospinal fluid and spinal cord. Furthermore, additional characterization demonstrated that the NPCs grafted into the spinal cord and form symmetrical synapses with α-motor neurons and transneuronal transfer between host and grafted neurons was observed, demonstrating functional neural circuitry [91].

Given that the combination of both cellular and trophic intervention provides added benefit in ALS model systems, the efficacy of cellular therapies engineered to produce increased levels of growth factors has also been examined. The protective effects of NPCs derived from human cortical tissue, which were engineered to produce increased amounts of GDNF and primarily give rise to astrocytes upon differentiation, have been examined for their ability to protect motor neurons in the ALS rat [90]. While nonengineered cells did not provide protection, production of GDNF enriched the spinal cord environment and protected motor neurons from cell death. Neuromuscular contacts, however, were still lost and disease onset and survival were not improved. An additional study also supports the transplantation of human NPCs producing either GDNF or IGF-I into the SOD1-G93A mouse, and demonstrates attenuation of motor neuron loss, but did not affect overall survival [92]. To address the potential for distal intervention, however, Suzuki et al. utilized human MSCs as a shuttle to deliver GDNF to the neuromuscular contacts in SOD1-G93A rats [93]. They demonstrated that GDNF delivery to muscle maintained

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### Table 1. Stem cell technology utilization in motor neuron disease.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Species</th>
<th>Genetic modification</th>
<th>Model</th>
<th>Delivery; treatment age</th>
<th>Outcome</th>
<th>Additional notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMA</td>
<td>Mouse</td>
<td>ES</td>
<td>Intra-thal injection; 1 day</td>
<td>N/A</td>
<td>N/A</td>
<td>Neural (including MN) and astrocyte differentiation, MN and NMJ protection, axon projection toward ventral root</td>
</tr>
<tr>
<td>ES</td>
<td>Mouse</td>
<td>Hb9-GFP, ALDHhiSSCsc, neural primed</td>
<td>Intra-thal injection; 1 day</td>
<td>N/A</td>
<td>N/A</td>
<td>Neural (including MN) and astrocyte differentiation, MN and NMJ protection. Growth factor production</td>
</tr>
<tr>
<td>ES</td>
<td>Mouse</td>
<td>Smm-/-, SMN2+/-/+/-, differentiaion</td>
<td>Intra-thal injection; 1 day</td>
<td>N/A</td>
<td>N/A</td>
<td>Neural (including MN) and astrocyte differentiation, MN and NMJ protection, growth factor production, axon projection toward ventral root</td>
</tr>
</tbody>
</table>

**Abbreviations:**
- SMA: Spinal Muscular Atrophy
- ES: Embryonic stem
- MN: Motor neuron
- NMJ: Neuromuscular junction
- NPCs: Neural progenitor cells
- GDNF: Glial-derived neurotrophic factor
- BDNF: Brain-derived neurotrophic factor
- GFP: Green fluorescent protein
- SMN: Survival motor neuron protein
- MSC: Mesenchymal stem cell
- UBC: Umbilical cord cell
- N/A: Not applicable

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**References:**
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- [90] Suzuki et al.
- [91] ALS and Stem Cells - 52
- [92] 2011 Feldman Laboratory Publications
- [93] ALS and Stem Cells - 52

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**Table 1. Stem cell technology utilization in motor neuron disease.**

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Outcome</th>
<th>Additional notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>[95]</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>[94]</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>[93]</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
neuromuscular contacts, which not only promoted increased motor neuron survival, but also an increase in lifespan of up to 28 days. Overall, these results support the need for both cellular and axonal motor neuron treatments for ALS, and provide evidence for the potential efficacy of stem cell therapies for ALS.

### Stem cell efficacy for SMA

Stem cells have recently been examined for their potential for the treatment of SMA (Table I). Mouse ES cell-derived NPCs were examined in mice for efficacy on SMA disease progression [94-95]. ES cells, which were predifferentiated into NPCs and motor neuron progenitors, incorporated into the ventral horn of SMN2g/+;Smn-/-;SMNAT-/- SMA mice after intrathecal injection. The integrated NPCs produced an array of growth factors including GDNF, BDNF, TGF-α and neurotrophin-3. Engrafted animals demonstrated a 39% increase in lifespan which coincided with improved body weight and grip strength, decreased motor neuron and muscle loss and maintained neuromuscular contacts. Given the short time the stem cells have to integrate and mature in the spinal cord, it is somewhat surprising that they had such a dramatic effect. These promising results bode well for the extrapolation of stem cell therapies to larger animals and eventually humans.

Stem cell intervention for motor neuron diseases is most likely to provide support when the cellular therapy can be administered earlier in the disease course. Supporting the existing motor neurons is likely the most critical component to halt or decrease disease progression. In 2009, California Stem Cell, Inc. (CA, USA) announced the completion of a pre-IND meeting with the FDA for a Phase I/II trial to inject human-grade motor neurons derived from human ES cells as a therapy for SMA. ES-derived motor neurons are proposed to alleviate disease by cell replacement and muscle innervation, effects which may be more viable in younger patients, and through cellular support. Any potential stem cell treatment for SMA faces similar issues to those described for ALS with respect to motor neuron replacement; however, neuronal support combined with upregulation of SMN2 may provide an effective avenue for therapy development. Given the genetic background of the disease, treatment combining stem cells and gene therapy may enhance stem cell function and improve therapeutic efficacy.

### Translating stem cell therapy from bench to bedside

Given the increasing body of *in vitro* and *in vivo* data supporting the beneficial nature of NPCs to protect motor neurons in both ALS and SMA, we are at the cusp of the development of new cellular therapies for both diseases. But what does it take to get neuroprotective stem cells from bench to bedside? Here we describe the steps required to make that leap.

### Hurdles to cellular transplantation therapy

While there are abundant data supporting the potential efficacy of cellular replacement therapies in motor neuron disease, there are certain issues that must be considered before designing a trial and translating therapies to patients [96-98]. Selection of the appropriate cell type is imperative to achieve the desired neuroprotective outcomes; appropriate amounts of cells must be accessible and available, and they must exhibit the characteristics that are predicted to confer neuroprotection while avoiding the potential for tumor formation. Furthermore, issues regarding cell graft survival must be examined within the transplanted microenvironment, as well as immune rejection potential. Though some transplantation options such as MSCs and iPS cells involve the use of autologous cells and could circumvent the possibility of rejection, immunosuppression must be utilized for most cellular transplantation therapy options to prevent cellular rejection. Finally, careful attention must be paid to the technical aspects of cell delivery within the nervous system to avoid adverse reactions and surgical complications.

### Supporting data & rationale

Human NPCs are currently being utilized in a Phase I safety trial in ALS patients. The NPCs are derived from a single fetus donated after elective abortion. The upper thoracic region of the spinal cord was dissected out and the cells were expanded to provide a population of human spinal cord stem cells (HSSCs). These HSSCs have been shown to effectively enhance recovery of lost motor function in rats after spinal cord ischemic injury [99] and improve survival up to 20 days in SOD1-G93A rats [4]. Approximately 70% of the HSSCs injected in to the ventral horn of the spinal cord are of a neural lineage and express both glutamatergic and GABAergic neurotransmitter markers [100]. Procedural and HSSC safety was also assessed in a larger animal,
the Gottingen-Minnesota minipig [98,101]. Using the minipig, a dose of 30,000 cells/2–6 µl for injection into the spinal cord was determined to be well tolerated. Delivery of the cells into the spinal cord was accomplished utilizing an innovative device designed to deliver HSSCs that reduces surgical complexity while providing advanced safety and accuracy [98, 102].

The encouraging results of these and other supporting in vitro and in vivo studies provide the rationale and foundation for the first translational trial of HSSCs in ALS patients. The current trial is a Phase I trial with the overall objective of validating the feasibility and safety of direct intraspinal transplantation of stem cells for ALS. While the safety of intraspinal injection and examination of the toxicity of HSSC transplantation are the primary objectives of the current trial, validation of this approach will pave the way for future studies examining the translational efficacy of stem cell transplantation therapy for ALS, and potentially other motor neuron and neurological diseases. We hypothesize, based on the supporting studies, that engrafted cells will provide neuroprotection to diseased motor neurons in ALS patients through direct cellular support, as a source of neurotrophic support, and by maintaining a suitable spinal cord microenvironment for remaining motor neurons (Figure 2B).

**Trial design & surgical procedures**

In total, 18 ALS patients with varying degrees of disease will receive unilateral or bilateral injections of HSSCs into the ventral horn of the lumbar or cervical enlargements of the spinal cord (Figure 2A). Patients are divided into five cohorts (Groups A–E) according to a paradigm we term ‘Risk Escalation’, which maintains the most conservative path with respect to risk to patients as the trial progresses (Figure 2C). Subjects undergo surgery to receive direct intraspinal transplantation of HSSCs following a carefully optimized protocol based on collective results of large animal and human data [98,101,103–107]. Briefly, the surgical procedure includes a laminectomy, mounting of an innovative apparatus designed by our group to stabilize the injection device, controlled injection of HSSCs, and then regimented treatment.

**Figure 2. Clinical trial design examining intraspinal transplantation of human spinal cord stem cells in amyotrophic lateral sclerosis patients.** (A) Amyotrophic lateral sclerosis (ALS) patients will receive unilateral or bilateral human spinal cord stem cell injections in the lumbar or cervical enlargements of the spinal cord (arrows). (B) Proposed mechanism of neuroprotection conferred by grafted cells in ALS patients. As motor neurons normally residing in the spinal cord of ALS patients start to degenerate, injected human spinal cord stem cells are hypothesized to integrate into the spinal cord to provide a source of cellular support, maintain a healthy microenvironment within the spinal cord, and provide neurotrophic support to the remaining motor neurons. (C) The trial will follow a ‘risk escalation’ paradigm, which reflects the gradual increase in risk between the different cohorts. ALS patients are divided into groups A–E based on ambulatory ability, number of injections (unilateral vs bilateral), and injection location (lumbar [L] vs cervical [C]).
postoperative recovery and evaluation. At this point, FDA approval has been obtained for the first 12 patients and progression through the trial groups will occur as subsequent subjects continue to present without major adverse side effects.

**Outcomes & expectations**
To date, the first eight patients have successfully received intraspinal HSSC transplants. The first six patients in Group A, while nonambulatory, did not experience transplant-related loss of sensory or remaining motor function. Similarly, the two patients in Group B did not experience any major adverse effects of the surgery and maintained ambulatory ability. These results are promising, and support the safety of the procedural technique and intraspinal cellular transplantation. While multiple questions remain to be addressed with advancement of the field, we are optimistic that progression through the trial groups and successful completion of the trial will support the utilization of cellular transplantation for therapeutic efficacy in ALS.

**Future perspective**
With the recent advances in stem cell biology, we are poised to move safely, yet rapidly, towards cellular therapies for neurodegenerative disorders. The development of new human models for disease via the generation of patient-specific iPS cells will allow us to more closely relate cellular dysfunction with disease mechanisms. Given the multiple complex mechanisms that can contribute to disease progression in ALS, comparison of individual patient iPS cells may reveal hierarchical targets or subdivisions of disease that can be differentially targeted for pharmacological drug development. Furthermore, safety trials are already complete for MSCs in ALS [108], and with the completion of further safety trials using different cell lines, we will begin to distill which components of stem cell therapies are most advantageous for targeting different aspects of disease. Given what we know regarding the complex mechanisms and consequences of motor neuron loss in ALS, combinational therapies with additional factors or reagents and cell cocktails including NPCs with a propensity to give rise to both glia and neurons will likely afford maximal effectiveness. We must also consider systemic treatment to maintain neuromuscular contacts in conjunction with spinal treatments, since both may be essential for a more complete efficacious therapy. Finally, we must develop better ways of monitoring and visualizing stem cells in situ within the human body to fully understand long-term effects. With a greater understanding of the process of cellular transplantation for motor neuron diseases, we will develop streamlining methods for translational stem cell therapies.

Convincing results in animal models and safety trials for stem cell therapy in humans currently underway are fueling the drive to translate stem cell therapies from the bench to the bedside for multiple neurodegenerative diseases. While the current review discusses the development of stem cell therapies specifically for motor neuron diseases, it should be noted that the principles discussed also extend to the development of stem cell treatments for other neurological disorders and diseases as well. The progress of stem cell technology for neurodegenerative diseases in general has been reviewed elsewhere [61,109,110] and stem cells have been extensively studied for Alzheimer’s disease, multiple sclerosis, Parkinson’s disease and spinal cord trauma [111–113]. In all cases, the cells impact the same three rationales outlined above: to replace lost cells, augment and support cells, or enrich the cellular microenvironment. Indeed, ES-derived oligodendrocytes are currently being utilized in a human trial for spinal cord injury where the proposed mechanisms include cell replacement and environmental enrichment for neuronal growth [114]. Overall, we have made great strides in the translation of stem cell therapies from the bench to patients for motor neuron diseases, and the field of cellular transplantation technology for multiple diseases is primed for continued advancement.

**Acknowledgements**
We thank M Marsala, M Heffran, J Riley, B Raore and J Taub for their technical assistance with the preclinical studies for the trial and we acknowledge the valuable input of K Johe and the Data Safety Monitoring Board of the current trial. We thank M Polak and C Kelly for their clinical assistance and collection of clinical data. We are grateful to the Emory Amyotrophic Lateral Sclerosis Center, patients, and families for their participation.

**Financial & competing interests disclosure**
NM Boulis is the inventor of devices to enable safe and accurate injection of the human spinal cord. NeuralStem Inc. has purchased an exclusive license to this technology. NM Boulis received an inventors share of this fee, and has the right to royalty payments for distribution of this technology. Preclinical studies were funded in part by the A Alfred Taubman Medical
Stem cells: the right tool for the right job
* Individual stem cell lines may have different innate abilities to provide support, and growth factor production may be critical to their function.
* The strength of stem cell therapy lies in its ability to combat multiple aspects of disease treatment in a single therapy.

Exploration of motor neuron disease mechanisms
* The amyotrophic lateral sclerosis (ALS) disease phenotype is complex and motor neuron loss is likely the result of contributions from multiple mechanisms including loss of neurotrophic support and glial dysfunction.
* In spinal muscular atrophy, motor neuron loss is associated with significant decreases in survival motor neuron (SMN)1 protein levels that could potentially be restored by altering the levels of compensatory SMN2 expression.
* Induced pluripotent stem cells provide a system to examine disease mechanisms and test potential therapeutics in a patient-specific manner.

Stem cell efficacy for motor neuron diseases
* A combination of maintenance in the spinal cord and at neuromuscular junctions may be required for a systems approach for the treatment of ALS.
* Stem cell therapy in combination with gene therapy to upregulate SMN2 protein levels may provide cellular support in spinal muscular atrophy.

Translating stem cell therapy from bench to bedside
* In vitro and in vivo data support the efficacy of stem cells for the treatment of ALS, and data from large animals and humans provide an innovative means for human spinal cord stem cell transplantation.
* A Phase I trial is currently underway examining the feasibility and safety of direct intraspinal transplantation of human spinal cord stem cells into ALS patient spinal cords.
* Results to date on the initial patient groups are promising, and with continued success, the trial paves the way for the translation of stem cell therapies for motor neuron diseases and other neurodegenerative diseases as well.

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Papers of special note have been highlighted as:
* of interest
** of considerable interest


** Demonstrates the therapeutic efficacy of human neural stem cells in the amyotrophic lateral sclerosis rat model, which provides support for the use of these cells in the current trial.


 Jablonka S, Schrank B, Krzewski M, Rossoll W, Sendtner M: Reduced survival motor neuron (smn) gene dose in mice leads to motor neuron degeneration: an animal


** Describes the first generation of induced pluripotent stem cells utilizing the Yamanaka factors.


** Demonstrates the generation of induced pluripotent stem cells from an amyotrophic lateral sclerosis patient.


** Demonstrates the generation of induced pluripotent stem cells from a patient with spinal muscular atrophy.


Describes the use of mesenchymal stem cells to deliver therapeutic growth factors in an amyotrophic lateral sclerosis model. It also demonstrates the efficacy of using stem cells in combination with growth factor therapy to protect both motor neurons and neuromuscular junctions.


Demonstrates the use of neurons derived from embryonic stem cells to improve the lifespan of a spinal muscular atrophy mouse model.


Cervical Multilevel Intraspinal Stem Cell Therapy

Assessment of Surgical Risks in Gottingen Minipigs

Bethwel Raore, MD,* Thais Federici, PhD,* Jason Taub, MD,* Michael C. Wu, PhD,† Jonathan Riley, MD,* Colin K. Franz, PhD,* Michele A. Kliem, PhD,* Brooke Snyder, PhD,* Eva L. Feldman, MD,‡ Karl Johe, PhD.§ and Nicholas M. Boulis, MD*

Study Design. Assessment of long-term surgical risks from multiple intraspinal cell injections.

Objective. To prove that multilevel-targeted cell injection to the spinal cord can be a feasible and safe procedure.

Summary of Background Data. Neural cell transplantation has been proposed as a treatment for a variety of neurologic disorders, including degenerative, ischemic, autoimmune, and traumatic etiologies. Among these diseases, the lack of effective treatment for amyotrophic lateral sclerosis has prompted the search for cell-based neuroprotection or motor neuron-replacement therapies.

Methods. Fifteen female minipigs, divided into 3 experimental groups, underwent either 5 or 10 unilateral injections of neural stem cells or 10 vehicle injections into the C3–C5 segments of the spinal cord, using a device and technique developed for safe and accurate injection into the human spinal cord. All animals received intravenous Tacrolimus (0.025 mg/kg) BID during the course of the study. Sensory and motor functions as well as general morbidity were assessed for 28 days. Full necropsy was performed and spinal cords were analyzed for graft survival. This study was performed under Good Laboratory Practice conditions.

Results. Neither mortality nor permanent surgical complications were observed within the 28-day study period. All animals returned to preoperative baseline showing full motor function recovery. Graft survival was demonstrated by immunohistochemistry.

Conclusion. Clinically acceptable neural progenitor survival, distribution, and density were achieved using the number of injections and surgical techniques specifically developed for this purpose.

Key words: microinjection, neural stem cells, spinal cord, cell therapy, pigs. Spine 2011;36:E164–E171

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### TABLE 1. Clinical Protocols for Intraspinal Cell Transplantation

<table>
<thead>
<tr>
<th>Year</th>
<th>Country/Sponsor</th>
<th>Cells</th>
<th>Indication</th>
<th>Delivery</th>
<th>Inclusion Criteria</th>
<th>Status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000–2003</td>
<td>Israel and Belgium (Proneuron biotech.)</td>
<td>Adult autologous macrophages</td>
<td>SCI</td>
<td>Syringe with a 30-gauge fixed needle Hand-held injections at the caudal border of the lesion</td>
<td>Acute complete SCI (ASIA Grade A) between C5 and T11 n = 8</td>
<td>Phase I. Completed</td>
<td>Knoller et al⁴</td>
</tr>
<tr>
<td>2001–2002</td>
<td>China</td>
<td>Cultured fetal OECs</td>
<td>SCI</td>
<td>Free-hand injections (personal communication)</td>
<td>Chronic SCI n = 16</td>
<td>38-mo follow-up report</td>
<td>Huang et al⁵</td>
</tr>
<tr>
<td>2001–2002</td>
<td>Italy</td>
<td>Adult autologous MSCs</td>
<td>ALS</td>
<td>18-gauge cannula Pump injector supported by a table-fixed arm</td>
<td>Definite ALS n = 9</td>
<td>Completed</td>
<td>Mazzini et al⁶</td>
</tr>
<tr>
<td>2003</td>
<td>China</td>
<td>Cultured fetal OECs</td>
<td>SCI</td>
<td>Free-hand injections (personal communication)</td>
<td>Chronic SCI (cervical, thoracic, or thoracolumbar) n = 171</td>
<td>2–8 wk postoperative follow-up report</td>
<td>Huang et al⁷</td>
</tr>
<tr>
<td>2003</td>
<td>China</td>
<td>Cultured fetal OECs</td>
<td>SCI</td>
<td>Hamilton syringe with a 28-gauge beveled needle Stabilized system mounted to the operating table</td>
<td>Chronic complete SCI (ASIA Grade A) between T4 and T10 n = 6</td>
<td>Phase I clinical trial. 1 year follow-up. Ongoing.</td>
<td>Feron et al⁸</td>
</tr>
<tr>
<td>2003–2006</td>
<td>China</td>
<td>Cultured fetal OECs</td>
<td>ALS</td>
<td>Free-hand injections (personal communication)</td>
<td>Probable or definite ALS n = 327</td>
<td>Short-term outcome (4 wk post-operative)</td>
<td>Chen et al⁹</td>
</tr>
<tr>
<td>2004</td>
<td>Australia</td>
<td>Cultured autologous OECs</td>
<td>SCI</td>
<td>Hamilton syringe with a 28-gauge beveled needle Stabilized system mounted to the operating table</td>
<td>Chronic complete SCI (ASIA Grade A) between T4 and T10 n = 6</td>
<td>Phase I clinical trial. 1 year follow-up. Ongoing.</td>
<td>Feron et al⁸</td>
</tr>
<tr>
<td>2007</td>
<td>Spain</td>
<td>Adult autologous BMCs</td>
<td>ALS</td>
<td>Intraspinal Not described.</td>
<td>ALS n = 10</td>
<td>Phase I/I clinical trial. Ongoing</td>
<td>Not published</td>
</tr>
<tr>
<td>2008</td>
<td>Turkey</td>
<td>Adult autologous BMCs</td>
<td>ALS</td>
<td>Intraspinal Not described.</td>
<td>ALS n = 13</td>
<td>1-year follow-up. Ongoing.</td>
<td>Deda et al¹⁰</td>
</tr>
<tr>
<td>2009</td>
<td>United States (Geron Corp.)</td>
<td>Human ESC-derived oligodendrocytes</td>
<td>SCI</td>
<td>Syringe positioning device that attaches to the frame of the operating room table</td>
<td>Subacute complete thoracic SCI (ASIA Grade A)</td>
<td>Phase I. Approved.</td>
<td>(<a href="http://www.geron.com">www.geron.com</a>)</td>
</tr>
</tbody>
</table>

SCI indicates spinal cord injury; MSCs, mesenchymal stem cells; ALS, amyotrophic lateral sclerosis; ASIA, American spinal injury association; OEC, olfactory ensheathing cells; BMCs, bone marrow cells; ESC, embryonic stem cells.

Percutaneous cordotomy and dorsal root entry zone (DREZ) procedures require spinal cord penetration and accurate lesion formation. Similarly, the treatment of syringomyelia requires the insertion of small chronically indwelling canulas into the spinal cord to drain the cyst cavity. Finally, the resection of vascular malformations and tumors often requires surgery in the highly eloquent tissue of the functioning spinal cord tissue.

Cord injections in humans have been performed in a free-hand injection fashion or using table-mounted operating devices. Free-hand intraparenchymal injection represents an ill-advised delivery strategy in humans for a variety of reasons: (1) It cannot reliably reproduce anatomic targeting of the ventral horn; (2) It has an increased potential for spinal cord mass effect from uncontrolled pressure; (3) Movement of the unsteady needle can shear white matter tracts; (4) Finally, it provides an imprecise rate of infusion predisposing the injection to reflux up the catheter. Table-mounted devices are usually designed in combination with micromanipulators and microinjectors, offering better stability, better control of volume and speed of injection, and anatomic precision through 3-dimensional positioning. Despite these advantages,
these systems allow for movement of the patient with respect to the injection needle both during respiration in the prone position, and as a result of inadvertent jostling of the patient. Each of these cases carries significant potential to promote suboptimal efficacy and the generation of significant neurologic morbidity.

Clinical translation of spinal cord approaches still requires optimized means for intraspinal delivery. Limited literature exists addressing intraspinal surgical techniques in large animals.17–19 We have previously demonstrated that single injections of cell suspensions20,21 and viral vectors22 can be safely and accurately performed into the healthy porcine cervical spinal cord, using a microinjection platform specifically designed for this purpose. Our injection platform fixes to the patients, spine rather than the operating table. The platform allows for rostrocaudal displacement to accommodate multilevel/multiple injections. In our studies, surgical risks and morbidity have been assessed in healthy large animals. The size and morphologic similarity of the swine and human spines and spinal cords renders the pig optimal for reliable safety and feasibility studies of grafting approaches in the spinal cord. In the present study, we validate the safety and accuracy of multiple unilateral cervical intraspinal injections and demonstrate postoperative graft survival in Gottingen minipigs.

**MATERIALS AND METHODS**

This study was conducted under Good Laboratory Practices conditions. A Contract Research Organization assured the quality of the data. Surgical procedures, animal care, and collection of tissue were performed at the Saint Joseph’s Translational Research Institute, in Norcross, GA following protocol approved by the Institutional Animal Care and Use Committee at the same institution.

**Study Design**

The study design is summarized in Table 2. Briefly, 15 to 20 kg female Gottingen minipigs (Marshall BioResources, North Rose, NY), divided into 3 groups (n = 5/group), underwent multiple unilateral injections of cells or vehicle (hibernation buffer) into the spinal cord. Groups 1 and 2 received 5 and 10 unilateral injections of cells, respectively. Group 3 received 10 unilateral injections of vehicle.

**Cells**

A human neural stem cell line, NSI-566RSC, derived from the cervical-thoracic cord of a single 8-week human fetus23 was supplied by NeuralStem Inc. (Rockville, MD). Cells from passage 12 were used in this study.

At no more than 36 hours before the scheduled surgeries, live cell suspensions comprising the different densities (Table 2) were prepared in a current Good Manufacturing Practices facility and shipped overnight directly to Saint Joseph’s Translational Research Institute in an insulated shipping container held at 2 to 8°C. After arrival, cell density and viability were confirmed by the Trypan Blue exclusion method (0.4% solution, Sigma, St Louis, MO) and only cell suspensions with >85% viability were used.

**Immunosuppression**

Starting on the day of the surgery, Tacrolimus (Prograf—Astellas Pharma US Inc, Deerfield, IL) at a dose of 0.025 mg/kg, BID, was administered intravenously until euthanasia. A jugular vein 10 French chronic silicone catheter (Access Technologies—Norfolk Medical Inc, Skokie, IL) was placed under general anesthesia for drug administration.

**Additional Medication**

Cefazolin (500 mg/day, IM) was administered 1 day before the surgery and maintained until euthanasia. Buprenorphine (0.01 mg/kg, BID, IM) was given before the surgical procedure and maintained for 3 days. Methylprednisolone was administered as a bolus (125 mg, intravenous) just before the first cell injection and then daily (1 mg/kg, intravenous) during the first 7 postoperative days.

**Surgical Procedure**

**Anesthesia**

Animals were fasted 12 to 16 hours before surgery. Animal sedation and anesthesia induction consisted of a cocktail of IM ketamine 35 mg/kg, xylazine 1 mg/kg, and atropine 0.04 mg/kg. The animals were then intubated and maintained on oxygen and 1% to 3% isoflurane general anesthesia.

**Laminectomy**

Each animal underwent a C3–C5 laminectomy. Briefly, the animal was placed in prone position on a frame custom designed to mimic the patient positioning on a Jackson spinal surgical table which maximize exposure to the spine and minimize pressure on the abdomen and chest, therefore, minimizing epidural venous bleeding. The table uses adjustable slings that are placed under the chest and pelvis of the animal, allowing the abdomen to hang free. The design of the table was generously provided by Martin Marsala.24 The operative field was shaved, washed, and draped. The cervical multilevel C3–C5 laminectomy was then performed as previously described.21,22 Before dural opening, each animal received an intraoperative dose of intravenous methylprednisolone (125 mg). Following dural opening, the microinjection platform was fixed rostrally to the occiput and caudally to the C7 spinous process. Side rails were adjusted to fit the appropriate length between these 2 points. A gondola device bearing a Narishige microdrive

---

**TABLE 2. Experimental Design**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (Graft Site)</th>
<th>Cohort Size (n)</th>
<th>No. Injections</th>
<th>Dose (No. cells/volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C3–C5, unilateral</td>
<td>5</td>
<td>5</td>
<td>2 × 10⁹/µL; 6 µL/inj</td>
</tr>
<tr>
<td>B</td>
<td>C3–C5, unilateral</td>
<td>5</td>
<td>10</td>
<td>2 × 10⁹/µL; 6 µL/inj</td>
</tr>
<tr>
<td>C</td>
<td>C3–C5, unilateral</td>
<td>5</td>
<td>10</td>
<td>None; 6 µL/inj</td>
</tr>
</tbody>
</table>

*inj indicates injection.*

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model MO-97 (Narishige Scientific Instrument Lab, Tokyo, Japan) was placed on the rails (Figure 1). This gondola can be positioned at the appropriate position for the initial injection by sliding along the rails. It is then locked into position. A rigid cannula was placed onto the microdrive and secured approximately 5-mm above the dorsal surface of the spinal cord. The gondola is equipped with a turntable and screw adjustment to allow for positioning at the desired point on the radius of the circle. Thus, any point within the circle created by the rotation of the turntable with the screw set at the maximum radius can be reached. This device allows for microadjustment in the x (mediolateral) and y (rostrocaudal) directions. Finally, the gondola is equipped with a universal joint that allows for correction of the sagittal and coronal angles.10

Cell Injection
The right DREZ was identified under 3.5× surgical loupe magnification and penetrated on an orthogonal trajectory to the cord surface at a point <1 mm medial to the DREZ. The injection rate was programmed to 6 μL/min, using a precalibrated MINJ-PD microINJECTOR pump (Tritech Research, Inc Los Angeles, CA). Doses were administered through the pump connected to a 250 μL Hamilton syringe (Hamilton Co, Reno, NV) and a cannula assembly consisting of a 30-gauge beveled needle attached to 1 meter of silicone tubing. The cell suspension was infused at a depth of 4 mm from pial contact following initial cannula introduction to a depth of 5 mm. By passing to a depth of 5 mm and then pulling back 1 mm, a small reservoir is created to reduce reflux. This maneuver also serves to correct the position of the surface of the cord which is depressed by approximately 1 mm as the cannula passes through the pia in a downward direction. Unilateral microinjections were performed at 2-mm intervals along the rostrocaudal axis at C3–C5 in the 10 injection group, and at 4-mm intervals in the 5 injection group. Following infusion, the cannula was left in place for 1 extra minute to minimize the potential for cell suspension reflux along the cannula track. Following watertight dural closure, muscular and fascial layers were closed using 2–0 and 0 Vicryl sutures, respectively. Skin closure was completed using 3–0 nylon suture.

Postoperative Care
Animals were extubated and monitored for 2 hours following anesthesia recovery. Next, they were transferred to individual cages and monitored at least once daily for food consumption, defecation, and micturition.

Behavioral Assessment
Clinical and behavioral observations were performed and recorded on days 1 through 7, 14, 21, and 28. Behavioral data were collected to assess neurologic morbidity. Sensory function was assessed by presence or absence of a withdrawal response to mechanical stimulus to the toes of front and hind limbs. Motor examination followed the Tarlov score system:

0 = Paralysis; no movement
1 = Perceptible tonus in the hind limbs; slight movement
2 = Movement in the hind limbs, but unable to sit or stand
3 = Ability to stand and walk but ataxic and for short periods
4 = Complete recovery; normal motor function

Euthanasia, Perfusion, and Tissue Collection
Animals were euthanized 28 days after surgery. Following euthanasia, transcardiac perfusion was performed with heparinized saline solution followed by buffered 4% paraformaldehyde solution. The appropriate spinal cord sections were collected for histologic analysis.

Histology
Spinal cords were embedded into gelatin matrix, postfixed, cryoprotected, and sectioned on a freezing-microtome at 40 μm. The transplantation area of the cervical segments and the 2 flanking areas were processed. Sections at 0.24 mm intervals (i.e., every sixth section) were stained with a monoclonal human-nuclei antibody (HuNu, Chemicon, MA) or a polyclonal antibody against a neuronal-specific marker, Doublecortin (DCX; Santa Cruz, CA). HuNu recognizes only human cell grafts and was used for graft identification. Doublecortin (DCX) is a marker of young neurons and was used to further characterize neuronal differentiation of the grafts. DCX is not expressed in adult porcine spinal cord.

Figure 1. Cervical intraspinal microinjection platform. A, The microinjection platform is demonstrated while attached rostrally to the occiput and caudally to the spinous process of C7. B, The platform and microdrive are demonstrated following a C3/4 laminectomy and dural opening before unilateral microinjection into the porcine cervical spinal cord. Adapted from Clinical and Translational Science 2009;2:2. Copyright John Wiley And Sons.

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No statistically significant differences were detected in the behavior outcome between the experimental groups (one-way ANOVA: POD 1 $P = 0.096$; POD 4 $P = 0.27$; POD 7 $P = 0.368$).

No deficits in micturition or defecation were noted.

### Histopathology

#### General Observations

There were a total of 125 needle injections into spinal cords of the 15 pigs from this study. Needle tracks and associated changes occurred primarily in the injected side of the spinal cords. There were no apparent differences among the groups. Responses in all but 2 pigs were generally relatively minor and consisted of changes that would be expected following multiple needle injections. Accordingly, needle tracks with associated hypercellularity were observed in all but 1 control pig, which consisted of microglial cells. Axonal dystrophy (axonal swelling) of minimal or mild severity was present in 3/5, 2/5, and 2/5 pigs in control, low, and high

Intervening slides were stained with antiglial fibrillary acidic protein (Thermo-Fisher, IL), a marker of astrocytes, anti-Iba-1 (WAKO, VA), a marker of microglia, and hematoxylin and eosin to reveal the general condition of the tissues.

### Statistical Analysis

Each experimental group contained 5 animals. Behavior scores of each animal were documented every day and expressed as mean ± standard deviation (SD) for each experimental group. Experimental conditions were compared with a one-way analysis of variance (ANOVA).

### RESULTS

#### Behavior Outcome

All animals returned to their preoperative neurologic baseline (Figure 2) by postoperative day (POD) 7 except for 1 animal in group B. Neither mortalities nor surgical complications were reported in the study.

#### Sensory Function

Sensory function was assessed on days 1 through 7 and then on POD 14, 21, and 28. The toes of the front and hind limbs were progressively compressed with a surgical forceps and the response was scored as present (+) or absent (−). All the animals had adequate withdrawal response before surgery and in the first postsurgical evaluation except for 1 animal in group A which showed an absent withdrawal response in the right hind limb. That animal recovered sensory function by POD 1 and did not show further deficits in sensory function over the 28-day period.

#### Motor Function

The Tarlov score system was used to evaluate motor function. The majority of animals (10 of 15) recovered full motor ability (score 4) by POD 2. The rest of the animals progressively recovered to preoperative baseline by POD 6. Only 1 animal (the same that experienced transient absent sensory response) had difficulty moving the right hind limb after surgery, but recovered to score 3 by POD 7 and completely recovered by POD 14. Despite some variations in the duration of transient neurologic deficits among the 3 groups, all the animals showed full motor function recovery which was maintained over the 28-day period (Figure 3).
dose groups, respectively, in the transplant area; in 1/5 low
dose pigs in the cervical cord cranial to the transplant area;
and in 1/5 and 1/5 pigs in the low and high dose groups,
respectively, in the cervical cord caudal to the transplant
area. More profound lesions occurred at 1 site in each of
2 pigs. These changes consisted of degeneration, necrosis,
and malacia which were present in the lateral and ventral
funiculi from the “region of interest” (ROI) of 1 control pig
and in the dorsal funiculi from the cervical region caudal
to the ROI in 1 high dose pig. They were present in the
white matter of both pigs and were associated with gliosis
and loss of neurons in the adjacent gray matter of the con-
trol pig apparently due to retrograde degeneration. Other
changes included mild nerve fiber degeneration in the cervi-
cal cord cranial to the cervical ROI in 1 low dose pig and
minimal gliosis in 1 low dose pig in the ROI. There were no
changes in the thoracolumbar segments. Glial cells (GFAP-
positive) in and surrounding needle tracks were detected
in all control and treated spinal cords (data not shown).
Reactive microglial cells (Iba-1-positive) were also identi-
cified in these sites. The microglial response was apparently
a reaction to injury from needle punctures, since positive cells
were present in the needle tracks from control cords as well
(data not shown).

**Graft Survival and Differentiation**
HuNu positive cells were present in the transplant areas in
0/5 controls, 5/5 low dose, and 3/5 high dose pigs where they
occurred primarily along needle tracks with some migration
to the immediately surrounding areas. The grafts were gener-
ally concentrated in the intermediate zone and a medial por-
tion of ventral horn (Figure 4A). The grafted cells appeared
to be migrating away from the injection site (Figure 4B).
Graft survival and its neuronal differentiation were also con-
firmed by staining the cord sections with anti-DCX antibody
since adult pig spinal cords do not express DCX. The HuNu
and DCX localization in the cord were identical (Figure 4A,
C). In the 2/5 high dose pigs where HuNu staining was ab-
sent (most likely due to epitope masking from overfixation),
presence of human graft was confirmed by staining with anti-
DCX antibody. Dense DCX stain was detected adjacent to
motor neurons in the ipsilateral ventral horn of the spinal
cord of treated animals (Figure 4D).

**DISCUSSION**
In this study, we have compared safety and feasibility of mul-
tiple intraspinal injections in the cervical spinal cord using
a stereotaxic device newly designed specifically for this pur-
pose. Stable human neural stem cells originally derived from
a fetal spinal cord tissue were injected 10 times at 2 mm or
5 times at 4 mm intervals along 1 side of C3–C5 segments.
There were no functional or pathologic differences between
animals receiving either 10 injections or 5 injections or be-
tween animals receiving 10 cell injections or 10 buffer injec-
tions. Most of the injections were within 1 mm of the intend-
ed target, at or near the ventral horn. Our data demonstrated
that we can safely and accurately target the ventral horn of
the spinal cord. Despite some transient motor dysfunction,
all the animals returned to normal function, demonstrating
the feasibility of multiple unilateral injections into the cer-
vical spinal cord. Histopathology evaluation revealed that,
as expected, there were minor tissue injuries associated with

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**Figure 4.** Graft identification and migration/ differentiation. Two adjacent coronal sec-
tions of a different animal from Group A,
stained with HuNu (A, B) or DCX antibod-
ies (C, D) are shown. B, HuNu staining—
Migrating edge of the grafted cells in the
gray matter; D, DCX staining—Note neurites
from the graft (arrows) extending out and
surrounding motor neurons (*) in the ventral
horn. Scale bars = 1 mm (A, C); 250 μm
(B, D).
Clinical trials have mainly focused on thoracic spinal cord injury (SCI) and amyotrophic lateral sclerosis (ALS) for SCI and ALS. The development of safe and accurate means for spinal cord stem cell transplantation will pave the way for a wide array of therapies for spinal cord conditions, such as SCI, ALS, and multiple sclerosis (MS).

With the emerging promise of stem cell therapies, several trials for SCI and ALS have been launched around the world. Clinical trials have mainly focused on thoracic intraspinal injections based on the sole premise that Phase I safety trials can demonstrate safety and tolerability of the biologic product. In SCI trials to date, human spinal cord injection has been targeted below the level of complete injury. This approach has the advantage of minimizing the potential for exacerbating the neurologic deficit of the patient. It has the disadvantage, however, of making it impossible to detect any injury associated with injection. Thus, these “safety” trials are only capable of detecting formation of tumors, but not deterioration of white or gray matter function at the segmental level of injection. Because SCI is not a terminal disease, histologic evaluation of the graft sites will occur sporadically at best. Thus, these phase I trials are not designed to detect segmental damage. In contrast, in the vulnerable spinal cords of ALS, partial spinal cord injury, and MS patients, segmental damage will be immediately apparent.

Use of large animal models is critical for evaluating safety of combined surgical procedure, cell injection device, and the candidate cell products before clinical trials. Large animal models are also crucial for the understanding of graft rejection. For evaluating safety of intraspinal cell transplantation, minipigs were quite useful due to anatomic similarity and size compared to the adult human spines and spinal cords. While much preclinical proof-of-principle work has been done in rodents, the tolerance of the rodent spinal cord for trauma, and its native capacity for recovery vastly exceeds that of humans. The porcine spinal cord mimicked closely to the minimal tolerance of the human spinal cord to injuries. The healthy minipigs also provided the means to: (1) evaluate sensory, motor, and urinary function after surgery; (2) assess the impact of spinal cord pulsation observed in vivo; (3) address the impact of epidural and intraparenchymal vasculature on the risk of hemorrhage; and (4) evaluate the risk of infection and tolerance of immunosuppressive regimens.

Feasibility of intraspinal cell transplantation is being investigated for treatment of SCI and ALS. Macrophage cell therapy has been suggested to modulate the immune response at the site of the spinal cord injury. Olfactory ensheathing cells and oligodendrocyte precursor cells have been postulated to support myelination of regrowing axons in SCI. Mesenchymal stem cells, which can differentiate into neural cells, as well as gliarestricted precursors have been demonstrated to secrete growth factors and modulate gliosis in ALS. Finally, it has been demonstrated that human embryonic stem cell-derived motor neurons and human neural stem cells are able to differentiate into neurons and form axons and synapses in rodent models of SCI and ALS. In the current study, we used stable neural stem cells (NSI-566RSC) that have been proven to induce growth factor expression and provide segmental neuroprotection as well as functional benefits in animal models of SCI and ALS. In these previous studies, grafted cells formed synaptic contacts on host MN and expressed multiple growth factors, including glial-derived neurotrophic factor, brain-derived neurotrophic factor, and insulin-like growth factor-1. Detailed characterization of survival and neuronal differentiation of NSI-566RSC after intraspinal transplantation in minipigs has been undertaken in previous studies and was not the primary aim of this study. However, after 28 days, there were quantitative survival and robust neuronal differentiation in all grafts. Consistent with the previous studies, each graft occupied approximatively 1-mm sphere in the tissue. Thus, 2-mm graft intervals used in this study represented the maximum frequency reasonable from therapeutic and safety perspectives.

This study has shown the safety of multiple unilateral cervical spinal cord injections. Ideally cell therapy for ALS would require the treatment of the whole motor neuron columns, however, given that death in ALS occurs secondary to respiratory failure and the loss of upper airway control, our rationale is aimed at prolonging life by preserving the phrenic motor neurons and strengthening the proximal upper extremities. We were able to establish clinically acceptable levels of cell density and number of injections using the surgical techniques and devices previously described. Overall, up to 10 unilateral injections of NSI-566RSC at 2 mm intervals into the C3–C5 cervical spinal cord proved to be feasible and safe. Additional refinements of the injection device are ongoing to accommodate lumbar spinal cord injections and perform the injections in a minimally invasive fashion.

Key Points

- Free-hand injections and table-attached devices are currently being used for spinal cord injections but carry significant potential for inconsistent graft targeting leading to suboptimal efficacy in addition to causing neurologic morbidity.
- We have developed a spinal cord microinjection platform designed to facilitate safe and accurate administration of cellular and molecular therapeutics to humans.
- Validation in large animal models is a required step in progressing towards a clinical trial.
- The size and morphologic similarity in spines of the swine and of human beings renders the pig optimal for safety studies in the spinal cord.
- We can accurately target the ventral horn of the spinal cord. Multiple unilateral injections of cells into the cervical spinal cord of minipigs proved to be feasible and safe.
Acknowledgments
The authors thank Neuralstem, Inc. and ALS Association NINDS SK02NS059416.

References
The identification of gene expression profiles associated with progression of human diabetic neuropathy

Junguk Hur,1,2 Kelli A. Sullivan,2 Manjusha Pande,2,3 Yu Hong,2 Anders A. F. Sima,4,5 Hosagrahar V. Jagadish,1,3,6 Matthias Kretzler1,3,7 and Eva L. Feldman1,2,3

Diabetic neuropathy is a common complication of diabetes. While multiple pathways are implicated in the pathophysiology of diabetic neuropathy, there are no specific treatments and no means to predict diabetic neuropathy onset or progression. Here, we identify gene expression signatures related to diabetic neuropathy and develop computational classification models of diabetic neuropathy progression. Microarray experiments were performed on 50 samples of human sural nerves collected during a 52-week clinical trial. A series of bioinformatics analyses identified differentially expressed genes and their networks and biological pathways potentially responsible for the progression of diabetic neuropathy. We identified 532 differentially expressed genes between patient samples with progressing or non-progressing diabetic neuropathy, and found these were functionally enriched in pathways involving inflammatory responses and lipid metabolism. A literature-derived co-citation network of the differentially expressed genes revealed gene subnetworks centred on apolipoprotein E, jun, leptin, serpin peptidase inhibitor E type 1 and peroxisome proliferator-activated receptor gamma. The differentially expressed genes were used to classify a test set of patients with regard to diabetic neuropathy progression. Ridge regression models containing 14 differentially expressed genes correctly classified the progression status of 92% of patients (P < 0.001). To our knowledge, this is the first study to identify transcriptional changes associated with diabetic neuropathy progression in human sural nerve biopsies and describe their potential utility in classifying diabetic neuropathy. Our results identifying the unique gene signature of patients with progressive diabetic neuropathy will facilitate the development of new mechanism-based diagnostics and therapis.

Keywords: biomarkers; diabetic neuropathy; classification model; sural nerve; gene expression

Abbreviations: DAVID = Database for Annotation, Visualization and Integrated Discovery
Introduction

Twenty-five million Americans have diabetes and the incidence is increasing by 5% annually (http://www.diabetes.org). Peripheral neuropathy occurs in ~60% of these patients (Edwards et al., 2008). An additional 57 million Americans have impaired glucose tolerance, or pre-diabetes, and up to 30% of these patients exhibit peripheral neuropathy at diagnosis. Diabetic neuropathy is characterized by progressive loss of peripheral nerve axons, resulting in decreased sensation, pain and eventually complete loss of sensation (Edwards et al., 2008). In patients with diabetic neuropathy, nerve damage precedes the development of noticeable symptoms and diabetic neuropathy is almost never detected until clinically obvious symptoms and signs appear and irreversible damage has already occurred. Despite over 20 years of intense research, there are no effective treatment options to prevent, slow or reverse the progression of diabetic neuropathy other than control of blood glucose levels, and this is not always achievable even in a vigilant patient.

While modest regeneration has been documented in diabetic neuropathy, this regeneration fails over time with resulting loss of peripheral nerve fibres (Kennedy and Zochodne, 2005); therefore, our goal is to identify individuals at high risk of developing diabetic neuropathy in order to develop strategies to prevent or slow disease onset and progression. A number of dysregulated metabolic pathways have been documented in both human patients and experimental models of diabetic neuropathy including changes in osmotic balance within neurons and nerve fibres (Stevens et al., 1993), polyol synthesis and accumulation (Chung et al., 2003), oxidative stress (Vincent et al., 2004), changes in nerve blood flow (Cameron and Cotter, 2001), altered insulin and protein kinase C signalling (Yamagishi et al., 2002), formation of advanced glycation end products (Stern et al., 2002) and cellular inflammation (Wang et al., 2006). In the mid 1990s, many of these pathways were addressed in clinical trials including antioxidant therapies (Ford et al., 2001), aldose reductase inhibitors [tolrestat (Sima et al., 1993), epalrestat (Goto et al., 1995) and sorbinil (Krans, 1992)] and acetyl-carnitine supplementation; however, none were approved in the USA. While some biochemical and symptomatic effects were noted, none of these therapies reversed decreases in nerve conduction velocity or loss of myelinated fibre density.

We are in possession of a unique repository of human nerve biopsies harvested as part of one of these clinical trials. In the initial study, whole sural nerve biopsies, blood chemistries, electrophysiology and sensory data were collected from participants in a large randomized placebo-controlled clinical trial testing acetyl-l-carnitine for diabetic neuropathy treatment (Sima et al., 2005; Wiggins et al., 2009). Acetyl-l-carnitine treatment was efficacious in alleviating pain symptoms; however, no improvement in sural nerve conduction velocities, amplitudes or myelinated fibre density was observed (Sima et al., 2005). Our initial demographic analyses revealed that after correcting for baseline diabetic neuropathy severity and clinical factors, such as gender, age, duration and types of diabetes, insulin treatment, acetyl-l-carnitine treatment, and haemoglobin A1c, only elevated serum triglycerides measured at study onset correlated with diabetic neuropathy progression (Wiggins et al., 2009).

The acetyl-l-carnitine trial took place between 1995 and 1996 and was the first clinical trial to use high-throughput transmission electron microscopy to examine regenerating nerve fibres. At that time, molecular biology tools that are commonly used today were not available. In the current study, we combine modern microarray and bioinformatics techniques to examine the remaining biopsy material and extend the basic biochemical and anatomical analyses that were performed initially. We report the first high-throughput genome-wide expression study of human sural nerve biopsies obtained from patients with diabetic neuropathy to identify genes and pathways altered over the course of diabetes. Gene expression profiles were examined in sural nerve samples from two groups of patients with either fast progressing or slow/non-progressing diabetic neuropathy by high-throughput Affymetrix gene expression microarrays. A series of bioinformatics tools analysed differential gene expression profiles between the two groups and revealed gene networks linked to the progression of diabetic neuropathy. Computational predictive models, based on the expression profiles of selected genes, correctly classified patients as exhibiting either progressing or non-progressing diabetic neuropathy.

It is highly unlikely that future studies of diabetic neuropathy in patients will include the collection of sural nerve biopsies; therefore, it is imperative to fully examine these biopsies using the most current and advanced tools available. To our knowledge, the data presented here provide the first correlative measures of human diabetic neuropathy progression based on gene expression profiles and may be used to explore new pathways underlying disease pathogenesis. The identification of genes encoding secreted proteins may further provide a unique starting point for targeted biomarker development. Our goal is to identify patients at risk for rapid diabetic neuropathy progression and to someday intervene prior to diabetic neuropathy development.

Materials and methods

Human sural nerve samples

Human sural nerve biopsies were obtained during a double-blind, placebo-controlled, 52-week clinical trial of acetyl-l-carnitine treatment of diabetic neuropathy (Sima et al., 2005; Wiggins et al., 2009). Patient screening was described by Greene et al. (1999) and consisted of a medical history and physical examination, a detailed neurological examination and measures of nerve conduction velocity, vibration perception threshold and heart rate variability measurements (Greene et al., 1999). For study inclusion, patients had to be between 18 and 70 years of age, diagnosed with either type 1 or type 2 diabetes mellitus for at least 2 years, glycosylated haemoglobin levels between 7.1% and 14.0% on stable anti-diabetic therapy for 3 months or more and <200% of their ideal body weight with good nutrition (Greene et al., 1999). All patients demonstrated clinically evident, mild to moderate diabetic neuropathy as defined by the presence of at least two neurological findings: (i) symptoms consistent with a distal symmetric polyneuropathy; (ii) signs consistent with a distal symmetric polyneuropathy; and (iii) abnormal nerve function tests including abnormal nerve conduction velocity in at least two
neural or abnormal vibration perception threshold or abnormal heart rate (Greene et al., 1999). Bilaterally detectable sural sensory nerve action potentials and vibration perception threshold in the great toes were required for study inclusion in order to exclude subjects with advanced nerve damage (Greene et al., 1999). Subjects were also excluded for known non-diabetic causes of peripheral neuropathy, unstable pre-proliferative or proliferative retinopathy, serious systemic disease including vasculitis or inflammatory disease, history of carcinoma in the last 5 years, serum creatinine >2.0 mg/dl, creatinine clearance <50 ml/min, proteinuria >3 g/day, or serum uric acid >10 mg/dl (American Diabetes Association, 1988; Greene et al., 1999).

Prior to treatment, whole sural nerve biopsies (5–6 cm, Week 0; denoted as the primary sample) were harvested from the distal calf ~10 cm proximal to the lateral malleolus. The nerve sample was immediately divided into five 1-cm segments; three segments frozen in liquid nitrogen and two segments fixed in 0.1 M cacodylate buffered (pH 7.3) with 2.5% glutaraldehyde and shipped overnight to the University of Michigan Nerve Biopsy Laboratory. Following 52 weeks of treatment, measures of diabetic neuropathy were re-assessed and a second whole sural nerve biopsy from the contralateral leg was harvested (Week 52; denoted as the secondary sample). Frozen nerve segments not used in the initial study were stored at −80°C.

A blood sample was collected at the time of patient enrolment and the following measures were recorded: haemoglobin A1c, haematocrit, serum triglycerides and cholesterol and albumin. These samples were exhausted by the initial analysis and no further blood or urine samples were collected at study conclusion.

Our post hoc analysis classified the patient samples into two groups, progressors and non-progressors, based on myelinated fibre density. Patient samples in the progressor group lost >500 fibres/mm², while patient samples in the non-progressor group lost <100 fibres/mm² over 52 weeks (Wiggin et al., 2009). Primary and secondary biopsies from 36 patients (18 progressors and 18 non-progressors) were used in this study. The selection of patient samples from each group was adjusted for myelinated fibre density at trial onset, insulin treatment, gender and type of diabetes. The use of the human sural nerve samples was approved by the Institutional Review Board for Human Subject Research at the University of Michigan.

RNA preparation for microarray

Total RNA was isolated from a 1-cm segment of each sural nerve biopsy using a commercially available kit (RNeasy® Mini Kit; QIAGEN, Inc.), including an on-column deoxyribonuclease digestion and 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.). Fifty samples (14 primary and 36 secondary) with a minimum RNA integrity number of 6.5 were used for microarray hybridization (Fig. 1).

Affymetrix microarrays

Total RNA (75 ng) from each sample was amplified and biotin labelled using the Ovation Biotin-RNA Amplification and Labelling System (NuGEN Technologies, Inc.) according to the manufacturer’s protocol. The University of Michigan Comprehensive Cancer Centre Affymetrix and Microarray Core Facility (University of Michigan) performed the amplification and hybridization using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Intensities of target hybridization to respective probe features were detected by laser scan of the array. Image files were generated by Affymetrix GeneChip software (MAS5).

Data analysis

Quality assessment and data preprocessing

The Affymetrix CEL files were analysed using a local version of the GenePattern genomic analysis platform from the Broad Institute (http://www.broadinstitute.org/cancer/software/genepattern) (Reich et al., 2006). The samples were Robust Multi-array Average normalized using the BrainArray Custom CDF HGU133Plus2_Hs_ENTREZG version 12 (http://brainarray.mhri.med.umich.edu) (Dai et al., 2005).

Microarray quality was assessed using the probe-level modelling and quality metrics provided by the Affy package of BioConductor (Bolstad et al., 2003; Irizarry et al., 2003; Gautier et al., 2004). Three outlier arrays (two from the primary progressor group and one from the secondary non-progressor group) that did not cluster with other

![Fig. 1](brain.oxfordjournals.org/doi/fig/10.1244/braa.2010.0621)

**Figure 1** Primary and secondary biopsy selection. Primary and secondary biopsies of 36 patients with diabetic neuropathy (DN) were included in this study. Samples with a minimum RNA integrity number (RIN) of 6.5 were used for microarray hybridization. Microarrays of 12 primary (five progressors and seven non-progressors) and 35 secondary (18 progressors and 17 non-progressors) samples were used for differential gene expression analysis and diabetic neuropathy progression modelling. btw = between; P = progressor; NP = non-progressor.
arrays in principal component analysis results were excluded from further analyses.

Identification of differentially expressed genes

Two independent analysis platforms were employed to identify differentially expressed genes between different biological groups (secondary biopsies; progressors and non-progressors): the GenePattern platform using the standard robust multi-array average-based probe set approach and ChipInspector (CI; version 2.1; Genomatix Software GmbH). The robust multi-array average approach averages normalized expression levels across all probes for the gene (probe set level analysis) whereas Genomatix ChipInspector calculates the change in each probe (probe level analysis) (Cohen et al., 2008). Genes were deemed as ‘differentially expressed genes’ using Cyber-T (Baldi and Long, 2001), based on a Bayesian regularized t-test, $P < 0.05$ in the robust multi-array average approach and a false discovery rate (FDR) $< 0.1\%$ using ChipInspector (Cohen et al., 2008) with a minimum of four probes per transcript.

Functional enrichment analyses

The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov) (Huang da et al., 2009a, b) and ConceptGen (http://conceptgen.ncbi.org) (Sartor et al., 2010) were used to identify over-represented biological functions and pathways among the differentially expressed genes.

Network analysis

A gene co-citation network of the differentially expressed genes was generated by Genomatix BiblioSphere (Genomatix Software GmbH) using a sentence level co-citation filter. This network allows visualization of the differentially expressed genes and their potential associations with each other identified in the literature. The topology of the network was further analysed using the Fast Greedy community-structure identification algorithm, implemented in the Cytoscape plug-in Glay (http://brainarray.mbi.uﬂ.edu/sugang/glay) (Su et al., 2010) to identify coherent subnetworks. Identified subnetworks were subjected to functional enrichment analyses by DAVID to reveal over-represented biological functions within each subnetwork.

Computational modelling using gene expression profiles

Differentially expressed gene expression was evaluated for the ability to classify progressors versus non-progressors using ridge regression modelling (Shedden et al., 2008; Ju et al., 2009; Yoshihara et al., 2010). The gene expression profiles of the secondary samples, excluding those samples paired with primary samples, were used as the training set (13 progressors and 11 non-progressors). The expression profiles of the primary samples were used as the testing set (five progressors and seven non-progressors). Three different sets of differentially expressed genes were used in the initial models; Set 1 included all 532 differentially expressed genes, Set 2 contained 63 differentially expressed genes with a minimum fold-change of 1.5, and Set 3 included 10 differentially expressed genes with a minimum fold-change of 2. To identify a set of genes with the least number of genes but with high classification accuracy, the genes from Set 2 were added to Set 3 one at a time until the accuracy of the expanded set reached the maximum level. The statistical significance of each model was evaluated by repeating the classification procedures 1000 times by randomizing class labels.

Array data validation using real-time reverse transcriptase–polymerase chain reaction

The expression of eight differentially expressed genes identified by microarray was confirmed by real-time reverse transcriptase–polymerase chain reaction performed on 10 independent samples from each secondary group (progressor and non-progressor). Reverse transcription was performed using iScript™ cDNA Synthesis kit (Bio-Rad). Real-time polymerase chain reaction amplification and SYBR® green fluorescence detection were performed using iCycler IQ™ Real-time Detection System (Bio-Rad Laboratories). The fluorescence threshold value ($C_T$) was calculated using iCycler iQ™ system software and the levels were normalized to an endogenous reference gene: TATA box binding protein. A Pearson correlation coefficient was calculated for each gene between the log$_2$-transformed expression values as measured by microarray and the negative of the $C_T$ by reverse transcriptase–polymerase chain reaction (Gyorffy et al., 2009).

Results

Sample information

Patient information regarding type and duration of diabetes, gender, body mass index, circulating lipids and measurement of neuropathy (the O’Brien score and myelinated fibre density) is provided in Table 1. The only significant difference between the progressor and non-progressor groups was the change in myelinated fibre density over 52 weeks. Eighty per cent of the study participants had type 2 diabetes and 61% were treated with insulin.

Microarray quality assessment and identification of differentially expressed genes

Fifty samples [14 primary (seven progressors and seven non-progressors) and 36 secondary (18 progressors and 18 non-progressors) samples] met the RNA quality criteria and were hybridized to Affymetrix gene expression microarrays. Excluding two outliers and one mislabelled sample identified during the quality assessment process, 47 microarrays [12 primary (five progressors and seven non-progressors) and 35 secondary (18 progressors and 17 non-progressors)] were used in our analyses (Fig. 1).

The changes in gene expression described below represent changes between secondary biopsies from the progressor ($n = 18$) and non-progressor ($n = 17$) groups. In at least one sample, 14 885 genes were expressed above background. Among them, 558 genes had a Bayesian $P < 0.05$, while 4899 genes had a ChipInspector FDR $< 0.1\%$. Only 532 genes deemed as differentially expressed genes by both methods were included for further analyses. Real-time reverse transcriptase–polymerase chain reaction demonstrated a positive correlation with the microarray data in all of the eight selected differentially expressed genes (Supplementary Table 1).
Table 1 Patient characteristics (n = 36)

<table>
<thead>
<tr>
<th></th>
<th>Non-progression</th>
<th>Progression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Diabetes type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Type 2</td>
<td>15</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Acetyl-l-carnitine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-l-carnitine</td>
<td>11</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Placebo</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Insulin treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.7 ± 12.9</td>
<td>52.2 ± 10.3</td>
<td>0.524</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>10.8 ± 7.2</td>
<td>12.0 ± 7.3</td>
<td>0.622</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>30.0 ± 5.7</td>
<td>31.6 ± 10.3</td>
<td>0.568</td>
</tr>
<tr>
<td>Haemoglobin A1C (%)</td>
<td>8.9 ± 1.6</td>
<td>9.2 ± 1.4</td>
<td>0.449</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.8 ± 0.8</td>
<td>2.7 ± 1.9</td>
<td>0.088</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.5 ± 0.8</td>
<td>5.5 ± 0.9</td>
<td>0.938</td>
</tr>
<tr>
<td>O’Brien score</td>
<td>4179.7 ± 772.2</td>
<td>3854.5 ± 860.1</td>
<td>0.241</td>
</tr>
<tr>
<td>MFD base (fibres/mm²)</td>
<td>5133.2 ± 1139.2</td>
<td>5132.8 ± 1450.8</td>
<td>0.999</td>
</tr>
<tr>
<td>MFD 52 Weeks (fibres/mm²)</td>
<td>5256.8 ± 1200.0</td>
<td>4066.6 ± 1538.7</td>
<td>0.014*</td>
</tr>
<tr>
<td>MFD change (fibres/mm²)</td>
<td>123.6 ± 209.2</td>
<td>–1066.2 ± 391.1</td>
<td>3.84E-13***</td>
</tr>
</tbody>
</table>

Continuous variables are reported as mean ± standard deviation. P-values were calculated by two-sample t-test for continuous variables and Fisher’s exact test for categorical variables (*P < 0.05, ***P < 0.001). MFD = myelinated fibre density.

While acetyl-l-carnitine had no effect on myelinated fibre density or nerve conduction velocity, an assessment of potential effects on gene expression was performed. One hundred and forty-eight and 859 differentially expressed genes were identified between the acetyl-l-carnitine-treated and the non-treated samples in the non-progressing and progressing groups, respectively. Among these, 18 and 73 genes were also detected in the set of 532 differentially expressed genes identified between the progressing and non-progressing groups (~3.4 and 13.7%, respectively). Four genes were common between these sets of genes; however, none exhibit consistent directional change, i.e. either up- or downregulation in both non-progressors and progressors (Supplementary Table 2). We conclude that it is unlikely that the initially identified 532 differentially expressed genes are significantly affected by acetyl-l-carnitine treatment.

Functional enrichment analyses

Functional enrichment analyses of the 532 differentially expressed genes were performed to identify over-represented biological functions using Gene Ontology terms and pathways. DAVID identified 31 and 168 over-represented biological functions among the up- and downregulated differentially expressed genes in the progressor group, respectively (DAVID P < 0.05). Table 2 lists a selected subset of the over-represented biological functions; the upregulated genes in progressors (i.e. upregulated genes in non-progressors) were enriched in ‘extra cellular region’, ‘defence response’ and ‘inflammatory response’ (Table 3), while downregulated genes in progressors (i.e. upregulated genes in non-progressors) were enriched in energy metabolism-related functions such as ‘glucose metabolic process’, ‘peroxisome proliferator-activated receptor (PPAR) signalling pathway’ and ‘regulation of lipid metabolic process’ (Table 4).

ConceptGen identified similar sets of over-represented Gene Ontology terms and pathways, whose associations are visually represented (Fig. 2). ConceptGen also identified Medical Subject Headings terms highly associated with the differentially expressed genes. The downregulated differentially expressed genes were enriched with Medical Subject Headings terms such as lipids, fatty acids, triglycerides, cholesterol and insulin, suggesting decreased energy metabolism in rapidly progressing diabetic neuropathy.

Network analysis

A literature-derived gene network of the differentially expressed genes was created by Bibliosphere based on sentence level co-citations of differentially expressed genes to examine their potential relationships (Fig. 3). This network is composed of subnetworks centred on the five most connected genes: the transcription factor jun (JUN), leptin (LEP), serpin peptidase inhibitor E Type 1 (SERPINE1), apolipoprotein E (APOE) and peroxisome proliferator-activated receptor gamma (PPARγ). The complete network was further analysed by Fast Greedy algorithm (Clauset et al., 2004), implemented in the Cytoscape plug-in GLay (Su et al., 2010) (Supplementary Fig. 1), to cluster the genes into subgroups based on their network structure. Six clusters with a minimum of eight
genes were identified. DAVID identified representative biological functions within each cluster: cell death and inflammatory response (Cluster 1), glucose and lipid metabolism (Cluster 2), cell projection and axonogenesis (Cluster 3), cellular homeostasis and cofactor metabolic process (Cluster 4), cytoskeletal protein binding (Cluster 5), and Wnt receptor signalling pathway (Cluster 6). A simplified network including only nodes with a minimum of five connections is illustrated in Fig. 4.

**Computational modelling of diabetic neuropathy progression**

Ridge regression models were developed to classify the status of patient samples as progressors/non-progressors based on the gene expression profiles (Fig. 5). Both models using differentially expressed gene Set 1 (532 genes) and Set 2 (63 genes) achieved a classification accuracy of 92% (11/12) (Supplementary Table 3). In order to create a model using the smallest set of differentially expressed genes but with the same accuracy, individual differentially expressed genes from Set 2 were sequentially added to Set 3 until the new set achieved the original classification accuracy (92%). Four models of 14 genes achieved an accuracy of 92% (Supplementary Table 4); each model includes the 10 base differentially expressed genes from Set 3 and four additional genes from Set 2 (Table 5). All four models demonstrated $P$-values $<0.001$ from the 1000 repeated classification procedures using randomly shuffled class labels. The positive predictive values of these models ranged from 83% to 100%, and the negative predictive values were from 86% to 100% (Supplementary Table 4).

**Discussion**

Diabetic neuropathy is the most common diabetic complication, affecting up to 60% of patients and contributes significantly to pain, injury, poor wound healing and lower extremity amputation.

### Table 2 Over-represented biological functions in differentially expressed genes

<table>
<thead>
<tr>
<th>Biological function</th>
<th>Gene count</th>
<th>P-value</th>
<th>Enrichment fold</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated in progressors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracellular region</td>
<td>58</td>
<td>6.86E-07</td>
<td>1.9</td>
</tr>
<tr>
<td>Prostanoid metabolic process</td>
<td>4</td>
<td>2.24E-03</td>
<td>14.9</td>
</tr>
<tr>
<td>Defence response</td>
<td>18</td>
<td>5.99E-03</td>
<td>2.1</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>12</td>
<td>6.24E-03</td>
<td>2.6</td>
</tr>
<tr>
<td>Regulation of axonogenesis</td>
<td>5</td>
<td>8.34E-03</td>
<td>6.2</td>
</tr>
<tr>
<td>Response to wounding</td>
<td>15</td>
<td>1.75E-02</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Downregulated in progressors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical homeostasis</td>
<td>20</td>
<td>8.25E-06</td>
<td>3.3</td>
</tr>
<tr>
<td>Glucose metabolic process</td>
<td>11</td>
<td>1.21E-05</td>
<td>6.1</td>
</tr>
<tr>
<td>Glycerolipid metabolic process</td>
<td>11</td>
<td>2.01E-05</td>
<td>5.8</td>
</tr>
<tr>
<td>PPAR signalling pathway</td>
<td>8</td>
<td>7.04E-05</td>
<td>7.6</td>
</tr>
<tr>
<td>Regulation of lipid metabolic process</td>
<td>9</td>
<td>4.93E-05</td>
<td>6.8</td>
</tr>
<tr>
<td>Response to insulin stimulus</td>
<td>8</td>
<td>1.67E-04</td>
<td>6.8</td>
</tr>
</tbody>
</table>

DAVID identified 31 and 168 over-represented biological functions among the up- and downregulated differentially expressed genes (DAVID $P < 0.05$). The table lists a subset of over-represented biological functions regulated in the progressors.

### Table 3 Differentially expressed genes related to defence response and inflammatory response (upregulated genes in progressors)

<table>
<thead>
<tr>
<th>Entrez ID</th>
<th>Symbol</th>
<th>Description</th>
<th>P-value</th>
<th>Fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Defence response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>ADORA2B</td>
<td>Adenosine A2b receptor</td>
<td>0.0133</td>
<td>1.4</td>
</tr>
<tr>
<td>2788</td>
<td>GNG7</td>
<td>Guanine nucleotide binding protein (G protein), gamma 7</td>
<td>0.0336</td>
<td>1.2</td>
</tr>
<tr>
<td>7033</td>
<td>TFF3</td>
<td>Trefoil factor 3 (intestinal)</td>
<td>0.0127</td>
<td>1.5</td>
</tr>
<tr>
<td>23601</td>
<td>CLEC5A</td>
<td>C-type lectin domain family 5, member A</td>
<td>0.0207</td>
<td>1.7</td>
</tr>
<tr>
<td>57817</td>
<td>HAMP</td>
<td>Heparin antimicrobial peptide</td>
<td>0.0025</td>
<td>2.4</td>
</tr>
<tr>
<td>81035</td>
<td>COLEC12</td>
<td>Collectin subfamily member 12</td>
<td>0.0152</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Inflammatory response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>ADORA3</td>
<td>Adenosine A3 receptor</td>
<td>0.0327</td>
<td>1.3</td>
</tr>
<tr>
<td>624</td>
<td>BDKR82</td>
<td>Bradykinin receptor B2</td>
<td>0.0273</td>
<td>1.2</td>
</tr>
<tr>
<td>3075</td>
<td>CFH</td>
<td>Complement factor H</td>
<td>0.0010</td>
<td>1.2</td>
</tr>
<tr>
<td>4282</td>
<td>MIF</td>
<td>Macrophage migration inhibitory factor (glycosylation-inhibiting factor)</td>
<td>0.0282</td>
<td>1.1</td>
</tr>
<tr>
<td>4973</td>
<td>OLR1</td>
<td>Oxidized low density lipoprotein (lectin-like) receptor 1</td>
<td>0.0003</td>
<td>1.6</td>
</tr>
<tr>
<td>7852</td>
<td>CXCR4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
<td>0.0388</td>
<td>1.3</td>
</tr>
<tr>
<td>10344</td>
<td>CCL26</td>
<td>Chemokine (C-C motif) ligand 26</td>
<td>0.0004</td>
<td>3.2</td>
</tr>
<tr>
<td>10630</td>
<td>PDPN</td>
<td>Podoplanin</td>
<td>0.0202</td>
<td>1.2</td>
</tr>
<tr>
<td>25824</td>
<td>PRDX5</td>
<td>Peroxiredoxin 5</td>
<td>0.0200</td>
<td>1.1</td>
</tr>
<tr>
<td>53833</td>
<td>IL2R8</td>
<td>Interleukin 20 receptor beta</td>
<td>0.0185</td>
<td>1.3</td>
</tr>
<tr>
<td>57834</td>
<td>CYP4F11</td>
<td>Cytochrome P450, family 4, subfamily F, polypeptide 11</td>
<td>0.0372</td>
<td>1.4</td>
</tr>
<tr>
<td>148022</td>
<td>TICAM1</td>
<td>Toll-like receptor adaptor molecule 1</td>
<td>0.0379</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Upregulated genes in progressors were enriched in biological functions such as ‘extracellular region’, ‘defence response’ and ‘inflammatory response’. The table lists 18 differentially expressed genes related to ‘defence response’, including 12 ‘inflammatory response’ differentially expressed genes.
Figure 2 A network of over-represented biological concepts identified by ConceptGen. The concepts (gene sets) over-represented in the upregulated genes (A) and downregulated genes (B) in progressors. The centre nodes in violet, titled as ‘GP-CI-Common-SP-SN...’ refer to the differentially expressed genes. DEGs = differentially expressed genes; MeSH = Medical Subject Headings.

Table 4 Differentially expressed genes related to lipid metabolism and PPAR signalling pathway (downregulated genes in progressors)

<table>
<thead>
<tr>
<th>Entrez ID</th>
<th>Symbol</th>
<th>Description</th>
<th>P-value</th>
<th>Fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td>348</td>
<td>APOE</td>
<td>Apolipoprotein E</td>
<td>0.0266</td>
<td>0.8</td>
</tr>
<tr>
<td>2180</td>
<td>ACSL1</td>
<td>Acyl-CoA synthetase long-chain family member 1</td>
<td>0.0379</td>
<td>0.8</td>
</tr>
<tr>
<td>3952</td>
<td>LEP</td>
<td>Leptin</td>
<td>0.0099</td>
<td>0.7</td>
</tr>
<tr>
<td>5140</td>
<td>PDE3B</td>
<td>Phosphodiesterase 3B, cGMP-inhibited</td>
<td>0.0126</td>
<td>0.7</td>
</tr>
<tr>
<td>5468</td>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
<td>0.0426</td>
<td>0.7</td>
</tr>
<tr>
<td>8660</td>
<td>IRS2</td>
<td>Insulin receptor substrate 2</td>
<td>0.0118</td>
<td>0.9</td>
</tr>
<tr>
<td>9370</td>
<td>ADIPOQ</td>
<td>Adiponectin, C1Q and collagen domain containing 2</td>
<td>0.0094</td>
<td>0.7</td>
</tr>
<tr>
<td>51085</td>
<td>MLXIPL</td>
<td>MLX interacting protein-like</td>
<td>0.0083</td>
<td>0.7</td>
</tr>
<tr>
<td>57104</td>
<td>PNPLA2</td>
<td>Patatin-like phospholipase domain containing 2</td>
<td>0.0206</td>
<td>0.8</td>
</tr>
<tr>
<td>100129500</td>
<td>LOC100129500</td>
<td>Hypothetical LOC100129500</td>
<td>0.0485</td>
<td>0.7</td>
</tr>
</tbody>
</table>

- **Regulation of lipid metabolic process**
  - Entrez ID: 348, Symbol: APOE, Description: Apolipoprotein E, P-value: 0.0266, Fold-change: 0.8
  - Entrez ID: 2180, Symbol: ACSL1, Description: Acyl-CoA synthetase long-chain family member 1, P-value: 0.0379, Fold-change: 0.8
  - Entrez ID: 3952, Symbol: LEP, Description: Leptin, P-value: 0.0099, Fold-change: 0.7
  - Entrez ID: 5140, Symbol: PDE3B, Description: Phosphodiesterase 3B, cGMP-inhibited, P-value: 0.0126, Fold-change: 0.7
  - Entrez ID: 5468, Symbol: PPARγ, Description: Peroxisome proliferator-activated receptor gamma, P-value: 0.0426, Fold-change: 0.7
  - Entrez ID: 8660, Symbol: IRS2, Description: Insulin receptor substrate 2, P-value: 0.0118, Fold-change: 0.9
  - Entrez ID: 9370, Symbol: ADIPOQ, Description: Adiponectin, C1Q and collagen domain containing 2, P-value: 0.0094, Fold-change: 0.7
  - Entrez ID: 51085, Symbol: MLXIPL, Description: MLX interacting protein-like, P-value: 0.0083, Fold-change: 0.7
  - Entrez ID: 57104, Symbol: PNPLA2, Description: Patatin-like phospholipase domain containing 2, P-value: 0.0206, Fold-change: 0.8
  - Entrez ID: 100129500, Symbol: LOC100129500, Description: Hypothetical LOC100129500, P-value: 0.0485, Fold-change: 0.7

- **PPAR signalling pathway**
  - Entrez ID: 948, Symbol: CD36, Description: CD36 molecule (thrombospondin receptor), P-value: 0.0373, Fold-change: 0.7
  - Entrez ID: 2180, Symbol: ACSL1, Description: Acyl-CoA synthetase long-chain family member 1, P-value: 0.0379, Fold-change: 0.8
  - Entrez ID: 4199, Symbol: ME1, Description: Malic enzyme 1, NADP(+)-dependent, cytosolic, P-value: 0.0181, Fold-change: 0.8
  - Entrez ID: 5105, Symbol: PCK1, Description: Phosphoenolpyruvate Carboxykinase 1 (soluble), P-value: 0.0497, Fold-change: 0.8
  - Entrez ID: 5346, Symbol: PLIN, Description: Perilipin, P-value: 0.0092, Fold-change: 0.7
  - Entrez ID: 5468, Symbol: PPARγ, Description: Peroxisome proliferator-activated receptor gamma, P-value: 0.0426, Fold-change: 0.7
  - Entrez ID: 6319, Symbol: SCD, Description: Stearoyl-CoA desaturase (delta-9-desaturase), P-value: 0.0241, Fold-change: 0.7
  - Entrez ID: 9370, Symbol: ADIPOQ, Description: Adiponectin, C1Q and collagen domain containing 2, P-value: 0.0094, Fold-change: 0.7

Downregulated genes in progressors were over-represented with energy metabolism-related functions such as ‘glucose metabolic process’, ‘PPAR signalling pathway’ and ‘regulation of lipid metabolic process’. The table lists differentially expressed genes related to ‘regulation of lipid metabolic process’ and ‘PPAR signalling pathway’.
The pathogenesis of diabetic neuropathy is complex and includes hyperglycaemia-induced oxidative stress and deranged polyol metabolism, changes in nerve microvasculature, decreased growth factor support and dysregulated lipid metabolism (Edwards et al., 2008; Figueroa-Romero et al., 2008). Addressing these deficits alone or in combination has yet to result in effective diabetic neuropathy treatment, confirming that an increased understanding of the mechanisms underlying the onset and progression of diabetic neuropathy is of prime importance.

The current study takes an important first step towards this goal by identifying specific sets of genes whose expression accurately classifies patient samples with regard to diabetic neuropathy progression and by analysing their interactions within known cellular pathways. Identifying common elements in these complex networks will yield novel insights into disease pathogenesis, provide new therapeutic targets and identify potential diabetic neuropathy biomarkers. The genes identified in the current study confirm data gathered from experimental models of diabetes and provide a comprehensive picture of the expression of multiple targets in a single human tissue sample.

Our initial analyses of this data set classified the patient samples based on myelinated fibre density and found that two large groups emerged; those with a loss of myelinated fibre density ≥500 fibres/mm² over 52 weeks (progressors) and those whose myelinated fibre density was relatively stable (myelinated fibre density loss ≤100 fibres/mm² over 52 weeks, non-progressors) (Wiggin et al., 2009). We examined sural nerve biopsies from two groups of diabetic neuropathy patients (progressors and non-progressors) to discover differences in gene expression that could account for the differences in their clinical course. Gene expression profiling in damaged peripheral nerves by diabetic neuropathy or axotomy has been explored in experimental rodent models (Renaud et al., 2005; Bosse et al., 2006; Price et al., 2006; de Preux et al., 2007; Karamoysoyli et al., 2008) but has yet to be examined in humans. These studies indicate...
that changes in gene expression in injured peripheral nerves are similar to the over-represented biological functions (inflammation and energy metabolism) reported in the current study.

Multiple cell types are affected in diabetic complication-prone tissues. Peripheral nerves contain the cellular extensions (axons and dendrites) of both sensory and motor neurons and their ensheathing glia, the Schwann cells. Other components include fibroblasts, capillary endothelial cells and a complex extracellular matrix. The majority of messenger RNA isolated from any peripheral nerve will be that generated by Schwann cells. Schwann cells are a target of hyperglycaemia and diabetes results in Schwann cell damage in part due to altered axon integrity and defective growth factor signalling (Yu et al., 2008; McGuire et al., 2009). In addition, inflammatory pathways including advanced glycation end products/receptor for advanced glycation end products (AGE/RAGE) signalling in axons and Schwann cells are reported to contribute to nerve damage (Lukic et al., 2008).

The upregulated differentially expressed genes in progressors were enriched with ‘defence response’ and ‘inflammatory response’. Inflammation-associated molecules such as chemokines and cytokines are implicated in the development and progression of both diabetic nephropathy and diabetic neuropathy (Rivero et al., 2009). Among these inflammation genes, bradykinin receptor B2 (BDKRB2) is of particular interest. BDKRB2 regulates the expression of genes involved in progressive glomerulosclerosis such as transforming growth factor beta 1 (TGF-β1) and p53 (Kakoki et al., 2006). We recently reported that type 1 diabetic mice with dysregulated BDKRB2 developed enhanced nephropathy and diabetic neuropathy (Kakoki et al., 2010). Membrane-associated adenosine A3 receptor (ADORA3), is also implicated in the pathogenesis of diabetic nephropathy (Pawelczyk et al., 2005). Thus, the upregulation of cytokines, chemokines and genes such as DBKRB2 and ADORA3 in our study (Table 3) suggests enhanced inflammation and dysregulated defence responses, thus contributing to more substantial nerve damage in patients.

Figure 4 Gene co-citation network clustered by fast-greedy community structuring algorithm. The complete co-citation network of the differentially expressed genes was clustered based on network topology by Fast Greedy algorithm implemented in the Cytoscape GLay plug-in (Supplementary Fig. 1). The figure contains only the nodes with a minimum of five connections (edges) in the complete network. The size of node represents the number of edges. Nodes (genes) highlighted in red or yellow refer to the highly connected genes; red for the central genes in the initial BiblioSphere co-citation network (Fig. 3) and yellow for additionally identified highly connected genes in the GLay-identified clusters.
with progressive diabetic neuropathy. It is not yet clear if these cytokines and chemokines are expressed by the Schwann cells or by infiltrating macrophages (Sommer et al., 2005), which may interact with each other in injury and demyelinating diseases (Martini et al., 2008). Regardless, our data raise the intriguing idea that the inflammatory response should be further explored as a new therapeutic target for diabetic neuropathy.

The downregulated differentially expressed genes in the progressors were enriched with biological functions related to energy metabolism including ‘glucose metabolic process’ and ‘PPAR signalling pathway’ (Table 4). Among these differentially expressed genes, PPARγ, encoding a nuclear receptor for glitazones, plays a key role in regulating glucose and lipid metabolism (Duan et al., 2009). Agonists of PPARγ are effective in ameliorating diabetic neuropathy and nephropathy in animal models (Maeda et al., 2008; Yamagishi et al., 2008). Another key gene is APOE, encoding an apolipoprotein, which regulates the normal catabolism of triglycerides and cholesterol. A polymorphism of this gene is linked to the progression of diabetic nephropathy (Li et al., 2010). Decreased levels of PPARγ and APOE as well as other lipid metabolism-related differentially expressed genes correlates with the increased levels of serum triglycerides confirming our recent

Table 5 The gene content of the four computational models with 14 genes

<table>
<thead>
<tr>
<th>Model#</th>
<th>EntrezID</th>
<th>Symbol</th>
<th>Description</th>
<th>Cyber-T P-value</th>
<th>Fold-change</th>
<th>Detectable biofluids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>1469</td>
<td>CST1</td>
<td>Cystatin SN</td>
<td>0.0349</td>
<td>10.0</td>
<td>Saliva</td>
</tr>
<tr>
<td></td>
<td>10804</td>
<td>GJB6</td>
<td>Gap junction protein, beta 6, 30 kDa</td>
<td>0.0011</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10344</td>
<td>CCL26</td>
<td>Chemokine (C-C motif) ligand 26</td>
<td>0.0004</td>
<td>3.2</td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td>10647</td>
<td>SCGB1D2</td>
<td>Secretoglobin, family 1D, member 2</td>
<td>0.0033</td>
<td>2.9</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>57817</td>
<td>HAMP</td>
<td>Hepcidin antimicrobial peptide</td>
<td>0.0025</td>
<td>2.4</td>
<td>Blood, urine</td>
</tr>
<tr>
<td></td>
<td>6036</td>
<td>RNASE2</td>
<td>Ribonuclease, RNase A family, 2</td>
<td>0.0088</td>
<td>2.4</td>
<td>Blood, urine</td>
</tr>
<tr>
<td></td>
<td>3860</td>
<td>KRT13</td>
<td>Keratin 13</td>
<td>0.0315</td>
<td>2.4</td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td>4741</td>
<td>NEFM</td>
<td>Neurofilament, medium polypeptide</td>
<td>0.0036</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1412</td>
<td>CRYBA2</td>
<td>Crystallin, beta A2</td>
<td>0.0065</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80763</td>
<td>C12orf39</td>
<td>Chromosome 12 open reading frame 39</td>
<td>0.0057</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Additional</td>
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<td>1381</td>
<td>CRABP1</td>
<td>Cellular retinoic acid binding protein 1</td>
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<td>1.8</td>
</tr>
<tr>
<td></td>
<td>11341</td>
<td>SCRGR1</td>
<td>Stimulator of chondrogenesis 1</td>
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<td>1.7</td>
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<tr>
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<td>4</td>
<td>163933</td>
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<td>Olfactomedin 4</td>
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<td>1.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>55825</td>
<td>PECR</td>
<td>Peroxisomal trans-2-enoyl-CoA reductase</td>
<td>0.0043</td>
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<td></td>
<td>1</td>
<td>51085</td>
<td>MLXIPL</td>
<td>MLX interacting protein like</td>
<td>0.0083</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>153918</td>
<td>FAM164B</td>
<td>Family with sequence similarity 164, member B</td>
<td>0.0052</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3112</td>
<td>HLA-DOB</td>
<td>Major histocompatibility complex, class II, DO beta</td>
<td>0.0356</td>
<td>0.6</td>
</tr>
<tr>
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<td>1</td>
<td>56605</td>
<td>ERO1LB</td>
<td>ERO1-like beta (S. cerevisiae)</td>
<td>0.0014</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>225</td>
<td>ABCD2</td>
<td>ATP-binding cassette, subfamily D (ALD), member 2</td>
<td>0.0054</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Each model demonstrating a classification accuracy of 92% includes 10 base differentially expressed genes from Set 3 and 4 combinations of 11 differentially expressed genes from Set 2. The Metabolomics analysis package (Biomarker Filter) from Ingenuity Pathway Analysis (http://www.ingenuity.com) revealed that nine of them are detectable in saliva, blood or urine.
finding that altered lipid metabolism may play a role in the progression of diabetic neuropathy (Wiggin et al., 2009). Further experimental work is required to determine how altered lipid metabolism influences the progression of diabetic neuropathy.

We have demonstrated that increased glucose metabolism results in increased oxidative stress, mitochondrial dysfunction and cell death in both in vitro and in vivo models of diabetic neuropathy (Vincent et al., 2004; Russell et al., 2008). In the current study, genes involved in glucose metabolism are downregulated in progressors, which is counter-intuitive to what we and others have reported for sensory neurons. Yet, considering that the majority of sural nerve RNA originates from Schwann cells, these data do support what we and others have reported regarding Schwann cells i.e. that they are resistant to hyperglycaemia-induced cell death and exhibit an enhanced antioxidant capacity (Delaney et al., 2001; Vincent et al., 2009). Our data also imply that sensory neurons and Schwann cells likely employ different pathways when presented with metabolic stressors such as hyperglycaemia.

Although functional enrichment analyses identify overrepresented biological functions, they do not reveal how these genes interact with each other. To obtain a global view of the network, we examined gene interaction networks based on literature-derived co-citation information (Figs 3 and 4). Although co-citation of two genes in a single sentence does not necessarily indicate there is a direct interaction, this process may reveal novel associations and lend new insights into function (Schmelzer et al., 2008). In the current study, the BiblioSphere co-citation network demonstrated potential interactions among differentially expressed genes and identified five major subnetworks centred on the following genes: PPARγ, APOE, SERPINE1, JUN and LEP.

The majority of the key genes identified in our network analyses are implicated in the pathogenesis of diabetes and diabetic complications (mainly diabetic nephropathy) (Supplementary Table 5). As discussed above, PPARγ and APOE are downregulated in progressors. Downregulation of either gene in adipocytes leads to a decrease in serum lipid uptake with subsequent hyperlipidaemia (Duan et al., 2009) and a predisposition towards developing diabetic neuropathy (Wiggin et al., 2009). A fibrinolysis regulating gene, SERPINE1 encodes plasminogen activator inhibitor 1 (PAI-1), whose elevated levels are associated with higher incidences of diabetes (Festa et al., 2002) and knocking out PAI-1 ameliorated diabetic nephropathy in mice (Nicholas et al., 2005). A recent study suggested leptin’s therapeutic effect in a combinatorial treatment with insulin in type 1 diabetic mice (Wang et al., 2010). The cell cycle controlling JUN might be also involved in progression of diabetic neuropathy through its close interacting partners c-Jun N-terminal kinases (JNKs). JNKs are key signalling molecules linking inflammation and insulin resistance and are significantly activated in multiple tissues including the sural nerve of patients with type 1 and 2 diabetes (Yang and Trevillyan, 2008). Thus, the enriched biological functions and the networks of the differentially expressed genes reflect current theories with regard to dysregulation in diabetes and its complications, suggesting their expression changes may be related to the development of diabetic neuropathy.

To fully incorporate all of the co-citation connections among the differentially expressed genes, we applied the Fast Greedy algorithm, a community structure identification algorithm, to the entire co-citation network. Fast Greedy grouped LEP and PPARγ together within the context of glucose and lipid metabolism and JUN and SERPINE1 within the context of cell death and inflammation. Three other subnetworks were identified with noteworthy key genes: ‘cell projection and axonogenesis’ with nerve growth factor receptor (NGFR), ‘cellular homeostasis and inflammatory response’ with thioredoxin and ‘cytoskeletal protein binding’ with stathmin 1 (STMN1).

Nerve growth factor receptor exerts protection against nerve damage and the expression of nerve growth factor receptor protein in plasma correlates with diabetic neuropathy progression in diabetic rats (Chilton et al., 2004). Thioredoxin, which regulates cellular oxidative stress, is also implicated in diabetes. Thioredoxin’s antioxidant activity is significantly inhibited by hyperglycaemia, suggesting its important role in vascular oxidative stress and inflammation in diabetes (Schulze et al., 2004). No direct implication of stathmin 1, a major regulator of microtubule dynamics, in diabetes is currently known. We and others have reported regenerative changes in response to diabetic neuropathy (Dyck et al., 1986; Sullivan et al., 2003). The expression of genes involved in axonal extension may reflect these changes and an individual’s ability to recover from nerve damage.

Our next goal was to use observed differentially expressed gene expression to classify a separate subset of biopsies using Ridge regression modelling. Regression modelling using gene expression data has proven extremely useful in predicting the progression of cancer and diabetic nephropathy (Shedden et al., 2008; Ju et al., 2009). In the current study, gene expression profiles from secondary biopsy samples with known myelinated fibre density (progressors or non-progressors) were compared and used in training the models. The models were then used to classify the expression profiles of a set of primary biopsies for their progression endpoint 12 months later. The best classification models included 14 genes and correctly identified 11 out of 12 patients with respect to their identification as progressors versus non-progressors. This classification accuracy (92%) is much higher than our previous naïve Bayes-based classification model’s accuracy (63%) using only physiological and demographic data of these patients (Wiggin et al., 2009). Our data demonstrate that the gene expression profile from sural nerve biopsies of patients with diabetic neuropathy achieve a higher prediction accuracy (92%) than the clinical parameters alone and are better predictors of diabetic neuropathy progression.

We hypothesize that the genes identified in our classification models (Table 5) represent products or ‘genetic biomarkers’ of the biological networks involved in diabetic neuropathy onset and progression. This idea is reinforced by the fact that several of the genes have known associations with diabetes or diabetic complications. We are particularly interested in CST1, whose expression was increased by 10-fold in progressors. CST1, encoding a cysteine protease inhibitor, was initially implicated in gastric and colorectal tumourigenesis (Choi et al., 2009; Yoneda et al., 2009). Another member of this protein family, cystatin C (CST3), has been identified as a prime predictor of diabetic nephropathy.
Acknowledgements

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Supplementary material

Supplementary material is available at Brain online.

References


Diabetic neuropathy: cellular mechanisms as therapeutic targets

Andrea M. Vincent, Brian C. Callaghan, Andrea L. Smith and Eva L. Feldman

Abstract | In patients with diabetes, nerve injury is a common complication that leads to chronic pain, numbness and substantial loss of quality of life. Good glycemic control can decrease the incidence of diabetic neuropathy, but more than half of all patients with diabetes still develop this complication. There is no approved treatment to prevent or halt diabetic neuropathy, and only symptomatic pain therapies, with variable efficacy, are available. New insights into the mechanisms leading to the development of diabetic neuropathy continue to point to systemic and cellular imbalances in metabolites of glucose and lipids. In the PNS, sensory neurons, Schwann cells and the microvascular endothelium are vulnerable to oxidative and inflammatory stress in the presence of these altered metabolic substrates. This Review discusses the emerging cellular mechanisms that are activated in the diabetic milieu of hyperglycemia, dyslipidemia and impaired insulin signaling. We highlight the pathways to cellular injury, thereby identifying promising therapeutic targets, including mitochondrial function and inflammation.

Introduction

Neuropathy is the most common complication of diabetes, affecting approximately 50% of patients over the course of their disease.1–3 Patients with diabetes can develop several peripheral nerve disorders, but the vast majority exhibit a distal, symmetric polyneuropathy that starts in the feet and progresses proximally.4 Neuropathy occurs in both type 1 diabetes (in which the pancreas does not produce enough insulin) and type 2 diabetes (in which either the production of or cellular response to insulin is impaired).5 Typical symptoms of diabetic neuropathy include pain, numbness, tingling, weakness, and difficulties with balance. The disease is associated with substantial morbidity, including depression, susceptibility to foot or ankle fractures, ulceration and lower-limb amputations.6–8

Recent reviews have focused on progress in clinical trials and how to apply current knowledge to the development of rational treatment regimens for patients with diabetes in order to prevent or improve neuropathy. In this article, we aim to move the research forward by discussing our understanding of how metabolic factors influence the development of neuropathy, how the various mechanisms interact, and the potential implications of these insights for future therapeutic interventions.

Pathophysiological features

Typical diabetic sensory peripheral neuropathy is characterized pathologically as an axonopathy with distal predominance.9 The disease first affects the longest axons, which innervate the feet; by the time symptoms reach the knees, the fingers are often affected.10 Axonal changes are present in both myelinated and unmyelinated fibers, with early development of ‘honeycombed’ Schwann cell–axon networks, and later axonopathy with corrugated myelin breakdown.11 Regenerating axons are present in human sural nerve and skin biopsy samples but, over the course of the disease, regeneration fails.12 Reduced blood flow through loss of autonomic nerve functions may contribute to the progression of diabetic neuropathy,13 and alterations in microvessels, similar to the pathogenic neovascularization described in diabetic retinopathy and nephropathy, also are observed in peripheral nerves.14

Biochemical features

Reduced glycemic control is clearly associated with the development of diabetic neuropathy: both direct glucose measures and levels of glycosylated hemoglobin correlate with the occurrence of neuropathy.9,15,16 However, the cause of diabetic neuropathy is more complex than dysregulated glucose levels alone. Several contributing factors have been postulated and have received differing degrees of acceptance. In this section of the Review, we emphasize the importance of glucose-mediated injury in the pathogenesis of diabetic neuropathy. However, the presence of many of these mechanisms—such as accumulation of sorbitol, oxidative stress, and 12/15-lipoxygenase activation—before the development of overt hyperglycemia and diabetes in patients with the metabolic syndrome indicates that additional factors must link diabetes with peripheral neuropathy.17

Oxidative stress has been considered the final common pathway of cellular injury in hyperglycemia18 but, as highlighted in this Review, the mechanisms leading to
Injury to neurons, Schwann cells and microvascular endothelial cells in the diabetic milieu contributes to the pathogenesis of neuropathy.

Key points
- Multiple metabolic imbalances underlie the development of diabetic neuropathy
- Hyperglycemia, dyslipidemia and cardiovascular dysfunction are each independent risk factors for neuropathy
- Targeting risk factors as well as cellular oxidative stress and inflammation will be important in future treatment approaches
- Injury to neurons, Schwann cells and microvascular endothelial cells in the diabetic milieu contributes to the pathogenesis of neuropathy

**Figure 1** | Hyperglycemia and hyperlipidemia activate multiple injury mechanisms in sensory neurons. Glucose and lipoproteins interact with various receptors on neurons and microvascular endothelial cells. Diabetes-modified (that is, oxidized and glycated) proteins and lipoproteins bind additional receptors. These receptors include transporters that internalize glucose and lipids, which can accumulate intracellularly and disrupt mitochondrial metabolic pathways. The receptors also initiate inflammatory signaling mechanisms that directly produce oxidative stress and increase expression and activity of oxidative and nitrosative enzymes. Oxidative stress damages mitochondria and other cellular components, leading to neuronal injury. Abbreviations: AGE, advanced glycation end product; GLUT glucose transporter; LDLR, LDL receptor; LOX1, oxidized LDL receptor 1; oxLDL, oxidized LDL; RAGE, receptor for advanced glycation end products.

**Formation of advanced glycation end products**
Advanced glycation is a nonenzymatic chemical modification of proteins, lipids and nucleic acids via attachment of reactive carbohydrate groups to exposed sites. In diabetes, the oxidizing environment and increased carbohydrate accumulation accelerate the formation of advanced glycation end products (AGEs). Furthermore, clearance of AGEs from plasma is reduced in diabetic patients with renal impairment.

Protein AGEs produce diabetic neuropathy through two major mechanisms. First, advanced glycation tends to decrease the biological function of proteins, thus inhibiting neuronal activity. Second, extracellular lipid and protein AGEs bind to cell surface receptors, particularly the receptor for AGE (RAGE), initiating an inflammatory signaling cascade that further increases

**Hyperglycemia**
Disposal of excess glucose
Several mechanisms of hyperglycemia-induced cellular injury were first described in the vascular endothelium. Subsequently, these mechanisms were observed in peripheral sensory neurons. Glucose uptake is less rapidly regulated in neurons than in endothelial cells, which may account for the high susceptibility of neurons to glucose-mediated injury.

Intracellular glucose is principally removed through the process of glycolysis, which generates pyruvate for mitochondrial catabolism to form ATP. Excess pyruvate from glycolysis is thought to injure neurons through two mechanisms. First, the overload of metabolites to the mitochondrial electron transfer chain leads to increased generation of reactive oxygen species, which inhibit the activities of key mitochondrial components such as coenzyme Q, NADH oxidase and complex I, resulting in mitochondrial dysfunction. Second, excess pyruvate is shunted to the lactate pathway when oxygen is limiting, with continued formation of lactate, which may account for the high susceptibility of neurons to oxidative stress.

If glycolysis does not adequately dispose of intracellular glucose, a number of alternative pathways are activated. Aldose reductase reduces glucose to sorbitol, and sorbitol dehydrogenase oxidizes sorbitol to fructose. These activities increase cellular osmolarity and deplete NADPH, both of which lead to oxidative stress. Activation of this osmotic stress pathway, involving increased expression of the taurine transporter, is evident in cultured human Schwann cells exposed to hyperglycemia. Excess glucose can also be shunted to the hexose pathway, in which the glycolytic intermediate fructose-6-phosphate is converted, via glucosamine-6-phosphate, to uridine diphosphate-N-acetylgalactosamine. This molecule modifies serine and threonine residues of specific transcription factors, such as Sp1. These transcription factors are implicated in hyperglycemic inflammatory injury in endothelial basement membranes and pancreatic β-cells. Further evidence suggests that Sp1 is activated in sural nerves of patients with diabetes.
The expression of RAGE is elevated in peripheral epidermal axons, sural axons, Schwann cells and dorsal root ganglia neurons, following the pattern of electrophysiological and structural abnormalities associated with neuropathy.44

The importance of AGEs in the development of diabetic neuropathy has been largely confirmed using AGE inhibitors such as aminoguanidine and benfotiamine65 and through studies of RAGE-knockout mice.44 The AGE–RAGE axis seems to mediate a sustained cellular proinflammatory response that is involved in chronic injury in diabetic complications.46 These sustained changes involve long-term activation of the proinflammatory transcription factor nuclear factor κB, and upregulation of RAGE expression following initial RAGE activation. Inhibition or genetic deletion of RAGE significantly reduces diabetic neuropathy in mice and may be a viable therapeutic target in humans, although the physiological role of RAGE is not known.

**Dyslipidemia**

Increased plasma lipids, particularly triglycerides and cholesterol, are a feature of the metabolic syndrome, which can lead to diabetes. It has long been known that increased plasma lipids, known as dyslipidemia, are a major risk factor for cardiovascular disease. Clinical epidemiological studies have now demonstrated a similar strong association between dyslipidemia and microvascular complications, including neuropathy in both type 1 and type 2 diabetes. Dyslipidemia is, therefore, an important modifiable parameter in the prevention and treatment of neuropathy in diabetes.47–49 The mechanisms by which plasma lipids produce neuronal injury are not fully known, but several factors that play a part in lipid-mediated neuropathy have been identified.

Some evidence exists that neuropathy, particularly when it involves loss of autonomic control of the cardiovascular system, is closely associated with vascular disease factors, including obesity, high plasma levels of cholesterol and triglycerides, and high blood pressure.50,51 In a small study of patients with type 1 diabetes, cardiac autonomic neuropathy was associated with impaired ventricular function but not associated with systemic markers of vascular endothelial dysfunction, suggesting that vascular disease itself may not directly lead to neuronal injury.52 However, a more thorough examination of risk factors and complications in more than 1,400 patients with type 1 diabetes revealed that a decreased vibration perception threshold, which predicts foot ulceration and amputation, was strongly associated with a previous history of cardiovascular disease.53 Further work is needed to determine whether elevated lipids have direct effects on peripheral neurons and/or Schwann cells.

Studies of patients with type 2 diabetes more frequently demonstrate a correlation between peripheral sensory neuropathy and peripheral vascular disease than do studies of patients with type 1 diabetes.54 In rodents, a high-fat diet leads to accumulation of sorbitol, oxidized lipids and poly ADP-ribose polymerase (PARP), and activation of lipoxygenases in peripheral nerves before the development of diabetes.55 Metabolic pathways that might mediate neuronal injury in dyslipidemia are described below and illustrated in Figure 1. Cell culture studies suggest that downstream of inflammation and oxidative and nitrosative stress, protein damage may lead to mitochondrion-mediated activation of cell death mechanisms in neurons.55,56 These molecular modifications also activate the endoplasmic reticulum unfolded-protein response in many cell types, which can lead to cell death through endoplasmic reticulum stress.57,58

**Free fatty acids**

Free fatty acids cause lipotoxicity in cultured neuronal and Schwann cell lines.59 This toxicity is mediated through lysosomal dysfunction via mechanisms that are not well understood, but are thought to involve permeabilization of the lysosomal membrane by cathepsin L, leading to oxidative stress and mitochondrion-activated injury.55,60 Palmitic acid may be the primary free fatty acid that promotes injury of cultured Schwann cells.61

In addition to directly affecting cells of the PNS, elevated plasma levels of free fatty acids produce systemic effects that may also promote diabetic neuropathy. Cultured adipocytes and tissue macrophages release inflammatory cytokines that are known to produce peripheral nerve inflammation.59 Furthermore, increased intramuscular free fatty acids in humans promotes insulin resistance via impaired insulin signaling, thereby blocking glucose disposal.62 This suggests a mechanism whereby plasma lipids promote hyperglycemia, leading to a synergy of dyslipidemia and hyperglycemia that increases diabetic complications.

**Oxidized and glycated LDLs**

In diabetes, plasma lipoproteins are subject to an oxidizing environment. Peripheral sensory neurons, like vascular endothelial cells, express scavenger receptors for oxidized LDLs (oxLDLs), including oxidized LDL receptor 1 (LOX1) and Toll-like receptor 4.21,63–65 These neurons also express RAGE, which binds glycated LDL.62 The receptors internalize oxLDL and glycated LDL, releasing potentially injurious triglycerides and fatty acids, and initiate an inflammatory signaling pathway that results in activation of NADPH oxidase.51,64,65 This enzyme produces substantial cellular oxidative stress by generating superoxide radicals and by depleting NADPH levels. Oxidative stress in diabetes leads to increased expression of oxLDL and RAGE via p38 mitogen-activated protein kinase (MAPK) signaling, producing a positive-feedback mechanism of injury.44,64

**Oxysterols**

An oxidizing environment increases the oxidation of cholesterol to oxysterols. Oxysterols are known to accumulate in the brain in neurodegenerative diseases such as Alzheimer disease.66 Recent studies in PC-12 cells have
shown that oxysterol derivatives of cholesterol cause neurotoxicity through mitochondrion-mediated cell death pathways. The concept that oxysterols increase neuronal injury and could have a pathological role in diabetic complications is gaining traction, as oxysterols readily form in patients with diabetes, although there is little clinical evidence to date.

**Insulin resistance**

Insulin resistance is the hallmark of type 2 diabetes. Although neurons do not depend on insulin signaling for glucose utilization, a growing body of evidence suggests that peripheral insulin resistance contributes to neuropathy. Notably, neuropathy frequently occurs in patients with impaired glucose tolerance, before the development of diabetes. Insulin resistance can also develop in type 1 diabetes and is associated with the presence of microvascular complications such as neuropathy. Free fatty acids cause insulin resistance in liver and muscle, and are also responsible for cellular inflammation and endoplasmic reticulum stress. Both inflammation and endoplasmic reticulum stress have been observed in neurons under the influence of various pathogenic mechanisms. We suggest, therefore, that free fatty acids that accumulate as a result of insulin resistance may injure peripheral neurons. Peripheral insulin resistance induced by a high-fat diet in rats is mirrored by decreased insulin receptor activity in neurons in the brain, which leads to neuronal stress and injury, and a loss of neurotrophic signaling.

**Current therapeutic strategies**

Therapies aimed at blocking the symptoms of painful neuropathy are available, but few options target the root causes of the disease. The immense physical, psychological and economic cost of diabetic neuropathy underscores the need for causally targeted therapies. Antioxidant strategies have been most widely explored, but only α-lipoic acid, which is part of the standard of care for diabetes in Germany, has shown promise, albeit limited.

**Targeting hyperglycemia**

The most informative studies of patients with type 1 diabetes are the Diabetes Control and Complications Trial (DCCT) and the subsequent Epidemiology of Diabetes and its Complications (EDIC), which involved follow-up evaluations of the same cohort of 1,300 patients for over 20 years. Data from these studies have provided important insights into the optimal approach to glycemic control for type 1 diabetes. In the DCCT, intensive insulin therapy early in the disease course to maintain a mean hemoglobin A1c (HbA1c) of 7.2% reduced the cumulative incidence of diabetic neuropathy by 60% at 5 years compared with conventional treatment, which involved less-intensive glycemic therapy (mean HbA1c 9.0%).

EDIC, a 20-year follow-up study of the patients in the DCCT, yielded unanticipated results. Within 1 year of beginning EDIC, glycemic control in the two treatment groups equalized to an average HbA1c of 8%. All 1,300 patients were assessed annually for diabetic neuropathy. After 8 years, patients from the intensive-therapy DCCT cohort had a lower incidence of diabetic neuropathy than patients from the conventional-therapy DCCT cohort, despite 8 years of comparable glycemic control. These findings underscore the importance of early, intensive glucose control for the prevention of complications, including neuropathy, in diabetes. This study led researchers to propose the idea of glucose or metabolic memory, because early intensive glycemic control results in fewer complications decades later, despite reducing the level of control. Metabolic memory is the subject of ongoing research and probably arises from long-term alteration of gene expression owing to epigenetic changes or onset of vascular disease.

The UK Prospective Diabetes Study, which followed a cohort of more than 3,000 patients with type 2 diabetes for over 15 years, provides important insights into the optimal approach to glycemic control in this form of diabetes. In this study, patients who received intensive glycemic therapy (insulin or sulfonylurea) experienced a reduction in the incidence of neuropathy—as measured with a biothesiometer—of approximately 12% compared with patients assigned to a conventional regimen. The difference between the two treatment groups was even greater when all microvascular complications were considered. In type 2 diabetes, however, attempts to intensively control glycemia are associated with increased severe myocardial events and are not recommended for standard care.

**Pain management**

Pain is the most severe consequence of neuropathy in terms of patient quality of life, yet it remains undertreated. Often, patients are unaware that pain is a symptom of diabetic neuropathy and fail to report it. In addition, therapies remain variable, and only one-third of patients report at least 50% reduction in pain with therapy. The prevalence of painful diabetic neuropathy is estimated to be 18% in type 2 diabetes and 6% in type 1 diabetes, and the incidence increases with age and diabetes duration. An evidence-based review of clinical trial data established that pregabalin is reasonably effective for treating pain in diabetic neuropathy, and showed that venlafaxine, duloxetine, amitriptyline, gabapentin, valproate, opioids and capsaicin are probably effective and should be considered for patients who are unresponsive to pregabalin.

Current first-line therapies involve monotherapies or drug combinations that target the specific type of pain, and comprise anticonvulsants (gabapentin and pregabalin), serotonin–noradrenaline reuptake inhibitors (duloxetine), and tricyclic antidepressants (amitriptyline, nortriptyline and desipramine). In clinical trials, these medications have shown a similar number needed...
to treat (between three and six patients) to observe a 50% reduction in pain.87–90

Clearly, more-efficacious and neuropathy-specific medications are needed. New analgesics show some promise for improved efficacy against pain symptoms. One such compound is tapentadol, a dual-action compound that acts as a μ-opioid receptor agonist and noradrenaline reuptake inhibitor. Compared with placebo, extended-release tapentadol significantly improved pain and was well-tolerated in 588 randomly assigned patients with type 1 and type 2 diabetes who were dissatisfied with at least 3 months of prior treatment with opioid and/or non-opioid analgesics.91 A treatment algorithm for the management of pain has been developed and is becoming accepted as the standard of care in patients with painful diabetic neuropathy (Figure 2).85

**New therapeutic targets and strategies**

A substantial proportion of patients with diabetes develop neuropathy despite intensive glycemic control. This was clearly shown in the Steno-2 study, in which patients treated with a multifactorial intervention including aspirin, statins, renin–angiotensin blockers, glycemic control, and lifestyle modifications nevertheless developed diabetic complications at a high rate.92 An unmistakable need exists, therefore, for new treatment strategies. A selection of the most promising of these strategies is discussed below, and a summary of compounds in development is presented in Table 1. Most clinical trials have produced disappointing results, but they have often been confounded by a high rate of improvement in the placebo group, or other unanticipated effects.93 Furthermore, failure of new drugs in the long term probably results from the multiple mechanisms that contribute to neuronal injury in diabetes (Figure 1).85

**Cytoprotective therapies**

**Reducing cell death**

Opinion in the field of diabetic neuropathy is divided on whether neuronal loss occurs and, if it does, whether cell death involves apoptosis, nonapoptotic programmed cell death, or necrosis. Evidence of apoptosis has been demonstrated in both rodent and cell culture models of neuropathy,22,60,94–98 but other studies failed to reproduce these findings.99 The involvement of mitochondria in neuronal injury is generally accepted, implicating an apoptotic mechanism, despite limited evidence for a decrease in the number of dorsal root ganglion neurons in vivo.99 Both lysosomes and mitochondria are implicated in fatty-acid-induced neuronal injury via cathepsin-mediated membrane permeabilization.100 Activation of caspase 3, a key molecular component of the apoptotic pathway, is generally observed in models of diabetic neuropathy, and several protective compounds with different mechanisms of action, including lipid receptors or antioxidants, block the activation of this apoptosis effector enzyme.85,100

**Inhibiting poly ADP-ribose polymerase**

PARP is activated following oxidative damage to DNA. This enzyme adds poly(ADP-ribose) subunits to DNA strand breaks, converting DNA damage into intracellular signals that activate either DNA repair by the base-excision pathway, or cell death.101 Inhibition of PARP may slow the progression of diabetic neuropathy by blocking the activation of cell death and by preventing PARP-mediated depletion of NAD+ and ATP.102,103 This potential is particularly interesting because PARP inhibitors are currently in clinical trials for cancer treatment104–106 and, if effective, could rapidly translate to the diabetes clinic.

**Providing trophic support**

Recent studies have used gene and stem cell transfer in rodent models of diabetes to provide neuronal trophic support or promote neovascularization in order to prevent or improve diabetic neuropathy. Clinical data are not yet available, although trials are in progress. In mice with streptozotocin-induced diabetes, gene transfer of neurotrophin-3 using herpes simplex viral delivery into the footpads provided long-term protection against neuropathy.107 After 5.5 months, mice that had received the construct displayed preservation of action potential amplitudes and conduction velocity in sensory and motor nerves, response to a paw heat stimulus, pilocarpine-induced sweating, and intraepidermal nerve fiber density, unlike mice that received a control herpes simplex viral vector.
**Table 1 | Recent drug trials in diabetic neuropathy**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Proposed mechanism</th>
<th>Preclinical studies</th>
<th>Clinical trial results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aleglitazar</td>
<td>Dual PPARα/γ agonist</td>
<td>In rats, decreased plasma glucose and LDL cholesterol levels; increased glucose clearance and HDL cholesterol levels; improved insulin resistance120</td>
<td>Reduced glycemia in phase II trials; currently in phase III trial for diabetic cardiovascular end points121</td>
</tr>
<tr>
<td>L-arginine</td>
<td>Improves circulation in microvessels</td>
<td>Produces vasodilatation of isolated vessels of all species122</td>
<td>No effect on endothelial function or neuropathy score123</td>
</tr>
<tr>
<td>Zemarestat, epalrestat, ranirestat, fidarestat and five related compounds</td>
<td>Aldose reductase inhibitors</td>
<td>Zenarestat prevented abnormal neurotrophin receptor expression;124 fidarestat prevented oxidative stress and neuropathy in diabetic rats125</td>
<td>Epalrestat is well-tolerated long term126,127 and approved in Japan;128 most compounds produce modest improvements in nerve conduction and pain scores; ranirestat seems to improve motor nerve function in mild to moderate disease;129 fidarestat showed some adverse effects in long-term treatment33</td>
</tr>
<tr>
<td>α-Lipoic acid</td>
<td>Antioxidant; pyruvate dehydrogenase activator; other unknown mechanisms</td>
<td>Improved nerve and cardiac disorders in diabetic rats129</td>
<td>Approved for standard of care in Germany;127 some evidence that the compound decreases oxidative stress,130 prevents AGE formation131 and improves neuropathic deficits; US trials remain inconclusive132</td>
</tr>
<tr>
<td>Actovegin</td>
<td>Increases cellular metabolism through an unknown mechanism; increases glucose and oxygen uptake and use; increases ATP turnover</td>
<td>Improved brain metabolic defects in rats with experimental stroke133</td>
<td>Sequential intravenous and oral delivery over 160 days improved neuropathic symptoms, vibration perception threshold, sensory function, and quality of life134</td>
</tr>
<tr>
<td>Fibrates</td>
<td>Lipid lowering</td>
<td>Fenofibrate improves insulin sensitivity135 and other parameters that affect neuropathy, such as vascularization136 and lipid metabolism137</td>
<td>Clofibrate decreases neuropathy;138 fenofibrate decreases eye and kidney complications;138 fenofibrate decreases risk of amputation in patients with diabetes but without macrovascular disease139</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>GABA analogue that blocks new synapse formation140</td>
<td>No preclinical data or known mechanism; use of anticonvulsants based on similarities between pathophysiology of diabetic neuropathy and epilepsy141</td>
<td>Blocks pain and improves symptoms of cardiac autonomic neuropathy142</td>
</tr>
<tr>
<td>Acetyl-carnitine</td>
<td>Restoring possibly depleted levels in diabetes; required for mitochondrial function</td>
<td>Improved blood flow and sciatic motor nerve conduction velocity in rats with type 1 diabetes143</td>
<td>Early treatment may decrease pain; one of two large studies suggested improvement in NCV and nerve regeneration144</td>
</tr>
<tr>
<td>Pentoxifyline and pentosan polysulphate</td>
<td>Improves circulation in microvessels by blocking phosphodiesterase; antioxidant Cilastazol, another phosphodiesterase inhibitor, improved NCV in rats with type 1 diabetes145 but was ineffective in humans146</td>
<td>In combination, these compounds improved cardiovascular autonomic function and vibration perception in type 2 diabetes147</td>
<td></td>
</tr>
<tr>
<td>Benfortamine</td>
<td>Blocks AGE formation</td>
<td>Decreased AGE levels and diabetic complications in rats148,149</td>
<td>Reviews propose testing in patients, but clinical trials have not been instigated140</td>
</tr>
<tr>
<td>C-peptide</td>
<td>Lacking in type 1 diabetes; binds to a G protein-coupled receptor and alters metabolism150</td>
<td>Improved blood flow and early neuropathy in rats with type 1 diabetes151,152</td>
<td>Short-term use (&lt;3 months) decreased early evidence of NCV slowing, sensory deficits and autonomic neuropathy in patients with type 1 diabetes153</td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>Neurotrophic factor</td>
<td>Decreased neuropathy in rats154 and mice;155 however, the endogenous form may be responsible for pain in neuropathy156</td>
<td>Some efficacy against sensory deficits, but produced painful adverse effects157,158</td>
</tr>
<tr>
<td>Ruboxistaurin</td>
<td>Akt inhibitor</td>
<td>Decreased microvascular complications in rodents157</td>
<td>Seems to be effective against diabetic retinopathy, but no effect on neuropathy in phase III trials159</td>
</tr>
<tr>
<td>Basic fibroblast growth factor</td>
<td>Stimulates angiogenesis and nerve cell regeneration</td>
<td>Intravenous administration in rats modestly improves blood flow, NCV deficits and hypoalgesia160</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

**Abbreviations**: AGE, advanced glycation end product; GABA, γ-aminobutyric acid; NCV, nerve conduction velocity; PPAR, peroxisome proliferator-activated receptor.

Two studies have investigated the effect of intramuscular injection of endothelial progenitor cells into the hindlimbs of streptozotocin-treated rodents.108,109 In both cases, the treatment increased neovascularization and blood flow, and preserved sciatic nerve function. In one of the studies, the progenitor cells were obtained from the bone marrow of C57Bl/6J mice and injected into other C57Bl/6J mice that had been diabetic for 12 weeks.108 Loss of microvessels was reversed, and the injected cells preferentially engrafted into peripheral nerves and increased the expression of angiogenic neurotrophic factors. Autologous transplants would avoid the risk of rejection by the recipient, making this technique particularly attractive for the reversal of neuropathy in patients.

**Inhibiting NADPH oxidase**

As highlighted in Figure 1, NADPH oxidase is a key mediator of oxidative and nitrosative stress in diabetic...
neuropathy, and inhibition of this enzyme could, therefore, be a therapeutic strategy to reduce the cellular injury that may underlie this disease. Moreover, numerous studies in rodents have suggested a role for NADPH oxidase in diabetic neuropathy. In hypertensive rats, expression of the NADPH oxidase 4 isoform (NOX4; also known as renox) is increased in the kidney in diabetic nephropathy. Administration of angiotensin blocks NOX4 activation, oxidative injury and renal dysfunction without altering blood pressure, suggesting that NOX4 might mediate pathological changes independently of blood pressure.110 We have demonstrated increases in NADPH oxidase activity in rodent dorsal root ganglion neurons following hyperglycemia, or RAGE or LOX1 activation,21,22,42 and we recently found that expression of both NOX2 and NOX4 is increased in vitro and in vivo in states of hyperglycemia and dyslipidemia (A. M. Vincent et al., unpublished work). We are further exploring the pathological role and regulation of these NADPH oxidase isoforms in diabetic mice.

NADPH oxidase inhibitors are not yet available for clinical use. Nonspecific NADPH oxidase inhibitors— for example, diphenylene iodonium and apocyanin—inhibit other flavoenzymes such as nitric oxide synthase and xanthine oxidase.111 Apocyanin has been used as an experimental inhalant to assess airway disease but has no therapeutic applications.112 In cell culture and rodent models of experimental diabetes, apocyanin and diphenylene iodonium can prevent the development of diabetic complications,113–115 but the lack of specificity of these compounds limits conclusions regarding the direct role of NADPH oxidase in these effects. Development of a specific inhibitor is needed to enable investigation of the therapeutic potential of targeting NADPH oxidase in diabetic neuropathy.

Reducing inflammation

Figure 1 indicates a central role for inflammatory mechanisms in neuropathy. We predict, therefore, that successful treatment or prevention of diabetic neuropathy will require inflammation to be blocked at the systemic and cellular levels. Activation of receptor-mediated inflammatory signaling by AGE and oxidized lipoproteins leads to oxidative and nitrosative stress, which can cause microvascular disease. Consistent with a key role for inflammation in diabetic neuropathy, sciatic and sural nerve blood flow and conduction velocities were protected by the anti-inflammatory effects of etrhopoetin in diabetic rats.116 Pain pathways in diabetic neuropathy involve the inflammatory mediator p38 MAPK. Recent clinical trials of a novel p38 MAPK inhibitor in neuropathic pain demonstrated rapid pain relief,117 with decreased systemic inflammation after 14 days of treatment. These findings suggest that blocking of inflammation is feasible and could form an effective component of strategies to treat diabetic neuropathy. However, extended treatment with a different p38 MAPK inhibitor did not effectively block systemic or chronic inflammation in patients with rheumatoid arthritis, suggesting that the role of this kinase in cellular inflammation is complex.118 Selective inhibition of the proinflammatory enzyme cyclo-oxygenase-2 prevents cardiac autonomic neuropathy in mice with type 1 diabetes,119 providing further support for the use of anti-inflammatory agents to prevent neuropathy.

Conclusions

Crucial advances in our understanding of and approach to the treatment of diabetic neuropathy have been made. It is now widely recognized that physicians and patients must aim to counteract multiple risk factors in order to improve both daily care and clinical trial outcomes. Standardization of assessment methods for monitoring disease progression will improve current weaknesses in patient care and clinical trial design (Box 1). Particular attention to dyslipidemia and cardiovascular risk factors, in addition to hyperglycemia, is likely to improve all diabetic macrovascular and microvascular complications. In the future, novel therapeutic targets at the level of mitochondrial metabolic control and inflammatory pathways are likely to further decrease the incidence of diabetic neuropathy.

Review criteria

The National Library of Medicine PubMed database was searched for the term “diabetic neuropathy”. Information was extracted from full-text versions of articles published in English. Mechanistic data obtained since 1985, and data from clinical trials conducted since 2004, were included.


Author contributions
A. M. Vincent researched data for the article. All authors contributed to discussions of the content, writing of the article, and review and/or editing of the manuscript before submission.
Nerve growth factor/p38 signaling increases intraepidermal nerve fiber densities in painful neuropathy of type 2 diabetes

Hsinlin T. Cheng a,⁎, Jacqueline R. Daucha a, John M. Hayes a, Brandon M. Yanik b, Eva L. Feldmana

a Department of Neurology, University of Michigan Medical Center, Ann Arbor, Michigan, USA
b College of Literature, Science, and the Arts, University of Michigan, Ann Arbor, Michigan, USA

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ABSTRACT

Painful diabetic neuropathy (PDN) is a common, yet devastating complication of type 2 diabetes. At this time, there is no objective test for diagnosing PDN. In the current study, we measured the peptidergic intraepidermal nerve fiber densities (IENFD) from hind paws of the db/db mouse, an animal model for type 2 diabetes, during the period of mechanical allodynia from 6 to 12 weeks of age. Intraepidermal nerve fibers (IENF) of the hind footpads were identified by protein gene product (PGP) 9.5 immunohistochemistry. The peptidergic IENF were determined by double immunofluorescence using anti-PGP9.5 and antibodies against tropomyosin-receptor-kinase (Trk) A. We observed a significant increase in PGP9.5-positive IENFD at 8 and 10 weeks of age. Similarly, Trk A-positive peptidergic IENF, which also express substance P and calcitonin gene related peptide in db/db mice, were observed to be elevated from 1.5 to 2 fold over controls. This upregulation ended at 16 weeks of age, in accordance with the reduction of mechanical allodynia. Anti-NGF treatment significantly inhibited the upregulation of peptidergic IENFD during the period of mechanical allodynia, suggesting that increased neurotrophism may mediate this phenomenon. In addition, SB203580, an inhibitor of p38, blocked the increase in peptidergic IENFD in db/db mice. The current results suggest that peptidergic IENFD could be a potential diagnostic indicator for PDN in type 2 diabetes. Furthermore, the inhibition of NGF-p38 signaling could be a potential therapeutic strategy for treating this painful condition.

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Introduction

Diabetes mellitus affects over 20 million Americans. About 60% of patients diagnosed with diabetes develop diabetic neuropathy (DN). Painful diabetic neuropathy (PDN) is an early manifestation of DN, which frequently presents in the pre-diabetic states of impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) (Boulton et al., 2005; Feldman et al., 2005; Ziegler et al., 2009). This devastating complication can be found in 40% to 50% of patients with diabetes that have documented neuropathies (Galer et al., 2000). Regrettably, the quality of life for diabetic patients with PDN is significantly diminished (Dworkin et al., 2005, 2007; Jensen et al., 2006).

Patients with PDN experience mechanical allodynia and thermal hyperalgesia, which are frequently described as continuously burning, tingling, electric-like, crampy, or achy pain. Allodynia occurs when normally non-painful stimuli become painful, whereas hyperalgesia is an increase in sensitivity to normally painful stimuli. PDN begins in the feet and progresses proximally over time. Symptoms of neuropathic pain are generated by small-calibered Aδ and C fibers that innervate the skin.

At present, there is no available diagnostic tool to objectively determine the severity of PDN. Clinicians must rely on subjective reports from patients concerning their personal experiences of pain, which are frequently influenced by their psychosocial conditions and potential secondary gain from illness. As a result, such measures often lack credibility. In order to objectively test for DN, nerve conduction study and skin biopsy are most commonly used. Unfortunately, nerve conduction study is not a reliable method to diagnose PDN due to its inability to detect small fiber pathology. Skin biopsy can detect small fiber pathology, and thus has been widely used to determine intraepidermal nerve fiber density (IENFD) in DN (Catalan et al., 2007), in addition to the diagnosis of other small-fiber neuropathies. However, its value for determining or predicting the development of PDN is still under debate (Sorensen et al., 2006a,b).

The current method of IENFD measurement uses protein gene product (PGP) 9.5 to identify all IENF in the skin (Johnson et al., 2008; Sullivan et al., 2007; Zandecki et al., 2008). Furthermore, PGP9.5 IENFD is considered a standard method to determine small fiber sensory neuropathies including DN (Lauria et al., 2005). Many published animal

Abbreviations: CGRP, calcitonin gene related peptide; CSF, cerebrospinal fluid; DMSO, dimethyl sulfoxide; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; IENFD, intraepidermal nerve fiber densities; NGF, nerve growth factor; PDN, painful diabetic neuropathy; PBS, phosphate buffered saline; PGP, protein gene product; SP, substance P; STZ, streptozotocin; Trk, tropomyosin-receptor-kinase.

⁎ Corresponding author at: University of Michigan, Department of Neurology, 109 Zina Pitcher Place, 5015 BSRB, Ann Arbor, Michigan 48109-200. Fax: +1 734 763 7275.
E-mail address: chengt@umich.edu (H.T. Cheng).
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and human studies have demonstrated a decrease of PGP9.5 IENFD in DN (Kennedy et al., 1996; Shun et al., 2004). Such a decrease of PGP9.5 IENFD is detected early in the course of diabetes and has been found in patients with IGT (Sumner et al., 2003). Nevertheless, the value of PGP9.5 IENFD for the diagnosis of PDN has yet to be determined.

In the present study, our aim was to determine if there is evidence of a correlation between IENFD and mechanical allodynia specific to PDN. Additionally, we intended to further explore our hypothesis that there is enhanced axonal sprouting or regeneration present at the initial stage of PDN to mediate mechanical allodynia in the db/db mouse. Based on previous findings, which report that nerve growth factor (NGF) mediates mechanical allodynia in db/db mice, we focused on subgroups of IENF called peptidergic fibers that express neurotrophins involved in the mediation of nociception. The development of these fibers is dependent on NGF, and these peptidergic fibers are positive for tropomyosin-receptor-kinase A (Trk A), the high affinity receptor for NGF. Two of the major neurotrophins expressed by these peptidergic fibers are substance P (SP) and calcitonin gene related peptide (CGRP). In order to quantify the peptidergic IENFD, we performed double immunofluorescent staining with Trk A and either SP or CGRP on footpads from hind paws of the db/db mouse, an animal model of type 2 diabetes. Additionally, we applied anti-NGF and SB203580, a p38 inhibitor, to db/db mice to gain insight on the molecular mechanisms underlying the changes in IENFD during the early phase of mechanical allodynia.

Previously, we reported that mechanical allodynia is detected in the db/db mouse from 6 to 12 weeks of age. Additionally, we reported an upregulation of SP and NGF at 8 and 10 weeks of age in the db/db mouse (Cheng et al., 2009). Here, we observe a 1.5- to 2-fold elevation in SP and CGRP expression compared to the control values in Trk A- positive peptidergic IENF. Furthermore, we report a cessation of the increase in peptidergic IENFD at 16 weeks of age, corresponding to the reduction of pain behaviors at this time point.

Anti-NGF treatment and SB203580 treatment significantly inhibit the increase in peptidergic IENFD during the period of mechanical allodynia, suggesting that increased neurotrophism may mediate PDN. These results suggest that quantification of peptidergic IENFD could be a potential diagnostic indicator for PDN in type 2 diabetes, and that inhibiting NGF-p38 signaling could be a potential therapeutic strategy for the treatment of PDN.

Materials and methods

Animals

Male C57BLKS db/db (stock number 000642) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The homozygous (Lepr<sup>ob</sup>/Lepr<sup>ob</sup>, or db/db) mice were used as a model of type 2 diabetes, while heterozygous mice (Lepr<sup>ob+</sup>/+, or db/+) served as nondiabetic controls. Analyses and procedures were performed in compliance with protocols established by the Animal Models of Diabetic Complications Consortium (AMDCC) (http://www.amdccc.org) and were approved by the Use and Care of Animals Committee at the University of Michigan. All possible efforts were made to minimize the animals’ suffering and the number of animals used.

Intraepidermal nerve fiber (IENF) measurement

IENF quantification was performed using 4 db/+ mice and 4 db/db mice at 5, 8, 10, and 16 weeks time points. Prior to perfusion, both right and left hind foot pads were collected from the plantar surface of the hind paw, immersed for 6–8 h at 4 °C in Zamboni’s fixative (2% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer), rinsed in 30% sucrose in PBS solution overnight, cryosectioned at 30 μm thickness before being processed for immunohistochemistry.

Tissue sections were processed for PGP9.5, Trk A, SP, CGRP, and TNF-α immunohistochemistry (Polydefkis et al., 2001). Sections were incubated at 4 °C for 16–24 h with primary antibodies: PGP9.5 (1:1000, Millipore, Billerica, MA), Trk A (1:500, R&D Systems, Minneapolis, MN), SP (1:200, Abcam, Cambridge, MA), CGRP (1:500, Sigma-Aldrich, St. Louis, MO), and TNF-α (1:1000, Abcam). Sections were then rinsed 3 times in phosphate buffered saline (PBS) and incubated with secondary antiserum conjugated with different fluorophores (AlexaFluor 488, 594, or 647, Invitrogen, Carlsbad, CA). Sections were rinsed and mounted with ProLong® Gold antifade reagent (Invitrogen). In order to confirm that there were no nonspecific immunoreactions, sections were incubated with primary or secondary antisera alone. Fluorescent images were collected on an Olympus Fluoview 500 confocal microscope using a 20×1.2 water immersion objective at a resolution of 1024×1024 pixels. The optical section thickness was 0.5 μm. Approximately forty images per stack were flattened using the MetaMorph (Molecular Devices, Sunnyvale, CA, version 6.14) arithmetic option. Six sections were measured for each footpad. IENFD data were presented as the mean number of fibers per linear mm of epidermis from a total of 12 sections per animal (Christianson et al., 2003).

Anti-NGF treatment

In order to inhibit NGF action during the period of allodynia, we administered anti-NGF (10 mg/kg, mouse monoclonal antibody clone AS21, Exalpha Biologicals, Maynard, MA) or control IgG intraperitoneally. Treatment was performed once weekly at the beginning of 6 weeks of age for 2 weeks (Wild et al., 2007). Hind foot pads were collected for immunohistochemistry at the end of treatment at 8 weeks of age.

SB203580 treatment

An osmotic minipump (Alzet minipump model 1007D, Duent Corporation, Cupertino, CA) was used for continuous intrathecal infusion into the lumbar spinal cord region. The 100 μl volume minipump is designed with a 0.51 μl/h infusion rate. The minipumps were filled with artificial cerebrospinal fluid (CSF) that contained 10% dimethyl sulfoxide (DMSO) with or without SB203580 (1 mg/ml, EMD Chemicals, Gibbstown, NJ). The minipumps were implanted into the dorsal subcutaneous space between the shoulder blades of each mouse at 7 weeks of age under sterile conditions. A caudally directed polyethylene cannula (Becton Dickinson and Company, Sparks, MD) was threaded subcutaneously at the level of the L5 spinal process. The L5 spinal process was removed and the tip of the cannula was then inserted into the subarachnoid space at the L5 level. The intrathecal infusion lasted for 1 week, after which hind foot pads were collected for immunohistochemistry.

Data presentation and statistical analyses

All data are presented as group means ± SEM. The data between db/+ and db/db mice of the same age were analyzed using the Mann–Whitney test. Statistical comparisons between different age groups were made by a one-way ANOVA test followed by a post hoc Tukey’s multiple comparison test. A p-value of less than 0.05 was considered statistically significant.

Results

PGP9.5 and Trk A-positive IENFD increase in diabetic animals during the period of mechanical allodynia

Previously, we reported that the db/db mouse, an animal model of type 2 diabetes, develops mechanical allodynia at 6–12 weeks of age.
In our current study, we examined the temporal correlation between the change of peptidergic IENFD and the development of mechanical allodynia using the hind foot pads from animals characterized in previous studies (Cheng et al., 2009, 2010). We performed immunohistochemistry of a pan-neuronal marker, PGP 9.5, on hind footpads from 5, 8, 10, and 16 weeks of age (Fig. 1). The 5 week group was prediabetic; 8 and 10 week groups experienced mechanical allodynia, and the 16 week group was diabetic with significant sensory neuropathy but no PDN (Cheng et al., 2009). As demonstrated in Fig. 1, PGP9.5 immunohistochemistry labeled the horizontal plexus of nerve fibers underneath the epidermis (Fig. 1A, asterisks). IENF originate from the dermal plexus and extend into the epidermis (Fig. 1A, arrow and arrowhead). PGP9.5 immuno-positive IENF are either Trk A-positive (Fig. 1 arrows) or Trk A-negative (Fig. 1, arrowheads). The representative confocal double immunofluorescent images from 8 weeks db/+ (Figs. 1A, B, C) and db/db mice (Figs. 1D, E, F) demonstrated the presence of both PGP9.5- and Trk A-positive IENF in both animals. Quantitative analysis revealed that there was a significant 1.4-fold increase in PGP9.5-positive IENFD in db/db mice at 8 and 10 weeks of age (Fig. 1G). In contrast, no significant difference was detected at 5 and 16 weeks of age (Fig. 1G). In accordance with PGP9.5, Trk A-positive IENFD was not changed in the db/db mouse at 5 weeks of age but significantly increased at 8 and 10 weeks of age (Fig. 1H). There was a 1.5-fold increase of Trk A-positive IENF in db/db mice at 8 weeks of age and a >2-fold increase at 10 weeks of age. By 16 weeks of age, the upregulation of Trk A-positive IENFD in diabetic animals diminishes and reverts to that of the control level (Fig. 1H). In order to demonstrate the fiber-type specific upregulation of Trk A-positive IENFD out of the general increase of PGP9.5-positive IENFD, we calculated the percentages of Trk A-positive/PGP9.5-positive fibers in each sample and normalized the data to the means of db/+ for each age group (Fig. 1J). We found that there was a 1.5-fold increase in the ratio of TrkA/PGP9.5 between db/db and db/+ mice at 8 and 10 weeks of age db/+.

**SP and CGRP-positive IENFD are increased in diabetic animals during the period of mechanical allodynia**

Trk A-positive IENF are peptidergic nerve fibers that express SP and CGRP. Confocal double immunofluorescent studies demonstrated most SP immunoreactivity colocalize with Trk A-positive IENFD in both db/+ and db/db mice (Figs. 2A–C, D–F) respectively. Quantitative analysis demonstrated increased SP-positive IENFD at 8 and 10 weeks in db/db mice compared to db/+ control mice (Fig. 2G). Similar to Trk A-positive IENF, a 1.5-fold increase at 8 weeks and 2-fold increase at 10 weeks of SP-positive IENF were detected in db/db mice (Fig. 2G). In parallel, CGRP-positive IENFD were detected in both db/+ and db/db mice (Figs. 3A–C, D–F, respectively). The CGRP IENFD staining was higher in number than SP-positive IENFD, but was only detected in Trk A-positive IENFD (Figs. 3A–F). Up to 1.5-fold elevation of CGRP-positive IENFD was detected in db/db mice at 8 and 10 weeks of age (Fig. 3G). Consistent with other peptidergic IENFD, the upregulation of CGRP-positive IENFD diminished by 16 weeks of age (Fig. 3G).

**Fig. 1.** Increased IENFD in db/db mice during the period of mechanical allodynia. A–C: Immunohistochemistry studies from the hind paw skin of 8 week-old db/+ mice (A–C) demonstrate PGP9.5 (A) and Trk A (B)-positive IENF. Each counted IENF is labeled with a white dot. A: IENF (arrow and arrowhead) extend from the dermal plexus (asterisks) into the epidermis in db/+ mice. B: Trk A immunoreactive fibers are also positive for PGP9.5 (arrow). C: The merged picture demonstrates both Trk A-positive (arrow) and -negative (arrowhead) IENF. D–F: Immunohistochemistry studies from the hind paw skin of 8 week-old db/db mice demonstrated PGP9.5 (D) and Trk A (E)-positive IENF. D: PGP9.5-positive IENF (arrow) in epidermis of db/+ mice. E: Trk A-positive IENF (arrow) in db/db mice. F: The merged picture demonstrates most PGP9.5 immunoreactive fibers are also positive for Trk A (arrow). Quantification analysis of IENFD demonstrates increased PGP9.5 (G) and Trk A (H) at 8 and 10 weeks of age. I: Fold changes of the ratio of Trk A-positive fibers and PGP9.5-positive IENF in each animal to db/+ mice of the same age. There was a 1.5 fold increase of TrkA/PGP9.5-positive IENFD ratio in db/db mice when compared to db/+ mice of the same age. ***, p < 0.001, N = 4. Bar = 20 μm.
Continuation of TNF-α-positive IENFD increase in diabetic animals beyond the period of mechanical allodynia

To determine whether this upregulation of peptidergic IENF was a general phenomenon involving all the IENF, we measured TNF-α-positive IENF from 5, 8, 10, and 16 weeks of age. In db/+ mice, TNF-α-positive IENF include both Trk A-positive and -negative fibers (Figs. 4A–C). In db/db mice, the TNF-α-positive IENF increase at 8 weeks of age in both Trk A-positive and -negative fibers (Figs. 4D–F). Quantitative studies detected up to a 1.5-fold increase of TNF-α-positive IENFD at 8 and 10 weeks of age (Fig. 4G). In contrast to peptidergic IENF, TNF-α-positive IENFD remain elevated at 16 weeks of age (Fig. 4G).

Anti-NGF inhibited the upregulation of PGP9.5-positive and peptidergic IENFD in diabetic animals

The observation of increased peptidergic IENF suggests that there is NGF-dependent neurotrophism in the skin that mediates mechanical allodynia. In order to test this hypothesis, we administered anti-NGF intraperitoneally to antagonize NGF actions weekly for 2 weeks starting at 6 weeks of age. Previously, we reported that this treatment effectively inhibited the development of mechanical allodynia in the db/db mouse at 8 weeks of age (Cheng et al., 2009). In contrast to peptidergic IENF, TNF-α-positive IENFD remain elevated at 16 weeks of age (Fig. 4G).

SB203580 treatment inhibited the upregulation of PGP9.5-positive and peptidergic IENFD in diabetic animals

We previously reported that NGF-mediated p38 activation is an important mechanism for the development of mechanical allodynia (Cheng et al., 2009). In order to further understand its role, we tested whether p38 could mediate the effects of NGF on increasing peptidergic IENFD. SB203580, a p38 inhibitor, was administered via an intrathecal minipump for 1 week to animals at 7 weeks of age as described previously (Cheng et al., 2009). SB203580 treatment significantly decreased PGP9.5, Trk A, SP, and CGRP-positive IENFD in db/db mice at 8 weeks of age (Fig. 6). SB203580 treatment did not affect IENFD in db/+ mice (Fig. 6).

Discussion

The results from our current study support the use of peptidergic IENFD measurement for the diagnosis of PDN of type 2 diabetes in clinical practice. As was previously mentioned, PGP9.5 has been established as the best pan-neuronal marker across species to localize IENF (Karanth et al., 1991). However, PGP9.5 IENFD measurement has not been proven to be a useful method for the diagnosis of pain. Similar to the current protocol for human IENFD measurement, PGP9.5 immunohistochemistry has been used to determine IENFD in db/db mice (Polydefkis et al., 2001). We previously reported that decreased IENFD correlated with the decrease of nerve conduction velocities in db/db mice at 24 weeks of age (Sullivan et al., 2007). In addition, Wright et al. also reported no change of IENFD in db/db mice at 15 weeks of age, similar to our findings at 16 weeks of age (Wright et al., 2007). In the current study, a significant increase in PGP 9.5-
positive IENFD was detected in db/db mice at 8 and 10 weeks of age, during the period of mechanical allodynia. This study suggests that early pain phenotypes are associated with increased IENFD in the animal model of type 2 diabetes. This phenomenon has not been previously characterized. Consistent with our current findings, Karanth et al., 1990, 1991 reported increased PGP9.5 IENFD in STZ treated animals (Christianson et al., 2003). Johnson et al., 1997 found similar results, they reported that PGP9.5 IENFD decreased at early stages of diabetes. Conversely, instead of an increase in IENFD, several recent reports demonstrated a loss of PGP9.5-positive IENFD in animals and humans with PDN from both type 1 and type 2 diabetes (Holland et al., 1997; Polydefkis et al., 2001; Sorensen et al., 2006a,b).)

Among these studies, Holland and colleagues correlated the severity of PGP9.5-positive IENFD loss in patients with mixed groups of painful sensory neuropathy including PDN (Holland et al., 1997; Polydefkis et al., 2001; Sorensen et al., 2006a,b). Among these studies, Sorensen et al., 2006b demonstrated a greater percentage increase (up to 3 fold) of peptidergic fibers in total PGP9.5-positive IENFD in db/db mice during the period of mechanical allodynia. Our data demonstrated a positive correlation between the peptidergic IENFD with PDN at the early stages of type 2 diabetes. Using a STZ model of type 1 diabetes, Karanth et al. reported an early increase in CGRP-positive IENFD in diabetic rats (Karanth et al., 1990). Unfortunately, it is unclear in this study if the upregulation is associated with pain behaviors. Apart from this inquiry, most other reports demonstrated decreased CGRP- and SP-positive IENFD in STZ treated animals (Christianson et al., 2003). Johnson et al.
demonstrated an early loss of peptidergic IENF in the diabetic mouse following STZ injection with insensate diabetic neuropathy (Johnson et al., 2008). The peptidergic IENF loss occurs as early as 4 weeks after STZ injection, without the prior development of pain behaviors. Levy et al. also observed reduced CGRP immunoreactive areas in diabetic skin, suggesting that the diminution of CGRP immunopositivity is associated with neuropathy (Levy et al., 1992). However, neither of these reports correlated the loss of CGRP immunoreactivity with PDN. In general,
these reports mainly focus on the late phase of DN, not the phase with PDN (Christianson et al., 2003). We believe that the upregulation of NGF could be a phenomenon specific to the PDN period of type 2 diabetes.

Our results demonstrated increased TNF-α-positive IENFD in db/db mice from 8 to 16 weeks of age. This finding is consistent with our previous published data that there is a NGF-p38-mediated upregulation of TNF-α in DRG of db/db mice at 8 weeks of age (Cheng et al., 2010). However, this increment was not correlated with the diminution of mechanical allodynia at 16 weeks of age. Unfortunately, similar results have not been reported in rodents or human skin. Our data suggest that TNF-α might not be a good indicator for PDN, but rather an indicator for the progressive nerve damage from DN. A similar phenomenon was reported in a chronic nerve constriction model by Schaefers and colleagues. After sciatic nerve constriction, increased TNF-α expression was detected in the skin and muscle nerve afferents as well as their associated DRG neurons (Schaefers et al., 2003). This upregulation of TNF-α in peripheral nerves is most likely associated with enhanced anterograde axonal transport after nerve injury (Schaefers et al., 2002).

Based on our findings, there is no initial loss of IENFD before or during the early stages of PDN. In actuality, increased IENFD are observed. We believe that this phenomenon is most likely based on increased axonal sprouting, rather than regeneration. It is well known that unmyelinated nociceptive fibers reinervate the skin after denervation, which is similar to the reestablishment of neuromuscular junctions after degeneration in myelinated fibers (Diamond and Forster, 1992; Nixon et al., 1984). Similar to our model of PDN, Griffin et al. detected nerve regeneration or axonal sprouting after nerve injury (Griffin et al., 2010). The current measurement of IENFD only includes axons that penetrate the dermal–epidermal junction. We believe that increased axonal sprouting likely occurs at the dermal plexus, prior to axonal innervation of the epidermis. There is a plethora of evidence that indicates the subepidermal plexus and basal skin layers are sources of neurotrophic factors (Cheng et al., 2009; English et al., 2005). Additionally, our findings from the anti-NGF experiment demonstrate that NGF could contribute to the increase of IENFD during PDN.

We observed that anti-NGF treatment significantly decreased SP and CGRP-positive IENFD. The same experimental paradigm also inhibited the development of mechanical allodynia in the db/db mouse (Cheng et al., 2009), indicating that increased neurotrophism is an important mechanism to mediate PDN in type 2 diabetes. NGF is a promoter for both SP and CGRP expression in cultured DRG neurons. Our data suggests increased NGF action during the initial period of mechanical allodynia could increase the regeneration of TrkA positive IENFD which express neuropeptides such as SP and CGRP. In the literature, increased NGF action has been reported to mediate several chronic painful conditions (Pezet and McMahon, 2006). The current data are in agreement with our previous report that revealed increased cutaneous NGF action of db/db mice during mechanical allodynia (Cheng et al., 2009). In addition to our findings, Christianson and colleagues reported that exogenous NGF administration enhanced the cutaneous nerve sprouting and protected against DN, indicating NGF could promote IENF regeneration (Christianson et al., 2003). Additionally, NGF treatment enhances the expression of pain-specific sodium channel expression in peripheral nerves and DRG neurons, including SNS, a tetrodotoxin-resistant (TTX-R) sodium channel (Dib-Hajj et al., 1998). In further support of our conclusions, increased NGF gene expression has been detected in the calf skin of patients with diabetes (Dielm et al., 1999). Taken together, these enhanced NGF actions in DN of type 2 diabetes could be responsible for the increased peptidergic IENFD during the development of PDN.

We previously reported that the NGF-p38 pathway in DRG mediated the development of mechanical allodynia in db/db mice. In our current study, SB203580 inhibited the upregulation of IENFD during the period of mechanical allodynia. In support of our findings, SB203580, but not the ERK pathway blocker U0126, inhibited the ability of PC12m32 cells to induce neurite outgrowth in response to osmotic shock (Kano et al., 2007). This p38-mediated neurite outgrowth requires CREB and paxillin phosphorylation (Huang et al., 2004; Kano et al., 2007). The current findings suggest similar mechanisms could also mediate increased IENFD during mechanical allodynia in db/db mice.

Finally, our current results suggest increased regeneration of peptidergic nerve fibers in the skin during the period of mechanical allodynia. Our findings suggest that there is increased innervation in skin from large-sized DRG neurons in db/db mice. Multiple studies have suggested NGF-related nerve regeneration in animal models of diabetic neuropathy (Yasuda et al., 2003). In our previous report, we demonstrated the conversion of large-sized DRG neurons to NGF- and SP-positive phenotypes during the period of mechanical allodynia (Cheng et al., 2009). These large-sized DRG neurons normally extend...
myelinated Aβ fibers, which do not innervate skin nor mediate nociception. Combining our current and previous findings, we propose a hypothesis that there could be increased skin innervation from those large DRG neurons to enhance mechanical sensitivity. This hypothesis is supported by Christianson and colleagues who reported that NGF increases myelinated cutaneous innervation in diabetic animals and restores sensory dysfunction from DN (Christianson et al., 2007).

Conclusions

In conclusion, the upregulation of peptidergic IENFD via NGF-p38 signaling is observed in an animal model of type 2 diabetes during PDN. This phenomenon is associated with and better correlated with the development of mechanical allodynia than the changes in PGP9.5-positive IENFD. These findings not only support the measurement of peptidergic IENFD for the diagnosis of PDN in type 2 diabetes, but also identify the NGF-p38 signaling pathway as a possible pharmacological target for treating this devastating condition.

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References


Hyperinsulinemia Induces Insulin Resistance in Dorsal Root Ganglion Neurons

Bhumsoo Kim, Lisa L. McLean, Stephen S. Philip, and Eva L. Feldman
Department of Neurology, University of Michigan, Ann Arbor, Michigan 48109-2200

Insulin resistance (IR) is the major feature of metabolic syndrome, including type 2 diabetes. IR studies are mainly focused on peripheral tissues, such as muscle and liver. There is, however, little knowledge about IR in neurons. In this study, we examined whether neurons develop IR in response to hyperinsulinemia. We first examined insulin signaling using adult dorsal root ganglion neurons as a model system. Acute insulin treatment resulted in time- and concentration-dependent activation of the signaling cascade, including phosphorylation of the insulin receptor, Akt, p70S6K, and glycogen synthase kinase-3β. To mimic hyperinsulinemia, cells were pretreated with 20 nM insulin for 24 h and then stimulated with 20 nM insulin for 15 min. Chronic insulin treatment resulted in increased basal Akt phosphorylation. More importantly, acute insulin stimulation after chronic insulin treatment resulted in blunted phosphorylation of Akt, p70S6K, and glycogen synthase kinase-3β. Interestingly, when the cells were treated with phosphatidylinositol 3-kinase pathway inhibitor, but not MAPK pathway inhibitor, chronic insulin treatment did not block acute insulin treatment-induced Akt phosphorylation. Insulin-induced Akt phosphorylation was lower in dorsal root ganglion neurons from BKS-db/db compared with control BKS-db+ mice. This effect was age dependent. Our results suggest that hyperinsulinemia cause IR by disrupting the Akt-mediated pathway. We also demonstrate that hyperinsulinemia increases the mitochondrial fission protein dynamin-related protein 1. Our results suggest a new theory for the etiology of diabetic neuropathy, i.e., that, similar to insulin dependent tissues, neurons develop IR and, in turn, cannot respond to the neurotrophic properties of insulin, resulting in neuronal injury and the development of neuropathy. (Endocrinology 152: 3638–3647, 2011)
damage to supporting Schwann cells, contributing to the pathogenesis of diabetic neuropathy. Insulin is a well-documented growth factor for neurons (7, 8), and its receptors are widely expressed in both the peripheral and central nervous systems (9). Although neurons are not insulin dependent, they are insulin responsive (7–9). Therefore, insulin deficiency in type 1 diabetes and hyperinsulinemia in type 2 diabetes may contribute to the development of diabetic neuropathy.

Akt, a serine/threonine kinase-activated downstream of phosphatidylinositol 3-kinase (PI3-K), is a critical signaling molecule mediating cell survival, growth, and energy homeostasis (10, 11). Full activation of Akt requires phosphorylation of Thr308 in the catalytic domain by phosphoinositide dependent protein kinase (PDK)1 and Ser473 in the C-terminal hydrophobic domain by mammalian target of rapamycin complex (mTORC)2 (10). Considering the important roles of Akt in insulin signaling, it is not surprising that alterations in Akt activity are found in various cells in diabetes and insulin-resistant states. In obese rats, insulin-stimulated Akt1 activity is decreased in muscle and adipose tissue but increased in liver and vice versa for Akt2 (12). Akt phosphorylation is reduced in adipocytes and skeletal muscle of type 2 diabetic patients (13, 14).

IR studies are mainly focused on peripheral tissues, such as muscle and liver. Chronic insulin treatment induces IR with decreased Akt signaling and glucose transporter expression in muscle and adipocytes (15–17). There is, however, little knowledge about IR in neurons. In the late 1990s, our laboratory introduced the idea that glucose-mediated oxidative stress injures sensory neurons, leading to eventual development of diabetic neuropathy (18). Recently, we started to investigate whether hyperinsulinemia-induced IR causes or contributes to similar neuronal injuries. We speculate that like metabolic tissues, such as fat and muscle, neurons develop a form of IR, making them susceptible to glucose-mediated damage. In the current study, we examined possible molecular mechanisms for neuronal IR. We present evidence of chronic stimulation-mediated blunting of Akt and its downstream effectors in adult dorsal root ganglion (DRG) neuron culture. We provide the evidence that hyperinsulinemia results of disruption in mitochondrial biogenesis. We also demonstrate that insulin-mediated Akt phosphorylation is reduced in DRG neurons prepared from a mouse model of type 2 diabetes. We conclude that chronic stimulation of Akt may contribute to reduced insulin signaling in DRG neurons and underlie, in part, the pathogenesis of diabetic neuropathy.

Materials and Methods

Antibodies and chemicals

Antibodies against insulin receptor (InsR), InsR substrate (IRS)-1, and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antiglyceraldehyde-3-phosphate dehydrogenase antibody was from Chemicon (Temecula, CA) and antidynamin-related protein (Drp)1 was from Abnova (Walnut, CA). All other antibodies were purchased from Cell Signaling (Beverly, MA). Inhibitors (LY294002 and U0126) were purchased from Calbiochem (La Jolla, CA). Palmitic acid (PA) was purchased from Nu-Chek Prep, Inc. (Elysian, MN). All other chemicals were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

Adult DRG neuron preparation

DRG neurons were prepared as previously described from adult (>6 wk old) C57Bl6J mice or Sprague Dawley rats (19). BKS-db/db and db+ (BKS.Cg-m+/- Leprdb/db), JAX Mice stock no. 00642) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 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. All culture reagents were from Gibco (Grand Island, NY) unless stated otherwise. The neurons were dissociated in 0.2% collagenase for 30 min followed by 1% trypsin for 15 min. Cells were plated on collagen-coated 12-well tissue culture plates in feed media (1:1 mix of DMEM:F-10 media containing 1× B27 additives without antioxidant, 7 μM aphydicolin, and 1000 U/ml penicillin/streptomycin/neomycin) (d 0). The media were changed to fresh feed media on d 2. On d 3 (24 h before treatment), to exclude the effect of insulin contained in B27, culture media were changed to treatment media (feed media without B27). Chronic treatment with insulin, glucose, or palmitate was achieved by adding these agents on d 3 in treatment media for 24 h. Inhibitors were also added to the media of some cultures at the same time, for 24 h.

Western immunoblotting

DRG neuron cultures were lysed in radioimmunoprecipitation assay (RIPA) buffer (Pierce, Rockford, IL) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were collected, briefly sonicated, and centrifuged at 13,000 rpm for 15 min at 4 C. Western immunoblotting was performed as described previously (20). Tris-buffered saline with 0.01% Tween 20, and 5% fat-free milk was used for blocking and antibody dilution. 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 (Amersham Bioscience, Piscataway, NJ) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Images were captured using the Chemidoc XRS system and analyzed by Quantity One software (Bio-Rad Laboratory, Hercules, CA) (20). All experiments were repeated at least three times, and representative results are presented in the figures. In some experiments, the nitrocellulose membranes were incubated at 60 C for 15 min in stripping solution [2% sodium dodecyl sulfate, 100 mM dithiothreitol, and 100 mM Tris (pH 6.8)], whereupon they were used for immunoblotting with another antibody.
Immunohistochemistry (IHC)

Adult DRG neurons cultured on laminin-coated coverslips were permeabilized with PBS containing 3% BSA, 0.1% Triton X-100, and 1% normal donkey serum (IHC buffer). The cells were incubated with the primary antibodies diluted in IHC buffer at 4°C overnight. After rinsing with PBS three times for 5 min each, the cells were incubated with the antirabbit IgG secondary antibody conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR) for 2 h at room temperature. After rinsing with PBS, the coverslips were mounted with ProLong antifade mounting media containing 4',6-diamidino-2-phenylindole (Molecular Probes). The digital images were captured using a Spot-RT camera (Diagnostic Instrument, Inc., Sterling Heights, MI) attached to Nikon Microphot-FXA microscope (Nikon, Melville, NY).

Statistical analysis

All experiments were repeated at least three times and presented as the mean ± SEM. Statistical analysis was performed by either one-way ANOVA with Tukey’s post hoc analysis or Student’s t test depending on the number of comparison groups, using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Statistical significance was defined as P < 0.05.

Results

Insulin signaling influences the development of the nervous system by promoting neurogenesis, survival, and differentiation (21–24). The MAPK and PI3-K/Akt-pathways are the major intracellular signaling cascades that mediate insulin’s effects (9, 25). As a first step, insulin signaling in adult DRG neurons was examined by Western immunoblotting after treatment with 20 nM insulin for 0–2 h. Insulin stimulation induced a time-dependent increase in the phosphorylation (i.e., activation) of intracellular signaling molecules, including InsR, Akt, and glycogen synthase kinase-3β (GSK-3β) (Fig. 1A). ERK exhibited high background phosphorylation, and insulin stimulation had a minimal effect on its phosphorylation. Each blot was stripped and reprobed with antibodies against total protein as well as actin to confirm equal protein loading. As expected, the PI3-K pathway inhibitor LY294002 reduced insulin-induced Akt phosphorylation, and the MAPK inhibitor U0126 decreased basal ERK phosphorylation (Fig. 1B). We find similar results using rat adult DRG neurons (data not shown).

We next examined the localization of the signaling molecules in DRG neurons using IHC. IR and IRS-1 display a punctate pattern of localization within the cytoplasm and along the neurites. Nuclear localization was not detected (Fig. 2). In contrast, Akt1 and Akt2 display more diffuse distribution within the cytoplasm and nucleus. We did not detect any quantifiable changes in the localization of the signaling molecules by insulin treatment (data not shown).

IR may be induced by hyperglycemia, dyslipidemia, or hyperinsulinemia, which are the characteristics of type 2 diabetes and the metabolic syndrome (1, 26, 27). Therefore, we next explored whether these factors contribute to the development of IR in DRG neurons. To create an in vitro model of the metabolic syndrome and assess IR, adult DRG neurons were treated with 25 mM added glucose, 0.2 mM PA, or 20 nM insulin for 24 h. The media were then replaced with fresh treatment media for 30 min, and the cells were stimulated with 20 nM insulin for another 15 min. Akt was phosphorylated after 15 min of acute insulin treatment in DRG neurons not given a 24-h pretreatment. Interestingly, when cells were chronically pretreated with 20 nM insulin for 24 h, Akt phosphorylation by subsequent acute insulin treatment was significantly reduced (Fig. 3A). In contrast, 24 h of glucose or PA treatment had no effect on the activation of Akt in response to short-term insulin treatment. Densitometric analysis confirmed that only insulin pretreatment significantly decreased Akt phosphorylation (Fig. 3B). The effect of chronic insulin pretreatment on Akt phosphorylation was concentration-dependent from 2 to 200 nM (Fig. 3C). Maximal Akt phosphorylation by acute insulin treatment was significantly
lower in insulin-pretreated cells compared with non-
treated cells. There was also a concentration-dependent
increase in basal Akt phosphorylation, but this trend did
not reach statistical significance. When the cells were pretreated with 20 or 200 nM insulin,
acute insulin treatment failed to elicit any signif-
ificant increase in Akt phosphorylation over
basal phosphorylation (Fig. 3D). Insulin treatment
did not induce any noticeable changes in
ERK phosphorylation regardless of pretreat-
ment insulin concentration. InsR phos-
phorylation was not significantly affected by up to 20
nM insulin pretreatment (Fig. 3C). InsR phos-
phorylation and expression were decreased
only at the highest pretreatment insulin con-
centration (200 nM). These results suggest that
development of IR is mainly induced by hyper-
insulinemia, rather than hyperglycemia or hy-
perlipidemia, in DRG neurons.

We next examined the detailed changes in
insulin signaling by insulin pretreatment. As
shown in the previous figure, IR phosphoryla-
tion and protein expression levels were not af-
fected by insulin pretreatment (Fig. 4Aa). IRS-1 phosphor-
ylation was measured by phosphor-specific antibodies at

FIG. 2. Cellular localization of insulin signaling molecules. Adult rat DRG neurons
were cultured on laminin-coated coverslides and immunolabeled with the indicated
antibodies (red). Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI)
(blue). Scale bar, 10 μm.

FIG. 3. Chronic insulin stimulation mimicking hyperinsulinemia down-regulates the ability of DRG neurons to respond to acute insulin stimulation. A, DRG neurons were incubated with 25 mM glucose (Glu), 0.2 mM PA, or 20 nM insulin (Ins) for 24 h, washed in fresh treatment media for 30 min, and then stimulated with 20 nM insulin for 15 min. B, Densitometric analysis of Akt phosphorylation of A. *, P < 0.05 by one-way ANOVA compared with the other acute insulin-treated conditions. Akt phosphorylation in Glu and PA pretreated cells was not statistically significant compared with control cells. C, DRG neurons were incubated with 0–200 nM insulin for 24 h and then stimulated with 20 nM insulin for 15 min. D, Densitometric analysis of Akt phosphorylation of C. Statistical significance was analyzed by Student’s t test. Cell lysates were prepared in RIPA buffer and immunoblotted with the indicated antibodies. Antiactin immunoblot demonstrates the equal loading of the proteins. Ctl, Control; p, phosphorylated form.
Ty1222 and Ser636/639 sites. Insulin stimulation induced slight phosphorylation at both locations, and like IR, insulin pretreatment did not affect the phosphorylation (Fig. 4Ab). Full activation of Akt requires sequential phosphorylation of Thr308 and Ser473 by PDK1 and mTORC2, respectively (10). Twenty-four-hour insulin treatment decreased the phosphorylation at both of these sites (Fig. 4Ac). Insulin pretreatment or acute insulin stimulation did not affect the protein expression levels of Akt1 or Akt2 isoforms or total Akt (Fig. 4Ad). The Akt3 isoform was not expressed in DRG neurons. p70S6K and GSK-3β are two important signaling molecules mediating Akt’s physiological effects on survival, neuronal differentiation, and metabolism (10, 11). In parallel with the effect on Akt phosphorylation, 24-h chronic insulin pretreatment decreased acute insulin-stimulated p70S6K and GSK-3β phosphorylation (i.e. activation of p70S6K and inactivation of GSK-3β) (Fig. 4Ac). Insulin and IGF-I share common signaling pathways for their physiological actions (9, 25). Even though insulin pretreatment significantly reduced Akt phosphorylation by acute insulin treatment, it did not affect IGF-I-stimulated Akt phosphorylation (Fig. 3B), suggesting that the effect of insulin pretreatment is specific to insulin signaling.

We next examined the possible mechanism behind the reduction in Akt activation after chronic insulin treatment using the inhibitors for PI3-K/Akt (LY294002) or ERK (U0126) signaling pathways. During the 24-h chronic insulin pretreatment, DRG neurons were incubated without or with these inhibitors. The cells were then washed with Hank’s balanced salt solution and incubated in fresh treatment media (without insulin or inhibitors) for 30 min before stimulation with 20 nM insulin for 15 min. As in the previous experiments, acute insulin treatment-induced Akt phosphorylation was significantly reduced in DRG neurons chronically pretreated with insulin (Fig. 5A). Combined treatment with insulin and LY294002 restored Akt phosphorylation by acute insulin treatment to control levels (Fig. 5A, compare lanes 2, 4, and 5). U0126 had no effect on Akt phosphorylation. Densitometric analysis (Fig. 5B) confirms that LY294002 treatment (lane 5) restored Akt phosphorylation, which was significantly higher than in cells treated without inhibitors (lane 4) or with U0126 (lane 6) treatment, and displayed no statistically significant difference compared with control insulin-treated neurons (lane 2). Insulin-induced IRS-1 serine phosphorylation was not affected by chronic insulin or by LY294002 or U0126. mTOR/p70S6K inhibitor, rapamycin, reduced and proteasome inhibitor, MG132, increased IRS-1 phosphorylation. However, Akt phosphorylation was not changed by rapamycin or MG132 treatment (Fig. 5C). As in the previous experiments, ERK phosphorylation was not significantly affected by a 24-h insulin treatment or by the inhibitors. These results suggest that chronic hyperactivation of Akt by insulin prevents further activation by acute insulin treatment.

These observations were confirmed using adult DRG from an animal model of type 2 diabetes, the BKS-db/db mouse. The BKS.Cg-m-/+ Lep(ab-/-), commonly known as BKS-db/db, expresses a homozygous mutation of the leptin receptor and demonstrates typical characteristics of type 2 diabetes, including obesity, hyperinsulinemia, and hyperglycemia (http://jaxmice.jax.org/strain/000642.html). BKS-db+ mice are heterozygous for this mutation and serve as a control. Adult mouse DRG neurons prepared from different ages were cultured in vitro for 4 d and stimulated with 20 nM insulin for 0–60 min. DRG neurons prepared from 5 wk (1 wk diabetes) db+ and db/db mice demonstrated the increased Akt phosphorylation by insulin treatment, which

FIG. 4. Chronic insulin treatment reduces insulin-stimulated Akt phosphorylation and downstream signaling molecules. A, The cells were treated without or with 20 nM insulin for 24 h and stimulated with 20 nM insulin for 15 min. B, The cells were pretreated with insulin for 24 h and then stimulated with 20 nM insulin or IGF-I for 15 min. Cell lysates were prepared in RIPA buffer and immunoblotted with the indicated antibodies. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. panAkt, Total Akt; Ins, insulin; p, phosphorylated form.

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was maintained up to 60 min (Fig. 6A). However, DRG neurons from 24-wk db/db, but not db/H11001/H11021, mice displayed decreased Akt phosphorylation at 60 min. This effect was age dependent, with decreased Akt phosphorylation beginning at approximately 16 wk of age (12-wk diabetes) (Fig. 6B).

Mitochondria play important roles in cellular energy metabolism and ATP generation (28). High metabolic activity of mitochondria results in the generation of reactive oxygen species (29). We demonstrated the critical role of mitochondria in the progression of hyperglycemia-mediated neuronal damage and diabetic neuropathy (30–32). Previous work from our laboratory implicated Drp1, a GTPase mediating mitochondrial fission, in hyperglycemia-mediated mitochondrial damage in sensory neurons (33, 34). For this experiment, we used rat adult DRG. Consistent with mouse DRG, rat adult DRG neurons display a similar decrease in insulin-induced Akt phosphorylation at 60 min. This effect was age dependent, with decreased Akt phosphorylation beginning at approximately 16 wk of age (12-wk diabetes) (Fig. 6B).

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Discussion

IR due to hyperinsulinemia is one of the main characteristics of type 2 diabetes and other metabolic syndromes (1). IR studies are mostly focused on metabolic tissues, such as adipocytes and muscle. We hypothesized that neurons, similar to metabolic tissues, may develop IR that contributes to the development of neuropathy in diabetic patients. In the current study, we demonstrate that insulin-induced Akt phosphorylation was severely blunted in adult DRG neurons that had been previously exposed to insulin for a long period (24 h). Interestingly, acute Akt signaling was restored when the PI3-K inhibitor, LY294002, was added with 24-h insulin. We also observed that adult DRG prepared from the animal models of type 2 diabetes display reduced insulin-stimulated Akt phosphorylation. Our results suggest that chronic stimulation of PI3-K/Akt pathway is partly responsible for the development of IR.

Stimulation of adult DRG with physiologic levels of insulin resulted in the activation of signaling pathways, including the phosphorylation of InsR, Akt, and its downstream signaling molecules, GSK-3β and p70S6K. We have previously reported the activation of Akt by IGF-I and its critical role in DRG survival (35). In contrast, ERK phosphorylation was only slightly affected by insulin treatment, which was in agreement with the previous reports by ours (35) and other researchers (36, 37). Even though InsR phosphorylation was quickly decreased after approximately 60 min of insulin stimulation, Akt phosphorylation was maintained up to 2 h. Akt is activated downstream of PI3-K. However, it has been suggested that full activation of PI3-K is not necessary for maximal Akt activation (38). Akt activity in skeletal muscle from obese...
Zucker rats is only slightly decreased, even though PI3-K activity is significantly reduced compared with lean control rats (12). As expected, PI3-K and MAPK signaling pathway inhibitors LY294002 and U0126 decreased insulin-stimulated as well as basal Akt and ERK phosphorylation, respectively. Our results suggest that the Akt-mediated signaling pathway plays a major role in conducting insulin’s action in DRG neurons.

Type 2 diabetes and the metabolic syndrome are characterized by hyperglycemia, dyslipidemia, and hyperinsulinemia, all of which contribute to the development of IR (1, 26, 27). In the late 1990s, our laboratory introduced the idea that glucose-mediated oxidative stress injures sensory neurons, leading to eventual death and loss of neurons and supporting Schwann cells and the development of diabetic neuropathy (5). We demonstrated that 20–25 mM added glucose induces programmed cell death in DRG neurons (32, 33, 39, 40). Our recent results suggest that dyslipidemia also contributes to diabetic neuropathy by increasing plasma oxidized low density lipoprotein, which directly leads to oxidative stress and injury in DRG neurons via lectin-like oxidized low density lipoprotein receptor (LOX)-1 (41, 42). Even though no data are available for neurons, it has been shown that long-term exposure to insulin suppresses mitochondrial biogenesis and function and leads to increased production of mitochondrial reactive oxygen species in primary hepatocytes (43).

After establishing the normal insulin signaling pathway in DRG neurons, we next examined the possible contributors to IR by treating the neurons with high glucose, fatty acid, or insulin. Of these, only chronic insulin treatment resulted in significant inhibition of Akt activation and was insulin pretreatment concentration dependent. Phosphorylation of both Ser473 and Thr308 sites was severely inhibited by chronic insulin treatment. The total protein level of Akt was not affected by insulin pretreatment. Insulin-induced phosphorylation of downstream signaling molecules GSK-3β and p70S6K was also reduced by insulin pretreatment. This effect was specific to insulin signaling, because IGF-I-induced Akt phosphorylation was not affected by chronic insulin treatment. Our results are in agreement with a recent report using primary hepatocytes (44). When primary hepatocytes were chronically pretreated with insulin, acute insulin-induced Akt phosphorylation was greatly reduced. Glucose pretreatment, however, did not affect insulin-stimulated Akt phosphorylation. In the same report, the authors also demonstrated that continuous treatment of nonobese diabetic mice (NOD/ShiLtj) with a long-acting human insulin analog, insulin detemir, resulted in the development of IR with reduced mitochondrial production and increased lipid accumulation and oxidative stress. Therefore, our results strongly suggest that hyperinsulinemia is the main contributor for the development of IR in DRG neurons.

Insulin-stimulated Akt phosphorylation was examined in DRG neurons prepared from an animal model of type 2 diabetes, the BKS-db/db. These mice are obese and be-
stimulated with 20 nM insulin for 15 min. B, Densitometric analysis of Akt phosphorylation of corresponding no 15-min insulin treatment (bars 1 and 3); *, P < 0.05 compared with no 24-h insulin and 15-min insulin treatment (bar 2). C, DRG neurons were cultured from BKS-db/db compared with DRG neurons from BKS-db/db mouse were cultured for 4 d in normal (without high glucose or insulin) media before insulin treatment.

The specific role of PI3-K/Akt signaling for the induction of IR in neurons was confirmed by blocking this pathway with PI3-K inhibitor, LY294002. When DRG neurons were exposed to insulin and LY294002 together for 24 h, acute insulin-induced Akt phosphorylation was restored. Our results are in agreement with a previous report in the L6 muscle cell line (15). Recently, Liu et al. (46) reported similar results using the mice fed a high-fat diet. When fed a high-fat diet, the mice develop IR along with increased oxidative stress. The mice display hyperinsulinemia with normal fasting glucose levels, again suggesting that IR is mainly induced by hyperinsulinemia rather than hyperglycemia (44). The adverse effect of a high-fat diet on oxidative stress and IR were reversed when the mice were treated with LY294002 during the day (when mice usually do not eat). These results and our own suggest that constant insulin signaling increases basal Akt phosphorylation, rendering the target tissues desensitized to insulin and develop IR.

Serine phosphorylation of IRS-1 triggers its degradation; this step is generally considered a negative regulator of insulin signaling that contributes to the development of IR (25). Chronic insulin treatment that resembles IR induces IRS-1 degradation in 3T3-L1 adipocytes (47, 48). PI3-K pathway-mediated reduction in IRS-1/2 expression is responsible for the decreased Akt phosphorylation after chronic insulin stimulation in L6 muscle cells (15). Although IRS-1 serine phosphorylation generally inhibits insulin signaling, recent reports suggest that it may have positive roles. Ser302 phosphorylation is implicated in nutrient-mediated cell growth and mitogenesis (49). Phosphorylation at Ser636, which has negative effect on insulin signaling, is decreased by Ser629 phosphorylation, resulting in enhanced insulin signaling (50). However, our results demonstrate little correlation between IRS-1 phosphorylation/expression and Akt phosphorylation, suggesting that, unlike peripheral tissues, signaling molecule(s) downstream of IRS are responsible for the decreased Akt phosphorylation after chronic insulin treatment in DRG neurons. Full activation of Akt requires phosphorylation of Thr308 in the catalytic domain by PDK1 and Ser473 in the C-terminal hydrophobic domain by mTORC2 (10). The mTORC1 complex regulates cellular homeostasis and is under the control of energy and nutrient input (51). mTOR is the key component of both mTORC1 and mTORC2 complexes and is essential for cell metabolism, growth, and survival (51). AMP-activated protein kinase (AMPK) is cellular energy sensor that is regulated by the AMP/ATP ratio (52). AMPK regulates lipid and glucose metabolism in liver and muscle and, thus, considered a potential pharmacological target for diabetes and metabolic syndrome.
Kim
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Address all correspondence and requests for reprints to: Bhumsoo Kim, Ph.D., University of Michigan, Department of Neurology, 109 Zina Pitcher Place, 5371 BSRB, Ann Arbor, Michigan 48109-2200. E-mail: bhumsoo@umich.edu.

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Transcriptional Profiling of Diabetic Neuropathy in the BKS db/db Mouse
A Model of Type 2 Diabetes

Manjusha Pande,1,2 Junguk Hur,1,3 Yu Hong,1 Carey Backus,1 John M. Hayes,1 Sang Su Oh,1 Matthias Kretzler,2,3,4 and Eva L. Feldman1,2,3

OBJECTIVE—A better understanding of the molecular mechanisms underlying the development and progression of diabetic neuropathy (DN) is essential for the design of mechanism-based therapies. We examined changes in global gene expression to define pathways regulated by diabetes in peripheral nerve.

RESEARCH DESIGN AND METHODS—Microarray data for 24-week-old BKS db/db and db/+ mouse sciatic nerve were analyzed to define significantly differentially expressed genes (DEGs); DEGs were further analyzed to identify regulated biological processes and pathways. Expression profile clustering was performed to identify coexpressed DEGs. A set of coexpressed lipid metabolism genes was used for promoter sequence analysis.

RESULTS—Gene expression changes are consistent with structural changes of axonal degeneration. Pathways regulated in the db/db nerve include lipid metabolism, carbohydrate metabolism, energy metabolism, peroxisome proliferator–activated receptor signaling, and the Wnt signaling pathway. Promoter sequences of lipid metabolism–related genes exhibit evidence of coregulation of lipid metabolism and nervous system development genes.

CONCLUSIONS—Our data support existing hypotheses regarding hyperglycemia-mediated nerve damage in DN. Moreover, our analyses revealed a possible coregulation mechanism connecting hyperglyceremia and axonal degeneration. Diabetes 60:1981–1989, 2011

A ccording to statistics published by the American Diabetes Association in 2007, 23.6 million people, approximately 8% of the U.S. population, have diabetes and 1.6 million new cases are diagnosed every year. Type 2 diabetes is the most common type of diabetes, accounting for 90–95% of all cases, and is characterized by hyperglycemia, insulin resistance, and dyslipidemia. In addition, an estimated 57 million American adults exhibit impaired glucose tolerance, which frequently develops into type 2 diabetes (1).

The hyperglycemia that defines diabetes is a major factor in the development of micro- and macrovascular complications, which severely affect patients’ longevity and quality of life. Microvascular complications include retinopathy, nephropathy, and neuropathy. From 60 to 75% of diabetic patients develop diabetic neuropathy (DN), the most common and debilitating complication of diabetes (1). DN is the leading cause of nontraumatic lower-extremity amputations, and the annual cost of DN management is estimated to be more than $10 billion (2).

DN results from length-dependent axonal loss affecting distal portions of extremities first and progressing proximally in a stocking-glove pattern (3). Signs and symptoms of DN vary, and though both sensory and motor nerve fibers may be involved, sensory manifestations appear earlier and are more prevalent. Although development of DN correlates with the severity and duration of diabetes, there is a great variability in the onset and progression of symptoms. Some diabetic patients with poorly controlled hyperglycemia do not display signs and symptoms of DN, whereas others with good glycemic control develop DN, suggesting the involvement of factors besides hyperglycemia. We contend that a better understanding of the molecular mechanisms underlying the development and progression of DN is needed for early diagnosis and intervention as well as for understanding the failure of existing treatments. Identification of potential nonglycemic mechanisms is critical for better prediction of progression and for designing preventive therapies (4). For example, obesity and dyslipidemia have recently been identified as additional risk factors in the development of microvascular complications and may play an important role in the pathogenesis of DN (5–7).

Because of a mutation in the leptin receptor, BKS db/db mice are hyperphagic and obese, develop severe type 2 diabetes with marked hyperglycemia, and serve as an experimental model of type 2 diabetes. These mice develop diabetes by 4 weeks and DN by 8 weeks of age, with prolonged thermal latencies, slowed nerve conduction velocities (NCVs), and loss of intraepidermal nerve fiber (IENF) density (8,9). In addition, db/db mice exhibit high plasma triglyceride and lipoprotein levels and may serve as a model for diabetic dyslipidemia (10). Heterozygous (db/) mice do not develop diabetes and are used as nondiabetic controls in experimental DN (11).

Using DNA microarrays, we compared global gene expression in the sciatic nerve of db/db mice with that of db/+ mice at 24 weeks of age (20 weeks of diabetes) to study changes in gene expression in peripheral nerves. Bioinformatic analysis of microarray gene expression data identifies regulated genes and biological processes and provides new insights into the underlying pathogenesis of the disease process (12).
RESEARCH DESIGN AND METHODS

Mice. BKS.Cg-+/-Leprdb/J mice (stock number 000642) were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained at the University of Michigan in a pathogen-free environment and cared for following the guidelines of the University of Michigan Committee on the Care and Use of Laboratory Animals. Mice were given continuous access to food (Purina 5001, Purina Mills LLC, St. Louis, MO) and water.

Measurement of blood glucose, GHb, cholesterol, and triglyceride levels. Fasting blood glucose levels were measured every 4 weeks. After a 6 h fast, tail blood was analyzed using a standard glucometer (One Touch Profile, LIFESCAN, Inc., Milpitas, CA). At 24 weeks, GHb was measured using the Helena Laboratories Test Kit, Glyco-Tek affinity column method (Helena Laboratories Corp., Beaumont, TX). Plasma samples from six mice per group (db/db and db/+ ) were pooled, and each pool was analyzed by fast-protein liquid chromatography. The fractions were assayed for cholesterol and triglyceride levels. These assays were performed as previously published (13).

Assessment of neuropathy. Hind paw withdrawal time and tail flick time were recorded as measures of thermal latency (14). Sural and sciatic NCVs were recorded and IENF density in footpads of the mice was measured as previously described (8,15).

Tissue harvest and RNA preparation. At 24 weeks of age, mice were killed by sodium pentobarbital overdose and tissues were harvested as previously described (8). The left sciatic nerve was dissected and stored in 30 μL RNA-later (Ambion, An Applied Biosystems Business, Austin, TX). Total RNA was isolated from sciatic nerve using a commercially available silica gel-based isolation protocol (RNeasy Mini Kit, QIAGEN Inc., Valencia, CA), including an on-column DNase digestion following the manufacturer's protocol. RNA quantity and quality were measured by microfluid electrophoresis using the RNA 6000 Pico LabChip on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Affymetrix microarrays. RNA isolated from 10 db/db and 9 db/+ mice was used for hybridization. Of the total RNA, 75 ng from each sample was amplified and biotin labeled using the Ovation Biotin-RNA Amplification and Labeling System (NuGEN Technologies Inc., San Carlos, CA) according to the manufacturer's protocol. RNA amplification and hybridization was performed in two batches (batch 1: db/db [n = 6], db/+ [n = 5]; batch 2: db/db [n = 4], db/+ [n = 4]) by the University of Michigan Comprehensive Cancer Center Affy-metrix and Microarray Core Facility using the GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). Hybridization intensities for the probe features on the chip were detected by laser scan. Image files were quantified using Affymetrix GeneChip software (MAS5) to generate CEL files.

Data analysis. Body weight, blood glucose and GHb levels, plasma cholesterol and triglyceride levels, thermal latency measures, IENF density, and NCVs of the mice from the two groups were compared using a t test. Data for the mice corresponding to outlier microarrays (see below) were excluded from these analyses, leaving six db/db and seven db/+ animals.

The Affymetrix CEL files were processed using a local copy of GenePattern, a bioinformatics platform from the Broad Institute (16), and standard libraries from the BioConductor project (http://www.bioconductor.org) and the R project (http://www.r-project.org). Array quality assessment was performed using BioConductor SimpleAffy and AffyQC-Report packages. Six arrays (two db/db- and four db/+ identified as outliers were removed from further analysis based on the array quality assessment.

Array intensities were normalized with the robust multichip average (RMA) algorithm. The robust multichip average background correction (RMA) and hybridization intensities for the probe features on the chip were detected by laser scan. Image files were quantified using Affymetrix GeneChip software (MAS5) to generate CEL files.

RESULTS

Diabetes, dyslipidemia, and neuropathy phenotyping. Table 1 lists phenotyping parameters for the db/db and db/+ mice measured at 24 weeks of age. The db/db mice are significantly heavier and exhibit higher levels of fasting blood glucose and GHb than the db/+ mice at 24 weeks of age. Plasma cholesterol and triglyceride levels are significantly elevated in the db/db mice, confirming dyslipidemia in these mice. Thermal latency in the hind paw and tail is significantly increased in db/db mice, indicative of sensory loss. Both IENF density and sciatric motor nerve conduction velocity (SMNCV) are significantly lower in db/db mice. Increased thermal latency, loss of IENF density, and decreased SMNCV confirm the development of DN in the db/db mice at 24 weeks of age.

Differential gene expression. Figure 1 outlines the steps in the transcriptional analysis of the microarray data. Using intensity-based moderated t test false discovery rate <0.05 as a threshold, we identified 4,017 DEGs; 2,122 genes were significantly deregulated.
upregulated and 1,895 genes were downregulated in the *db/db* relative to the *db/+* samples. Microarray data were validated using qRT-PCR for 5 selected DEGs, which demonstrated high correlation between microarray and qRT-PCR expression levels (Supplementary Table 1).

**Functional enrichment.** Enrichment analysis identifies functional categories that have a higher representation in the regulated genes and, thus, are likely to be active in the experimental condition. Table 2 lists Gene Ontology (GO) terms overrepresented in DEGs identified using DAVID. Overrepresented biological processes with a higher number of upregulated genes are cell cycle, lipid metabolic process, lipid transport, carbohydrate metabolic process, apoptosis, response to stress, and axonogenesis. Biological processes with a majority of genes downregulated are axonogenesis and cell adhesion. Overrepresented cellular component terms are mitochondrion and axon, with a majority of mitochondrial genes upregulated and a majority of axonal genes downregulated.

**Network analysis.** Gene network analysis is useful for exploring both published and novel gene associations that may be involved in the process being studied. ConceptGen is a gene set relation mapping tool that identifies relationships (significant overlap) between an experimental gene set and curated gene sets in sources such as GO terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Medical Subject Heading (MeSH) terms (20). ConceptGen was used to analyze 717 DEGs annotated with the GO biological processes shown in Table 1. The mouse gene identifiers were converted by ConceptGen Homology conversion tool to 695 human homologs. Relationship among selected concepts (GO biological processes, KEGG pathways, and MeSH terms) associated with the gene set were visualized using ConceptGen network graph tool (Fig. 2). KEGG pathways with significant overlap with the DEG set include fatty acid metabolism, peroxisome proliferator–activated receptor (PPAR) signaling, and extracellular matrix–receptor interaction. Concepts related to the DEG set clustered in three groups: lipid metabolism, apoptosis, and axonogenesis (Fig. 2).

BiblioSphere generates a network of genes based on their cocitation in PubMed abstracts and identifies functionally related subnetworks based on known regulatory relationships and pathway annotation. Figure 3A illustrates a BiblioSphere network of DEGs centered on tumor necrosis factor–alpha (TNF–α) with gene–gene connections restricted to expert-curated connections. TNF–α was chosen as the central node because of its importance in type 2 diabetes and lipid metabolism (22). Central nodes of subnetworks and associated pathways include Hif1a (apoptosis), Serpine1 (p53 signaling), App (axonogenesis), Lyn (Fc ε RI signaling), and Pparg (PPAR signaling and fatty acid oxidation) (Fig. 3B).

PPI network of DEGs was constructed using Cytoscape MiMI plug-in (23). Subnetworks were identified based on pathway annotation of genes in MiMI (Fig. 4). Regulated pathways identified by PPI network are carbohydrate metabolism (glycolysis and tricarboxylic acid cycle [TCA]), energy metabolism (oxidative phosphorylation), lipid metabolism (fatty acid metabolism and glycerolipid metabolism), glutathione metabolism, cell development (cell cycle, apoptosis, axon guidance, and neurotransmitter signaling), cell communication (cell adhesion and extracellular matrix–receptor interaction), and signal transduction pathways

![FIG. 1. Transcriptional data analysis workflow. RNA samples obtained from the sciatic nerve (SCN) of *db/db* and *db/+* mice were hybridized to Affymetrix GeneChip microarrays. Data quantified from scanned microarrays were analyzed to identify DEGs. Functional enrichment analysis of DEGs was performed to identify overrepresented biological categories and pathways. Network analysis identified functionally related subgroups of DEGs. Expression profile clustering identified subgroups of coexpressed DEGs. Promoter sequence analysis identified overrepresented TFBS modules in the promoter sequences of coexpressed genes.](image-url)

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**Table 2**

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<th>Category</th>
<th>Term</th>
<th>Number of genes (up/down)</th>
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<td>GO_BP</td>
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</table>

Functional terms significantly overrepresented in the DEGs are listed. BH P value, Benjamini-Hochberg corrected P value of enrichment in DAVID; GO_BP, GO biological process; GO_CC, GO cellular component.

Supplementary Table 2 lists DEGs in the pathways regulated in the db/db nerve. The majority of DEGs in the glycolysis, TCA, oxidative phosphorylation, fatty acid metabolism, glycerolipid metabolism, mitochondrial fatty acid elongation, lipid transport, adipocytokine signaling, PPAR signaling, and apoptosis pathways are upregulated, whereas most of the axonogenesis-related genes are downregulated in db/db nerve.

Expression profile clustering and promoter sequence analysis. Clustering of gene expression profiles identifies potentially co-regulated and functionally related subgroups of genes. CRC, a model-based Bayesian clustering tool, grouped the DEGs into 18 clusters based on positive as well as negative correlation among their expression profiles. We performed enrichment analysis for gene sets from individual clusters to identify overrepresented functional categories (Supplementary Table 3). Figure 5A illustrates a cluster of 275 DEGs enriched in “mitochondrion,” “lipid metabolic process,” and “axonogenesis.” Gene regulatory sequence elements are organized into motifs of TFBSs known as promoter modules, possibly suggesting coordinated regulation by multiple transcription factors (TFs) (24). We used Genomatix FrameWorker tool to analyze 41 promoter sequences of 22 genes annotated with “lipid metabolism” from the above cluster (Fig. 5B) to look for significantly overrepresented TFBS motifs. Five of these genes are also annotated with “mitochondrion.” Table 3 lists the top five most significantly overrepresented promoter modules consisting of three vertebrate TF matrix families (groups of functionally similar TFs) (25). The HOXF family TFs, the HBOX family TF Meox1, the LHXF family TFs, and the HOMF family TF Msx1 are downregulated, whereas the ETSF family TFs are upregulated in the db/db nerve (Supplementary Table 4).

DISCUSSION

How the metabolic dysregulation caused by insulin resistance leads to local nerve fiber damage in DN is unclear. In this study, we performed global gene expression profiling of sciatic nerve from 24-week-old db/db mice, which exhibit hyperglycemia, dyslipidemia, and DN typical of type 2 diabetes. We detected alterations in the expression of genes involved in carbohydrate and lipid metabolism, lipid transport, stress responses, and apoptosis. Furthermore, promoter sequences of coexpressed lipid metabolism–related DEGs exhibited significantly overrepresented TFBS motifs and are likely to share a common regulatory mechanism relevant to the development of DN.
Morphological and electrophysiological changes in the BKS db/db mice closely mimic the changes observed in DN patients (8,9,26–28). However, the db/db mouse model does not fully duplicate human DN pathology; not all structural changes observed in human DN are seen in the db/db mouse (27,29). Duration of diabetes in mice is likely not long enough for the development of severe long-term degeneration associated with human DN. The development of hyperglycemia and DN in the BKS db/db mice are considered to be consequences of hyperphagia and insulin resistance resulting from impaired leptin signaling in the hypothalamus (8). However, the role of leptin signaling in peripheral nerve is not understood, and whether leptin receptor mutation and obesity have a compounding effect on the development of DN in these mice is not known.

Schwann cells (SC) are major contributors to the mRNA in the sciatic nerve biopsies, with a small contribution coming from axons, epineurial fibroblasts, adipocytes, and vascular endothelial cells (30). Our analyses revealed that genes encoding myelin structural proteins (P0, Pmp22, Connexin-32, Mag, E-Cadherin, and Peri-axin), myelin structural lipid synthesis genes (Plp, Elovl6, and Apolipoprotein D), as well as Sox10, a TF required for myelination, are downregulated in db/db nerve. Segmental demyelination has been observed in human DN (31,32); mouse studies, however, do not report evidence of demyelination (27,29). Downregulation of myelin protein-encoding genes and myelin lipid synthesis genes may represent SC abnormalities preceding the structural changes.

The TFs c-Jun and Krox-24, and other genes expressed by denervated SC to promote axonal regeneration (GAP-43, Ncam, and LI) (33), are downregulated, as are positive regulators of axonogenesis (Cdhl4, Nefl, and Mapt) and axon guidance pathway genes (Netrin G1 and Fyn), suggesting a lack of axonal regeneration. These changes are consistent with the axonal degeneration observed in morphological studies in the db/db mice (27,29).

Loss of neurotrophic signal in DN may impair nerve generation and cause dying back of axons (3,34). Neurotrophin Ngf; NT-3 receptor TrkC; Gdnf receptors Gfra1, Gfra3, and Gfra4; neuregulin receptor Erbb; and neurotrophin signaling pathway genes (Akt3 and Mapk10) are downregulated, indicating impaired neurotrophic support in the db/db nerve. Downregulation of Ngf, TrkC, and Gfra1 is consistent with the results of studies in diabetic rats (35–37). Other neurotrophins are not differentially regulated in our data. Some studies in rats with streptozotocin-induced diabetes report reduced expression of Bdnf, NT-3, NT-4/5, Iglf-1, Iglf-II, Cntf, Gdnf, and neurotrophin receptors p75LNGFR and TrkB approximately 12 weeks after the induction of diabetes (35–40), whereas others report no change in NT-3 expression in rat sciatic nerve (41). The difference in the animal models used in the studies (rat model of type 1 diabetes vs. mouse model of type 2 diabetes) may explain this discrepancy.

The SC marker S100β as well as antiapoptotic genes (Mapk8ip1 and TGF-β2) are downregulated, whereas proapoptotic genes (Fas, TNF-α, Casp8, and Brcal) are upregulated in the db/db samples (Supplementary Table 2). The downregulation of SC marker and antiapoptotic genes along with upregulation of proapoptotic genes suggests SC apoptosis in db/db nerve; however, no studies to date have demonstrated SC death in DN.

The causes of axonal and SC degradation in DN are not well understood, but several hypotheses have been
developed in regard to the pathogenesis of the nerve injury (3,34). Our analyses indicate increased glucose, energy, and lipid metabolism in the \( \text{db/db} \) nerve and support the roles of hyperglycemia- and hyperlipidemia-induced oxidative stress, inflammatory response, and vascular ischemia in DN. In addition, our data indicate impaired axonal transport, neurotrophic signal, cell adhesion, and cell communication. In this study, we focused on hyperglycemia- and dyslipidemia-related gene expression changes in the nerve.

Hyperglycemia is a major factor in the development and progression of DN; current hypotheses suggest the effect of hyperglycemia is likely to be vascular, metabolic, or a combination of both (34,42). The metabolic hypothesis of axonal and SC damage in diabetes suggests that activation of glucose metabolism pathways in hyperglycemia results in oxidative stress. In our data, glycolysis, TCA, and oxidative phosphorylation genes are upregulated (Supplementary Table 2), suggesting activation of glucose and energy metabolism in the \( \text{db/db} \) nerve. Upregulation of oxidative phosphorylation genes and downregulation of mitochondrial H\(^+\) transporting ATP synthases in the \( \text{db/db} \) nerve suggest increased superoxide production (43). The mitochondrial proton carrier Ucp2, known to be upregulated in response to elevated reactive oxygen species (ROSs) (44), is highly upregulated; the oxidative stress-induced growth inhibitor Osgin1 is also upregulated in the \( \text{db/db} \) nerve. Upregulation of antioxidant genes (\( \text{Sod2}, \text{Peroxiredoxins}, \) and \( \text{Catalase} \)) suggests cellular response to increased ROS production. Edwards et al. (3) hypothesized that increased cellular oxidative stress results in activation of the Poly (ADP-ribose) polymerase (PARP) pathway, which in turn induces inflammatory responses in the nerve. Upregulation of PARP, inflammatory response, and MAPK signaling pathway genes in the \( \text{db/db} \) nerve support the PARP pathway–mediated inflammatory response hypothesis. Nuclear factor-\( \kappa \)B (NF-\( \kappa \)B) induced–inflammatory response is also implicated in demyelination and axon degeneration (3); however, NF-\( \kappa \)B and genes regulated by NF-\( \kappa \)B, such as \( \text{iNOS} \), are not differentially regulated in our data.

The vascular hypothesis of nerve damage in diabetes suggests that activation of glucose metabolism pathways causes functional and structural changes in the neuronal vasculature leading to endothelial hypoxia and axonal ischemia (45). Bradley et al. (46) noted thickening of perineurial cell basal lamina and increased endoneurial collagenization around SC in the sural nerve of DN patients. Activation of protein kinase C by hyperglycemia induces expression of vascular endothelial growth factor (VEGF)—an angiogenic gene and permeability factor—inhibits production of nitric oxide and alters Na\(^+\)-K\(^+\)-ATPase activity. Activation of glucose metabolism pathways induces TGF-\( \beta \)1 and Serpine1 expression,
FIG. 5. Gene expression profiles generated by CRC. Log$_2$ expression levels in seven db/+ and six db/db samples. A: Expression profile cluster of 275 DEGs enriched in “mitochondrion,” “lipid metabolic process,” and “axonogenesis.” B: Expression profiles of 22 “lipid metabolism” genes selected for promoter sequence analysis.
which results in endothelial fibrosis and thickening of vascular membranes (3), VEGF-C, TGF-β1, and Serpine1 are upregulated; Na+-K+-ATPases (Atp1b2 and Atp1b3) are downregulated in the db/db nerve. Uptregulation of hypoxia-inducible factor Hif1a may indicate cellular response to ischemia (47). These changes in gene expression support the hypothesis of neuronal ischemia and hypoxia. Imbalance in expression of nitric oxide synthase (Nos) family TFs may modulate this coregulation. Further investigation into the signal mediated by these TFs is likely to provide more insight into dyslipidemia-induced nerve injury.

In conclusion, gene expression changes suggest roles of multiple etiological factors in the development of DN. The changes are consistent with pathological characteristics of DN, such as axonal degeneration and potential loss of neurotrophic signal. Our findings support the role of hyperglycemia-induced oxidative stress and ischemia in nerve injury. Our results also support the hypothesis of oxidized lipid-mediated nerve injury and increased mitochondrial oxidative stress in dyslipidemia. In addition, our analyses revealed possible coregulation of lipid metabolism, stress response, and axonal degeneration genes and identified TFs that may modulate this coregulation. Further investigation into the signal mediated by these TFs is likely to provide more insight into dyslipidemia-induced nerve injury.

ACKNOWLEDGMENTS

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

M.P. researched data and wrote the manuscript. J.H. researched data and reviewed and edited the manuscript. Y.H. performed PCR validation. C.B., J.M.H., and S.S.O. conducted animal experiments. M.K. contributed to bioinformatic analysis design. E.L.F. designed and directed the study, contributed to discussion, and reviewed the manuscript.

Table 3

<table>
<thead>
<tr>
<th>TFBS module</th>
<th>Strand</th>
<th>Common to sequences (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMF_HOMF_ETSF</td>
<td>+ + +</td>
<td>51</td>
<td>6.58E-07</td>
</tr>
<tr>
<td>HOXF_LHXF_HOXF</td>
<td>+ + +</td>
<td>58</td>
<td>6.22E-04</td>
</tr>
<tr>
<td>HOXF_HBOX_HOXF</td>
<td>+ + -</td>
<td>56</td>
<td>1.47E-03</td>
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<tr>
<td>HBOX_LHXF_HOXF</td>
<td>+ + -</td>
<td>51</td>
<td>2.01E-03</td>
</tr>
<tr>
<td>OCT1_LHXF_HOXF</td>
<td>+ + +</td>
<td>51</td>
<td>2.41E-03</td>
</tr>
</tbody>
</table>

Five promoter modules consisting of three elements were significantly overrepresented in the promoter sequences of lipid metabolism–related genes. Module matrix families, strand orientation, percent of promoter sequences containing the module, and P values of enrichment are shown. The P value is the probability of finding the module in a set of randomly selected promoters and is determined by scanning a background promoter sequence set of 5,000 human promoters.
Parts of this study were presented in abstract/poster form at the 18th Annual International Conference on Intelligent Systems for Molecular Biology, Boston, Massachusetts, 11–13 July 2010, and at the NCIBI 5th Annual Research Conference 2010 at the University of Michigan, Ann Arbor, Michigan, 20–21 April 2010.

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REFERENCES

Cortical Neurons Develop Insulin Resistance and Blunted Akt Signaling: A Potential Mechanism Contributing to Enhanced Ischemic Injury in Diabetes

Bhumsoo Kim, Kelli A. Sullivan, Carey Backus, and Eva L. Feldman

Abstract

Patients with diabetes are at higher risk of stroke and experience increased morbidity and mortality after stroke. We hypothesized that cortical neurons develop insulin resistance, which decreases neuroprotection via circulating insulin and insulin-like growth factor-I (IGF-I). Acute insulin treatment of primary embryonic cortical neurons activated insulin signaling including phosphorylation of the insulin receptor, extracellular signal-regulated kinase (ERK), Akt, p70S6K, and glycogen synthase kinase-3β (GSK-3β). To mimic insulin resistance, cortical neurons were chronically treated with 25 mM glucose, 0.2 mM palmitic acid (PA), or 20 nM insulin before acute exposure to 20 nM insulin. Cortical neurons pretreated with insulin, but not glucose or PA, exhibited blunted phosphorylation of Akt, p70S6K, and GSK-3β with no change detected in ERK. Inhibition of the phosphatidylinositol 3-kinase (PI3-K) pathway during insulin pretreatment restored acute insulin-mediated Akt phosphorylation. Cortical neurons in adult BKS-db/db mice exhibited higher basal Akt phosphorylation than BKS-db+ mice and did not respond to insulin. Our results indicate that prolonged hyperinsulinemia leads to insulin resistance in cortical neurons. Decreased sensitivity to neuroprotective ligands may explain the increased neuronal damage reported in both experimental models of diabetes and diabetic patients after ischemia-reperfusion injury. Antioxid. Redox Signal. 14, 1829–1839.

Introduction

Stroke is the third leading cause of death in the United States behind heart disease and cancer (24). It is clear from epidemiological studies that diabetes exacerbates and/or is a principal cause of both stroke and myocardial infarction (31, 43, 46). Ischemic stroke is a major macrovascular complication of diabetes and diabetic patients consistently exhibit poorer outcomes and prognoses than nondiabetic patients after a stroke (27, 31). Over the last 15 years, our laboratory has extensively explored how peripheral sensory neurons respond to hyperglycemia (16, 38, 62). Recently, we began examining the effects of hyperglycemia on neurons of the central nervous system. These studies arose because of our growing interest in macrovascular disease and diabetes, with an emphasis on stroke.

While neurons are not insulin dependent, they are insulin responsive (5). Autoradiographic studies detect insulin receptors (InsR) in the brain of rodents and humans, and during development, insulin facilitates glucose metabolism during periods of neuronal growth (5). Increases in peripheral insulin lead to parallel increases in the brain via InsR-mediated uptake across the blood–brain barrier (12). The role of insulin in the brain is not fully known, but insulin acutely alters brain glucose utilization in a region-specific manner and alters short-term memory (11). Moreover, insulin is a well-documented growth factor for neurons of both the peripheral and central nervous systems (66).

Insulin resistance is a state of decreased responsiveness of target tissues to normal circulating levels of insulin and is a major feature of type 2 diabetes, glucose intolerance, obesity, dyslipidemia, and hypertension; that is, metabolic syndrome (7). Recent epidemiological evidence suggests that the insulin resistance associated with type 2 diabetes is a risk factor for stroke (31, 46). Patients with diabetes show a two- to sixfold increase in the risk of stroke compared to nondiabetic individuals. Population-based cohort studies demonstrate that otherwise healthy individuals with metabolic syndrome demonstrate a significant increase in stroke as well as cardiovascular mortality (43). Insulin resistance also increases...
the risk of stroke recurrence and, cumulatively, a poorer outcome and increased mortality (25). While these studies clearly document the correlation of diabetes and stroke, the underlying mechanism has yet to be identified.

Multiple factors, including hyperglycemic neuronal injury and insulin resistance, may contribute to the reported increase in mortality after stroke in diabetic patients. The contributions of each component of the metabolic syndrome to stroke vary and are controversial. Studies concerning the role of hyperglycemia on stroke demonstrate conflicting results; some conclude there is increased stroke risk with chronic hyperglycemia (1, 37), whereas other work finds no such relationship (45). The UK Prevention in Diabetes Study failed to demonstrate significantly reduced risk of stroke in patients treated with tight glucose control compared to conventional diet therapy (21). Even though hyperlipidemia is a high risk factor for cardiovascular disease (CVD), its contribution to stroke is also unclear, with some studies reporting a beneficial effect of cholesterol reduction, whereas others find no variation by lipid profile among diabetic status (23, 36, 54). In contrast, studies consistently demonstrate the relationship between hyperinsulinemia and stroke, though less than the association with CVD. The Atherosclerosis Risk in Communities Study reported that hyperinsulinemia increased stroke risk by 1.19-fold/50 pM increment in fasting insulin level (18). A 22-year follow-up study of healthy Finnish men also demonstrated that hyperinsulinemia carried a 2.1-fold increase in stroke risk after adjustment for age (48). Analysis of the data from the Third National Health and Nutrition Survey demonstrates independent association of insulin resistance with stroke (odds ratio 1.06) after adjustment with age, hypertension, myocardial infarction, claudication, physical activity, and HbA1C (6). Ultimately, our understanding of the impact of individual elements of the metabolic syndrome has the most impact on the increased risk of stroke observed in diabetic patients requiring more investigation at both the clinical and basic science levels.

Experimental studies of stroke and brain ischemia document the contribution of neuronal apoptosis after stroke and brain ischemia, and Akt signaling is a key regulator of these processes (71). Akt, a serine/threonine kinase activated downstream of phosphatidylinositol 3-kinase (PI3-K), is a critical signaling molecule in eukaryotic cells (71). Akt phosphorylation decreases immediately after global brain ischemia, followed by a dramatic increase within 24 h before returning to basal levels within 48 h (47). Preventing the initial decrease of Akt phosphorylation by insulin-like growth factor-I (IGF-I) treatment reduced neuronal death (30), whereas inhibition of Akt and glycogen synthase kinase-3β (GSK-3β) by the PI3-K inhibitor, LY294002, facilitated DNA fragmentation in hippocampal neurons (17). These results suggest that precise regulation of Akt is critical for neuronal survival after brain injury.

The BKS.Cg-m+/+Leprdb/J mouse (BKS-db/db) is a spontaneous model of type 2 diabetes. Due to the expression of a mutant leptin receptor, these mice are hyperphagic, which results in severe obesity hyperinsulinemia and insulin resistance beginning at ~4 weeks of age (http://jaxmice.jax.org/strain/000642.htm). These animals exhibit increased serum lipids and hyperglycemia similar to that seen in human patients and are the most commonly used model of type 2 diabetes (58).

In the current study, we examined possible molecular mechanisms regarding chronic insulin stimulation-mediated blunting of Akt and its downstream effectors. We demonstrated evidence of insulin resistance in an in vitro model of hyperglycemia and cortical neurons from a mouse model of type 2 diabetes. We conclude that chronic stimulation of Akt may contribute to blunted insulin signaling in cortical neurons and underlie, in part, the poor prognosis seen in diabetic patients with stroke.

Materials and Methods

Antibodies and chemicals

All antibodies were purchased from Cell Signaling (Beverly, MA) except Tau5 (Biosource International, Camarillo, CA), anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; Chemicon, Temecula, CA), and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Inhibitors (LY294002 and U0126) were purchased from Calbiochem (La Jolla, CA). PA was purchased from Nu-Chek Prep, Inc. (Elysian, MN). All other chemicals were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

Cortical neuron preparation

Cortical neurons were harvested 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 coverslips coated with PLL in 24-well culture plates. Cells were maintained in the feed medium (Neurobasal medium; Invitrogen, Grand Island, NY) supplemented with 1 × B27 without antioxidant (Invitrogen), antibiotics (penicillin, streptomycin, and neomycin; Sigma), 2.5 µg/ml albumin, 10 µg/ml apo-transferrin, 0.1 µg/ml biotin, 15 µg/ml D-galactose, 7 ng/ml progesterone, 16 µg/ml putrescine, 4 ng/ml selenium, 3 ng/ml µ-estradiol, 4 ng/ml hydrocortisone, 3 µg/ml catalase, and 2.5 µg/ml superoxide dismutase. Neurobasal medium contains 25 mM of glucose. The medium was replaced with a fresh feed medium on days 1 and 3. On day 6 (24 h before treatment), to exclude the effect of insulin contained in B27, the culture medium was changed to treatment medium (feed medium without B27). Insulin, glucose, or palmitate was added on day 6 to the treatment medium for 24 h treatment.

Mouse brain preparation

BKS-db/db and db+ (BKS.Cg-m+/+Leprdb/J, JAX Mice stock No. 000642) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were euthanized at 24 weeks of age (20 week of diabetes). 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 (38, 62). Brains were cut in half and the cortex minced, and divided equally into microcentrifuge tubes in the cortical neuron treatment medium. The tubes were left at 37°C for 45 min to stabilize before stimulation with insulin or IGF-I.

Western immunoblotting

Cortical neuron cultures were lysed in RIPA buffer (Pierce, Rockford, IL) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Mouse cortex was homoge-
nized using a plastic pestle in a microcentrifuge tube in T-PER tissue protein extraction reagent (Pierce) containing protease inhibitor cocktail (Roche Diagnostics). Lysates were collected, briefly sonicated, and centrifuged at 13,000 rpm for 15 min at 4°C. Western immunoblotting was performed as described previously (33). TBS with 0.01% Tween-20 and 5% fat-free milk was used for blocking and antibody dilution. The incubations with primary and secondary antibodies were carried out either at RT for 2 h or at 4°C overnight. The signal was observed using enhanced chemiluminescence reagents (ECL; Amersham Bioscience, Piscataway, NJ) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Images were captured using the Chemidoc XRS system and analyzed by Quantity One software (Bio-Rad Laboratory, Hercules, CA) (33) and the statistical analysis was performed by Prism software (GraphPad, Inc., La Jolla, CA). All experiments were repeated at least 3 times and representative results are presented in the figures. In some experiments, the nitrocellulose 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.

**Immunohistochemistry**

The cortical neurons cultured on PLL-coated coverslips were permeabilized with phosphate-buffered saline (PBS) containing 3% bovine serum albumin, 0.1% Triton X-100, and 1% normal goat serum (IHC buffer). The cells were incubated with Tau5 antibody diluted in IHC buffer in a humidified chamber at 4°C overnight. After rinsing with PBS three times for 5 min each, the cells were incubated with the anti-mouse IgG secondary antibody conjugated with AlexaFluor 488 (Molecular Probes, Eugene, OR) for 2 h at RT. After rinsing with PBS, the coverslips were mounted with the ProLong antifade mounting medium containing DAPI (Molecular Probes). The digital images were captured using a Spot-RT camera (Diagnostic Instrument Inc., Sterling Heights, MI) attached to Nikon Microphot-FXA microscope.

**Statistical analysis**

All experiments were repeated at least 3 times and presented as the mean ± SEM. Statistical analysis was performed by one-way analysis of variance with Tukey’s post analysis or Student’s *t*-test using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Statistical significance was defined as *p* < 0.05.

**Results**

Cortical neuron cultures were maintained for 3 and 6 days in the standard feeding medium (see Materials and Methods section). IHC for Tau5, a neuron-specific marker, confirmed the cell’s neuronal phenotype (Fig. 1A). From day 3 to 6 in vitro, cortical neurons extended neurites and appeared more mature (Fig. 1A). Treatment of the cortical neurons with insulin or IGF-I after 6 days in vitro did not result in any noticeable effects on cell size or neurite extensions (data not shown). It is possible that the cells already have so many neurites that we could not detect any measurable changes. Both insulin and IGF-I influence the development of cortical and hippocampal neurons by promoting neurogenesis, survival, and differentiation (3, 14, 52). Intracellular signaling cascades that mediate the effect of insulin and IGF-I include the mitogen-activated protein kinase (MAPK) and PI3-K/Akt residues.
pathways (5). To examine insulin and IGF-I signaling in our system, cortical neurons were treated with 20 nM insulin or IGF-I for 0–2 h followed by Western immunoblotting. Insulin stimulation induced a time-dependent increase in the phosphorylation (i.e., activation) of intracellular signaling molecules, including InsR, and its downstream targets, Akt, GSK-3β, and p70S6K (Fig. 1B). Phosphorylation of extracellular signal-regulated kinase (ERK) was not prominent and, in many cases, not different from the control (untreated neurons). Each blot was stripped and reprobed with antibodies against total protein as well as GAPDH to confirm equal protein loading. IGF-I treatment also resulted in a similar response; however, activation of Akt and p70S6K was generally stronger compared to insulin stimulation.

Hyperglycemia, dyslipidemia, and hyperinsulinemia are characteristic of type 2 diabetes and the metabolic syndrome, all of which may contribute to insulin resistance (7). We next examined the contribution of each of these factors to insulin resistance in cortical neurons. To create an in vitro model of the metabolic syndrome and assess insulin resistance, cortical neurons were treated with 25 mM added glucose, 0.2 mM PA, or 20 nM insulin for 24 h. The medium was then replaced with a fresh treatment medium for 30 min and the cells were exposed to 20 nM insulin for 15 min. Twenty-four hour insulin treatment resulted in reduced InsR phosphorylation, whereas 24 h glucose and PA treatment resulted in an increase in InsR phosphorylation (Fig. 2A). In untreated cortical neurons, Akt is activated after 15 min of insulin treatment. Twenty-four hour insulin treatment reduced this response to subsequent short-term insulin treatment. In contrast, 24 h of glucose or PA had no effect on the activation of Akt in response to short-term insulin treatment. An increase in basal Akt phosphorylation was noted after 24 h insulin treatment (Fig. 2A). Densitometric analysis confirmed that only insulin pretreatment resulted in a statistically significant decrease in insulin-stimulated Akt phosphorylation (Fig. 2B).

Full activation of Akt requires sequential phosphorylation of Thr308 and Ser473 by PDK1 and mammalian target of rapamycin complex 2 (mTORC2), respectively (71). The 24 h insulin treatment decreased the phosphorylation at both of these sites (Fig. 3A). Densitometric analysis demonstrates a statistically significant suppression of Akt phosphorylation after 24 h insulin pretreatment (Fig. 3B). p70S6K and GSK-3β are two important signaling molecules mediating Akt’s role in survival, neuronal differentiation, and metabolism (71). In parallel with the effect on Akt phosphorylation, 24 h insulin treatment also reduced short-term insulin-stimulated p70S6K and GSK-3β phosphorylation (i.e., activation of p70S6K and inactivation of GSK-3β). When p70S6K is phosphorylated, it displays slower migration (34). We observed a slowing migrating band of p70S6K in insulin-stimulated samples without pretreatment but not in samples pretreated with insulin for 24 h (Fig. 3A). Total protein levels of p70S6K and GSK-3β were not affected (Fig. 3A). In contrast, IGF-I-stimulated Akt phosphorylation was less affected by insulin pretreatment even up to 48 h without a reduction in total Akt protein (Fig. 3C). Of the three treatments tested, these results suggest that hyperinsulinemia, rather than hyperglycemia or hyperlipidemia, is the main factor responsible for the development of insulin resistance in cortical neurons.

We next explored the possible mechanism behind the reduction in Akt activation after chronic insulin treatment. During 24 h insulin treatment, cortical neuron cultures were incubated without or with inhibitors of Akt (LY294002) or ERK (U0126) signaling pathways. After 24 h the cells were washed with HBSS and incubated in a fresh treatment medium (without insulin or inhibitors) for 30 min. The cultures were then treated with 20 mM insulin for 15 min. As in the previous experiments, cortical neurons chronically pretreated with insulin followed by acute insulin treatment exhibited reduced Akt phosphorylation (Fig. 4A). Combined treatment with insulin and the PI3-K inhibitor, LY294002, restored Akt phosphorylation by acute insulin treatment to control levels. The MAPKK inhibitor, U0126, had no effect on Akt phosphorylation. Densitometric analysis (Fig. 4B) confirms that LY294002 treatment (lane 5) restored Akt phosphorylation, which was significantly higher than without inhibitor (lane 4) or U0126 (lane 6) treatment and displayed no statistically significant difference compared to untreated neurons (lane 2). As in the previous experiments, ERK phosphorylation was not significantly affected by 24 h insulin treatment or by either inhibitor. These results suggest that chronic hyperactivation of Akt by insulin prevents further activation by acute insulin stimulation.
Insulin treatment-induced InsR phosphorylation was not affected by the chronic insulin, LY294002, or U0126 treatments (Fig. 5). The phosphorylation pattern of p70S6K and GSK-3β paralleled that of Akt phosphorylation (Fig. 5). p70S6K displayed decreased mobility when phosphorylated (lanes 2 and 5) (34). These results confirm the upstream requirement of Akt for the activation of these signaling molecules.

These observations were confirmed in an animal model of type 2 diabetes, the BKS-db/db mouse. The BKS.Cg-m+/+ Leprdb/J, commonly known as BKS-db/db, expresses a homozygous mutation of the leptin receptor and demonstrates typical characteristics of type 2 diabetes, including obesity, hyperinsulinemia, and hyperglycemia (http://jaxmice.jax.org/strain/000642.html). BKS-db+/ mice are heterozygous for this mutation and serve as a control. At 24 weeks of age (20 weeks of diabetes), cortex was harvested from BKS-db/db and db+/mice and treated with 20 nM insulin for 0–15 min. Insulin stimulation resulted in a time-dependent increase in Akt phosphorylation in BKS-db+/ cortex (Fig. 6A). In contrast, db/db cortex displayed higher basal Akt phosphorylation, and insulin treatment did not increase this phosphorylation. Densitometric analysis demonstrates statistically significant increases in Akt phosphorylation in BKS-db+ cortex but no such changes from db/db cortex (Fig. 6B). These results confirm our in vitro observations and demonstrate that hyperinsulinemia may induce insulin resistance in vivo by the same mechanism, that is, reduced Akt activation.

Discussion

Hyperinsulinemia resulting from systemic insulin resistance is characteristic of type 2 diabetes and is also part of the constellation of symptoms associated with the metabolic syndrome (7). Because insulin crosses the blood–brain barrier, its levels are also increased within the central nervous system (15, 57). We hypothesized that increased insulin signaling would result in insulin resistance within the central nervous system, contributing to the increased neuronal damage seen in patients with type 2 diabetes after ischemia-reperfusion injury. In neurons, the PI-3K/Akt pathway is normally activated by insulin and IGF-I and is critical for translating the protective effects of these ligands (14, 52). In the current study, we found that long-term (24 h) exposure to insulin increased Akt activation and severely blunted this response after subsequent short-term insulin treatment. Acute Akt signaling was restored when the PI3-K/Akt pathway was normally activated by insulin and IGF-I and is critical for translating the protective effects of these ligands (14, 52). In the current study, we found that long-term (24 h) exposure to insulin increased Akt activation and severely blunted this response after subsequent short-term insulin treatment. We hypothesized that increased insulin signaling would result in insulin resistance within the central nervous system, contributing to the increased neuronal damage seen in patients with type 2 diabetes after ischemia-reperfusion injury. In neurons, the PI-3K/Akt pathway is normally activated by insulin and IGF-I and is critical for translating the protective effects of these ligands (14, 52). In the current study, we found that long-term (24 h) exposure to insulin increased Akt activation and severely blunted this response after subsequent short-term insulin treatment. Acute Akt signaling was restored when the PI3-K/Akt pathway was normally activated by insulin and IGF-I and is critical for translating the protective effects of these ligands (14, 52). In the current study, we found that long-term (24 h) exposure to insulin increased Akt activation and severely blunted this response after subsequent short-term insulin treatment.
of these ligands as demonstrated by activation of Akt and ERK1/2 and downstream targets of Akt, GSK-3β, and p70S6K. These downstream effectors are activated within 15 min of treatment. Insulin signaling was followed for 2 h at which time both the total levels of InsR and its activated form are diminished. This may be due to InsR recycling (10) or to saturation of intracellular substrates.

Once a normal signaling pattern was established, we examined the impact of long term insulin exposure on insulin signaling. Epidemiological data reveal that diabetic patients have an increased risk for stroke and poorer outcomes after stroke including increased infarct size and increased hemorrhage after initial ischemia (31, 43, 46). Many of these studies cite insulin resistance as the major risk factor; therefore, we examined cortical neuron cultures after chronic insulin treatment as a model of neuronal insulin resistance. As discussed above, cortical neurons respond to insulin and IGF-I stimulation with activation of Akt within 15 min. To examine the factors associated with insulin resistance, cortical neurons were treated with insulin, glucose, or PA to mimic hyperinsulinemia, hyperglycemia, and hyperlipidemia, respectively.

There are few direct reports regarding insulin resistance in neurons of the central nervous system; however, several studies document increased neuronal damage when insulin signaling is blocked. Neuron-specific InsR knock-out mice exhibit decreased Akt and GSK-3β activation and increased tau phosphorylation, but with no memory dysfunction or neuronal loss (53). In contrast, diet-induced insulin resistant mice exhibited similar decreases in Akt signaling coupled with increased amyloid plaques and decreased spatial performance (26). Both the BKS-db/db and ob/ob mice are insulin resistant and sustain greater infarct areas and increased behavioral deficits than their nondiabetic littermates (60). Although these studies did not specifically address insulin signaling, they confirm data collected from human patients regarding the negative impact of diabetes on ischemia-reperfusion injury.

Neurons preferentially use glucose as their main energy source. The neurobasal (NB+) medium contains 25 mM, which is optimal for maintaining neuronal health and axonal outgrowth (51). In our previous work examining the effects of diabetes on peripheral sensory neurons, we demonstrated that 20–25 mM (total 50 mM, 280 mg/dl) added glucose induces oxidative stress, mitochondrial depolarization, and programmed cell death (38, 62). These levels are within the range of that detected 2 h postprandial (200 mg/dl, 11.1 mM) in a patient with poorly regulated diabetes (42). With regard to the central nervous system, brain glucose levels are two-to threefold lower than the plasma glucose levels. Silver and Erecinska (57) demonstrated that brain glucose levels are 3-fold lower both under control (2.4 mM brain vs. 7.6 mM blood) and hyperglycemic (4.5 mM brain vs. 15.2 mM blood) conditions. Most importantly, however, brain glucose levels exhibit a linear correlation with peripheral blood glucose levels (15, 57); that is, an increase in peripheral blood glucose directly increases brain glucose. Although it is almost impossible to create primary culture conditions that exactly replicate in vivo conditions, we believe that our in vitro experiments accurately reflect diabetic conditions within the central nervous system as long as we maintain the ratio of glucose increase. Our findings demonstrate that hyperglycemia had no significant effect on insulin signaling.

To examine the effects of hyperlipidemia, cortical neurons were treated with PA for 24 h. Our recent results in peripheral
sensory neurons suggest that dyslipidemia contributes to diabetic neuropathy by increasing plasma-oxidized low-density lipoprotein, which directly leads to oxidative stress and injury in dorsal root ganglion neurons via LOX-1 (61). In the current study, exposure to PA had no effect on insulin signaling. Excessive exposure to insulin, rather than hyperglycemia, has been suggested as the primary instigator of insulin resistance in a type 1 diabetes mouse model (40). Of these parameters tested in the current study (high glucose, PA, or insulin), only chronic insulin treatment prevented activation of Akt. Specifically, phosphorylation of Akt at Thr308 and Ser473 was blocked. In addition to reduced Akt activation, the phosphorylation of downstream effectors, GSK-3β and p70S6K, were diminished after chronic insulin treatment. This observation is specific for insulin signaling, as acute IGF-I activation of Akt was not affected by chronic insulin treatment.

Based on our in vitro findings and the reports outlined above in diabetic mice, neuronal insulin resistance was examined in the BKS-db/db mouse. These mice are obese, become hyperinsulinemic at 10–14 days of age and similar to human diabetic patients, experience more damage after ischemia-reperfusion injury to the brain (60). Because of their extreme insulin resistance, it is very difficult to separate the effects of hyperglycemia from chronic insulin exposure in these animal models. Our observation that neurons dissected from BKS-db/db mice at 24 weeks of age (20 week of diabetes) are unable to phosphorylate Akt in response to acute insulin treatment argues for hyperinsulinemia. Further examination of these animals or other type 2 models of diabetes is warranted.

The absence of leptin signaling in the db/db mouse is responsible for hyperphagia; the dramatic obesity that follows nonstop eating results in insulin resistance and diabetes in this model. Type 2 diabetes may also be induced by feeding mice a Western style high-fat diet (65). While our data clearly support a role for insulin resistance in decreased Akt signaling, leptin also decreases Akt activation in the rat brain (8). In our studies, the lack of leptin receptor should minimize the effects of leptin signaling in this regard. Leptin receptors are expressed in the hypothalamus, cerebellum, and cerebral cortex (8) and are neuroprotective after ischemic injury (69). Further studies of diet-induced type 2 models with intact leptin signaling are an important step toward examining the combined role of insulin resistance and leptin signaling with regard to neuronal survival after ischemia-reperfusion injuries.

The beneficial effects of insulin treatment after ischemic injury have been demonstrated in in vitro and in vivo models, and in all cases reported depend on Akt activation. In vitro induction of oxidative stress decreases constitutive tyrosine phosphorylation of the insulin and IGF-I receptors and would normally result in neuronal apoptosis. Insulin treatment restores baseline activation of these receptors and increases activation of Akt and subsequent maintenance of Bcl-2 and inhibition of caspase 3 (14). In vivo, a single bolus of insulin given at the onset of re-perfusion activated Akt, protected hippocampal neurons from apoptosis, and spared learning and memory (52). Akt also plays a key role in neuroprotection...
by baicalein (39), simvastatin (9), and humanin (67), which are all implicated in neuronal survival after ischemic injury. Therefore, suppression of Akt phosphorylation (i.e., activation) due to insulin resistance can profoundly affect neuronal survival after ischemic injury and stroke. The specificity of the PI-3K/Akt pathway in the induction of insulin resistance in cortical neurons is underscored by blocking this pathway with LY294002. When cortical neurons were exposed to insulin and LY294002, Akt activation was prevented and acute insulin signaling activated the expected range of downstream proteins. Similar results are reported in mice fed with a high-fat diet. These mice develop insulin resistance with increased basal Akt phosphorylation and display increased oxidative stress (41). When the mice were treated with LY294002 during the day (when mice usually do not eat), all these changes were reversed with increased insulin sensitivity. Therefore, Akt may be the key factor connecting increased risk of ischemia and stroke as well as poor prognosis after stroke in patients with diabetes and other metabolic syndromes. Several signaling molecules regulated by Akt, including Bad, forkhead transcription factor (FKHR), and GSK-3β, are implicated in ischemia/reperfusion-mediated neuronal injury (71) and are discussed below.

Bad is a proapoptotic member of the Bcl family of proteins (13). After cerebral ischemia, Bad translocates to the outer membrane of the mitochondria and dimerizes with Bcl-xL (2). This process triggers cytochrome c release and caspase activation. Akt-induced phosphorylation inactivates Bad and prevents cell death (13). Peroxisome proliferator-activated receptor (PPAR)γ is a ligand-modulated transcription factor activated by insulin-sensitizing thiazolidinediones and a therapeutic target for treating type 2 diabetes (22). Rosiglitazone, troglitazone, and pioglitazone have all demonstrated protective effects against ischemia-reperfusion–induced myocardial damage and cerebral infarction in animal studies (55, 59, 68). These effects were mediated by preventing ischemia-induced degradation of Akt, Bcl-2, and Bcl-xL and subsequent phosphorylation and inactivation of Bad (19).

In response to apoptotic stimuli (including ischemia) FKHR translocates to the nucleus and initiates the expression of proapoptotic proteins such as FAS. Akt phosphorylates FKHR, leading to suppression of its apoptotic activity and promoting cell survival (50). The vanadyl compound bis(1-oxy-2-pyridinethiolato)oxovanadium(IV) [VO(OPT)] is neuroprotective in a mouse ischemic model via Akt activation and subsequent phosphorylation of FKHR (56).

Active (i.e., dephosphorylated) GSK-3β is increased after ischemic injury and GSK-3β inhibitor reduces ischemic infarction (35). Akt phosphorylates and thus inactivates GSK-3β (63). Lithium, traditionally used as a mood stabilizer, displays neuroprotective effects after stroke by increased expression of brain-derived neurotrophic factor and Bcl-2 and inhibition of GSK-3β (64). Induction of focal ischemic stroke by middle cerebral artery occlusion in diabetic mice exacerbated ischemia-induced cognitive deficits and brain infarction (70). These changes were accompanied with increased tau phosphorylation and decreased GSK-3β phosphorylation.

Even though less studied, p70S6K is also involved in preventing neuronal cell death after ischemic brain injury. Phosphorylation of p70S6K was decreased during transient focal ischemia (29) and the protective effect of thrombin against focal cerebral ischemia was accompanied with increased phosphorylation of p70S6K (28). Our current report demonstrates the regulation of GSK-3β and p70S6K phosphorylation by Akt in cortical neurons. Impairment of Akt activity by hyperinsulinemia-induced insulin resistance affects the downstream signaling molecules involved in ischemic/stroke injury and increases neuronal cell death. We are currently investigating the signaling downstream of Akt in both cortical neurons in vitro and in the BKS-db/db mice. Our hypothesis is summarized in Figure 7.

In summary, our experiments reveal that chronic insulin stimulation in vitro and in vivo results in a decrease in acute insulin-stimulated Akt activation, a form of insulin resistance in cortical neurons. Insulin resistance (or metabolic syndrome) is a risk factor for stroke (31) and its recurrence (25). Six out of nine recent epidemiological studies provide evidence that insulin resistance is associated with stroke (31). Insulin resistance was observed in up to 50% of the patients with transient ischemic attack or stroke (32). The prevalence of metabolic syndrome was almost twice as high (43.5% vs. 22.8%) in people with a self-reported history of stroke.
comparing those without a history of vascular disease (46). Individuals with metabolic syndrome, even without diabetes or CVD, are at increased risk of long-term cardiovascular outcomes, suggesting the importance of identifying metabolic syndrome early to intervene in the possible development of CVD and stroke (43). Precise regulation of Akt phosphorylation is critical for neuronal survival during brain ischemia (20, 47, 71). Our results suggest for the first time that decreased responsiveness of Akt phosphorylation due to hyperinsulinemia may explain the increased neuronal damage reported in both experimental models of diabetes and diabetic patients (4, 44, 49). Experiments are ongoing to determine the role(s) of InsR turnover and the signaling capacity of intracellular substrates responsible for insulin signal transduction. The inability of neurons to respond to circulating growth factors, including insulin or IGF-I, provides a potential explanation for the increased neuronal damage observed in diabetic human patients after stroke.

Acknowledgments

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Author Disclosure Statement

The authors state that no competing financial interests exist.

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Effect of mild hyperglycemia on autonomic function in obstructive sleep apnea

Amanda C. Peltier · Kanika Bagai · Kay Artibee · André Diedrich · Emily Garland · Thomas Elasy · Yaping Shi · Lily Wang · Eva L. Feldman · David Robertson · Beth A. Malow

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Abstract

Introduction Obstructive sleep apnea (OSA) has been hypothesized to cause a hypersympathetic state, which may be the mechanism for the increased incidence of cardiovascular disease in OSA. However, there is a high prevalence of hyperglycemia in OSA patients, which may also contribute to autonomic dysfunction.

Methods Thirty-five patients with OSA and 11 controls with average body mass index (BMI) of 32.0 ± 4.6 underwent polysomnography, glucose tolerance testing, autonomic function tests, lying and standing catecholamines, overnight urine collection, and baseline ECG and continuous blood pressure measurements for spectral analysis. A linear regression model adjusting for age and BMI was used to analyze spectral data, other outcome measures were analyzed with Kruskal–Wallis test.

Results Twenty-three OSA patients and two control patients had hyperglycemia (based on 2001 American Diabetes Association criteria). Apnea–hypopnea index (AHI) correlated with total power and low frequency (LF) power ($r = 0.138, 0.177, p = 0.031$; and $r = 0.013$) but not with the LF/high frequency (HF) ratio ($p = 0.589$). Glucose negatively correlated with LF systolic power ($r = -0.171, p = 0.038$) but not AHI ($p = 0.586$) and was marginally associated with pnn50, total power, LF, and HF power ($p$ ranged from 0.07 to 0.08).

Conclusion These data suggest that patients with OSA and mild hyperglycemia have a trend towards lower heart rate variability and sympathetic tone. Hyperglycemia is an important confounder and should be evaluated in studies of OSA and autonomic function.

Keywords Sleep apnea obstructive · Hyperglycemia · Autonomic nervous system

Introduction Obstructive sleep apnea (OSA) is a common disorder affecting 2–4% of the United States population. OSA confers an increased risk in cardiovascular disease and stroke. One mechanism attributed to OSA is hyperactivity of the sympathetic nervous system secondary to chronic intermittent hypoxia occurring during sleep [1–3]. Multiple
studies have demonstrated evidence of hypersympathetic activity and altered autonomic function in patients with sleep apnea [4–6].

Obstructive sleep apnea is also highly linked with diabetes mellitus and increased insulin resistance, with a high prevalence of prediabetes and undiagnosed diabetes observed in our previously published cohort [7] as well as other cohorts [8–10]. In addition, a high number of patients have hyperglycemia which is not detected by fasting glucose [7]. There is significant evidence that even mild hyperglycemia alters autonomic function [11–13] and that hypersympathetic activity can be observed in early diabetes [14].

Previous studies of autonomic function in OSA have either not measured glucose (relying on patient self-report of diabetes), or have only used fasting glucose as a screen to rule out hyperglycemia in their cohort. We hypothesized that hyperglycemia may significantly affect measures of autonomic function such as heart rate variability, catecholamine levels, and other autonomic function tests independent of OSA status. We assessed glycemic status, autonomic function and peripheral nerve function in mildly obese patients with a new diagnosis of OSA and followed them after treatment with continuous positive airway pressure (CPAP) to determine if there were any changes after treatment. We report here the initial cross-sectional results.

Methods

Participants

Volunteers with symptoms of disturbed sleep such as snoring and excessive daytime sleepiness were recruited for this longitudinal study. Subjects recruited with normal polysomnograms (PSGs) in the Vanderbilt Sleep Disorders Center were recruited as normal controls. Study participants were between 21 and 75 years of age, with no known history of diabetes or other disorders known to affect the autonomic system. All patients on antihypertensive, anticholinergic, or other medications known to affect autonomic testing were requested to hold medication for at least 3–5 half lives and to withhold caffeine use. Patients unable to hold medications safely for this time period were excluded from the study. To avoid treatment bias, previous treatment of OSA was also an exclusion criteria. Because the study included repeated measurements after CPAP treatment to follow both glucose and autonomic function measures, patients could not be on hypoglycemic medication. All patients on antihypertensive, anticholinergic, or other medications known to affect autonomic testing were requested to hold medication for at least 3–5 half lives and to withhold caffeine use. Patients unable to hold medications safely for this time period were excluded from the study. To avoid treatment bias, previous treatment of OSA was also an exclusion criteria. Because the study included repeated measurements after CPAP treatment to follow both glucose and autonomic function measures, patients could not be on hypoglycemic medication. Therefore, patients with glucose levels consistent with diabetes were excluded from the study for safety concerns. To avoid obesity as a confounding effect, participants with a body mass index (BMI) of 25–40 were included in the study.

Definitions

Obstructive sleep apnea was defined as an apnea–hypopnea index (AHI) greater than five events/h with either 3% desaturation or EEG arousal present [15]. Glucose status was defined using the 2004 American Diabetes Association criteria (fasting glucose <100 mg/dL normal, 100–125 mg/dL impaired fasting glucose, >125 mg/dL diabetic, 2-h glucose <140 mg/dL normal, 140–199 mg/dL impaired glucose tolerance, ≥200 mg/dL diabetic [16]).

Procedures

This study was approved by the Vanderbilt University Internal Review Board and subjects were consented according to the Declaration of Helsinki. The study was posted on clinicaltrials.gov (NCT00681161). All subjects received a complete physical examination with a detailed neurological exam. Eligible subjects came to the Vanderbilt Clinical Research Center for PSG and for 3-h glucose tolerance testing (OGTT) the following morning. Urine was collected overnight for catecholamine measurement. Participants were kept fasting overnight during their PSG. Study participants with OSA and glucose levels below the diabetic range were invited back for baseline autonomic testing and nerve conduction studies. Study participants were instructed to avoid food and caffeine for 3 h prior to the autonomic testing. Medications known to interfere with autonomic testing were held for 24–48 h prior to testing.

Autonomic evaluation

Recording of ECG, and hemoglobin saturation (SpO2), were measured with a patient monitor with Nellcor technology (Vital-Guard 450C, Ivy Biomedical Systems, Inc., Branford, CT, USA).

Continuous blood pressure was measured by volume clamp method [17] on the middle finger of the non-dominant hand (Nextfin, BMYE B.V., Amsterdam, The Netherlands) and verified using oscillometric determined brachial BP (Vital-Guard 450C, Ivy Biomedical Systems, Inc.) on the contralateral arm. After a period of at least 30 min in supine position, 10 min of resting supine heart rate and blood pressure data were recorded for spectral analysis. The degree of sinus arrhythmia was assessed during controlled breathing (5 s inhalation and 5 s exhalation (6 cycles/min) during 90 s). The sinus arrhythmia ratio was calculated as the ratio of the longest to the shortest RR interval. Responses of blood pressure fall and heart rate acceleration was determined during 30 s of hyperventilation. A Valsalva maneuver was performed where the patient blows through a mouthpiece connected to a closed pipe with a pressure transducer and a small
leakage (22 gauge hole) for 15 s. Blood pressure fall during early phase two, blood pressure recovery during late phase 2, and blood pressure overshoot were determined. The Valsalva ratio of heart rate was calculated. The blood pressure increase to isometric exercise during squeezing a handgrip dynamometer (Model 76618, Lafayette Instrument Co., IN, USA) with the dominant hand at 30% of maximal voluntary contraction for 3 min was determined. A cold pressor test was performed where the patient put the dominant hand into ice water for 1 min and the blood pressure increase at 1 min was recorded.

Orthostatic vital signs were determined by measuring heart rate and brachial blood pressure (Dinamap 1846SX, Critikon, Tampa, FL, USA) on the non-dominant arm and following at least 30 min supine and at 1, 3, 5, and every 5 min until 30 min after standing.

Blood for catecholamine determination during supine and upright were drawn through intravenous catheter on the contra-lateral arm.

QSART QSART testing was performed using the QSweat device (WR Electronics, Rochester, MN, USA) using the technique of Low et al. [18]. Capsules were placed at four standard sites: distal forearm, proximal leg, distal leg, and foot. 10% acetylcholine solution was iontophoresed using a current of 2.0 mA. Results were recorded in microliters of sweat volume. A study was determined to be abnormal if one or more sites were below the fifth percentile for age and gender using previously published normative data, or if there was a proximal distal gradient with a distal site <1/3 the volume of the proximal leg site.

Spectral analysis Signals were digitized at a sampling rate of 500 Hz (DI720USB, DATAQ Instruments, Akron, OH, USA), and then processed with user software written in PV-Wave (Visual Numerics Inc., Houston, TX, USA).

Heart rate variability in the time domain Standard deviation (SD RRI), root mean square of successive differences of R–R intervals (RMSSD RRI) and percentage of interval changes greater than 50 ms to normal sinus R–R intervals (PNN50) were calculated [19].

Spectral analysis Beat-to-beat values of detected R–R intervals and BP values were linear interpolated, low-pass filtered (cutoff 0.5 Hz) and re-sampled at 4 Hz. Data segments of 300 s were used for spectral analysis. Linear trends were removed and power spectral density was estimated with the FFT-based Welch algorithm using segments of 256 data points with 50% overlapping and Hanning window [20]. The power in the frequency range of low frequencies (LF 0.04–0.15 Hz) and high frequencies (HF 0.15–0.40 Hz) was calculated following Task Force recommendations [19].

Baroreflex Gain was defined as the mean magnitude value of the transfer function between BP and RRI in the low-frequency (BRS LF) and high-frequency (BRS HF) band with negative phase and squared coherence value greater than 0.5.

Spontaneous Baroreflex Slope was calculated as the slope of the linear regression line between the systolic BP and the subsequent R–R intervals using sequences defined as an episode of at least three heart beats with more than 0.5 mmHg systolic BP changes and 5 ms R–R interval changes. The averaged value of all slopes with a correlation coefficient greater than 0.85 was calculated for sequences with rising (BRS SEQUP) and falling BP (BRS SEQDOWN) [21].

Polysomnography

Subjects underwent polysomnography (PSG) in the Vanderbilt Clinical Research Center (CRC). Subjects were studied with standard polysomnographic techniques, using four EEG channels (C3, C4, O1, O2 by the International 10–20 system), three chin electromyogram (EMG) leads, two electrooculogram (EOG) leads, two electrocardiogram (ECG) leads, snoring sound, respiratory effort using the chest and abdomen, airflow at the nose and mouth using thermocouples and nasal pressure cannulas, two bilateral surface EMG electrodes (placed over the anterior tibialis muscles) were recorded. Oxyhemoglobin saturation (SaO₂) was monitored by pulse oximetry.

Experienced polysomnographic technologists used standard techniques to score manually all recordings for sleep stages, respiratory events and limb movements. PSG measures followed the American Academy of Sleep Medicine recommendations for sleep staging and respiratory scoring [15]. Studies were then reviewed by one of the authors (KB), a board-certified sleep specialist.

Glucose and insulin regulation

Subjects were kept fasting for a minimum of 8 h before glucose evaluation. Fasting samples were drawn for glucose and insulin levels for the HOMA-IR calculation (homeostasis model approximation for determining insulin resistance, HOMA IR = G0I0/22.5). Adults <35 with normal BMI and insulin resistance have a HOMA of 1.0, with increasing values indicating greater insulin resistance [23, 24]. A 3-h oral glucose tolerance test (OGTT) with 75 g glucose was performed. Blood samples for glucose and insulin were drawn every 30 min during a 3-h OGTT for a composite insulin sensitivity index (ISI) measurement derived by DeFronzo’s calculation: 10,000/ (Gavg/I avg) 0.5. This has been shown to approximate insulin sensitivity calculated from the insulin clamp (R = 0.76). Decreasing values indicate loss of insulin sensitivity [25]. Fasting insulin levels above 40 U/mL were considered abnormal.
Statistical methods

For patient baseline demographics, catecholamine levels, spectral analysis of heart rate variables and autonomic function tests, data were summarized with mean ± standard deviation for continuous variables and with percentages for categorical variables. Unadjusted comparisons between control patients, hyperglycemic, and normoglycemic OSA patients, and between hyperglycemic and normoglycemic OSA patients were conducted using Kruskal–Wallis test and Wilcoxon rank sum test for continuous variables and the Pearson chi-square test for categorical variables.

Linear regression model was used to examine the effects of glucose and AHI on heart rate and blood pressure variability. For each outcome variable, logarithm transformation was used to obtain normally distributed values when needed, and two models were constructed: simple linear model for estimating crude effect and multiple linear regression model for estimating adjusted effect by additionally including pre-specified confounding variables age and BMI.

Results

Patient characteristics

Forty-six patients were recruited, 35 patients met diagnostic criteria for OSA and 11 had normal polysomnograms without significant breathing disturbances. Of the 46 patients included in the final analyses, 22 patients had hyperglycemia based on baseline oral glucose tolerance test results (19 had OSA), with either elevated fasting glucose (n = 11 subjects), impaired glucose tolerance (n = 10 subjects), or both (n = 1 subject). There were more women than men in all groups but there were no differences between percentage of women in either OSA or hyperglycemia groups (p = 0.76, 0.92, respectively). There were no significant differences between patients with elevated fasting glucose or impaired glucose tolerance for any variable. Therefore, these groups were combined for all subsequent analyses, we define this combined group the hyperglycemic group.

Patients with OSA (n = 35) were significantly older than the control patients (n = 11, mean ± SD; 46.9 ± 9.2 vs. 36.5 ± 11.7 years, p = 0.009) and were more obese (32.9 ± 4.2 vs. 28.9 ± 3.5 kg/m², p = 0.008), but comparing patients with (n = 22) and without hyperglycemia (n = 24) did not demonstrate any differences in age or BMI (hyperglycemic patient mean age 46.0 ± 8.6 vs. normoglycemic patients age 43.0 ± 12.2 years, p = 0.79, and hyperglycemia patient BMI 32.3 ± 3.8 vs. normoglycemic patient BMI 31.7 ± 4.9 kg/m², p = 0.35) (Table 1).

The insulin sensitivity index was not different between hyperglycemic and normoglycemic patients (4.3 ± 2.4 vs. 5.7 ± 2.9, p = 0.10), but HOMA-IR was (2.8 ± 1.4 vs. 2.0 ± 1.5, p = 0.05), although both were significantly different between control patients and OSA patients (ISI 4.6 ± 2.5 vs. 7.7 ± 2.5, p = 0.01 and HOMA-IR 1.7 ±

Table 1 Characteristics of 46 patients with and without hyperglycemia (impaired fasting glucose or impaired glucose tolerance)

<table>
<thead>
<tr>
<th></th>
<th>Impaired fasting glucose patients (n = 10)</th>
<th>Impaired glucose tolerance patients (n = 12)</th>
<th>Normoglycemic patients (n = 24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>47 ± 7</td>
<td>45 ± 10</td>
<td>44 ± 12</td>
<td>0.93</td>
</tr>
<tr>
<td>Female</td>
<td>68 (15)</td>
<td>75 (9)</td>
<td>70 (16)</td>
<td>0.38†</td>
</tr>
<tr>
<td>African-American</td>
<td>10 (1)</td>
<td>8 (1)</td>
<td>0 (0)</td>
<td>0.34‡</td>
</tr>
<tr>
<td>Body mass index</td>
<td>31.4 ± 3.2</td>
<td>33.2 ± 4.3</td>
<td>31.2 ± 4.7</td>
<td>0.41</td>
</tr>
<tr>
<td>AHI</td>
<td>16 ± 18</td>
<td>23 ± 21</td>
<td>11 ± 11</td>
<td>0.23</td>
</tr>
<tr>
<td>REM AHI</td>
<td>26 ± 24</td>
<td>23 ± 33</td>
<td>14 ± 15</td>
<td>0.29</td>
</tr>
<tr>
<td>Minimum O₂ saturation</td>
<td>85 ± 6</td>
<td>83 ± 5</td>
<td>85 ± 7</td>
<td>0.22</td>
</tr>
<tr>
<td>Epworth Sleepiness Scale</td>
<td>7 ± 6</td>
<td>9 ± 4</td>
<td>10 ± 6</td>
<td>0.73</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>109 ± 5</td>
<td>91 ± 7</td>
<td>89 ± 5</td>
<td>0.001*</td>
</tr>
<tr>
<td>2-h glucose (mg/dL)</td>
<td>119 ± 16</td>
<td>157 ± 11</td>
<td>111 ± 19</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.4 ± 0.9</td>
<td>3.2 ± 1.7</td>
<td>2.2 ± 28</td>
<td>0.12</td>
</tr>
<tr>
<td>ISI</td>
<td>4.2 ± 1.3</td>
<td>4.4 ± 3.2</td>
<td>5.7 ± 2.9</td>
<td>0.23</td>
</tr>
</tbody>
</table>

P values were reported for comparisons between normoglycemic, impaired fasting glucose and impaired glucose tolerance patients. Fasting glucose above 100 mg/dL and 2 h glucose above 140 mg/dL were used in accordance with the 2009 American Diabetes Association criteria % (N); or mean ± standard deviation (SD)

AHI apnea–hypopnea index, REM rapid eye movement sleep, HOMA-IR homeostasis model approximation for determining insulin resistance, ISI insulin sensitivity index

† Chi-squared test used, P values for other variables were based Kruskal–Wallis test; * p value <0.05
Triglycerides were not significantly higher in the hyperglycemic group (129 ± 78 vs. 124 ± 62.5, p = 0.629) (Table 2).

### Autonomic function test results

#### Autonomic function tests

There were no significant differences between hyperglycemic and normoglycemic patients after analysis of standard autonomic function tests. There was a trend to a lower heart rate among control patients compared to OSA patients (61 ± 12 vs. 68 ± 8 and 70 ± 13) but this was not significant (p = 0.14). There were no differences between healthy controls and OSA patients in baseline heart rate, blood pressure, sinus arrhythmia, heart rate or blood pressure response to Valsalva maneuver, isometric hand grip, or cold pressor test. There was no difference between the groups in sweat volumes at any site (Table 3).

#### Heart rate and blood pressure variability

Heart rate variability was calculated from 34 patients who had artifact-free ECG segments while supine before testing began. Age, BMI, glucose, and AHI were analyzed independently and then stepwise. The glucose value used was the 2 h glucose level from their initial OGTT. BMI did not have a significant effect alone on any of the main variables but was kept in the model as a variable of interest. Gender did not have a significant effect on heart rate variability in

### Table 2 Characteristics of 46 patients with and without obstructive sleep apnea (OSA) N (%); or mean ± standard deviation (SD)

<table>
<thead>
<tr>
<th></th>
<th>OSA patients (n = 35)</th>
<th>Control patients (n = 11)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>47.1 ± 9.3</td>
<td>36.5 ± 11.7</td>
<td>0.009*</td>
</tr>
<tr>
<td>Female</td>
<td>69 (24)</td>
<td>64 (7)</td>
<td>0.76†</td>
</tr>
<tr>
<td>African-American</td>
<td>3 (1)</td>
<td>9 (1)</td>
<td>0.14†</td>
</tr>
<tr>
<td>Body mass index</td>
<td>32.8 ± 4.2</td>
<td>28.9 ± 3.5</td>
<td>0.008*</td>
</tr>
<tr>
<td>AHI</td>
<td>21 ± 17</td>
<td>1 ± 1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>REM AHI</td>
<td>26 ± 25</td>
<td>2 ± 2</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Minimum O₂ saturation</td>
<td>83 ± 6</td>
<td>85 ± 7</td>
<td>0.16</td>
</tr>
<tr>
<td>Epworth sleepiness scale</td>
<td>9 ± 5</td>
<td>Insufficient sample</td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>109 ± 5</td>
<td>89 ± 5</td>
<td>0.001</td>
</tr>
<tr>
<td>2-h glucose (mg/dL)</td>
<td>117 ± 15</td>
<td>112 ± 19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.4 ± 0.9</td>
<td>2.2 ± 1.6</td>
<td>0.058</td>
</tr>
</tbody>
</table>

AHI apnea-hypopnea index, REM rapid eye movement sleep, HOMA-IR homeostasis model approximation for determining insulin resistance, ISI insulin sensitivity index

† Chi-squared test used, other variables used Wilcoxon rank-sum test. Epworth Sleepiness scale not reported for controls because not all patients had scale at time of polysomnogram (PSG); * p value <0.05

1.4 vs. 2.7 ± 1.4, p = 0.029). Triglycerides were not significantly higher in the hyperglycemic group (129 ± 78 vs. 124 ± 62.5, p = 0.629) (Table 2).

### Table 3 Results of autonomic function tests comparing hyperglycemic versus normal glycemic patients

<table>
<thead>
<tr>
<th></th>
<th>Hyperglycemic patients (n = 22)</th>
<th>Normoglycemic patients (n = 24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline heart rate (bpm)</td>
<td>69 ± 13</td>
<td>66 ± 11</td>
<td>0.68</td>
</tr>
<tr>
<td>Baseline SBP † (mmHg)</td>
<td>113 ± 10</td>
<td>112 ± 13</td>
<td>0.36</td>
</tr>
<tr>
<td>Baseline DBP † (mmHg)</td>
<td>72 ± 7</td>
<td>69 ± 8</td>
<td>0.16</td>
</tr>
<tr>
<td>Sinus arrhythmia ratio</td>
<td>1.28 ± 0.14</td>
<td>1.28 ± 0.16</td>
<td>0.68</td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>1.56 ± 0.29</td>
<td>1.72 ± 0.43</td>
<td>0.28</td>
</tr>
<tr>
<td>Change in SBP after isometric handgrip (mmHg)</td>
<td>16 ± 9</td>
<td>10 ± 19</td>
<td>0.17</td>
</tr>
<tr>
<td>Change in SBP after cold pressor test (mmHg)</td>
<td>22 ± 14</td>
<td>24 ± 13</td>
<td>0.46</td>
</tr>
<tr>
<td>Forearm sweat volume (µL)</td>
<td>1.42 ± 0.81</td>
<td>1.30 ± 0.89</td>
<td>0.46</td>
</tr>
<tr>
<td>Proximal leg sweat volume (µL)</td>
<td>0.89 ± 0.53</td>
<td>0.85 ± 0.46</td>
<td>0.92</td>
</tr>
<tr>
<td>Distal leg sweat volume (µL)</td>
<td>0.90 ± 0.65</td>
<td>0.98 ± 0.64</td>
<td>0.77</td>
</tr>
<tr>
<td>Foot sweat volume (µL)</td>
<td>0.54 ± 0.37</td>
<td>0.45 ± 0.46</td>
<td>0.37</td>
</tr>
</tbody>
</table>
our data. Therefore, it was not included in the final model. Due to the small number of patient ECG data that could be analyzed, the model was limited to four variables to avoid over fitting of the model. The variables analyzed included age, BMI, AHI and 2 h glucose in mg/dL.

Age significantly affected Pnn50, and HF RRI, with a trend in TP RRI and LF RRI. All of the correlations measured were modest with no $r^2$ value greater than 0.2 but significantly different from zero. Unadjusted, glucose had no effect on any heart rate or blood pressure variables. However, after adjusting for age, BMI, and AHI, there was a correlation with lower LF RRI and TP RRI associated with abnormal glucose ($r^2 = -0.19$, $p = 0.03$; $r^2 = -0.15$, $p = 0.04$) and trend towards a lower low frequency of systolic blood pressure ($r^2 = -0.11$, $p = 0.06$) (Table 4). AHI was initially and after adjustment was not associated with any variable except LF RRI ($r^2 = 0.13$, $p = 0.05$) (Table 5).

### Table 4 Linear regression analysis of heart rate and blood pressure with glucose before and after adjusting for age, body mass index, and apnea–hypopnea index

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted</td>
</tr>
<tr>
<td>log(Pnn50)</td>
<td>-0.2251, -0.6220</td>
</tr>
<tr>
<td>log(LF RRI)</td>
<td>-0.1578, -0.3236</td>
</tr>
<tr>
<td>log(HF RRI)</td>
<td>-0.1525, -0.3459</td>
</tr>
<tr>
<td>log(TP RRI)</td>
<td>-0.1518, -0.2995</td>
</tr>
<tr>
<td>log(LF_HF ratio)</td>
<td>-0.0053, -0.1109</td>
</tr>
<tr>
<td>log(rmssd)</td>
<td>-0.0907, -0.1945</td>
</tr>
<tr>
<td>log(LF SYS)</td>
<td>-0.0652, -0.1683</td>
</tr>
<tr>
<td>log(BRS lfmn SYS)</td>
<td>-0.0470, -0.1455</td>
</tr>
<tr>
<td>log(sig sequp)</td>
<td>-0.0418, -0.1452</td>
</tr>
<tr>
<td>log(sig seqdown)</td>
<td>-0.0445, -0.1444</td>
</tr>
</tbody>
</table>

**Pnn50** number of normal sinus (NN) intervals with duration greater than 50 ms/number of NN intervals, **LF** low frequency, **RRI** R–R interval, **HF** high frequency, **TP** total power, **rmssd** root mean square successive difference in heart period, **SYS** systolic, **BRS lfmn SYS** baroreflex gain–mean magnitude value of the transfer function between bp and rri in low frequency band, **sig sequp** and **sig seqdown** refer to the baroreflex slope of the linear regression line between the systolic bp and subsequent R–R intervals using sequences with more than 0.5 mmHg systolic bp changes and 5 ms R–R interval changes.

### Table 5 Linear regression analysis of heart rate and blood pressure with AHI before and after adjusting for age, body mass index, and glucose

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>AHI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted</td>
</tr>
<tr>
<td>log(Pnn50)</td>
<td>-0.0551, -0.3604</td>
</tr>
<tr>
<td>log(LF RRI)</td>
<td>0.0325, -0.0973</td>
</tr>
<tr>
<td>log(HF RRI)</td>
<td>-0.0147, -0.1639</td>
</tr>
<tr>
<td>log(TP RRI)</td>
<td>0.0019, -0.1147</td>
</tr>
<tr>
<td>log(LF_HF ratio)</td>
<td>0.0472, -0.0322</td>
</tr>
<tr>
<td>log(rmssd)</td>
<td>-0.0207, -0.1011</td>
</tr>
<tr>
<td>log(LF SYS)</td>
<td>0.0297, -0.0506</td>
</tr>
<tr>
<td>log(Txy lfmn SYS)</td>
<td>-0.0075, -0.0811</td>
</tr>
<tr>
<td>log(sig sequp)</td>
<td>-0.0356, -0.1088</td>
</tr>
<tr>
<td>log(sig seqdown)</td>
<td>-0.0415, -0.1153</td>
</tr>
</tbody>
</table>

**Pnn50** number of normal sinus (NN) intervals with duration greater than 50 ms/number of NN intervals, **LF** low frequency, **RRI** R–R interval, **HF** high frequency, **TP** total power, **rmssd** root mean square successive difference in heart period, **SYS** systolic, **BRS lfmn SYS** baroreflex gain–mean magnitude value of the transfer function between bp and rri in low frequency band, **sig sequp** and **sig seqdown** refer to the baroreflex slope of the linear regression line between the systolic bp and subsequent R–R intervals using sequences with more than 0.5 mmHg systolic bp changes and 5 ms R–R interval changes.
Discussion

The results of this study suggest that mild hyperglycemia may affect heart rate variability, but does not independently contribute to autonomic dysfunction in patients with OSA. This finding suggests that more severe alterations in glucose may be needed before significant abnormalities are detectable in standard tests of autonomic function.

We obtained healthy overweight and obese controls (BMI over 25) to rule out potential effects of obesity on autonomic function [26]. However, either due to the small sample size or the mild OSA present, we did not detect a difference in autonomic function between our OSA patients and control patients which is different from previously published manuscripts. This could be due to the small sample size, but is more likely due to the mild severity of OSA in our patient sample.

There is increasing evidence that impaired glucose tolerance and pre-diabetes may contribute to peripheral nerve dysfunction [27, 28], and there is some evidence to support development of autonomic changes prior to development of diabetes [12]. A previous study demonstrated lower sweat volumes in asymptomatic patients with impaired glucose tolerance [13]. We did not observe similar findings, which may have been due to several factors, including a younger population and normal neurologic exams with no evidence of peripheral neuropathy, which may indicate that longer periods of sustained hyperglycemia are needed before sweating derangements are evident. It is not clear what degree of hyperglycemia is required or if there are other specific risk factors such as elevated triglycerides which may increase the prevalence of neuropathy in patients with mild hyperglycemia.

Spectral analysis of heart rate variability has become an attractive measure of autonomic function due to the lack of special equipment needed, and ease of obtaining data. Lower heart rate variability detected through analysis of 12-lead ECG data was significantly associated with higher cardiovascular mortality in subjects enrolled in the ACCORD study [29]. The neural control of heart rate is regulated at the sinoatrial node by interaction of sympathetic and vagal efferent discharge. High frequency fluctuations in heart rate have a significant vagal component and are tied to respiratory rate. The low frequency rhythmic fluctuations in heart rate are thought to be secondary to sympathetic modulation, but considerable evidence also suggests a significant vagal component as they are decreased after atropine as well as stellectomy. This ambiguity may limit the interpretation of frequency analyses as they do not always correlate with other measures of autonomic function [30]. However, in many instances, spectral analysis enables evaluation of autonomic input not easily obtained through other methods. Our study suggests that frequency analysis may be a more sensitive tool than standard autonomic function tests in detecting early involvement of the autonomic system compared to other standard autonomic tests. However, our results should be interpreted with caution given the small effect size, and multiple observations reported.

We did not observe evidence of hyper-sympathetic input of heart rate or in any other autonomic test performed, either in hyperglycemic patients or in patients with sleep apnea compared to controls, which may have been due to the mild sleep apnea present in our cohort. Previous studies have demonstrated a dose-dependent effect of OSA [22]. We also analyzed ECG data from our patients while awake for spectral analysis, whereas many of the studies previously performed analyzed ECG data from PSGs. The advantage of this method was to obtain data in a controlled environment, with patients not on any medications known to affect heart rate or autonomic function; this is not possible in ECG data abstracted from PSGs performed in large sleep laboratories.

We again have shown that approximately 50% of the OSA patients had hyperglycemia, and a significant correlation between hyperglycemia and sleep apnea. Hyperglycemia is highly prevalent in patients with sleep apnea, and conversely, sleep apnea is highly prevalent in diabetic populations independent of obesity and age. It is not known whether the presence of OSA is a risk factor for other diabetic complications.

In conclusion, mild hyperglycemia present in OSA may have an early effect on heart rate variability, and may contribute to autonomic dysfunction observed in these patients. However, major autonomic dysfunction consistent with diabetes was not observed in our hyperglycemic OSA patients. Future studies are needed to determine if measures of heart rate variability alone are superior to other measures of autonomic function in this cohort.

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References

Diabetic polyneuropathies: update on research definition, diagnostic criteria and estimation of severity

Peter J. Dyck1∗
James W. Albers2
Henning Andersen3
Joseph C. Arezzo4
Geert-Jan Biessels5
Vera Bril6
Eva L. Feldman2
William J. Litchy1
Peter C. O’Brien7
James W. Russell8 on behalf of
The Toronto Expert Panel on
Diabetic Neuropathy†

1Department of Neurology,
Mayo Clinic, Rochester,
MN, USA
2Department of Neurology,
University of Michigan,
Ann Arbor, MI, USA
3Aarhus University Hospital,
Aarhus, Denmark
4Department of Neuroscience,
Albert Einstein College of Medicine,
New York, NY, USA
5Rudolf Magnus Institute,
Utrecht, Netherlands
6Department of Medicine,
University of Toronto,
Toronto, Ontario, Canada
7Division of Biostatistics,
Mayo Clinic, Rochester,
MN, USA
8Department of Neurology,
University of Maryland,
Baltimore, MD, USA

∗Correspondence to: Peter J. Dyck,
Department of Neurology,
Mayo Clinic, 200 First Street SW,
Rochester, MN 55905, USA
E-mail: dyck.peter@mayo.edu

†See Appendix for Members of The
Toronto Consensus Panel on
Diabetic Neuropathy.

Summary

Prior to a joint meeting of the Neurodiab Association and International Symposium on Diabetic Neuropathy held in Toronto, Ontario, Canada, 13-18 October 2009, Solomon Tesfaye, Sheffield, UK, convened a panel of neurovascular experts to provide an update on polyneuropathies associated with diabetes (Toronto Consensus Panels on DPNs, 2009). Herein, we provide definitions of typical and atypical diabetic polyneuropathies (DPNs), diagnostic criteria, and approaches to diagnose sensorimotor polyneuropathy as well as to estimate severity. Diabetic sensorimotor polyneuropathy (DSPN), or typical DPN, usually develops on long-standing hyperglycaemia, consequent metabolic derangements and microvessel alterations. It is frequently associated with microvessel retinal and kidney disease – but other causes must be excluded. By contrast, atypical DPNs are intercurrent painful and autonomic small-fibre polyneuropathies. Recognizing that there is a need to detect and estimate severity of DSPN validly and reproducibly, we define subclinical DSPN using nerve conduction criteria and define possible, probable, and confirmed clinical levels of DSPN. For conduct of epidemiologic surveys and randomized controlled trials, it is necessary to pre-specify which attributes of nerve conduction are to be used, the criterion for diagnosis, reference values, correction for applicable variables, and the specific criterion for DSPN. Herein, we provide the performance characteristics of several criteria for the diagnosis of sensorimotor polyneuropathy in healthy subject- and diabetic subject cohorts. Also outlined here are staged and continuous approaches to estimate severity of DSPN. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords diabetic polyneuropathy; diabetic sensorimotor polyneuropathy; atypical diabetic polyneuropathy; classification; definitions

Abbreviations: DPN – diabetic polyneuropathy; DSPN – diabetic sensorimotor polyneuropathy; MNCV – motor nerve conduction velocity; NIS – Neuropathy Impairment Score.

Introduction and objectives

A summary of this, and the other updates reported here, has been published earlier [1]. This update begins with the consideration of classification of diabetic polyneuropathies (DPNs) and then provides definitions, minimal criteria for diagnoses, and estimation of severity of typical DPN, i.e. diabetic sensorimotor polyneuropathy (DSPN). Subsequently, atypical DPN is described and discussed only briefly. This update alludes only briefly to focal and multifocal varieties.
Classification of diabetic neuropathies

The neuropathies developing in patients with diabetes mellitus are known to be heterogeneous by their symptoms, pattern of neurological involvement, course, risk covariates, pathological alterations, and underlying mechanisms [2–4]. We accept the Thomas et al. [5,6] and Boulton et al. [7,8] separation of DPNs into generalized polyneuropathies (DPNs) and focal (e.g. CRIRI neuropathy and median neuropathy at the wrist from carpal tunnel syndrome) and multifocal varieties (e.g. multiple mononeuropathy, lumbosacral, thoracic, and cervical radiculoplexus neuropathies [5–9]). It is known that all patterns of neuropathy listed above also occur in patients without diabetes mellitus [10].

The evidence that generalized DPNs can be further classified into at least two major subgroups (typical and atypical) seems compelling [5–8]. Typical DPN is a chronic, symmetrical, length-dependent sensorimotor polyneuropathy and is thought to be the commonest variety of DPN, from cohort and population-based epidemiological studies [3]. It develops on a background of long-standing chronic hyperglycaemia, associated metabolic derangements, and cardiovascular risk factors [11–17]. It is postulated that metabolic derangements, secondary to chronic hyperglycaemia (polyol shunting, accumulation of advanced glycation end products, oxidative stress, lipid abnormalities among other metabolic derangements [17–20], and microvessel alterations [21–23]), are involved in the development of DSN. The pathological alterations of microvessels are similar to those observed in diabetic retinopathy and nephropathy. In cross-sectional and longitudinal epidemiological surveys of population-based cohorts of patients with diabetes mellitus, total hyperglycaemia has been shown to be an important risk covariate [14,16], but vascular risk factors have been emphasized in other studies [13,15]. Progression of DSN has been shown to be prevented or inhibited by rigorous glycaemic control [11,12,24,25]. DSN has been found to be statistically associated with retinopathy and nephropathy [3,26].

Atypical DPNs are different from DSN in several important features, i.e. onset, course, manifestations, associations, and perhaps putative mechanisms [5–8,27–31]. They appear to be intercurrent varieties, developing at any time during the course of a patient’s diabetes mellitus [30–32]. Onset of symptoms may be acute, subacute, or chronic, but the course is usually monophasic or fluctuating over time. Archer et al. [30] described a prototypic variety quite distinct from the usual course of DSN. Their nine cases had painful neuropathies that were preceded by weight loss, a feature emphasized by Ellenberg [29] but also characteristic of diabetic lumbosacral radiculoplexus neuropathy [9]. Burning pain and contact hyperalgesia were typical features. Sensory loss was mild and there was no or little weakness. With conventional treatment they improved. Symptoms disappeared in months. Retinopathy or nephropathy was not observed. Nerve conduction abnormalities, if present, were mild. Younger et al. [31] biopsied cutaneous nerves of patients having some features of the Archer et al. patients. They reported lymphocytic infiltrates, albeit small, but possibly suggestive of an inflammatory (perhaps immune) pathogenesis. It is important to note that many of these patients developed their symptomatic sensory and autonomic polyneuropathies shortly after rigorous control of hyperglycaemia had been achieved, making it unlikely that chronic hyperglycaemia is a putative risk covariate – different from the causative factors of typical DSN.

Investigators have described an increased prevalence of IFG or IGT in patients with small-fibre painful polyneuropathies [33–38]. Whether IFG or IGT causes an increased prevalence of DSN, and if it does, whether it causes typical or atypical DPN remains unsettled [34–42] for methodological reasons and contradictory results [39].

The focal and multifocal neuropathies associated with diabetes mellitus can be broadly subdivided into those in which repeated, mild, mechanical trauma, compression, or entrapment is causative and others possibly related to inflammation with or without associated ischaemia. The first group includes median neuropathy at the wrist, ulnar neuropathy at the elbow, and peroneal neuropathy at the knee. The second group may include mononeuropathy, e.g. cranial nerve III and multiple mononeuropathies, and radiculoplexus neuropathies of the lumbosacral (also called diabetic amyotrophy, Bruns Garland syndrome, and by other names), thoracic, and cervical segments. There is increasing evidence that inflammation, microvasculitis, and ischaemia are involved in these radiculoplexus neuropathies [9,43–46].

Typical DPN (i.e. diabetic sensorimotor polyneuropathy)

The San Antonio Conference defined DPN as ‘peripheral or autonomic nerve damage attributable solely to diabetes mellitus’ [47]. Boulton et al. [7] defined DPN as ‘presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after exclusion of other causes’. The case definition of distal symmetric polyneuropathy (of which DSN is a member) from the AAN, AAEM, and AAPMR report, and based on a formal review of the medical literature, states that ‘the highest likelihood of polyneuropathy occurs with a combination of neuropathic symptoms, multiple signs, and abnormal electrodiagnostic studies’ [48]. Whereas each of these definitions seems acceptable and intuitively correct, they do not separate typical DPN (i.e. DSN) from atypical DPN, which we judge to be needed. Here, we also emphasize more precise nerve conduction abnormality criteria for subclinical DSN (Stage 1a) and provide approaches useful for estimating the severity of DSN. In addition, proficiency of the clinical examination of signs and symptoms and of nerve conduction testing is emphasized.
We propose separate definitions for typical DPN (DSPN) and atypical DPN. DSPN is a symmetrical length-dependent sensorimotor polyneuropathy attributable to chronic hyperglycaemia, associated metabolic derangements, cardiovascular risk covariates, and microvessel alterations. An abnormality of nerve conduction which may be subclinical (asymptomatic and without signs or symptoms of polyneuropathy) appears to be the first objective and quantitative indication of DSPN and is a necessary condition for the confirmed diagnosis of DSPN. The occurrence of diabetic retinopathy and nephropathy in a given patient strengthens the case that a patient’s sensorimotor polyneuropathy is attributable to diabetes mellitus. However, the association among these complications is not strong enough to allow diagnosis of DSPN from knowing that diabetic retinopathy or nephropathy occurs in the same patient, i.e. other causes of polyneuropathy must be excluded.

For epidemiological surveys or controlled clinical trials of DSPN, we advocate the use of nerve conduction as an early and reliable indication of the occurrence of subclinical DSPN. To be reliable as the indicator of subclinical DSPN (Stage 1a), nerve conduction evaluation must be carried out rigorously using appropriate testing conditions and techniques using suitable criteria and reference values corrected for applicable variables of age, gender, height, and weight – the topic discussed in the next section. Volunteer symptoms and elicited signs are needed to confirm the diagnosis and to estimate severity. Other neurophysiological tests (e.g. quantitative sensation and autonomic tests) are useful in characterizing neuropathic expression. As for nerve conduction evaluations, so also for the clinical evaluation of signs and symptoms, careful attention needs to be given to the issue of proficiency of examiners. In a recent study of the proficiency of neuromuscular experts as compared to their 75% group diagnosis or as compared to confirmed nerve conduction abnormality, their diagnoses were more variable and less reproducible than usually assumed or desirable – indicating a need for careful instruction, consensus development, and quality assurance of the clinical evaluation, in conducting epidemiological surveys or randomized controlled clinical trials [49–51]. In an earlier but smaller study assessing proficiency with agreement on methods of examination and diagnosis and using confirmed nerve conduction abnormality as a guide in training, high levels of concordance among clinical examinations were achieved [52].

Atypical DPNs

Atypical DPN has not been as well characterized and studied as has typical DPNs (i.e. DSPN). It is possible that atypical DPN is actually not a single entity but several varieties. This condition appears to be an intercurrent and monosymphic or fluctuating disorder, tending to preferentially involve small sensory and autonomic nerve fibres, by not being closely associated with chronic hyperglycaemia or associated with the microvessel abnormalities found in DSPN.

Abnormality of nerve conduction as minimal criteria for the diagnosis of subclinical DSPN

The evidence that nerve conduction abnormality of limb nerves is the most objective and quantitative indication of DSPN comes from studies of DPN cohorts [53–57] and population-based study of healthy subject and diabetes mellitus cohorts [52,58–61].

For purposes of using nerve conduction studies for the research diagnosis of subclinical DSPN (Stage 1a), it is necessary that they are performed proficiently, using suitable criteria for abnormality based on adequately obtained reference values corrected for applicable variables and that the results are clearly presented and interpreted. In performance of nerve conduction studies, particular attention needs to be given to adequate maintenance of limb temperature, correct and exact placement of stimulating and recording electrodes, accurate measurement of distances, use of just supramaximal electrical stimulation, recognition of normal anatomic variations (e.g. nerve crossovers), avoidance of recording of spurious responses, and adequate documentation and record keeping. Assuming that nerve conduction values have been proficiently assessed, it is then necessary to express abnormality by comparison with adequately obtained reference values and to use these values to determine whether DSPN is present based on appropriate criteria for its diagnosis – the subject explored further in subsequent paragraphs.

The nerve conduction criteria, which might be used for the diagnosis of DSPN in epidemiological surveys, randomized controlled trials, and even for medical practice, were recently assessed in databases of previously studied healthy subjects and a population-based cohort of diabetic subjects (RDNS) – cohorts from Olmsted County, MN, USA [3,16,58]. Ideal nerve conduction criteria for DSPN would use attributes representative of neurophysiological abnormality in DSPN employing attributes that are frequently abnormal in the condition. In an HS cohort, use of the criterion should result in low frequency of abnormality, i.e. few false positives, providing values of abnormality near the set percentile abnormality, e.g. 2.5th or 1st percentile. In a population-representative cohort of patients with diabetes mellitus, the criterion should sensitively detect DSPN – in perhaps one third or more of the cases. In the Nerve Conduction Criteria Study, the authors were especially concerned about the frequency of false positives (type 1 error) because multiple attributes and multiple nerves are usually assessed in nerve conduction studies.

The Nerve Conduction Criteria Study [62] evaluated eight nerve conduction criteria for the potential diagnosis of DSPN and the frequencies of abnormalities were tabulated in the HS and diabetic subject cohorts. In the diabetic
subject cohort, the frequencies of nerve conduction abnormality using 2.5th/97.5th cut-offs were peroneal motor nerve conduction velocity (MNCV), 26.3%; sural amplitude, 25.4%; tibial MNCV, 24.8%; and ulnar MNCV, 21.3%; peroneal F-latency, 16.9%; and ulnar F-latency, 16.0%. Among the pairs of these six nerve conduction attributes, there was highly significant agreement for the diagnosis of DSPN.

Eight criteria for DSPN were compared (Table 1). Criterion 1 – ‘≥1 abnormality of any one attribute from any nerve’ did not perform well. It was inadequate in the following respects. Abnormality could be due to mononeuropathy. Use of this criterion resulted in an excessive number of false-positive diagnoses. In the HS cohort (expected to have no patients with DSPN) and using this criterion and percentiles of ≤5th/≥95th, ≤2.5th/≥97.5th, and ≤1st/≥99th, abnormality frequencies of 37.3, 17.3, and 9.1% resulted – a large type 1 error. Assuming specificity to be the same for the diabetic as they were for the HS (a reasonable assumption because the technique of testing and references values were the same), this criterion produced too high a frequency of abnormality among diabetic patients (Table 1).

Criterion 2 – ‘≥1 abnormal attributes in ≥2 separate nerves tested’. Inspection of the table shows that this criterion performs much better than Criterion 1. Using the ≤5th/≥95th percentile cut-off, the false positives are probably excessively high in both the HS and the diabetic subjects, but using lower percentile abnormality the error rate is acceptable. Criterion 3 – ‘an abnormality ≤1st/99th percentile of any attribute of two separate nerves, one of which must be the sural nerve’ (AAN, AAEM, and AAPMR). Using the ≤5th/≥95th percentile this criterion results in excessive false positives among HS and using the ≤1st/≥99th criteria in the diabetic subjects, too low a frequency of DSPN is obtained. Criterion 4 – ‘abnormality of peroneal MNCV and sural amplitude’. Use of this criterion results in low sensitivity in both healthy subject and diabetes mellitus cohorts (Table 1). Criteria 5–8 are composite scores of nerve conduction attributes (Table 1). Irrespective of which composite score was used, specificity was close to the preset percentile abnormality level in the HS cohort. In the diabetic subject cohort, good specificity and sensitivity were achieved, especially for Σ 2 nerve conduction normal deviates ≥97.5th, i.e. peroneal MNCV and sural amplitude.

From the results of the Nerve Conduction Criteria Study, the authors concluded that composite sum scores of normal deviates (from percentiles) and nerve conduction attributes performed best for diagnosing DSPN, although performance of Criterion 2, ‘≥1 abnormal attribute in ≥2 separate nerves’, and Criterion 3 (when modified) was also acceptable. In clinical practice, less rigid criteria may be justified (Table 1, footnote a).

### Estimating severity of DSPN

For medical practice and for conducting epidemiological surveys and randomized controlled clinical trials, measurement of the severity of DSPN in a given patient is

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Table 1. Nerve conduction abnormality in the RDNS and RDNS-HS cohorts using different criteria

<table>
<thead>
<tr>
<th>Criterion</th>
<th>RDNS-HS (N = 330)</th>
<th>RDNS (N = 456)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%) abnormal</td>
<td>Prevalence at first visit</td>
</tr>
<tr>
<td></td>
<td>5th/95th</td>
<td>2.5th/97.5th</td>
</tr>
<tr>
<td>Criteria 1: ≥1 of 12 nerve conduction attributes abnormal</td>
<td>123 (37.3)</td>
<td>57 (17.3)</td>
</tr>
<tr>
<td>Criteria 2: ≥1 abnormal in 2 separate nerves</td>
<td>37 (11.2)</td>
<td>8 (2.4)</td>
</tr>
<tr>
<td>Criteria 3: ≥1 abnormal in 2 separate nerves (1 is sural)</td>
<td>24 (7.3)</td>
<td>7 (2.1)</td>
</tr>
<tr>
<td>Criteria 4: Peroneal CV abnormal and sural amplitude abnormal</td>
<td>2 (0.6)</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>Criteria 5: Σ 2 nerve conduction normal deviates abnormal (peroneal CV and sural amplitude)</td>
<td>17 (5.2)</td>
<td>9 (2.7)</td>
</tr>
<tr>
<td>Criteria 6: Σ 2 nerve conduction normal deviates abnormal (peroneal CV and tibial CV)</td>
<td>17 (5.2)</td>
<td>9 (2.7)</td>
</tr>
<tr>
<td>Criteria 7: Σ 5 nerve conduction normal deviates abnormal</td>
<td>17 (5.2)</td>
<td>9 (2.7)</td>
</tr>
<tr>
<td>Criteria 8: Σ 6 nerve conduction normal deviates abnormal</td>
<td>17 (5.2)</td>
<td>9 (2.7)</td>
</tr>
</tbody>
</table>

*On the basis of theoretical considerations (Bonferonni’s modelling) the following abnormal frequencies would be expected based on type 1 error and lack of linkage among attributes studied.

*Of 330 × 12 = 3960 nerve attributes tested, 197 (5.0%) are abnormal at the 95th, 75 (1.9%) at the 97.5th and 36 (0.9%) at the 99th.
needed to estimate severity of symptoms, signs, neurophysiological test results, and overall severity of DSPN. This need is not met by simply tallying patients as having, or not having, DSPN – severity also needs to be ascertained. Two approaches have been described – staged [63] and continuous measurement approaches [64].

The staged approach

**Stage 0** = criteria for subclinical DSPN have not been met – an abnormality of nerve conduction not being present.

**Stage 1a** = criteria for subclinical DSPN have been met, but the patient does not have signs or symptoms of DSPN. If Criterion 3 (from previous sections) for DSPN is chosen, sural amplitude must be ≤1st percentile and any one other nerve conduction attribute assessed is ≤1st or ≥99th percentile with corrections made for applicable variables. If one of the composite scores is used (Criterion 5–8), the composite normal deviate score must be ≥97.5th or ≥99th percentile – whichever is chosen.

**Stage 1b** = criteria for subclinical neuropathy have been met and neuropathic signs without neuropathic symptoms are present.

**Stage 2a** = criteria for subclinical neuropathy have been met and the patient has neuropathic symptoms with or without neuropathic signs.

**Stage 2b** = criteria for subclinical neuropathy have been met and patient has unequivocal weakness of ankle dorsiflexion (Table 1, footnote a).

Continuous measurement of the severity of DSPN

An alternative method to assess the severity of DSPN is to use a continuous measure of neuropathic signs without or with nerve conduction or other neurophysiological test abnormalities. The Neuropathy Impairment Score (NIS) and NIS of the lower limb provide a sum score of scored weakness of a predetermined list of muscle groups, scored decrease of muscle stretch reflexes, and scored abnormality of sensory modalities of sensation of fingers and toes. Judgements are to be corrected for the influence of age, gender, anthropomorphic variables, and physical fitness. The NIS or NIS of the lower limb scores have been extensively described [65] and extensively used in epidemiological surveys and therapeutic trials of chronic inflammatory demyelinating and monoclonal gammopathies of undetermined significance neuropathies [52,66] and also in DSPN [65,67]. Paper and electronic forms of NIS and symptoms and disability scores have been extensively used in epidemiological surveys and randomized controlled clinical trials [68]. With some modifications, the MRC scale has been used to score overall muscle weakness [69]. Several other sum scores of impairment have been developed and published. The symptoms (and in some cases signs) of DSPN can be scored using the Neuropathy Symptoms Score [70], Neuropathy Symptoms and Change [68], the Michigan Score [71], and the Toronto Clinical Neuropathy Score [72–74].

Composite scores of representative attributes of nerve conduction have also been shown to be useful in estimating the severity of polyneuropathies [75–78]. It is not possible to develop a sum score of attributes of nerve conduction without some transformation of the data. Composite scores of nerve conduction can be derived if percentile values are expressed as normal deviates from percentiles corrected for applicable variables and abnormality is expressed in the same tail of the normal distribution. A composite score of neurophysiological tests is especially useful in epidemiological surveys and randomized controlled trials. Because DSPN is the summation of different symptoms, signs, and test abnormalities, use of composite normal deviate scores allows combining representative signs and test results. A further important use of composite scores is that it allows assessment of change in severity even within the range of normal and extending into abnormality. The percentile position of this composite measure must be independently set by studies of the composite score in reference populations. An example of such a composite score for use in DSPN is Σ 5 nerve conduction normal deviates. The Σ 5 nerve conduction normal deviate score is made up of peroneal nerve velocity, amplitude, distal latency, tibial distal latency, and sural amplitude, with the five nerve conduction attributes expressed as normal deviates. In a similar manner, it is possible to add other neurophysiological measures to the composite nerve conduction score. In Σ 7 NTs normal deviate, Σ 5 nerve conduction normal deviate is added to the normal deviate score of vibratory detection threshold of the toes and heart rate deep breathing decrease.

On the assumption that NIS abnormality correlates with neurophysiological test abnormalities, a composite score combining the two has been proposed and used in epidemiological surveys and controlled trials, e.g. NIS of the lower limb + Σ 7 NT normal deviate score.

Conclusions

The Neurodiabetes Consensus (Toronto) Group on DPNs supports the earlier classification of DPNs by Thomas et al. [5,6] and Boulton et al. [7,8] into generalized and focal and multifocal, and further separating DPNs into typical (DSPN) and atypical DPNs. For epidemiological surveys and controlled trials, we define DSPN as chronic, symmetric, length-dependent sensorimotor polyneuropathy developing from metabolic derangements and microvessel alterations related to chronic hyperglycaemia and cardiovascular risk factors. Metabolic derangements and microvessel alterations appear to be similar and common to those of retinopathy and nephropathy. As the pattern of DSPN is not unique in diabetes mellitus, other causes need to be excluded.
Diabetic Polyneuropathies

Minimal Criteria for DPN

Clinical Care

Possible Clinical DSPN
Probable Clinical DSPN
Confirmed Clinical DSPN
Subclinical DPN

Research

Figure 1. Minimal criteria for diabetic polyneuropathy

Atypical DPN are intercurrent generalized polyneuropathies having an acute or subacute onset and a monophasic or relapsing course that may develop at any time during a patient’s diabetes mellitus. These atypical neuropathies need further studies emphasizing natural history, classification, and outcome. Like DSPN, so also in atypical DPN other causes of neuropathy need to be excluded.

Definitions of minimal criteria for DSPN

1. Possible Clinical DSPN
   Symptoms or signs of DSPN. Symptoms may include: decreased sensation, positive neuropathic sensory symptoms (e.g. ‘asleep numbness’, ‘prickling’ or ‘stabbing’, ‘burning’ or ‘aching’ pain) predominantly in the toes, feet, or legs. Signs may include: symmetric decrease of distal sensation or unequivocally decreased or absent ankle reflexes.

2. Probable Clinical DSPN
   A combination of symptoms and signs of distal sensorimotor polyneuropathy with any two or more of the following: neuropathic symptoms, decreased distal sensation, or unequivocally decreased or absent ankle reflexes.

3. Confirmed Clinical DSPN
   An abnormal nerve conduction study and a symptom or symptoms or a sign or signs of sensorimotor polyneuropathy. Severity of DSPN can be assessed by staged or continuous approaches described above and by dysfunction and disability scores [65].

4. Subclinical DSPN (Stage 1a)
   No signs or symptoms of polyneuropathy. Abnormal nerve conduction, as described above, is present (Figure 1).

Atypical DPNs

Before further classification of atypical DPNs, setting minimal criteria for diagnosis and estimating severity, further characterization from epidemiological surveys and mechanistic studies are needed. The issue of painful, autonomic, and nerve morphological abnormalities are discussed in subsequent articles.

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Conflict of interest

The authors have no conflicts of interest.

Appendix

The Toronto Consensus Panel on Diabetic Neuropathy
James W Albers, MD, PhD, University of Michigan, Ann Arbor, MI, USA
Gérard Amarenco, MD, Service de Rééducation Neuropédiatrique et d’Explorations Périmétrales, Hôpital Rothschild, AP-HP, Paris, France
Henning Anderson, MD, Department of Neurology, Aarhus University Hospital, Aarhus, Denmark
Joe Arezzo, PhD, Albert Einstein College of Medicine, New York, NY, USA
Misha-Miroslav Backonja, MD, Department of Neurology, University of Madison-Wisconsin, Madison WI, USA
Luciano Bernardi, MD, Clinica Medica 1, Universita’ di Pavia, Pavia, Italy
Geert-Jan Biessels, MD, Department of Neurology, Rudolf Magnus Institute, Utrecht, Netherlands
Andrew J. M. Boulton, MD, Department of Medicine, University of Manchester, Manchester, UK
Vera Bril, MD, Department of Neurology, University of Toronto, Toronto, Ontario, Canada
Norman Cameron, PhD, University of Aberdeen, Aberdeen, UK
Mary Cotter, PhD, University of Aberdeen, Aberdeen, UK
Peter J Dyck, MD, Department of Neurology, Mayo Clinic, Rochester, MN, USA
John England, MD, Department of Neurology at Louisiana State University Health Sciences Center, New Orleans, LA, USA
Eva Feldman, MD, PhD, Department of Neurology, University of Michigan, Ann Arbor, MI, USA
Roy Freeman, MD, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA
Simona Frontoni, MD, Department of Internal Medicine, University of Tor Vergata, Rome, Italy
Jannik Hilsted, MD, Copenhagen University Hospital, Copenhagen, Denmark
Michael Horowitz, MD, PhD, Department of Medicine, University of Adelaide, Adelaide, Australia
Peter Kempler, MD, PhD, I Department of Medicine, Semmelweis University, Budapest, Hungary
Giuseppe Lauria, MD, Neuromuscular Diseases Unit, ‘Carlo Besta’ Neurological Institute, Milan, Italy

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Philip Low, MD, Department of Neurology, Mayo Clinic, Rochester, MN, USA
Rayaz Malik, MD, Division of Cardiovascular Medicine, University of Manchester, Manchester, UK
Peter C O’Brien, PhD, Mayo Clinic College of Medicine, Rochester, MN, USA
Rodica Pop-Busui, MD, PhD, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA
Bruce Perkins, MD MPH, Division of Endocrinology, University of Toronto, Toronto, Ontario, Canada
Gerry Rayman, MD, Diabetes Centre, Ipswich Hospital, Ipswich, UK
James Russell, MD, Department of Neurology and Neurophysiology, University of Maryland, Baltimore, MD, USA
Søren Sindrup, MD, Department of Neurology, Odense University Hospital, Odense, Denmark
Gordon Smith, MD, Department of Neurology, University of Utah, Salt Lake City, UT, USA
Vincenza Spallone, MD, PhD, Department of Internal Medicine, University of Tor Vergata, Rome, Italy
Martin Stevens, MD, Department of Medicine, University of Birmingham, Birmingham, UK
Solomon Tesfaye, MD, Diabetes Research Unit, Sheffield Teaching Hospitals, Sheffield, UK
Paul Valensi, MD, Service d’Endocrinologie-Diabétologie-Nutrition, Hôpital Jean Verdier, Bondy, France
Tamás Várkonyi, MD, PhD, First Department of Medicine, University of Szeged, Szeged, Hungary
Aristides Veves, MD, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA
Loretta Vileikyte, MD, PhD, Department of Medicine, University of Manchester, Manchester, UK
Aaron Vinik, MD, PhD, Strelitz Diabetes Research Institutes, Eastern Virginia Medical School, Norfolk, VA, USA
Dan Ziegler, MD, Institute for Clinical Diabetology, German Diabetes Center at the Heinrich Heine University, Leibniz Center for Diabetes Research; Department of Metabolic Diseases, University Hospital, Düsseldorf, Germany
Doug Zochodne, MD, Department of Clinical Neuroscience, University of Calgary, Calgary, Alberta, Canada
NIDDK observer – Teresa Jones, MD, NIDDK, Bethesda, MD, USA

References


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Clinical Diabetes Research - 147


Evidence-based guideline: Treatment of painful diabetic neuropathy

Report of the American Academy of Neurology, the American Association of Neuromuscular and Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation

V. Bril, MD, FRCP(C)
J. England, MD, FAAN
G.M. Franklin, MD, MPH, FAAN
M. Backonja, MD
J. Cohen, MD, FAAN
D. Del Toro, MD
E. Feldman, MD, PhD, FAAN
D.J. Iverson, MD, FAAN
B. Perkins, MD, FRCP(C), MPH
J.W. Russell, MD, MS, FRPC
D. Zochodne, MD

ABSTRACT
Objective: To develop a scientifically sound and clinically relevant evidence-based guideline for the treatment of painful diabetic neuropathy (PDN).

Methods: We performed a systematic review of the literature from 1960 to August 2008 and classified the studies according to the American Academy of Neurology classification of evidence scheme for a therapeutic article, and recommendations were linked to the strength of the evidence. The basic question asked was: “What is the efficacy of a given treatment (pharmacologic: anticonvulsants, antidepressants, opioids, others; and nonpharmacologic: electrical stimulation, magnetic field treatment, low-intensity laser treatment, Reiki massage, others) to reduce pain and improve physical function and quality of life (QOL) in patients with PDN?”

Results and Recommendations: Pregabalin is established as effective and should be offered for relief of PDN (Level A). Venlafaxine, duloxetine, amitriptyline, gabapentin, valproate, opioids (morphine sulfate, tramadol, and oxycodone controlled-release), and capsaicin are probably effective and should be considered for treatment of PDN (Level B). Other treatments have less robust evidence or the evidence is negative. Effective treatments for PDN are available, but many have side effects that limit their usefulness, and few studies have sufficient information on treatment effects on function and QOL.

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GLOSSARY
AAN = American Academy of Neurology; NNT = number needed to treat; PDN = painful diabetic neuropathy; QOL = quality of life; RCT = randomized controlled trial; SF-MPQ = Short Form–McGill Pain Questionnaire; SF-QOL = Short Form–Quality of Life; VAS = visual analog pain scale.

Diabetic sensorimotor polyneuropathy represents a diffuse symmetric and length-dependent injury to peripheral nerves that has major implications on quality of life (QOL), morbidity, and costs from a public health perspective.1,2 Painful diabetic neuropathy (PDN) affects 16% of patients with diabetes, and it is frequently unreported (12.5%) and more frequently untreated (39%).3 PDN presents an ongoing management problem for patients, caregivers, and physicians. There are many treatment options available, and a rational approach to treating the patient with PDN requires an understanding of the evidence for each intervention.

This guideline addresses the efficacy of pharmacologic and nonpharmacologic treatments to reduce pain and improve physical function and QOL in patients with PDN. The pharmacologic agents reviewed include anticonvulsants, antidepressants, opioids, anti-arrhythmics, cannabinoids, aldose reductase inhibitors, protein kinase...
C beta inhibitors, antioxidants (α-lipoic acid), transketolase activators (thiamines and allithiamines), topical medications (analgesic patches, anesthetic patches, capsaicin cream, clonidine), and others. The nonpharmacologic modalities include infrared therapy, shoe magnets, exercise, acupuncture, external stimulation (transcutaneous electrical nerve stimulation), spinal cord stimulation, biofeedback and behavioral therapy, surgical decompression, and intrathecal baclofen.

**DESCRIPTION OF THE ANALYTIC PROCESS**

In January 2007, the American Academy of Neurology (AAN), the American Association of Neuromuscular and Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation convened an expert panel from the United States and Canada, selected to represent a broad range of relevant expertise. In August 2008, a literature search of MEDLINE and EMBASE was performed in all languages using the MeSH term diabetic neuropathies and its text word synonyms and key words for the therapeutic interventions of interest (see appendix e-1 on the Neurology® Web site at www.neurology.org for a full list of search terms). The search identified 2,234 citations, the titles and abstracts of which were reviewed by at least 2 authors for relevance, resulting in 463 articles. All of these articles were reviewed in their entirety, and of these, the panel identified 79 relevant articles. Each of these articles was rated by at least 2 authors according to the AAN criteria for the classification of therapeutic articles (appendix e-2), and recommendations were linked to the strength of evidence (appendix e-3) and to effect size of the intervention. Disagreements regarding classification were arbitrated by a third reviewer.

Articles were included if they dealt with the treatment of PDN, described the intervention clearly, reported the completion rate of the study, and defined the outcome measures clearly. The panel also considered the side effects of the treatment and measures of function and QOL, if any. Case reports and review articles were excluded.

We anticipated that studies would use varying measures for quantifying pain reduction. For the purposes of this guideline we preferred the following outcome measures, listed in order of preference:

1. The difference in the proportion of patients reporting a greater than 30% to 50% change from baseline on a Likert or visual analog pain scale (VAS) as compared to no treatment (placebo) or the comparative treatment. The Likert scale is an 11-point linear scale ranging from 0 (no pain) to 10 (maximum pain), and the patient rates his or her pain level on this scale.\(^4\)\(^-\)\(^6\)
2. The percent change from baseline on a Likert or VAS as compared to no treatment (placebo) or the comparative treatment.\(^6\)
3. Any other quantitative measure of pain reduction provided by the investigators.

For studies reporting the difference in the proportion of patients reporting a greater than 30% to 50% reduction in pain, we considered a risk difference of >20% a large effect (number needed to treat [NNT] <5), a risk difference of >10% to 20% (NNT >5 to 10) a moderate effect, and a risk difference of ≤10% (NNT >10) a small effect, where risk difference is the reduction in pain in the active treatment group minus the reduction in the control group. For studies using a mean reduction from baseline on a Likert scale or VAS as compared to no treatment (placebo) or a comparative treatment, we considered a reduction difference of >30% a large effect, >15% to 30% a moderate effect, and ≤15% a small effect.

The panel recognized that older studies generally lacked measures of QOL and function compared to more recent studies. Furthermore, the panel was aware that a standardized QOL measure for PDN or a standardized assessment of function is not available, and multiple instruments were used to measure QOL, such as the SF-36° Health Survey, subsections of the SF-36, and function (such as sleep interference).

Studies with the highest levels of evidence for each intervention are discussed in the text, and data from other studies are shown in the tables. Details of Class I, II, and III studies are presented in the evidence tables.

**ANALYSIS OF EVIDENCE**

In patients with PDN, what is the efficacy of pharmacologic agents to reduce pain and improve physical function and QOL? Anticonvulsants.

We identified 20 articles relevant to anticonvulsants graded higher than Class IV (table e-1). Most of the randomized controlled trials (RCTs) rated as Class II instead of Class I had completion rates of less than 80% or the completion rate was not identified.

Four studies (3 Class I and 1 Class II) evaluated the efficacy of pregabalin.\(^7\)\(^-\)\(^10\) All studies found that pregabalin relieved pain, but the effect size was small relative to placebo, reducing pain by 11%–13% on the 11-point Likert scale in the Class I studies. A large dose-dependent effect (24%–50% reduction in Likert pain scores compared to placebo) was ob-
served in the Class II study.\textsuperscript{10} The NNT for a 50% reduction in pain was 4 at 600 g/day.\textsuperscript{7–10} In the QOL measures, social functioning, mental health, bodily pain, and vitality improved, and sleep interference decreased, all changes with \( p < 0.05 \).

Two studies (1 Class I and 1 Class II) evaluated the efficacy of gabapentin.\textsuperscript{11,12} In the Class I study,\textsuperscript{11} gabapentin had a small effect of net pain reduction from baseline of 11% on the 11-point Likert scale compared to the change in placebo-treated patients, while a Class II gabapentin study showed no effect.\textsuperscript{12} Gabapentin had no effect on overall QOL in the single study reporting this measure, but did show an improvement in subsets of mental health and vitality.\textsuperscript{11}

Two Class I trials evaluated the efficacy of lamotrigine.\textsuperscript{13,14} There was no difference in the primary outcome measures in the lamotrigine and placebo groups.

Two studies (both Class II) evaluated the efficacy of sodium valproate.\textsuperscript{15,16} Both showed a 27%–30% pain reduction (moderate) in the Short Form–McGill Pain Questionnaire (SF-MPQ) with sodium valproate compared to placebo, and QOL was not measured. Both studies were conducted by the same principal investigator at the same center but in separate populations with small numbers of patients; each study was remarkable for the lack of any change in placebo patients and for the lack of side effects typically attributed to sodium valproate. Treatment allocation concealment was not described.

One Class II study evaluated the efficacy of topiramate.\textsuperscript{17} The study reported a small effect compared to placebo, 7% net pain reduction on the VAS, and an NNT of 6.6 for >30% pain reduction.

Three Class II studies evaluated the efficacy of oxcarbazepine.\textsuperscript{18–20} Two studies showed no benefit,\textsuperscript{18,20} but a third showed a moderate benefit—17% more patients on oxcarbazepine had a >50% pain reduction compared to placebo, with an NNT of 6.02.\textsuperscript{19} The study showing a positive response had a slightly higher completion rate (73%\textsuperscript{19} compared to 67%).\textsuperscript{20} Short Form–Quality of Life (SF-QOL) scores were not improved.

Three Class III studies evaluated the efficacy of lacosamide.\textsuperscript{21–23} All the studies showed a small reduction in pain with 400 mg/day of lacosamide (3%, 6%, and 6% compared to placebo), but in 2 studies no significant differences compared to placebo were observed with 600 mg/day of lacosamide.\textsuperscript{22,23} In one study, benefits on general activity and sleep interference QOL measures were observed.\textsuperscript{21}

Conclusions. Based on consistent Class I evidence, pregabalin is established as effective in lessening the pain of PDN. Pregabalin also improves QOL and lessens sleep interference, though the effect size is small. Based on one Class I study, gabapentin is probably effective in lessening the pain of PDN. Based on 2 Class II studies, sodium valproate is probably effective in treating PDN. Lamotrigine is probably not effective in treating PDN. Based on Class II evidence, oxcarbazepine is probably not effective in treating PDN. There is conflicting Class III evidence for the effectiveness of topiramate in treating PDN. Based on Class III evidence, lacosamide is possibly not effective in treating PDN. The degree of pain relief afforded by anticonvulsant agents is not associated with improved physical function.

Recommendations

1. If clinically appropriate, pregabalin should be offered for the treatment of PDN (Level A).
2. Gabapentin and sodium valproate should be considered for the treatment of PDN (Level B).
3. There is insufficient evidence to support or refute the use of topiramate for the treatment of PDN (Level U).
4. Oxcarbazepine, lamotrigine, and lacosamide should probably not be considered for the treatment of PDN (Level B).

Clinical context. Although sodium valproate may be effective in treating PDN, it is potentially teratogenic and should be avoided in diabetic women of childbearing age. Due to potential adverse effects such as weight gain and potential worsening of glycemic control, this drug is unlikely to be the first treatment choice for PDN.

Antidepressants. We identified 14 articles relevant to antidepressants rated higher than Class IV (table e-2). Seventeen articles were excluded. Most of the RCTs rated as Class II instead of Class I had completion rates of less than 80%.

Two studies (1 Class I and 1 Class II) evaluated the efficacy of venlafaxine.\textsuperscript{24,25} The Class I study reported a moderate effect of venlafaxine, with 23% more pain relief than with placebo on the VAS-PI (0–100) scale and an NNT of 5.\textsuperscript{24} In the Class II study, venlafaxine plus gabapentin showed a moderate effect in relieving pain on the 11-point Likert scale in PDN, with 18% more relief than with placebo plus gabapentin.\textsuperscript{25} The QOL measures of bodily pain, mental health, and vitality improved on the SF-36.

Three studies (1 Class I and 2 Class II) evaluated the efficacy of duloxetine in PDN.\textsuperscript{26–28} The Class I study showed that duloxetine had a small effect compared to placebo, reducing pain by 8% on the 11-point Likert scale; QOL was not assessed. In 2 Class II studies, duloxetine reduced pain (measured by VAS) 13% more than placebo,\textsuperscript{27,28} but in one study, a moderate effect was shown in responder analysis, with 26% more responders on duloxetine.
120 mg/day (total 52%) than placebo (26%) (responders defined as those patients having 50% reduction in their 24-hour average pain score). The completion rate in both studies was about 75%. Duloxetine reduced interference with general activity and improved SF-36 and EQ-5D™ scores.

Three studies (1 Class I and 2 Class II) evaluated the efficacy of amitriptyline. The Class I study showed a large responder effect with amitriptyline, with 43% more responders with amitriptyline than with placebo (requiring at least 20% pain reduction for responder status). A third group in this study that was treated with maprotiline had 18% more responders than the placebo group. In 2 Class II studies, amitriptyline had a large effect, reducing pain by 63% and 58% more than placebo on a verbal 13-word scale converted to a numeric 5-point scale. In one of these Class II studies, an active placebo was used.

Two Class III trials evaluated other tricyclic antidepressants (imipramine and nortriptyline). One Class III study showed that 47% more subjects on imipramine improved on a global evaluation compared to the placebo group, but there was no difference on a 6-point symptom scale. Another Class III study showed a large effect with the combination of nortriptyline plus fluphenazine compared to placebo; 63% more patients had a 50% or greater VAS reduction in the combination group. One Class III study compared desipramine, amitriptyline, fluoxetine, and placebo and found a small effect (6% pain reduction) for both amitriptyline and desipramine but not for fluoxetine on a 13-word scale converted to 5 points.

Recommendations
1. Amitriptyline, venlafaxine, and duloxetine should be considered for the treatment of PDN (Level B). Data are insufficient to recommend one of these agents over the others.
2. Venlafaxine may be added to gabapentin for a better response (Level C).
3. There is insufficient evidence to support or refute the use of desipramine, imipramine, fluoxetine, or the combination of nortriptyline and fluphenazine in the treatment of PDN (Level U).

Opioids. We identified 9 articles relevant to opioids graded higher than Class IV (table e-3). Most of the RCTs rated as Class II instead of Class I had completion rates of less than 80%.

One Class I study showed that dextromethorphan relieved pain moderately by 16% more than placebo on a 20-point Gracely Box scale in PDN and improved SF-36 results. In one Class II study, dextromethorphan with benztpine reduced pain by 24% more than placebo on a 6-point scale, a moderate reduction.

A Class II study showed that morphine sulfate had a small effect and reduced pain from baseline by 15% on the SF-MPQ and improved SF-36 and Beck Depression Inventory results.

In 2 Class II studies, tramadol relieved pain moderately (16% and 20% more than placebo on a Likert scale) in PDN and improved physical function.

In 3 Class II studies, oxycodone controlled-release and Ultracet (tramadol + acetaminophen) relieved pain in PDN. Oxycodone had a small effect, with 9% more pain relief on the Pain Inventory than placebo. It also improved sleep quality by 7% more than placebo, but did not change SF-36 scores. Ulitracet improved pain relief by 13% on the VAS, a small effect, and also improved SF-36 scores by 10%. Oxycodone controlled-release had a moderate effect on pain (27% reduction in the VAS compared to placebo), improved disability by 10%, and improved most SF-36 subscores.

Conclusions. Based on one Class I study, dextromethorphan is probably effective in lessening the pain of PDN and improving QOL. Based on Class II evidence, morphine sulfate, tramadol, and oxycodone controlled-release are probably effective in lessening the pain of PDN. Dextromethorphan, tramadol, and oxycodone controlled-release have moderate effect sizes, reducing pain by 27% compared with placebo.

Recommendations. Dextromethorphan, morphine sulfate, tramadol, and oxycodone should be considered for the treatment of PDN (Level B). Data are insufficient to recommend one agent over the other.

Clinical context. The use of opioids for chronic non-malignant pain has gained credence over the last decade due to the studies reviewed in this article. Both tramadol and dextromethorphan were associated with substantial adverse events (e.g., sedation in 18% on tramadol and 58% on dextromethorphan, nausea in 23% on tramadol, and constipation in 21% on tramadol). The use of opioids can be associated with the development of novel pain syndromes such as rebound headache. Chronic use of opioids leads to tolerance and frequent escalation of dose.
Other pharmacologic agents. We identified 18 articles relevant to other pharmacologic agents rated higher than Class IV (table e-4). Thirteen other articles were excluded. Most of the RCTs rated Class II instead of Class I had completion rates of less than 80%, and those rated Class III often lacked predefined endpoints.

One Class I study of 0.075% capsaicin showed a large effect, with 40% more pain reduction on the VAS compared to vehicle cream.\(^5\) One Class II study showed that 0.075% capsaicin reduced pain in PDN with a small effect size of 13% in VAS compared to vehicle cream.\(^4\)

One Class I study of isosorbide dinitrate spray showed a moderate effect, with 18% more pain reduction on the VAS relative to placebo.\(^5\)

One Class I study of clonidine and pentoxifylline compared to placebo did not show an effect of these drugs on PDN.\(^6\)

One Class I study of mexiletine did not show an effect on PDN.\(^7\) Two Class II studies both showed pain reduction with mexiletine, one with a large effect (37% more pain reduction than placebo)\(^8\) and one with a small effect (5% difference compared to placebo).\(^9\) Sleep disturbance was reduced in the first Class II study\(^8\) but not in the second.\(^9\)

In a single Class I study of sorbinil, pain relief was not observed.\(^10\)

One Class I and 2 Class II studies showed benefit from \(\alpha\)-lipoic acid in reducing pain in PDN, but pain was not a predefined endpoint in these studies.\(^11-13\) The effect size in pain reduction was moderate (20%–24% superior to placebo).

In 2 Class III studies, IV lidocaine decreased pain relative to placebo infusion.\(^14,15\) In one study, a transient decrease of 75% was observed in a 5-point symptom scale, compared to a decrease of 50% with placebo infusion.\(^14\) In the other study, the McGill Pain Questionnaire improved by a small amount (9% reduction in present pain intensity) with lignocaine, and the differences with placebo were significant due to worsening in the placebo group.\(^15\) The baseline values were not provided.

In 2 Class III studies, the Lidoderm patch improved pain scores with a moderate to large effect (20%–30% reduction in pain scores from baseline and 70% of patients experienced more than a 30% decrease in pain).\(^16,17\)

Conclusions. Based on Class I and Class II evidence, capsaicin cream is probably effective in lessening the pain of PDN. Based on Class III studies, there is insufficient evidence to determine if IV lidocaine is effective in lessening the pain of PDN. Based on Class III evidence, the Lidoderm patch is possibly effective in lessening the pain of PDN. Based on Class I evidence, clonidine and pentoxifylline are probably not effective for the treatment of PDN. The evidence for the effectiveness of mexiletine is contradictory; however, the only Class I study of this agent indicates that mexiletine is probably ineffective for the treatment of PDN. There is insufficient evidence to determine whether vitamins and \(\alpha\)-lipoic acid are effective for the treatment of PDN. Based on Class I evidence, isosorbide dinitrate spray is probably effective for the treatment of PDN.

Recommendations
1. Capsaicin and isosorbide dinitrate spray should be considered for the treatment of PDN (Level B).
2. Clonidine, pentoxifylline, and mexiletine should probably not be considered for the treatment of PDN (Level B).
3. The Lidoderm patch may be considered for the treatment of PDN (Level B).
4. There is insufficient evidence to support or refute the usefulness of vitamins and \(\alpha\)-lipoic acid in the treatment of PDN (Level U).

Clinical context. Although capsaicin has been effective in reducing pain in PDN clinical trials, many patients are intolerant of the side effects, mainly burning pain on contact with warm/hot water or in hot weather.

In patients with PDN, what is the efficacy of nonpharmacologic modalities to reduce pain and improve physical function and QOL? We identified 11 articles relevant to nonpharmacologic treatment of PDN graded higher than Class IV (table e-5). Only articles on electrical stimulation, Reiki therapy, low-intensity laser therapy, and magnetized shoe insoles reached evidence levels sufficient for discussion in the text. Surgical decompression was addressed in a previous AAN practice advisory\(^18\) and will not be considered further in this article.

Electrical stimulation. One Class I study reported that percutaneous electrical nerve stimulation reduced pain in PDN by a large magnitude (42% on the VAS) compared with the reduction observed with sham treatment, and also improved sleep.\(^19\) One Class II study reported no effect with electrical stimulation.\(^20\) and one Class II study of frequency-modulated electromagnetic neural stimulation showed a small degree of pain relief (11% on the VAS) in a crossover design, but with no improvement in the placebo group.\(^21\)

One Class III study showed the addition of electrotherapy to amitriptyline was more effective than amitriptyline alone.\(^22\)

Magnetic field treatment. One Class I study using pulsed electromagnetic fields compared with a sham
device failed to demonstrate an effect in patients with PDN.23

One Class II study of the use of magnetized shoe insoles in patients with PDN showed a small effect (14% VAS decrease) at 4 months compared with that from nonmagnetized insoles, but the endpoint of burning pain was not predetermined.24

Other treatments. One Class I study on the use of low-intensity laser treatment compared to sham treatment did not show an effect on pain.25

Reiki therapy is defined as the transfer of energy from the practitioner to the patient to enable the body to heal itself through balancing energy. One Class I study of Reiki therapy did not show any effect on PDN.26

Other interventions such as exercise and acupuncture do not have any evidence for efficacy in treating PDN.

Conclusion. Based on a Class I study, electrical stimulation is probably effective in lessening the pain of PDN and improving QOL. Based on single Class I studies, electromagnetic field treatment, low-intensity laser treatment, and Reiki therapy are probably not effective for the treatment of PDN. There is not enough evidence to support or exclude a benefit of amitriptyline plus electrotherapy in treating PDN.

Recommendations
1. Percutaneous electrical nerve stimulation should be considered for the treatment of PDN (Level B).
2. Electromagnetic field treatment, low-intensity laser treatment, and Reiki therapy should probably not be considered for the treatment of PDN (Level B).
3. Evidence is insufficient to support or refute the use of amitriptyline plus electrotherapy for treatment of PDN (Level U).

Comparison studies. Studies with 2 active treatment arms and without a placebo arm were considered separately and graded using active control equivalence criteria (appendix e-2; table e-6). We identified 6 comparison studies of agents but did not find sufficient evidence to recommend one over the other.27-32 The comparisons were gabapentin to amitriptyline,2 venlafaxine to carbamazepine, nortriptyline + fluphenazine to carbamazepine, capsicain to amitriptyline, and benfothiamine + cyanocobalamin with conventional vitamin B. None of the studies defined the threshold for equivalence or noninferiority.

CLINICAL CONTEXT SUMMARY FOR ALL EVIDENCE It is notable that the placebo effect varied from 0% to 50% pain reduction in these studies. Adjuvant analgesic agents are drugs primarily developed for an indication other than treatment of PDN (e.g., anticonvulsants and antidepressants) that have been found to lessen pain when given to patients with PDN. Their use in the treatment of PDN is common.33 The panel recognizes that PDN is a chronic disease and that there are no data on the efficacy of the chronic use of any treatment, as most trials have durations of 2–20 weeks. It is important to note that the evidence is limited, the degree of effectiveness can be minor, the side effects can be intolerable, the impact on improving physical function is limited, and the cost is high, particularly for novel agents.

A summary of Level A and B recommendations for the treatment of PDN is provided in table 1.

RECOMMENDATIONS FOR FUTURE RESEARCH
1. A formalized process for rating pain scales for use in all clinical trials should be developed.
2. Clinical trials should be expanded to include effects on QOL and physical function when evaluating efficacy of new interventions for PDN; the measures should be standardized.
3. Future clinical trials should include head-to-head comparisons of different medications and combinations of medications.
4. Because PDN is a chronic disease, trials of longer duration should be done.
5. Standard metrics for side effects to qualify effect sizes of interventions need to be developed.
6. Cost-effectiveness studies of different treatments should be done.
7. The mechanism of action of electrical stimulation is unknown; a better understanding of its role, mode of application, and other aspects of its use should be studied.

DISCLOSURE
Dr. Bril has received research support from Talecris Biotherapeutics, Eisai Inc., Pfizer Inc, Eli Lilly and Company, and Johnson & Johnson. Dr. England serves on the speakers’ bureau for and has received funding for

Table 1 Summary of recommendations

<table>
<thead>
<tr>
<th>Recommended drug and dose</th>
<th>Not recommended</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level A</td>
<td></td>
</tr>
<tr>
<td>Pregabalin, 300–600 mg/d</td>
<td></td>
</tr>
<tr>
<td>Level B</td>
<td></td>
</tr>
<tr>
<td>Gabapentin, 900–3,600 mg/d</td>
<td>Oxcarbazepine</td>
</tr>
<tr>
<td>Sodium valproate, 500–1,200 mg/d</td>
<td>Lamotrigine</td>
</tr>
<tr>
<td>Venlafaxine, 75–225 mg/d</td>
<td>Lacosamide</td>
</tr>
<tr>
<td>Duloxetine, 60–120 mg/d</td>
<td>Clonidine</td>
</tr>
<tr>
<td>Amitriptyline, 25–100 mg/d</td>
<td>Pentoxifylline</td>
</tr>
<tr>
<td>Dextromethorphan, 400 mg/d</td>
<td>Mexiletine</td>
</tr>
<tr>
<td>Morphine sulphate, titrated to 120 mg/d</td>
<td>Magnetic field treatment</td>
</tr>
<tr>
<td>Tramadol, 210 mg/d</td>
<td>Low-intensity laser therapy</td>
</tr>
<tr>
<td>Oxycodone, mean 37 mg/d, max 120 mg/d</td>
<td>Reiki therapy</td>
</tr>
<tr>
<td>Capsaicin, 0.075% QID</td>
<td></td>
</tr>
<tr>
<td>Isosorbide dinitrate spray</td>
<td></td>
</tr>
<tr>
<td>Electrical stimulation, percutaneous nerve stimulation ×3–4 weeks</td>
<td></td>
</tr>
</tbody>
</table>
travel or speak on behalf of Takeda Biotherapeutics and Teva Pharmaceutical Industries Ltd.; served as an Associate Editor for "Current Treatment Options in Neurology;" receives research support from the NIH/NIH, Wyeth, AstraZeneca, and Pfizer Inc; holds stock/stock options in Wyeth and Takeda Biotherapeutics; and has served as an expert witness in a medico-legal case. Dr. Franklin serves on the editorial board of "Neuropadiology;" serves as a consultant for the New Zealand Accident Fund; and serves as a consultant for the Workers Compensation Research Institute. Dr. Backonja served on a Safety Monitoring Board for Medtronic, Inc.; serves on the editorial boards of "Clinical Journal of Pain, European Journal of Pain, Journal of Pain, Pain, and Pain Medicine;" is listed as author on a patent re: A hand-held probe for suprathermal testing in patients with neuropathic pain and other neurological sensory disorders; serves as a consultant for Allergan, Inc., Astellas Pharma Inc., Eli Lilly and Company, Medtronic, Inc., Merck Serono, NeurogesX, Pfizer Inc., and SK Laboratories, Inc.; and receives research support from NeurogesX. Dr. Cohen serves on an FDA Peripheral and Central Nervous System Drugs Advisory Committee; receives publishing royalties for "What Would You Do Now? Neuromuscular Disease" (Oxford University Press, 2009); estimates that he performs clinical neurophysiology testing as 50% of his clinical practice; and has given expert testimony, prepared an affidavit, and acted as a witness in a legal proceeding with regard to vaccine-related injuries and peripheral nerve injuries. Dr. Del Toro receives research support from the NIH. Dr. Feldman serves on a Data Safety and Monitoring Board for Novartis; serves on the editorial boards of "Anals of Neurology and the Journal of the Peripheral Nervous System;" receives publishing royalties from UpToDate; and receives research support from the NIH, the Taubman Research Institute, and the American Diabetes Association. Dr. Iverson serves as editor of "NeuroPI" and has been a treating expert witness with regard to a legal proceeding. Dr. Perkins has received research support from Medtronic, Inc., the Canadian Institutes of Health Research, the Juvenile Diabetes Research Foundation, and the Canadian Diabetes Association. Dr. Russell has received honoraria from Elsevier Inc. and Baxter International Inc.; and receives research support from Baxter International Inc., the NIH, the US Veterans Administration, the American Diabetes Association, and the Juvenile Diabetes Foundation. Dr. Zochodne serves on a scientific advisory board for and holds stock options in Aegera Therapeutics Inc.; has received honoraria from Ono Pharmaceutical Co. Ltd.; receives publishing royalties for "Neurobiology of Peripheral Nerve Regeneration" (Cambridge University Press, 2008); has received research support from the Canadian Institutes of Health Research, the Canadian Diabetes Association, the Juvenile Diabetes Research Foundation, the National Science and Engineering Research Council, the NIH, and the Alberta Heritage Foundation for Medical Research, Baxter International Inc., and Aegera Therapeutics Inc.; and has served as a co-PI on industry trials with Valeant Pharmaceuticals International and Pfizer Inc.

DISCLAIMER

This statement is provided as an educational service of the American Academy of Neurology. It is based on an assessment of current scientific and clinical information. It is not intended to include all possible proper methods of care for a particular neurologic problem or all legitimate criteria for choosing to use a specific procedure. Neither is it intended to exclude any reasonable alternative methodologies. The AAN recognizes that specific patient care decisions are the prerogative of the patient and the physician caring for the patient, based on all of the circumstances involved. The clinical context section is made available in order to place the evidence-based guideline(s) into perspective with current practice habits and challenges. No formal practice recommendations should be inferred.

CONFLICT OF INTEREST

The American Academy of Neurology is committed to producing independent, critical and truthful clinical practice guidelines (CPGs). Significant efforts are made to minimize the potential for conflicts of interest to influence the recommendations of this CPG. To the extent possible, the AAN keeps separate those who have a financial stake in the success or failure of the products appraised in the CPGs and the developers of the guidelines. Conflict of interest forms were obtained from all authors and reviewed by an oversight committee prior to project initiation. AAN limits the participation of authors with substantial conflicts of interest. The AAN forbids commercial participation in, or funding of, guideline projects. Drafts of the guidelines have been reviewed by at least three AAN committees, a network of neurologists, Neurology® peer reviewers, and representatives from related fields. The AAN Guideline Author Conflict of Interest Policy can be viewed at www.aan.com.

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REFERENCES


Assessing autonomic dysfunction in early diabetic neuropathy

The Survey of Autonomic Symptoms

ABSTRACT

Objective: Autonomic symptoms may occur frequently in diabetic and other neuropathies. There is a need to develop a simple instrument to measure autonomic symptoms in subjects with neuropathy and to test the validity of the instrument.

Methods: The Survey of Autonomic Symptoms (SAS) consists of 11 items in women and 12 in men. Each item is rated by an impact score ranging from 1 (least severe) to 5 (most severe). The SAS was tested in observational studies and compared to a previously validated autonomic scale, the Autonomic Symptom Profile (ASP), and to a series of autonomic tests.

Results: The SAS was tested in 30 healthy controls and 62 subjects with neuropathy and impaired glucose tolerance or newly diagnosed diabetes. An increased SAS score was associated with the previously validated ASP (rank order correlation = 0.68; p < 0.0001) and with quantitative measures of autonomic function: a reduced quantitative sudomotor axon reflex test sweat volume (0.31; p < 0.05) and an abnormal 30:15 ratio (0.53; p < 0.01). The SAS shows a high sensitivity and specificity (area under the receiver operating characteristic curve 0.828) that compares favorably with the ASP. The SAS scale domains had a good internal consistency and reliability (Cronbach α = 0.76). The SAS symptom score was increased in neuropathy (95% confidence interval [CI] 2.99–4.14) compared to control (95% CI 0.58–1.69; p < 0.0001) subjects.

Conclusions: The SAS is a new, valid, easily administered instrument to measure autonomic symptoms in early diabetic neuropathy and would be of value in assessing neuropathic autonomic symptoms in clinical trials and epidemiologic studies. Neurology® 2011;76:1099-1105

GLOSSARY

ASP = Autonomic Symptom Profile; AUC = area under the curve; CAN = cardiac autonomic neuropathy; CASS = Composite Autonomic Scoring Scale; CI = confidence interval; COMPASS = Composite Autonomic Symptom Scale; E1 = expiration:inspiration ratio; HRR = heart rate range; IENFD = intraepidermal nerve fiber density; IFG = impaired fasting glucose; IGR = impaired glucose regulation; IGT = impaired glucose tolerance; IRT = interscapular refractive test; IENFD = intraepidermal nerve fiber density; NCS = nerve conduction studies; OR = odds ratio; QST = quantitative sensory testing; ROC = receiver operating characteristic; SAS = Survey of Autonomic Symptoms; SSR = sympathetic skin response; TIS = total symptom impact score.

Impaired glucose regulation (IGR) is associated with peripheral neuropathy in at least 40% of cases. The neuropathy associated with IGR and early diabetes is a small-fiber neuropathy that is often accompanied by mild autonomic symptoms and abnormalities. A recent consensus statement by the American Diabetes Association recognizes that glycemic burden is a strong predictor of adverse outcomes and that IGR represents a continuum of risk. Thus, for the purposes of this study, subjects with neuropathy and prediabetes or newly diagnosed diabetes are described as having IGR or early diabetic neuropathy.

From the Department of Neurology (L.Z., A.A., J.W.R.), University of Maryland and Maryland VA Healthcare System, Baltimore; Department of Neurology (A.C.P.), Vanderbilt University, Nashville, TN; School of Health Sciences (P.A.W.), Oakland University, Rochester, MI; Departments of Neurology (A.G.S., J.R.S.) and Pathology (A.G.S.), University of Utah, Salt Lake City; Department of Neurology (E.L.F.), University of Michigan, Ann Arbor; and Department of Internal Medicine (N.B.A.), Division of Geriatric Medicine, Mobility Research Center, Institute of Gerontology, The University of Michigan, VA Ann Arbor Health Care System Geriatric Research, Education and Clinical Center, Ann Arbor.

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Disclosure: Author disclosures are provided at the end of the article.
There are few validated scores of autonomic symptoms and even fewer that have been evaluated in subjects with neuropathy. One validated scale is the Autonomic Symptom Profile (ASP). The Composite Autonomic Symptom Scale (COMPASS) was developed to provide an aggregate score of the autonomic symptoms in the ASP with weighting according to clinical relevance. When the ASP was used to assess subjects with neurogenic autonomic failure and control patients, the COMPASS scores correlated well with the Composite Autonomic Scoring Scale (CASS) obtained from autonomic testing. However, even though the correlation between autonomic symptoms and the CASS was better in type 1 than it was in type 2 diabetes, the correlation was weak overall. This observation was ascribed to mild autonomic symptoms in diabetic patients and thus a reduced strength of the association. Furthermore, this highlights the need for a sensitive, brief, and easy-to-use questionnaire for autonomic neuropathy symptoms that can be used in both research studies and clinical practice.

**METHODS** Standard protocol approvals, registrations, and patient consents. All subjects with neuropathy and normal subjects were consented according to the ethical standards committees on human experimentation (Michigan, Maryland, and Utah) and written informed consent was obtained on all participating subjects. Some of the subjects are participants in ClinicalTrials.gov NCT00780559.

**Questionnaire.** The questionnaire was designed to provide a succinct evaluation of autonomic symptoms in subjects with mild neuropathy. Questions were evaluated in subjects with early neuropathy and only symptoms that were found to be reproducibly present in these subjects were included. The Survey of Autonomic Symptoms (SAS) Scale assesses both the presence of symptoms and the degree of severity. The scale is intended to 1) provide assessment of the type of mild autonomic symptoms observed in early diabetic neuropathy and in autonomic neuropathies, 2) ensure that questions are unambiguous and can be easily understood by patients and research subjects, and 3) serve as an instrument that can be used in clinical trials, clinical practice, and large epidemiologic studies. The questions were developed from questions used routinely in practice in patients with neuropathy and then individual elements of the scale were evaluated for their reliability and sensitivity. From these questions, 11 (women) or 12 (men) questions were most frequently positive in subjects with early diabetic neuropathy. These questions assess the following autonomic symptom domains: orthostatic, sudomotor symptoms, vasomotor, gastrointestinal, urinary, and sexual dysfunction (table 1). The questions were designed to minimize ambiguity and required a yes or no response to symptoms occurring in the 6-month period prior to administration. The subject was then asked to indicate the degree of severity of the symptom, with 1 being the least severe and 5 the most severe, to determine the total symptom impact score (TIS).

**Study design.** Data in this study were obtained from subjects enrolled in the Impaired Glucose Tolerance Causes Neuropathy Study, the Improving Neuropathy and Mobility in Early Diabetes study, and the University of Maryland Neuromuscular and Department of Neurology Database. All subjects with polyneuropathy had IGR. IGR includes early type 2 diabetes mellitus (within 2 years of diagnosis), impaired glucose tolerance (IGT), and impaired fasting glucose (IFG) based on standardized ADA criteria. Subjects with IGR were evaluated with nerve conduction studies (NCS), other electrophysiologic tests such as quantitative sensory testing (QST) (vibration detection threshold and cold detection threshold), the quantitative sudomotor axon reflex test (QSART), and cardiac autonomic neuropathy (CAN) testing. Subjects also had skin biopsies performed at the calf and thigh and the intraepidermal nerve fiber density (IENFD) was measured. The criteria for inclusion within the study were IGR confirmed on at least 2 separate occasions, signs and symptoms of peripheral neuropathy, and an abnormality in at least one of the following: NCS, QST, or QSART, or IENFD. Subjects were excluded from the study if other causes of neuropathy existed as previously described.

**Table 1. Survey of Autonomic Symptoms**

<table>
<thead>
<tr>
<th>Symptom/health problem</th>
<th>Q1a. Have you had any of the following health symptoms during the past 6 months? (1 = Yes; 0 = No)</th>
<th>Q1b. If you answered yes in Q1a, how much would you say the symptom bothers you? (1 = Not at all; 2 = A little; 3 = Some; 4 = A moderate amount; 5 = A lot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Do you have lightheadedness?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>2. Do you have a dry mouth or dry eyes?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>3. Are your feet pale or blue?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>4. Are your feet colder than the rest of your body?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>5. Is sweating in your feet decreased compared to the rest of your body?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>6. Is sweating in your feet decreased or absent (for example, after exercise or during hot weather)?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>7. Is sweating in your hands increased compared to the rest of your body?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>8. Do you have nausea, vomiting, or bloating after eating a small meal?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>9. Do you have persistent diarrhea (more than 3 loose bowel movements per day)?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>10. Do you have persistent constipation (less than 1 bowel movement every other day)?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>11. Do you have leaking of urine?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>12. Do you have difficulty obtaining an erection (men)?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
</tbody>
</table>

*Number of symptoms reported: (sum of column A, 0–12 for men and 0–11 for women); total symptom impact score: (sum of column B, 0–60 for men and 0–55 for women).
Normal subjects were recruited as part of the University of Maryland Neuromuscular or Neurology Database. All normal subjects were examined by one of the authors (J.W.R. or L.Z.) and their medical records were carefully reviewed to exclude subjects with neurologic or autonomic disorders or those taking any medications which may induce autonomic changes. Both normal and IGR neuropathy subjects also completed the ASP.10 The ASP consists of 73 questions assessing the following 9 domains of autonomic symptoms: orthostatic (9 items); secretomotor, including sudomotor symptoms (8 items); male sexual dysfunction (8 items); urinary (5 items); gastrointestinal, including gastroparesis, diarrhea, and constipation (14 items); pupillomotor, including visual symptoms (7 items); vasmotor (11 items): reflex syncope (5 items); and sleep function (8 items) as previously described.2 This is scored to provide the COMPASS. The COMPASS is based on key scorable areas of the autonomic nervous system based on presence, severity, distribution, frequency, and progression of symptoms.3,10

Autonomic testing. When medically permissible, subjects with neuropathy were asked to discontinue any medications that could alter the results of their autonomic tests for 24 to 48 hours before testing and to refrain from consuming caffeine during this time. None of the control subjects were taking medications known to influence the results of autonomic tests. The QSART was purchased from WR Electronics (Stillwater, MN) and performed as previously described.4 CAN testing (WR Electronics) was used to assess the following. 1) Heart rate variability to deep breathing. The ratio of the heart rate response during expiration and inspiration, the expirationspiration (E:I) ratio, and heart rate range (HRR) were measured. 2) The Valsalva ratio and the beat-to-beat blood pressure measurements during the Valsalva maneuver. The subject was asked to maintain an expiration pressure of 40 mm Hg for 15 seconds. (3) Beat-to-beat blood pressure change during a 10-minute 70-degree head-up tilt compared to the resting supine blood pressure. (4) The 30:15 ratio. All these tests and their normative values have been previously described.5

CAN testing (WR Electronics) was used to assess the following. 1) Heart rate variability to deep breathing. The ratio of

Construct validity. The SAS was compared with the ASP/COMPASS and with the CASS to assess the degree of criterion validity. The CASS consists of a comprehensive battery of autonomic tests that has previously been shown to be quantitative, sensitive, specific, reproducible, and standardized.16,17 The SAS was then compared to measures of autonomic function including the QSART sweat response, 30:15 ratio, E:I ratio, HRR, and tilt response.

Statistical design. Analysis was performed using SPSS version 18. Pearson correlation coefficients were used to examine pairwise correlation between normally distributed variables. Spearman rank order correlations were used for data analysis for the ASP/COMPASS score and subscores and the CASS score and subscores because these scores are not normally distributed18 and a valid transformation of the data were not possible. Receiver operating characteristic (ROC) curves were calculated and compared as previously described.19 Internal consistency for the construct items was determined using Cronbach α. Statistical significance was defined as a 2-tailed p value <0.05, and data are presented as mean ± SEM.

RESULTS General clinical features of the subjects. A total of 93 subjects completed the SAS and the COMPASS. There were 38 women (mean age 59.37 ± 1.34 years) and 25 men (mean age 59.20 ± 1.63 years) with neuropathy. Of these, 94% had IGT or IFG, and 6% had early diabetes. In the control group, there were 18 women (mean age 56.94 ± 2.86) and 12 men (mean age 49.25 ± 1.78 years). Mean ages were not different between men and women with neuropathy and control women, but the mean age of control men was less than control women (p < 0.01). Despite the lower mean male control age, there was no difference between gender or age for the SAS symptom score or TIS in either controls or neuropathy subjects (figure 1). However, there was a difference between groups for both the SAS symptom score (p < 0.0001; 95% confidence interval 0.69-0.94) and the TIS (p < 0.05).
Table 2  Correlation of the SAS symptom score and total impact score with other autonomic measures

<table>
<thead>
<tr>
<th>Test</th>
<th>SAS symptom score</th>
<th>SAS total impact score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>Significance (p value)</td>
</tr>
<tr>
<td>ASP total score</td>
<td>0.68</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ASP secretomotor</td>
<td>0.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ASP vasomotor</td>
<td>0.43</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ASP orthostatic intolerance</td>
<td>0.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ASP bladder dysfunction</td>
<td>0.33</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ASP diarrhea</td>
<td>0.29</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ASP syncope</td>
<td>0.25</td>
<td>NS</td>
</tr>
<tr>
<td>ASP erectile dysfunction</td>
<td>0.27</td>
<td>NS</td>
</tr>
<tr>
<td>ASP gastric paresis</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td>QSART forearm volume</td>
<td>−0.31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>QSART foot volume</td>
<td>−0.31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CAN 30:15 ratio</td>
<td>−0.53</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CAN E:I ratio</td>
<td>−0.26</td>
<td>NS</td>
</tr>
<tr>
<td>CAN HR difference</td>
<td>−0.16</td>
<td>NS</td>
</tr>
<tr>
<td>CAN Valsalva ratio</td>
<td>−0.026</td>
<td>NS</td>
</tr>
<tr>
<td>CASS total score</td>
<td>0.32</td>
<td>NS</td>
</tr>
<tr>
<td>CASS sudomotor</td>
<td>0.19</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviations: ASP = Autonomic Symptom Profile; CAN = cardiac autonomic neuropathy; CASS = Composite Autonomic Scoring Scale; E:I = expiration/inspiration ratio; HR = heart rate; NS = not significant; QSART = quantitative sudomotor axon reflex test; SAS = Survey of Autonomic Symptoms.

Validation of SAS domains with other measures of autonomic function. The SAS showed a strong association with the ASP total score for all domains, and the secretomotor, vasomotor, and orthostatic intolerance ASP domains (table 2). There was a weaker association between the SAS and the ASP bladder dysfunction and diarrhea domains. There was no association between the SAS and the ASP erectile dysfunction, gastric paresis, and other domains. Importantly, an increased SAS symptom score or TIS was associated with a reduced forearm or foot sweat volume on the QSART (table e-2) and also with a reduced 30:15 ratio. However, there was no association between the SAS symptom or TIS and the E:I ratio, HRR, Valsalva ratio (table 2), or an abnormal tilt table response (symptom score: odds ratio [OR] 1.29, 95% CI 0.87–1.91, p = 0.19; TIS: OR 1.08, 95% CI 0.96–1.21, p = 0.19). The CASS total score or any of the CASS subscores (adrenergic, cardiogenic, or sudomotor) were not associated with the SAS using a nonparametric Spearman rank order analysis. Furthermore, when the CASS was expressed as a dichotomous variable and a logistic regression was performed, there was still no association between the CASS sudomotor score and the SAS symptom score (OR 1.19, 95% CI 0.95–1.50, p = 0.13) or the SAS TIS (OR 1.07, 95% CI 0.99–1.16, p = 0.08). There was also no association between the CASS total score and the SAS symptom score (OR 1.12, 95% CI 0.891–1.40, p = 0.33) and SAS TIS (OR 1.05, 95% CI 0.97–1.13, p = 0.20).

ROC for the SAS and ASP scores. In assessing autonomic symptoms in subjects with early diabetic neuropathy, the ROC sensitivity/specificity analysis indicated that the SAS symptom score showed a slightly greater sensitivity and specificity throughout its dynamic range than the ASP score (figure 2). The area under the curve (AUC) was as follows: SAS symptom score −0.828 (SEM 0.047, 95% CI 0.737–0.920) compared to the ASP score −0.812 (SEM 0.057, 95% CI 0.700–0.925). Based on the SAS symptom score ROC curve, a cutpoint of greater than zero would provide 95% sensitivity and 50% specificity and a cutpoint of greater than 3 would provide greater than 90% specificity and greater than 65% sensitivity in determining disease. Based on the SAS TIS ROC curve, a cutpoint of greater than 1 would provide greater than 90% sensitivity and greater than 50% specificity and a cutpoint greater than 7 would provide greater than 90% specificity and greater than 60% sensitivity. Using previously described methods,18 there was no difference between the SAS symptom score and ASP ROC curves (p = 0.682).

Internal consistency of internal reliability and frequency of the SAS domains. Internal consistency reliability testing using Cronbach α provided a value of 0.76, indicating that the scale domains are measuring the same overall construct. All items in the SAS showed a high degree of interitem correlation. However, of all the domains, item 10—“Do you have persistent constipation?”—added least to the overall reliability of the SAS. For subjects with neuropathy, the 3 items showing the greatest reliability were in order: 1) Item 5, “Is sweating in your feet decreased compared to the rest of your body?” 2) Item 4, “Are your feet colder than the rest of your body?” 3) Item 11, “Do you have leaking of urine?” The items addressing gastrointestinal function showed the lowest construct reliability in subjects with neuropathy.
most common symptoms reported by subjects with neuropathy are indicated in table 3. The most common items—“Do you have a dry mouth or dry eyes?” and “Are your feet colder than the rest of your body?”—have similar frequencies in both men and women.

**DISCUSSION**

The SAS was designed to assess autonomic symptoms in subjects with neuropathy and was found to have a slightly greater sensitivity and specificity in subjects with early diabetic neuropathy than the ASP. The questions in the SAS were designed to improve subject understanding and to reduce uncertainty in the responses. Currently available autonomic questionnaires are lengthy, complex, and take training and considerable time to score. In contrast, the SAS can be rapidly completed and scored. Furthermore, interpretation of the SAS is not dependent on age, gender, body mass index, and other factors. This provides for a flexible and more universally acceptable scale to assess autonomic function in subjects with neuropathy. However, further validation of the SAS is needed in blinded and longitudinal clinical studies.

There was a difference in both the SAS symptom score and the TIS between control and neuropathy subjects. This indicates that the SAS has power to distinguish between control subjects and those with peripheral neuropathy. The SAS symptom score and

<table>
<thead>
<tr>
<th>Item</th>
<th>% Affected</th>
<th>Mean TIS (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
</tr>
<tr>
<td>Lightheadedness?</td>
<td>50.00</td>
<td>38.00</td>
</tr>
<tr>
<td>Dry mouth or dry eyes?</td>
<td>77.78</td>
<td>70.83</td>
</tr>
<tr>
<td>Feet pale or blue?</td>
<td>22.22</td>
<td>33.33</td>
</tr>
<tr>
<td>Feet colder than the rest of your body?</td>
<td>66.67</td>
<td>70.83</td>
</tr>
<tr>
<td>Sweating in your feet decreased compared to the rest of your body?</td>
<td>33.33</td>
<td>25.00</td>
</tr>
<tr>
<td>Sweating in your feet decreased or absent (exercise/hot weather)?</td>
<td>16.67</td>
<td>20.83</td>
</tr>
<tr>
<td>Sweating in your hands increased compared to the rest of your body?</td>
<td>5.56</td>
<td>20.83</td>
</tr>
<tr>
<td>Nausea, vomiting, or bloating after eating a small meal?</td>
<td>5.56</td>
<td>16.67</td>
</tr>
<tr>
<td>Persistent diarrhea?</td>
<td>5.56</td>
<td>16.67</td>
</tr>
<tr>
<td>Persistent constipation?</td>
<td>11.11</td>
<td>25.00</td>
</tr>
<tr>
<td>Leaking of urine?</td>
<td>22.22</td>
<td>45.83</td>
</tr>
<tr>
<td>Difficulty obtaining an erection (men)?</td>
<td>55.56</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: NA — not available; TIS — total symptom impact score.
TIS are not affected by age, gender, body mass index, weight, or height. This indicates that the SAS would perform well across subject groups in a clinical trial or epidemiologic study and would be less likely to be affected by common confounding variables. The SAS demonstrated a strong association with the ASP total score and for the ASP domains of secretomotor, vasomotor, and orthostatic intolerance. This is consistent with the observation that symptoms that tested these 3 autonomic domains were most commonly reported by subjects with neuropathy and also showed the greatest internal consistency. Furthermore, both the SAS symptom score and TIS showed no association between the SAS and the ASP domains of gastrointestinal dysfunction and gastrointestinal symptoms showed the lowest internal consistency. These results may be explained by the fact that this study examined subjects with IGR and early diabetic neuropathy. Although patients with type 2 diabetes commonly have autonomic symptoms, the symptoms are usually mild and in one study the syncope and gastrointestinal symptom domains on the ASP did not differ from control subjects.

The SAS was further validated by an association between an increased SAS symptom score or TIS and a reduced forearm or foot sweat volume on QSART or a reduced 30:15 ratio. However, there was no association seen between the SAS symptom score or TIS and the E:I ratio, HRR, Valsalva ratio, abnormal tilt table response, CASS total score, or CASS subscores. These findings are consistent with a previous study that found only a weak association between autonomic symptom scores on the ASP and autonomic deficits on the CASS in patients with diabetes. Only a few subjects were taking a medication with significant anticholinergic properties that may reduce sweating, and these were discontinued for at least 24 hours prior to performing the QSART. Thus, these medications would not significantly affect the results in this study. These previous studies highlight the need to examine autonomic symptoms independently of autonomic deficits. The present study raises the possibility that early diabetic neuropathy is associated with a mild autonomic neuropathy that current autonomic tests are not sensitive enough to detect or that autonomic symptom scores overrate for the presence of autonomic neuropathy.

Compared to controls, subjects with IGR have been shown to have greater abnormalities in most cardiovascular reflex tests and greater heart rate variability characterized by the triangle index. In contrast, another study examined patients with newly diagnosed IGT with a battery of autonomic tests including heart rate variation variability, heart rate response to deep breathing, heart rate response to Valsalva maneuver, blood pressure response to standing up quickly, and skin sympathetic skin response (SSR) that evaluates postganglionic sympathetic sudomotor function but is less precise than the QSART. They found no difference compared to controls in measures of CAN. However, they did find lower amplitudes of the SSR in the IGT group compared to healthy controls that is consistent with the presence of a sudomotor autonomic neuropathy. The importance of abnormal sudomotor responses in subjects with IGR was also confirmed in other studies. This finding indicates that sudomotor fibers tend to be affected earlier in autonomic neuropathy in patients with IGR and that CAN may develop at a later stage or may require more sensitive tests to detect it than tests commonly used in the clinic.

In future studies the SAS could be adjusted to exclude the questions that individually were less reliable; however, excluding these questions would not affect the overall reliability of the test. A potential weakness of the study is the difference in the mean age of control men vs control women and individuals with neuropathy. This may be because it can be difficult to find age-matched male controls who have a normal neurologic examination, have no evidence of peripheral autonomic neuropathy, and are not on any medications that may even mildly affect autonomic functioning. However, despite the younger age of the control men, this had no affect on the validity of the study because there was no difference between age for the SAS symptom score or TIS in either control or neuropathy subjects. Future studies should address performance of the SAS in larger more diverse populations of subjects and in groups of subjects with other types of neuropathy. Despite these caveats, a validated questionnaire such as the SAS that is sensitive enough to detect mild autonomic neuropathy, is simple to complete, and performs consistently in subjects could potentially aid in the early detection and diagnosis of diabetic autonomic neuropathy.

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DISCLOSURE

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Triglycerides and Amputation Risk in Patients With Diabetes

Ten-year follow-up in the DISTANCE study

BRIAN C. CALLAGHAN, MD1
EVA FELDMAN, MD, PHD1
JENNIFER LIU, MPH1
KEVIN KERBER, MD1
RODICA POP-BUSUI, MD1
HOWARD MOFFET, MPH2
ANDREW J. KARTER, PHD2

OBJECTIVE—To determine the association between triglyceride levels and lower-extremity amputation (LEA) risk in a large diabetic cohort.

RESEARCH DESIGN AND METHODS—This is a 10-year survey follow-up study (from 1993–2006) of 28,701 diabetic patients with a baseline triglyceride measure. All patients were fully insured members of the Kaiser Permanente Medical Care Program and responded to a survey at baseline that included information on ethnicity, socioeconomic status, education, behavioral factors, and information required to determine type of diabetes. The relationship between triglycerides and time to incident nontraumatic LEA, defined by primary hospitalization discharge or procedures, was evaluated using Cox proportional hazards models.

RESULTS—Triglyceride level was an independent, stepwise risk factor for nontraumatic LEAs within this large diabetic cohort: triglycerides 150–199 mg/dL, hazard ratio (HR) 1.10 (95% CI 0.92–1.32); 200–499 mg/dL, 1.27 (1.10–1.47); >500 mg/dL, 1.65 (1.30–2.10) (reference <150 mg/dL).

CONCLUSIONS—Hypertriglyceridemia is a significant risk factor for LEA in diabetic patients even after controlling for known socioeconomic, health behavioral, and clinical factors. This previously unrecognized clinical risk needs to be further investigated to determine if treatment of triglycerides can reduce amputation risk.

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Diabetes is a common condition that damages the blood vessels of patients in a variety of ways. Patients with diabetes suffer from macrovascular complications such as myocardial infarction, stroke, and peripheral vascular disease. Moreover, they also develop microvascular disease that manifests as peripheral neuropathy (one of many mechanisms), retinopathy, and nephropathy. As a result of the combination of neuropathy and macroangiopathy in this population, patients with diabetes account for more than half of the nontraumatic lower extremity amputations (LEA) that occur worldwide (1,2). Tight glycemic control has been shown to decrease the number of patients that develop microvascular complications (3,4); however, a significant proportion of diabetic patients continue to develop these complications even with intensive glycemic control (5). Additionally, in the Steno-2 study, patients treated with a multifactorial intervention including aspirin, statins, renin angiotensin blockers, glycemic control, and lifestyle modifications still developed diabetic complications at a high rate (6). These surprising observations are leading investigators to reconsider other potentially modifiable risk factors.

Hypertriglyceridemia has received little attention of late and is typically not considered a primary clinical target. Elevated triglycerides in concert with low HDL is associated with the metabolic syndrome and increased cardiovascular risk in healthy populations (5). However, the relationship between triglycerides and noncardiovascular complications has received less attention. In the EURODIAB cohort study, patients with type 1 diabetes and elevated triglycerides were at an increased risk for the development of peripheral neuropathy in bivariate, but not multivariate analyses (7). Furthermore, elevated triglycerides were recently found to correlate independently with loss of sural nerve myelin fiber density, a marker for progression of diabetic neuropathy (8).

On the other hand, there have only been a few studies that have directly looked into the role of hypertriglyceridemia as a potential risk factor for LEA. In 1996, Humphries et al. (9) followed a cohort of 1,564 Nauruans with diabetes over 12 years to define potential risk factors for nontraumatic LEA. They found that fasting glucose levels, duration of diabetes, and male sex were independent risk factors. Triglyceride levels were higher in patients that sustained amputations, but the result was not statistically significant. In 2006, Davis et al. (10) conducted an observational cohort study, the Fremont Diabetes Study, that followed 1,294 patients with type 2 diabetes. They found that foot ulceration, a low arterial brachial index, elevated hemoglobin A1C level, and neuropathy were all independent predictors of LEA. Again, triglycerides were elevated in those who sustained amputations, but the association was not significant. Previous studies likely lacked sufficient power to detect a significant association of triglyceride level and risk of LEA. Therefore, we decided to investigate the hypothesis that there is a positive correlation between triglyceride and LEA risk in a larger population followed over a longer time frame.

RESEARCH DESIGN AND METHODS—Kaiser Permanente Medical Care Program (“Kaiser Permanente”) is a fully integrated, nonprofit, group practice health plan that provides...
Triglyceride and amputation risk

comprehensive medical services to over ~2.5 million members (January 1995) throughout Northern California (including the San Francisco Bay and Sacramento metropolitan areas) or ~25–30% of the surrounding population. The Kaiser Permanente membership closely approximates the general population ethnically and socioeconomically except for the extreme tails of the income distribution (11).

In 1993, Kaiser Permanente established the Kaiser Permanente Northern California Diabetes Registry. The registry is updated annually by identifying all health plan members with diabetes from automated databases for pharmacy, laboratory, hospitalization records, and outpatient diagnoses (12–14). The registry sensitivity is estimated to be 96% based on chart review (15). During 1994–1996, all noninstitutionalized registry members 19 years or older identified as having diabetes prior to 1 January 1996 (n = 90,302) were selected for participation in a health survey (self-administered questionnaire) or a computer-assisted telephone interview in English or Spanish. Eighty-three percent of eligible members participated in the survey. After removing those denying having diabetes or discontinuing membership, 70,748 respondents with diabetes remained. The survey collected information on, among other things, ethnicity, information needed to classify diabetes type (see Karter et al. for algorithm (16)), family history of diabetes, education, and behavioral risk factors. Survey respondents were ethnically diverse: 14% black, 12% Asian, 10% Latino, and 64% non-Latino White. Of those self-identifying as “Asian,” 44% were Filipino, 24% were Chinese, 12% were Japanese, and 19% were Korean, other Asian, or mixed race.

We compared the demographic composition (age, sex, socioeconomic status) of diabetes survey respondents to nonresponders in a previous study (14) and found no evidence to suggest responder bias. In addition to survey-derived data, we obtained measures of neighborhood-level socioeconomic status by linking each member’s address to the 1990 census block group-level average annual 1989 per capita income.

We used a survey follow-up study design to evaluate the incidence density of LEA and its predictors, restricting analysis to the 28,701 survey respondents who had triglyceride data within 1 year of baseline. Initial triglyceride levels were measured after overnight fasting. Baseline was defined as 1 January 1995 for all members surveyed in 1994 and the survey date (January 1995 to March 1997) for those surveyed after that date. The duration of follow-up (person-time) was tabulated through membership records and ended with an event censoring due to dropping of Kaiser Permanente membership for any period greater than 2 months, death, or the end of the study (31 December 2006). We excluded those with a history of prior LEA events noted in hospital discharge records during the 5 years prior to baseline. Prognostic, confounding, and stratifying variables (see below) were ascertained at or prior to baseline from automated records, the diabetes survey, and the 1990 census.

Study end points

Nontraumatic LEA procedures were identified from discharge codes (ICD-9-CM procedure codes 84.10–84.17). We included patients with any amputation of the lower extremity. In two separate validations of amputation procedures (A. Karter, personal communication, n = 109; and J.V. Selby and D. Zhang [17], n = 209), 99% of electronic hospital discharge records were confirmed by chart review.

Statistical analysis

Descriptive and multivariate analysis. Using direct standardization with the entire diabetes cohort as the population standard, we calculated age-adjusted sex- and ethnicity-specific rates for LEA. Proportional hazards regression (Cox) models were then used to calculate adjusted hazard ratios (HRs) as an estimate of the relative risk of elevated triglycerides (<150, 150–199, 200–499, and >500 mg/dL) associated with LEA. After detecting no violations of the proportionality assumption, we specified a series of Cox regression models, including a base demographic model (age-, race-, and sex-adjusted plus an indicator for elevated triglycerides, LDL, and HDL) and specified saturated models that added socioeconomic variables (race, individual-level education, average census block level income, and proportion in working class occupations), health behaviors (smoking status, alcohol intake, self-reported treatments for diabetes including diet and exercise, and frequency of self-monitoring of blood glucose), and clinical factors including type of diabetes, diabetes duration, type of diabetic medications, first-degree family history of diabetes, BMI (underweight, normal, overweight, obese) (18), height quartiles (19,20), hypertension (self-reported and pharmacy records of antihypertensive medication dispensing), peripheral neuropathy (self-report) (17,21), Alc (<7, 7–8, 8–10, >10 g/dL), LDL (<100, 100–129, 130–159, >160 mg/dL), HDL (<40, 40–59, >60 mg/dL), and use of statin or other lipid-lowering agents.

RESULTS—Patients who sustained an LEA were older (61 years [SD 11.2] vs. 59 years [11.2]), had a longer duration of diabetes (14 years [9.7] vs. 9 years [9.5]), and had a higher hemoglobin A1C (9.05 g/dL [2.12] vs. 8.36 g/dL [1.94]) compared with those who did not sustain an LEA (Table 1). The age- and sex-adjusted nontraumatic LEA incidence rate (95% CI) was 2.3 (1.5–3.1) per 1,000 person-years (Table 2). The sex-specific, age-adjusted rates (95% CI) were 1.4 (1.2–1.7) per 1,000 person-years for women and 3.2 (1.6–4.8) for men. The age- and sex-adjusted incidence rates (95% CI) for white, black, Hispanic, and Asians were 2.7 (1.5–4.0), 2.0 (1.6–2.5), 1.4 (1.1–1.8), and 0.9 (0.5–1.3), respectively.

Crude HRs for triglyceride, LDL, and HDL levels revealed a significant, stepwise relationship with triglycerides: 150–199 mg/dL, HR 1.10 (95% CI 0.92–1.32); 200–499 mg/dL, 1.27 (1.10–1.47); and >500 mg/dL, 1.65 (1.30–2.10) (reference <150 mg/dL). There was no significant association for LDL or HDL with amputation risk. Minimally adjusted Cox proportional hazards models including triglyceride, LDL, and HDL levels continued to demonstrate a stepwise association between triglycerides and LEA risk: 150–199 mg/dL, HR 1.07 (95% CI 0.89–1.29); 200–499 mg/dL, 1.20 (1.03–1.40); and >500 mg/dL, 1.39 (1.03–1.88) (reference <150 mg/dL). LDL was again not significantly associated with amputation risk, whereas HDL showed a protective effect between 40–59 mg/dL (HR 0.74 [95% CI 0.64–0.87]) but not at >60 mg/dL (1.21 [0.92–1.60]) (reference HDL <40 mg/dL). A further-adjusted Cox proportional hazards model (model 1 in Table 3) including triglycerides, LDL, HDL, age, sex, and race also revealed a stepwise association of triglycerides and LEA risk.

The stepwise association between triglycerides and LEA persisted despite further adjustment for socioeconomic (individual-level education, average census block level
Table 1—Baseline characteristics for 28,701 members with diabetes experiencing incident nontraumatic LEAs compared with those without incident LEA

<table>
<thead>
<tr>
<th></th>
<th>Incident LEA</th>
<th>No LEA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>981</td>
<td>27,720</td>
<td></td>
</tr>
<tr>
<td>Mean age (years) (SD)</td>
<td>60.8 (10.2)</td>
<td>59.4 (11.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Women (%)</td>
<td>35.0</td>
<td>46.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>62.6</td>
<td>58.8</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>14.1</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>5.6</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>10.1</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>Mixed/other</td>
<td>7.7</td>
<td>7.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lives in deprived neighborhood (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&gt;66% in census block group in working class occupation)</td>
<td>47.3</td>
<td>43.5</td>
<td>0.0847</td>
</tr>
<tr>
<td>Lives in deprived neighborhood (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&gt;20% of neighborhood with annual income below poverty level)</td>
<td>12.3</td>
<td>9.7</td>
<td>0.0027</td>
</tr>
<tr>
<td>Diabetes type (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>5.7</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Type 2</td>
<td>94.4</td>
<td>96.5</td>
<td>0.0009</td>
</tr>
<tr>
<td>Diabetes therapy (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin only</td>
<td>41.8</td>
<td>24.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OHA only</td>
<td>44.5</td>
<td>53.0</td>
<td></td>
</tr>
<tr>
<td>Combination</td>
<td>8.7</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Diet/exercise</td>
<td>4.1</td>
<td>15.2</td>
<td></td>
</tr>
<tr>
<td>No therapy</td>
<td>0.8</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Reported exercise as part of treatment (%)</td>
<td>45.2</td>
<td>54.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Daily self-monitoring of blood glucose (%)</td>
<td>47.3</td>
<td>48.2</td>
<td>0.5956</td>
</tr>
<tr>
<td>Mean A1C (g/dL) (SD)</td>
<td>9.05 (2.13)</td>
<td>8.36 (1.94)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A1C (g/dL) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;7</td>
<td>14.6</td>
<td>26.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>7–8</td>
<td>22.1</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>8–10</td>
<td>33.2</td>
<td>29.9</td>
<td></td>
</tr>
<tr>
<td>&gt;10</td>
<td>30.1</td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td>First-degree family history of diabetes (%)</td>
<td>57.5</td>
<td>57.1</td>
<td>0.8179</td>
</tr>
<tr>
<td>Duration of diabetes (&lt;10 years) (%)</td>
<td>38.9</td>
<td>65.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Uses statin treatment (%)</td>
<td>20.9</td>
<td>16.8</td>
<td>0.0007</td>
</tr>
<tr>
<td>Uses fibrates/niacin treatment (%)</td>
<td>9.7</td>
<td>9.3</td>
<td>0.6929</td>
</tr>
<tr>
<td>Uses antihypertensive treatment (%)</td>
<td>73.2</td>
<td>60.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Self-reported hypertension (%)</td>
<td>74.0</td>
<td>64.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Self-reported peripheral neuropathy (%)</td>
<td>58.1</td>
<td>29.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL (mg/dL) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100</td>
<td>12.2</td>
<td>12.6</td>
<td>0.0650</td>
</tr>
<tr>
<td>100–129</td>
<td>27.3</td>
<td>27.2</td>
<td></td>
</tr>
<tr>
<td>130–159</td>
<td>28.5</td>
<td>31.9</td>
<td></td>
</tr>
<tr>
<td>&gt;160</td>
<td>32.1</td>
<td>28.3</td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dL) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>67.9</td>
<td>62.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>40–59</td>
<td>25.8</td>
<td>32.7</td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>6.3</td>
<td>5.3</td>
<td></td>
</tr>
</tbody>
</table>

income, and proportion in working class occupations), health behavioral (smoking, BMI, drinking, adherence to guidelines for self-monitoring of blood glucose, and exercise), and clinical variables (statin medication, fibrates/niacin medication, family history of diabetes, height, duration of diabetes, A1C, type of diabetes and therapy, hypertension, neuropathy, retinopathy, stroke, heart attack, and end-stage renal disease [ESRD]) (model 2 in Table 3). As expected, duration of diabetes, A1C, height, hypertension, neuropathy, retinopathy, ESRD, stroke, and heart attack were also associated with amputation. Interestingly, statin medication use and fibrates/niacin medication use did not have statistically significant HRs. LDL >160 mg/dL and HDL >60 mg/dL were the only levels of these tests associated with an increased risk of amputation; it appeared to be a threshold effect for LDL, but there was no consistent or stepwise relationship for HDL.

Additionally, the rates of elevated triglycerides did differ substantially by race (21, 40, 46, and 46% among African Americans, Asians, Whites, and Hispanics, respectively; P < 0.001). However, we failed to detect a significant interaction between triglyceride level and race (P = 0.83), suggesting that the relationship between triglycerides and amputation risk did not differ by race. Furthermore, similar results were seen with triglyceride level and amputation risk using the entire diabetes cohort and utilizing a missing indicator for those who did not have triglyceride levels at baseline. We also found that, as expected, there was a significant inverse correlation between triglyceride and A1C levels (correlation = −0.24, P < 0.0001). However, this modest collinearity was not considered sufficient to distort our triglyceride effect estimates from the Cox regression models.

**CONCLUSIONS**—In this large, multiethnic cohort of diabetic patients, we found that elevated triglyceride levels are associated with LEA even after adjusting for a host of potential confounders. The consistency of the relationship between triglycerides and LEA across ethnic groups was further supportive. All models revealed a stepwise increase in amputation risk with increasing triglyceride levels. This is in contrast to low HDL, which was not associated with increased amputation risk. The threshold of LDL levels above 160 mg/dL was also associated with increased risk of amputation.
**Triglyceride and amputation risk**

Table 1—Continued

<table>
<thead>
<tr>
<th>Triglycerides (mg/dL) (%)</th>
<th>Incident LEA</th>
<th>No LEA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;150</td>
<td>32.7</td>
<td>37.8</td>
<td>0.0003</td>
</tr>
<tr>
<td>150–199</td>
<td>18.9</td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td>200–499</td>
<td>39.9</td>
<td>36.2</td>
<td></td>
</tr>
<tr>
<td>&gt;500</td>
<td>8.6</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>Mean BMI (kg/m²) (SD)</td>
<td>29.62 (5.67)</td>
<td>29.99 (6.22)</td>
<td>0.0600</td>
</tr>
<tr>
<td>Current drinker (%)</td>
<td>25.7</td>
<td>28.2</td>
<td>0.0028</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>12.6</td>
<td>11.2</td>
<td>0.0115</td>
</tr>
<tr>
<td>Obesity status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underweight (BMI &lt;18.5 kg/m²)</td>
<td>0.4</td>
<td>0.5</td>
<td>0.1143</td>
</tr>
<tr>
<td>Normal (BMI 18.5–24.9 kg/m²)</td>
<td>18.3</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>Overweight (BMI 25.0–29.9 kg/m²)</td>
<td>38.2</td>
<td>35.4</td>
<td></td>
</tr>
<tr>
<td>Obese (BMI &gt;30.0 kg/m²)</td>
<td>43.1</td>
<td>47.1</td>
<td></td>
</tr>
<tr>
<td>Height (in quartiles)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1</td>
<td>13.0</td>
<td>23.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21.8</td>
<td>24.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30.7</td>
<td>31.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>34.4</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
<td>Patients with ESRD (%)</td>
<td>4.6</td>
<td>0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Patients with history of heart attack (%)</td>
<td>17.0</td>
<td>10.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Patients with history of stroke (%)</td>
<td>8.0</td>
<td>3.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Patients with retinopathy (%)</td>
<td>1.9</td>
<td>0.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

OHA, oral hypoglycemic agent.

Dyslipidemia has been proposed as a potential risk factor in the development of diabetes complications such as retinopathy and neuropathy based on results utilizing the DCCT/EDIC cohort and the EURODIAB study (7,22). Our results suggest that high triglyceride levels, independent of the other major lipid components, may put patients at risk for one major diabetes complication—LEA. Whether this association applies more widely to other vascular diabetes complications is unknown.

These results add to the finding in prior studies that elevated triglyceride levels may be a risk factor for complications of diabetes (5,7,8), including LEA (9,10). However, the current study involved a larger population and found the association to be statistically significant even after adjusting the models for a multitude of variables that were not assessed in the prior studies.

Given the current state of the literature, the guidelines on triglyceride management do not advocate aggressive treatment. The National Cholesterol Education Program recommends diet and exercise for patients with triglyceride levels between 150–199 mg/dL, to consider treatment in high-risk patients with levels 200–499 mg/dL, and to use pharmacologic treatment when levels are 500 mg/dL or greater to prevent pancreatitis, but not necessarily to prevent microvascular and macrovascular complications. 

**Table 2—Incidence rates per 1,000 person-years (95% CIs) for LEA procedures**

<table>
<thead>
<tr>
<th>LEA</th>
<th>Total person-years follow-up</th>
<th>Number of incident events</th>
<th>Age- and sex-adjusted incidence rate per 1,000 person-years (95% CI)</th>
<th>Age-adjusted incidence rate per 100 person-years (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire cohort</td>
<td>218,027</td>
<td>981</td>
<td>2.3 (1.5–3.1)</td>
<td>1.4 (1.2–1.7)</td>
</tr>
<tr>
<td>White</td>
<td>127,272</td>
<td>614</td>
<td>2.7 (1.5–4.0)</td>
<td>3.2 (1.6–4.8)</td>
</tr>
<tr>
<td>African American</td>
<td>24,190</td>
<td>138</td>
<td>2.0 (1.6–2.5)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>23,305</td>
<td>99</td>
<td>1.4 (1.1–1.8)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>27,795</td>
<td>55</td>
<td>0.9 (0.5–1.3)</td>
<td></td>
</tr>
<tr>
<td>Mixed/other</td>
<td>15,465</td>
<td>75</td>
<td>2.6 (1.3–4.1)</td>
<td></td>
</tr>
</tbody>
</table>

(23). Clearly, further studies are needed to ascertain the role of hypertriglyceridemia in these diabetic sequelae. Our study gives further support to the notion that triglycerides may be one of the key modifiable risk factors in the development of amputations. If this association holds in clinical trials, then clinicians may have a target with the potential to improve important patient outcomes.

One of the main limitations of this study is that a large proportion of our population did not have data on triglyceride, HDL, and LDL levels during the year prior to baseline; at the time, these assessments were not done nearly as frequently or routinely as is common today. However, our sensitivity analysis using missing triglycerides as an indicator and evaluating the entire diabetes cohort suggests that missing data did not substantially bias our findings. Also, observational cohort studies, particularly cross-sectional designs, can make erroneous assumptions between cause and effect when suggestive variables correlate. In our study, the longitudinal study design and exclusion of patients with prevalent amputations at baseline precludes time-ordering violations (i.e., LEA preceding the triglyceride ascertainment). Another limitation is that observational studies are vulnerable to residual confounding; however, we were able to adjust for a wide array of confounders, which should greatly reduce this concern.

The major strengths of this study are its longitudinal cohort design, validated and complete capture of LEA events, and large sample size with rich array of clinical and behavioral data. Additionally, our diabetes registry captures patients who are not treated with diabetes medications (medical nutritional therapy) through laboratory findings and outpatient diagnosis records, providing a more representative diabetic sample than pharmacy-based registries that only capture patients receiving prescriptions or studies from diabetes specialty clinics that typically include patients with more severe disease.

In summary, elevated triglyceride level was associated with subsequent LEA independently of other lipid components and a wide range of potential confounders in this large, well-characterized diabetic cohort. Though specific guidelines exist for cholesterol (LDL and HDL levels) management in this patient population, only vague guidelines exist for triglyceride management (23). This observational study suggests that triglyceride levels are predictive of amputation risk
Table 3—Cox proportional hazards models with HRs (95% CIs) for nontraumatic LEA

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th></th>
<th>Model 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR 95% CI</td>
<td></td>
<td>HR 95% CI</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(reference: &lt;150)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150–199</td>
<td>1.16</td>
<td>0.97–1.40</td>
<td>1.29</td>
<td>1.07–1.55</td>
</tr>
<tr>
<td>200–499</td>
<td>1.35</td>
<td>1.15–1.58</td>
<td>1.40</td>
<td>1.19–1.65</td>
</tr>
<tr>
<td>&gt;500</td>
<td>1.38</td>
<td>1.17–2.14</td>
<td>1.65</td>
<td>1.22–2.24</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(reference: &lt;100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100–129</td>
<td>1.05</td>
<td>0.83–1.32</td>
<td>1.10</td>
<td>0.86–1.39</td>
</tr>
<tr>
<td>130–159</td>
<td>0.94</td>
<td>0.74–1.19</td>
<td>1.01</td>
<td>0.80–1.80</td>
</tr>
<tr>
<td>&gt;160</td>
<td>1.26</td>
<td>1.00–1.59</td>
<td>1.30</td>
<td>1.03–1.64</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(reference: &lt;40)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40–59</td>
<td>0.87</td>
<td>0.74–1.02</td>
<td>0.88</td>
<td>0.75–1.03</td>
</tr>
<tr>
<td>&gt;60</td>
<td>1.53</td>
<td>1.15–2.03</td>
<td>1.37</td>
<td>1.02–1.84</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(reference: 19–34)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>35–49</td>
<td>1.18</td>
<td>0.62–2.25</td>
<td>1.15</td>
<td>0.60–2.22</td>
</tr>
<tr>
<td>50–64</td>
<td>1.48</td>
<td>0.79–2.77</td>
<td>1.31</td>
<td>0.68–2.52</td>
</tr>
<tr>
<td>65–79</td>
<td>1.80</td>
<td>0.96–3.39</td>
<td>1.58</td>
<td>0.82–3.05</td>
</tr>
<tr>
<td>&gt;80</td>
<td>0.91</td>
<td>0.35–2.40</td>
<td>0.85</td>
<td>0.32–2.30</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.71</td>
<td>1.49–1.97</td>
<td>1.59</td>
<td>1.33–1.90</td>
</tr>
<tr>
<td>Race (reference: white)</td>
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</tr>
<tr>
<td>African American</td>
<td>1.36</td>
<td>1.12–1.65</td>
<td>1.04</td>
<td>0.85–1.28</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0.92</td>
<td>0.75–1.14</td>
<td>0.82</td>
<td>0.66–1.03</td>
</tr>
<tr>
<td>Asian</td>
<td>0.44</td>
<td>0.33–0.58</td>
<td>0.51</td>
<td>0.39–0.69</td>
</tr>
<tr>
<td>Mixed/Other</td>
<td>1.08</td>
<td>0.85–1.38</td>
<td>0.91</td>
<td>0.71–1.16</td>
</tr>
<tr>
<td>Duration of diabetes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(years) (reference: &lt;10 years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–19</td>
<td>1.94</td>
<td>1.65–2.28</td>
<td>2.38</td>
<td>1.96–2.88</td>
</tr>
<tr>
<td>&gt;20</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A1C (g/dL)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(reference: &lt;7)</td>
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<td></td>
</tr>
<tr>
<td>7–8</td>
<td>1.45</td>
<td>1.16–1.81</td>
<td>1.51</td>
<td>1.22–1.86</td>
</tr>
<tr>
<td>8–10</td>
<td>1.51</td>
<td>1.22–1.86</td>
<td>2.18</td>
<td>1.73–2.71</td>
</tr>
<tr>
<td>&gt;10</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Diabetes type and therapy (reference: diet only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>1.78</td>
<td>1.19–2.65</td>
<td>1.88–3.10</td>
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</tr>
<tr>
<td>Type 2 on insulin</td>
<td>2.41</td>
<td>1.28–2.05</td>
<td>1.88–3.10</td>
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<tr>
<td>Type 2 on OHA</td>
<td>1.62</td>
<td>1.28–2.05</td>
<td>1.28–2.05</td>
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</tr>
<tr>
<td>Statin medication</td>
<td>1.02</td>
<td>0.87–1.20</td>
<td>0.85</td>
<td>0.68–1.05</td>
</tr>
<tr>
<td>Fibrate/niacin medication</td>
<td>0.85</td>
<td>0.68–1.05</td>
<td>0.68–1.05</td>
<td></td>
</tr>
<tr>
<td>Smoking (reference: never)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Current</td>
<td>1.24</td>
<td>0.90–1.55</td>
<td>0.98</td>
<td>0.84–1.15</td>
</tr>
<tr>
<td>Former</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (reference: normal weight)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underweight</td>
<td>0.67</td>
<td>0.21–2.11</td>
<td>0.86</td>
<td>0.70–1.05</td>
</tr>
<tr>
<td>Overweight</td>
<td>0.86</td>
<td>0.65–0.98</td>
<td>0.65–0.98</td>
<td>0.65–0.98</td>
</tr>
<tr>
<td>Obese</td>
<td>0.80</td>
<td>0.65–0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (reference: 1st quartile)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd quartile</td>
<td>1.43</td>
<td>1.09–1.86</td>
<td>1.34</td>
<td>1.01–1.77</td>
</tr>
<tr>
<td>3rd quartile</td>
<td>1.34</td>
<td>1.01–1.77</td>
<td>1.98</td>
<td>1.48–2.66</td>
</tr>
<tr>
<td>4th quartile</td>
<td>1.51</td>
<td>1.27–1.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>2.60</td>
<td>2.23–3.04</td>
<td>1.85</td>
<td>1.15–2.98</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>1.85</td>
<td>1.15–2.98</td>
<td>1.27</td>
<td>1.06–2.52</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>1.27</td>
<td>1.06–2.52</td>
<td>1.97</td>
<td>1.55–2.50</td>
</tr>
<tr>
<td>Heart attack</td>
<td>1.97</td>
<td>1.55–2.50</td>
<td>4.29</td>
<td>3.00–6.03</td>
</tr>
<tr>
<td>Stroke</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESRD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Model 1: Triglycerides, LDL, HDL, age, sex, and race. Model 2: Model 1 + education, income, whether lives in working class neighborhood, smoking, alcohol use, BMI, height, adherence to guidelines for self-monitoring of blood glucose, exercise, statin medication, fibrate/niacin medication, family history of diabetes, duration of diabetes, A1C, type of diabetes and therapy, history of hypertension, neuropathy, retinopathy, ESRD, stroke, and heart attack. OHA, oral hypoglycemic agent.
Triglyceride and amputation risk

in a stepwise fashion. Despite standards of glycemic, blood pressure, and cholesterol control, patients continue to develop microvascular and macrovascular complications (combination of neuropathy and macroangiopathy is thought to cause LEAs), and triglyceride control may be an important additional primary prevention effort. More research is necessary to define a causal role of triglyceride levels on amputation risk in diabetic patients. Based on this current robust cohort study, such research should be a priority.

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

B.C.C. was involved in interpretation of the statistical analysis and wrote the manuscript. E.F. proposed the hypothesis and helped with the manuscript. J.L. performed the statistical analysis. A.J.K. proposed the hypothesis, helped with interpretation and contributed to the manuscript. A.J.K. was integrally involved in the creation of the cohort and was significantly involved in analysis and writing the manuscript.

Parts of this study were presented in abstract form at the American Neurologic Association meeting, San Francisco, California, 12–15 September 2010.

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