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Injury-Induced gp130 Cytokine Signaling in Peripheral Ganglia is Reduced in Diabetes Mellitus

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Abstract

Neuropathy is a major diabetic complication. While the mechanism of this neuropathy is not well-understood, it is believed to result in part from deficient nerve regeneration. Work from our laboratory established that gp130 family of cytokines are induced in animals after axonal injury and are involved in the induction of regeneration-associated genes (RAGs) and in the conditioning lesion response. Here, we examine whether a reduction of cytokine signaling occurs in diabetes. Streptozotocin (STZ) was used to destroy pancreatic β cells, leading to chronic hyperglycemia. Mice were injected with either low doses of STZ (5×60 mg/kg) or a single high dose (1×200 mg/kg) and examined after three or one month, respectively. Both low and high dose STZ treatment resulted in sustained hyperglycemia and functional deficits associated with the presence of both sensory and autonomic neuropathy. Diabetic mice displayed significantly reduced intraepidermal nerve fiber density and sudomotor function. Furthermore, low and high dose diabetic mice showed significantly reduced tactile touch sensation measured with Von Frey monofilaments. To look at the regenerative and injury-induced responses in diabetic mice, neurons in both superior cervical ganglia (SCG) and the 4th and 5th lumbar dorsal root ganglia (DRG) were unilaterally axotomized. Both high and low dose diabetic mice displayed significantly less axonal regeneration in the sciatic nerve, when measure *in vivo*, 48 h after crush injury. Significantly reduced induction of two gp130 cytokines, leukemia inhibitory factor and interleukin-6, occurred in diabetic animals in both ganglia 6 h after injury. These effects were accompanied by reduced phosphorylation of signal transducer and activator of transcription 3 (STAT3), a downstream effector of the gp130 signaling pathway. We also found decreased induction of several gp130-dependent RAGs, including galanin and vasoactive intestinal peptide. Together, these data suggest

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a novel mechanism for the decreased response of diabetic sympathetic and sensory neurons to injury.

Keywords

Diabetes; Neuropathy; Axon Regeneration; Axotomy; Peripheral Nervous System

Introduction

Neuropathy is the most significant clinical complication associated with diabetes. Dysfunction of sensorimotor or autonomic nerves plays a significant role in the morbidity and mortality of patients with type I and type II diabetes (Pasnoor et al., 2013; Said, 2007). Sensory and autonomic neuropathy can often coexist and lead to symptoms such as cardiovascular irregularities, loss of sensation, pain, and in extreme cases limb amputation (Boulton et al., 2005; Tesfaye et al., 2010; Verrotti et al., 2014). While the development and persistence of diabetic polyneuropathies have been investigated, the cause has yet to be determined.

Neurons in the peripheral nervous system (PNS) have the unique ability of regenerating their axons following injury (DeFrancesco-Lisowitz et al., 2014; Fawcett and Keynes, 1990; Zochodne, 2008). However, regeneration of PNS axons following injury is significantly impaired in diabetic patients and rodent models of diabetes (Bradley et al., 1995; Christie and Zochodne, 2013; Duran-Jimenez et al., 2009; Kennedy and Zochodne, 2000, 2005a; Longo et al., 1986; Zochodne et al., 2007). Much research has focused on deficits in trophic support as a cause for this regenerative failure; yet, the mechanisms responsible for the decreased regenerative capabilities found in diabetes are unknown (Chattopadhyay et al., 2005; Wu et al., 2011). The regenerative deficit found in diabetes has been posited to underlie the development of neuropathy (Ebenezer et al., 2011; Eckersley et al., 2001; Simmons and Feldman, 2002; Vinik et al., 2003).

Peripheral nerve regeneration is supported by both intrinsic and extrinsic mechanisms, which include broad neuronal gene expression changes, macrophage accumulation at the site of injury and near injured neuronal cell bodies, and the de-differentiation of Schwann cells to support the regrowth of axons (Barrette et al., 2008; Cattin and Lloyd, 2016; Niemi et al., 2013; Tedeschi and Bradke, 2017). Injury to peripheral nerves induces a complex and coordinated change in gene expression, a response that is not seen following injury to the central nervous system (CNS) and thus is thought to be one of the more important aspects of PNS axon regeneration (Boeshore et al., 2004; Chandran et al., 2016; Costigan et al., 2002; Ma and Willis, 2015). One such group of genes that has been identified to play a significant role in nerve regeneration after injury are the gp130 cytokines (Zigmond, 2011). The gp130 family of cytokines, also known as neuropoietic cytokines, includes, but is not limited to, leukemia inhibitory factor (LIF), interleukin (IL)-6, and ciliary neurotrophic factor (CNTF) (Cheon et al., 2011; Fischer and Hilfiker-Kleiner, 2008; Gadiant and Patterson, 1999; Hirano et al., 1997). The gp130 cytokines, *Lif* and *Il6*, are increased in peripheral ganglia following nerve injury and play a critical role in the conditioning lesion response of both sensory and

sympathetic neurons (Banner and Patterson, 1994; Cafferty et al., 2004; Gardiner et al., 2002; Habecker et al., 2009; Hyatt Sachs et al., 2010; Sun et al., 1994; Sun and Zigmond, 1996b).

Here, we show evidence that the injury induced gene and protein expression of IL-6 and LIF are significantly impaired in sensory and sympathetic ganglia in two different mouse models of type-I diabetes. Furthermore, downstream effectors of gp130 cytokine signaling, STAT3 and expression of various neuropeptides are also significantly reduced following peripheral nerve injury in diabetic mice compared to wild type controls. This defective gp130 signaling cascade could underlie the regenerative deficit and onset of autonomic and sensory neuropathy in diabetes.

Materials and Methods

Administration of Streptozotocin

All procedures were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. Eight- to 12 week-old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) were housed under a 12 h light/dark cycle with *ad libitum* access to food and water. One week after arrival, mice were fasted for 6 h. Mice were rendered diabetic by either a low dose, 60 mg/kg, *i.p.* STZ (MP Biomedicals Inc., Solon, OH, USA) injection given on 5 consecutive days or a single high dose, 200 mg/kg, *i.p.* injection. STZ was dissolved in sodium citrate buffer (pH 4.5) right before injections. Control mice were injected *i.p.* with sodium citrate buffer. Body weights and fasted blood glucose levels were monitored weekly from the tail vein. Mice with blood glucose levels >275 mg/dl within 1 week for high dose and 3 weeks for low dose following injection were considered diabetic.

Animal Surgeries

Mice underwent both unilateral SCG axotomy and unilateral sciatic nerve transection either 5 weeks (high dose groups) or 15 weeks (low dose groups) after diabetes onset (Fig. 1a-b). For SCG axotomy, the right SCG was exposed and the internal and external carotid nerves were cut where they exit the ganglion. The sciatic nerve was transected at mid-thigh level and a 1–2 mm piece of the distal nerve segment was removed. For *in vivo* regeneration, the right sciatic nerve was crushed with ultra-fine hemostats (Fine Science Tools, Foster City, CA, USA), effectively axotomizing a population of neurons of the L4 and L5 DRG (Rigaud et al., 2008). The contralateral SCG and sciatic nerves were exposed but not injured. The contralateral SCG and L4 and L5 DRG, and sciatic nerve were used as internal controls. At 6, 24, or 48 h after injury, the mice were sacrificed by CO₂ inhalation and both SCG, L4 and L5 DRG, sciatic nerves, and hind footpads were removed.

Measurement of Hemoglobin A1c Levels

Mouse glycohemoglobin A1c was measured using the Bio-Rad Total Glycated Hemoglobin Assay (Bio-Rad, Hercules, CA, USA). The measurement required that 10 µl of blood be harvested from the tail vein before the mouse was sacrificed. The assay was carried out following the manufacturer's guidelines. Glycohemoglobin A1c in whole blood specimens

was measured in a spectrophotometer at 531nm. The data were then represented as a percentage of total hemoglobin that was glycated.

Body Temperature Regulation Assay

To monitor the ability of the diabetic mice to regulate internal body temperature, mice were placed in a cold room (4°C) with each mouse in an individual plastic housing unit. Using a rectal thermometer, the mouse's body temperature was recorded. Temperatures were taken every 30 min for a total of 3 h. These measurements were taken one week prior to sacrificing the animals.

Sweat Assay

To measure sweating, a mold was made of the plantar surface of the hind paw with a silicone impression system using Silasoft N (Detax, Ettlingen, Germany; Vilches and Navarro, 2002). Diabetic and control mice were anesthetized with isoflurane and injected with 5 mg/kg pilocarpine nitrate (Sigma-Aldrich, St. Louis, MO, USA) subcutaneously to induce sweating. Approximately 0.2 ml of the silicone base and three to five drops of a liquid hardening catalyst, Silaplast (Detax), were mixed together and a thin layer was applied to the hind paw 10 min after pilocarpine injection. Since this material is immiscible with water, sweat droplets form impressions in the mold as it hardens. Each impression represents the activity of an individual sweat gland (Kennedy and Sakuta, 1984). The hardened mold was removed after 5 min. The molds were imaged and individual sweat droplets were counted under a dissecting microscope. Impressions in the mold were counted within 5 foot pads on each hind paw. Each hind paw was analyzed separately and five separate mice were used for each condition (control and diabetic).

Von Frey Test

To assess mechanical sensitivity, calibrated Von Frey monofilaments (Stoetling Co., Wood Dale, IL, USA) were used to measure the paw withdrawal threshold and frequency. Mice were allowed to acclimate for 3 min in an inverted Plexiglas chamber (10 cm × 7 cm × 8 cm) with a wire mesh grid bottom (1 cm spacing), allowing access to the plantar surface of the hind paws. When all four paws were touching the wire mesh, filaments (ranging from 0.16–8.0 g) were applied to the plantar surface of each hind paw for 1–2 s with an inter-stimulus interval of at least 10 s, being careful to avoid the toes, heel, and pads. Sudden paw withdrawal, sudden flinching, or sudden paw licking were considered a withdrawal response. The paw withdrawal threshold was calculated using the up-down method with 20 trials. Beginning in the middle of the filament series (1.4 g), ascending (if no response was observed a stronger stimulus was applied) or descending stimuli (if a response was observed). The resulting pattern of positive and negative responses was calculated using the 50% response threshold formulated from the mean of tabular values for the pattern of positive and negative responses. The frequency of withdrawal was determined using a standard monofilament (1.0 g; 4.08 mN) applied 10 times to the plantar hind paw. This data was then expressed as a response ratio to a 1.0 g stimulus. Eleven to twelve mice per condition were used to conduct this analysis.

Hot Plate Thermal Nociception Assay

The hot plate test was used to assess the response to thermal stimuli (Columbus Instruments, Columbus, OH, USA). The mouse was placed on a 53.5°C hot plate, and the latency to achieve a response in the hind paw (including licking, lifting, or shaking the paw, as well as attempts to jump off the surface) was measured. The mouse was removed immediately after a response was observed or if no response occurred within 30 s. A total of three trials were conducted per mouse with a 20 min inter-trial interval. The threshold was calculated as the mean latencies for the three trials. Eleven to twelve mice per condition were used to conduct this analysis.

In Vivo Regeneration

Two days after a unilateral sciatic nerve crush, animals were sacrificed and sciatic nerves were removed, cleaned, pinned down straight in a 35 mm dish, and fixed by immersion in 4% paraformaldehyde for 3 to 6 h. Nerves were cryoprotected in 30% sucrose, embedded in Tissue-Tek O.C.T. (Electron Microscopy Sciences, Hatfield, PA, USA), and sectioned. After blocking, 60 µm sections were incubated in SCG10 (1:4000; Novus Biologicals, Littleton, CO, USA) overnight at 4°C and then incubated in Alexa Fluor 555 secondary antibodies (1:400; Thermo Fisher, Waltham, MA, USA). Nerves were imaged on a Leica SP8 confocal microscope. The regeneration index was measured based on the method of Shin et al. (2014). Briefly, the amount of fluorescence was assessed using MetaMorph in a 100 pixel wide rectangle spanning the width of the nerve at the site of injury (identified by fluorescent microspheres, not shown) and another rectangle where the amount of fluorescence was 50% that of at the injury site. The distance between these two rectangles was measured and expressed as the regeneration index.

Real-time PCR

Six or 48 hours after axotomy, SCG and L4 and L5 DRG were removed, desheathed, and placed in RNALater (Thermo Fisher). Ganglia from two mice were pooled for each sample. The tissue was homogenized, and RNA was isolated using the RNAqueous Micro Kit (Thermo Fisher) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Samples were run on an ABI Step ONE Plus using Prevalidated Taqman Gene Expression Assays (Thermo Fisher) for *Lif*, *Il6*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH/Gapdh*), cholecystokinin (*CCK/Cck*), galanin, growth associated protein 43 (*GAP-43/Gap43*), pituitary adenylate cyclase-activating peptide (*PACAP/Adycap1*), and vasoactive intestinal polypeptide (*VIP/Vip*). Relative expressions were determined using the comparative Ct model (Ct) and normalizing to *GAPDH*.

Immunohistochemistry

Generally, SCG and L5 DRG from diabetic and control mice were removed 48 h after injury, and the ganglia were desheathed and fixed by immersion in 4% paraformaldehyde for 1 h. The tissues were cryoprotected in 30% sucrose and embedded in Tissue-Tek O.C.T. compound. IHC was performed on 10 µm cryostat sections. For quantification of STAT3, a rabbit monoclonal antibody to phosphorylated STAT3 (Y705; 1:100; Cell Signaling Technology, Danvers, MA, USA) was incubated with tissue sections overnight at room

temperature. After washing, the sections were incubated in Alexa Fluor 555 secondary antibody (1:400; Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA, USA). Adjacent sections were labeled with an antibody against HuC/HuD (Thermo Fisher) to label all neurons in the ganglia. In all experiments, sections not exposed to the primary antibody were included for each experimental group. Images were captured at 10x or 25x magnification using HCImage software (Hamamatsu Corporation; Bridgewater, NJ, USA). Cell counts were performed using FIJI is just ImageJ (FIJI) software and expressed as the percentage of neurons positively labeled with pSTAT3 in the nucleus.

Multiplex

IL-6 and LIF proteins were measured in both SCG and DRG using magnetic bead-based ProcartaPlex Mouse Myokine Panel for the Luminex platform (Affymetrix, Santa Clara, CA, USA). Control and diabetic mice were sacrificed 48 h after injury. SCG and L5 DRG were removed and flash frozen in liquid nitrogen. Tissue from two mice were pooled for each sample. Frozen samples were homogenized via sonication in T-Per buffer supplemented with Halt protease and phosphatase inhibitors (Thermo Fisher) and centrifuged at $1000 \times g$ for 10 min. Supernatants were collected for further analyses; pellets were re-suspended with 50–100 μ l of additional T-Per buffer and were centrifuged a final time ($1000 \times g$, 10 min, 4°C) with supernatants added to final sample volume. Multiplex analyses were conducted according to manufacturer's instructions. In brief, a solution containing antibody-coupled beads for LIF and IL-6 were pipetted into a 96-well microplate (25 μ l/well). Twenty-five microliters of assay diluent was then added, followed by 50 μ l of sample or appropriate standard. Plates were light-protected and incubated for 16–18 h on an orbital shaker (500–600 rpm) at 4°C . Following incubation, plates were washed 3x with manufacturer-provided detergent solution using a handheld magnet to keep beads in place. Twenty-five microliters of HRP-conjugated detection antibody was added to all wells and incubated at room temperature for 1 h (shaking at 500–600 rpm). Plates were washed 3x as described, and 25 μ l of streptavidin-RPE was added to wells. After a final wash, 100 μ l of Magpix drive fluid was added to wells, plates were vigorously shaken for 3 min, and then plates were read on a Magpix Luminex 200. Luminex Xponent® software was used to generate a standard curve for each analyte from which concentrations of unknown samples were calculated.

Statistical Analysis

Statistical analysis was performed using Sigma Plot (Version 14). Student's *t*-test was used for comparison of two groups without injury, two-way ANOVA using Tukey *post hoc* correction was used for comparison of two groups with the presence of injury. All tests performed are two-tailed, with an alpha level of $p > 0.05$ used to determine significance. Results are reported as mean $\pm$ SEM.

Results

STZ-Injected Mice Develop Hyperglycemia and Weight Loss

Many different rodent models are used to study type-I and type-II diabetes mellitus. One of the most well studied and characterized models is the STZ-induced type-I diabetic model (Eleazu et al., 2013; Islam and Loots, 2009). However, the dosing and time of onset for

neuropathy are not standardized for the model (Sullivan et al., 2007; Sullivan et al., 2008). Here we used two distinct STZ dosing paradigms to induce sustained hyperglycemia. Our low dose paradigm utilized five consecutive *i.p.* injections of STZ at 60 mg/kg (Fig. 1a) and has previously been shown to develop neuropathy within one to three months following injection (Sullivan and Feldman, 2005; Sullivan et al., 2007). Our high dose paradigm utilized a single *i.p.* injection of STZ at 200 mg/kg (Fig. 1b) and has been shown to develop neuropathy-like symptoms at 1 month after injection (Chen et al., 2010). For both paradigms, mice treated with STZ showed significantly lower body weights (Fig. 1c,d) and higher blood glucose levels (Fig. 1e,f) than their age-matched non-diabetic controls at all times examined after injection(s). The animals receiving low dose injections had a more gradual onset of hyperglycemia (low dose bars are averaged data over a three week period). The animals injected with a high dose of STZ became hyperglycemic within four days after injection. Mice with fasted blood glucose levels ≥ 275 mg/dl beginning one week after the final STZ injection were considered diabetic. Glycated hemoglobin is thought to be a better overall measure of increased glucose within the blood (Kilpatrick et al., 1998; Sullivan et al., 2008). The percentage of glycated hemoglobin was significantly increased in low dose (control: $3.68\% \pm 0.18\%$, diabetic – low: $6.78\% \pm 0.48\%$, ** $p < 0.001$; Fig. 1g) and in high dose (control: $3.87\% \pm 0.36\%$, diabetic – high: $5.44\% \pm 0.69\%$, * $p < 0.05$; Fig. 1h).

STZ Administration Induces Functional and Morphological Changes Associated with Diabetic Neuropathy

Diabetic neuropathy is a common and potentially life-threatening complication associated with all types of diabetes. Symptoms related to neuropathy are dependent upon the nerve(s) affected and can manifest in sensory or autonomic dysfunction (Said, 2007; Sharma et al., 2015; Vinik et al., 2003). To assess the possible development of diabetic neuropathy in our STZ diabetic models, we first measured intraepidermal nerve fiber density (IENFD) in the mouse hind paw. IENFD has been shown to measure accurately the presence or absence of diabetic sensory neuropathy in both rodent models and human patients with diabetes (Arimura et al., 2013; Beiswenger et al., 2008b; Navarro et al., 1995). 3 months after onset of hyperglycemia in the low dose diabetic mice and 1 month after onset of hyperglycemia in high dose diabetic mice, the IENFD was calculated by counting the number of beta-III-tubulin-positive axons projecting into the epidermal layer of the skin of the hind paw. The border between the epidermis and dermis is clearly delineated by the high density DAPI-staining seen (Fig. 2c-f). Significant reductions in IENFD were found in both low dose (Fig. 2a,c-d) and high dose (Fig. 2b, e-f) diabetic mice compared to their non-diabetic controls. This result is a direct measure of the presence of a dying-back diabetic neuropathy in these STZ mouse models.

To measure the presence of diabetic autonomic neuropathy, we performed a silicone mold-based sweat assay (Kennedy and Navarro, 1989; Vilches and Navarro, 2002; Vilches et al., 2012). Sweat assays are used in both rodent models and humans to measure the presence of autonomic dysfunction associated with diabetes mellitus (Bharali et al., 1988; Chattopadhyay et al., 2007; Diem et al., 2003; Gibbons et al., 2009). To assess the presence of autonomic dysfunction in our diabetic mouse models, mice were injected with the muscarinic agonist pilocarpine, which induces sweating. Silicone molds are placed on the

hind paws of anesthetized mice within 10 min after injection of pilocarpine. The molds harden and sweat droplets on the pads of the hind paw make indentations in the molds (ex. Fig. 2i-m). The indentations in the molds can then be counted and summed for each paw. This assay directly measures sympathetic-driven sweating which requires complete innervation of each sweat gland, as sympathetic denervation (through sciatic and saphenous nerve transection) of sweat glands completely ablates pilocarpine-induced sweating (Fig. 2g,j). Both low dose (Fig. 2g,i-k) and high dose (Fig. 2h,l-m) diabetic mice display significantly reduced sweating compared to non-diabetic controls.

Thermoregulation during cold exposure was also assayed as an additional measure of dysautonomia in diabetic mice (Bachman et al., 2002; Campanucci et al., 2010). It was previously shown that the ability of mice to regulate their internal body temperature is reliant on proper peripheral sympathetic synaptic transmission as silencing sympathetic neurons using an nicotinic acetylcholine receptor alpha 3 subunit knockout animal reveals significantly decreased body temperature regulation (Campanucci et al., 2010). Control mice placed at 4°C only showed a 1.8°C and 2.0°C drop in body temperature for the low and high dose groups respectively, over the 3 h trial (Fig. 2n-o). In contrast, both low dose (Fig. 2n) and high dose diabetic mice (Fig. 2o) had their internal body temperature drop by 6.7°C and 5.7°C over the 3 h cold exposure, respectively. These data are indicative of the presence of diabetic autonomic neuropathy in these mice.

Loss of sensation and the development of neuropathic pain are two possible consequences of diabetic neuropathy, and both have been reported to occur in STZ rodent models of diabetes (Sullivan et al., 2007; Sullivan et al., 2008). We used the Von Frey Test, to measure a possible loss of sensation, and the hotplate nociceptive pain assay, to measure a possible onset of painful neuropathy. Low dose diabetic mice displayed significantly reduced tactile sensation as measured by the increased filament weight used to yield a consistent response (Tactile Response Threshold; Fig. 3a) and the reduced paw withdrawal from a 1g monofilament over 10 trials (Response Ratio; Fig. 3c) in comparison to control mice. High dose diabetic mice also displayed a significant increase in the response threshold (Fig. 3b) and a significant decrease in the response ratio (Fig. 3d). This data indicates a loss of hind paw sensation in our STZ diabetic mouse models. However, we did not detect the presence of painful neuropathy as both the low dose (Fig. 3e) and high dose (Fig. 3e) did not show any differences in their latency to withdraw their paw from a hotplate. Taken together, the morphological and functional changes measured in the low and high dose STZ models reveals the presence of both sensory and autonomic diabetic neuropathy.

In Vivo Regeneration of Sensory Axons is Significantly Impaired in Diabetic Mice

Diabetes has long been associated with a reduction in peripheral nerve regeneration in response to an injury (Kennedy and Zochodne, 2000, 2005b; Maxfield et al., 1995). We measured regeneration at *in vivo* 48 h after crushing the sciatic nerve. To measure regeneration, regenerating axons were labeled with SCG10, a label which is rapidly down regulated distal to the injury site and accumulates in regenerating fibers of the sciatic nerve (Shin et al., 2014; Shin et al., 2012). The regeneration index used to quantify *in vivo* regeneration identifies the distance from the lesion site to the location where levels of

SCG10 are half of their levels at the crush site. This identifies the length to which approximately half of axons have regenerated (Shin et al., 2014). Low dose mice displayed significantly reduced regeneration compared to non-diabetic control mice (Fig 4a-b,e). High dose mice were trending towards a significant reduction in regeneration compared to control mice ($p = 0.053$; Fig. 4c-d,f).

Injury-Induced Expression of gp130 Cytokines are Reduced in STZ Diabetic Mice

To determine a possible reason for the impaired peripheral nerve regeneration seen in diabetes, we looked at the expression of two gp130 cytokines in the SCG and DRG 6 h after axotomy. LIF and IL-6 are highly upregulated in the SCG and DRG after injury and are required for the conditioning lesion response (Banner and Patterson, 1994; Cafferty et al., 2004; Cafferty et al., 2001; Habecker et al., 2009; Hyatt Sachs et al., 2010; Sun et al., 1994). Low dose diabetic mice showed a significant reduction in the expression of IL-6 and LIF in the SCG (Fig. 5a), 6h after transection of the postganglionic fibers, and in the DRG (Fig. 5c), 6 h after a sciatic nerve injury, compared to non-diabetic control mice. High dose diabetic mice displayed a significant reduction in the injury-induced expression of IL-6 and LIF in the SCG (Fig. 5b) and of IL-6 in the DRG (Fig. 5d) compared to control mice.

To ascertain the protein expression levels of IL-6 and LIF in SCG and DRG, tissue was collected 48 h after injury and a magnetic bead multiplex array was run. Low dose diabetic mice displayed significantly attenuated protein expression of both IL-6 and LIF in the SCG (Fig. 5e,g) and of IL-6 in the DRG (Fig. 5e) compared to control mice. High dose diabetic mice displayed a reduction in IL-6 protein expression in both the SCG and DRG after injury (Fig. 5f) compared to controls while LIF protein levels were not altered compared to control mice (Fig. 5h). Given the importance of these cytokines in peripheral axon regeneration (Zigmond, 2012), the deficit in IL-6 and LIF expression could be responsible for the reduced regenerative capabilities present in diabetes.

Downstream gp130 Signaling is impaired in SCG and DRG of STZ diabetic mice

IL-6 and LIF both signal through the gp130 receptor by activating Janus kinase 2 (JAK2) and causing the phosphorylation, dimerization, and nuclear translocation of the transcription factor STAT3 (Zigmond, 2012). STAT3 activation is necessary for peripheral nerve regeneration as inhibiting its activation in peripheral neurons significantly impairs their regenerative capabilities (Bareyre et al., 2011; Heinrich et al., 1998; Hyatt Sachs et al., 2010; Niemi et al., 2016). To measure STAT3 activation, SCG and DRG from diabetic and control mice were stained for phosphorylated STAT3 (pSTAT3) and the percentage of neurons expressing pSTAT3 in their nucleus was calculated using a neuronal counterstain, HuC/HuD. Low dose diabetic mice displayed a significantly lower percentage of neurons which express pSTAT3 in both the SCG (Fig. 6a) and the DRG (Fig. 6c) 48 h after injury compared to control mice. Similarly, high dose mice displayed reduced pSTAT3 expression in the SCG (Fig. 6b) and DRG (Fig. 6d) following injury. The reduced expression of pSTAT3 is likely a direct result of the reduced IL-6 and LIF expression, as it has previously been shown that gp130 signaling accounts for a majority of the STAT3 activation after injury (Hyatt Sachs et al., 2010).

gp130 signaling is known to drive the expression of the neuropeptides *Vip*, galanin, *Pacap/Adcyap1*, and *Cck* (Habecker et al., 2009; Rao et al., 1993a; Sun and Zigmond, 1996a; Zigmond et al., 1998; Zigmond, 2011; Zigmond et al., 1996). Galanin and *Pacap/Adcyap1* expression have been directly linked to axon regeneration, as a knockout or inhibition of these neuropeptides shows significantly impaired regeneration (Armstrong et al., 2008; Hobson et al., 2006; Holmes et al., 2000; Kerr et al., 2000). We measured the injury-induced mRNA expression of these neuropeptides, as well as the RAG GAP43, in SCG and DRG 48h after axotomy. Low dose diabetic mice displayed significant reductions in *Vip* in the SCG (Fig. 7a) and *Gap43* and *Vip* in the DRG (Fig. 7c) in response to injury compared to control mice. High dose diabetic mice showed significant reductions in *Vip*, galanin, and *Pacap/Adcyap1* in the SCG (Fig. 7b) and in GAP43, galanin, and PACAP in the DRG after injury (Fig. 7d). Taken together, deficits in the injury-induced expression, activation, and downstream signaling of gp130 in sensory and sympathetic ganglia may contribute to the regenerative deficits found in diabetes mellitus.

Discussion

The results of our study indicate that the injury-induced expression and downstream signaling of the gp130 cytokine IL-6 and LIF are significantly diminished in both low and high dose diabetic STZ mice. Deficits in gp130 signaling could underlie the regenerative deficit found in diabetes. For this paper, we chose to use two different doses of STZ, 60 mg/kg given on 5 consecutive days and a single injection of 200 mg/kg, to induce hyperglycemia in our mice. The onset of hyperglycemia and the duration of time it took to observe measureable neuropathy related deficits varied greatly between the two doses. An overview of the diabetic neuropathy literature reveals a wide range of STZ doses and length of hyperglycemia prior to onset of neuropathy have been observed in rodent models (Fox et al., 1999; Islam, 2013; Islam and Loots, 2009). While many of the functional deficits associated with neuropathy remain consistent in different STZ models, the tactile sensation and thermal nociception have yielded variable results (Fox et al., 1999). Using the Von Frey monofilament assay, STZ-induced diabetic rats more readily demonstrate hyperalgesia, while in STZ mouse models it is common to observe hypoalgesia, as we did here (Christianson et al., 2003a; Christianson et al., 2003b; Greene et al., 1992; Greene et al., 1999). Reports of changes in thermal nociception are far more variable, where hyperalgesia, hypoalgesia, or no change in withdrawal latency in diabetic rodent models (Beiswenger et al., 2008a; Fox et al., 1999; Kamei et al., 1991; Raz et al., 1988; Sharma et al., 2006). Thus, the lack of a thermal nociceptive phenotype in either low or high dose STZ diabetic mice is not uncommon.

In previous studies, changes in various components of the gp130 cytokine signaling in the intact sciatic nerve have been correlated with diabetic neuropathy. LIF receptor subunit and gp130 receptor subunit expression is significantly increased in the sciatic nerve and gastrocnemius muscles up to 4 weeks after onset of hyperglycemia in a streptozotocin-induced mouse model of diabetes (Toledo-Corral and Banner, 2011). Interestingly, systemic administration of IL-6 was found to significantly attenuate the development of diabetic neuropathy in an streptozotocin rat model (Andriambeloson et al., 2006). Daily injections of IL-6 resulted in significantly improved compound muscle action potentials and sciatic nerve

conduction velocity at 40 days after streptozotocin injection compared to vehicle treated diabetic rats (Andriambeloson et al., 2006).

The regenerative deficit of peripheral nerves in experimental diabetes is a well-documented aspect of neuropathy. Deficits in regeneration have been shown to significantly increase with the duration of diabetes (Ekström and Tomlinson, 1990). Alterations in the regenerative capabilities in diabetic rodent models affect both the initiation and elongation phases of axon regrowth after injury (Bisby, 1980; Ekstrom and Tomlinson, 1989). While the cause of the deficit in regeneration in diabetes remains unknown, many have hypothesized that the lack of regeneration might play a primary role in the development and onset of diabetic neuropathy. Eckersley et al. (2001) stated that “a reduced ability to regenerate peripheral axons may be partly responsible for diabetic neuropathy”, a statement echoed by others in the field (Ebenezer et al., 2011; Pittenger and Vinik, 2003; Simmons and Feldman, 2002). Interestingly, impaired nerve regeneration is a very well documented nervous system defect in diabetes in humans as well (Bengel et al., 2006; Bradley et al., 1995; Ebenezer et al., 2011; Polydefkis et al., 2004) and data indicates that diabetic patients are more susceptible to compression injuries of the nerve (Kennedy and Zochodne, 2000; Ma and Willis, 2015).

A major component of the axonal injury-induced regenerative response is the large scale gene expression changes that occur in peripheral neurons (Boeshore et al., 2004; Chandran et al., 2016; Ma and Willis, 2015; Smith and Skene, 1997). However, very few studies have measured RAG expression after injury in diabetic rodent models. Interestingly, our data showed that numerous neuropeptides, highly expressed in both SCG and DRG after axotomy in wild-type mice (Habecker et al., 2009; Hokfelt et al., 1987; Hokfelt et al., 1994; Hyatt-Sachs et al., 1996; Hyatt-Sachs et al., 1993; Mohny et al., 1994; Rao et al., 1993b; Sachs et al., 2007; Schreiber et al., 1994; Shadiack et al., 1995; Shadiack and Zigmond, 1998; Sun and Zigmond, 1996a; Zigmond et al., 1998; Zigmond, 1994), are expressed at significantly lower quantities in both a low and high dose diabetic mice. While it is clear that neuropeptide expression is significantly altered in our diabetic models after injury, the neuropeptides which displayed reduced expression were different between SCG and DRG and also between low dose and high dose STZ treatment. *Il6* and *Lif* expression were only reduced and not completely abolished in our diabetic models which might explain why only some but not all of the neuropeptides displayed reduced mRNA expression (Habecker et al., 2009). We also showed that the quintessential RAG, *Gap43*, was expressed at significantly lower than controls in the DRG. Previous studies have also displayed impaired *Gap43* expression in sensory neurons of diabetic rodents (Maeda et al., 1996; Pekiner et al., 1996; Scott et al., 1999). However, we were unable to detect *Gap43* expression in control or diabetic mouse SCG. Although, GAP43 protein expression has been measured in sympathetic neurons of the SCG after decentralization and in dissociated cell culture (Hou and Dahlstrom, 1995; Meiri et al., 1988), a microarray study did not detect expression of *Gap43* in the SCG after axotomy (Boeshore et al., 2004).

Here, we showed that two different dosing paradigms of STZ generate mouse models of diabetes with clear hyperglycemia and functional deficits which confirm the presence of both sensory and autonomic neuropathy. Both STZ mouse models also exhibit a reduction in the regeneration of axons following a sciatic nerve crush. The injury-induced upregulation of

the gp130 cytokines, *Il6* and *Lif*, normally seen at high levels in injured DRG and SCG, was significantly attenuated in both diabetic mouse models. Alterations in injury-induced gp130 downstream signaling was also observed as decreased pSTAT3 and neuropeptide expression were present in the SCG and DRG of both high dose and low dose diabetic mice. This data indicates that defective gp130 signaling in peripheral ganglia could underlie the regenerative failure seen in diabetes mellitus and identifies a possible new therapeutic target for the treatment of diabetic neuropathy.

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Abbreviations

CCK	cholecystokinin
CNTF	ciliary neurotrophic factor
DRG	dorsal root ganglia
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAP43	growth associated protein 43
gp130	glycoprotein 130
IENFD	intraepidermal nerve fiber density
IL-6	interleukin-6
JAK2	Janus kinase 2
LIF	leukemia inhibitory factor
PACAP/Adcyap1	pituitary adenylate cyclase-activating peptide
PNS	peripheral nervous system
RAG	regeneration associated gene
SCG	superior cervical ganglia
STAT3	signal transducer and activator of transcription 3
STZ	streptozotocin
VIP	vasoactive intestinal peptide

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Figure 1.

Low (60 mg/kg) and high (200 mg/kg) dose administration of streptozotocin (STZ) results in decreased weight and increased fasted blood glucose and hemoglobin A1c levels in mice. Low dose mice received 5 injections of STZ at 60mg/kg on consecutive days and were tested 3 months later (a). High dose mice received a single injection of STZ at 200mg/kg and were tested 1 month post-injection (b). Both low (c) and high (d) dose STZ injections resulted in significant weight loss compared to age-matched controls (n=15–30/group) with asterisks representing a significant weight difference between groups at the final time point. Sustained hyperglycemia was observed by testing the fasted blood glucose levels (mg/dl) on a weekly basis for low (e) and high (f) dose diabetic mice (n=15–30/group). Hemoglobin A1c was significantly elevated in low dose (g) and high dose (h) mice compared to their age matched controls (n=15/group). * = $p < 0.05$. ** = $p < 0.001$.

Figure 2.

Morphological and functional changes associated with diabetic sensory and autonomic neuropathy are displayed in low and high dose diabetic mice. Intraepidermal nerve fiber density (fibers/mm) of the hind paw was reduced in low dose (a) and high dose (b) diabetic mice compared to their age-matched controls (n=5/group). Representative images displaying beta-III-tubulin-positive axons crossing into the epidermal layer are shown for control (c,e), low dose (d) and high dose (f) diabetic mice. Arrows indicate fibers counted that projected into the epidermal layer of the hind paw. Pilocarpine-induced sweating was measured using silicone molds on the hind paws of mice. The total number of sweat droplets was counted per hind paw and both low dose (g) and high dose (h) mice displayed significantly impaired sweating compared to control mice (n=10/group). Representative images of sweat droplet imprints into the silicone mold are seen for control (i,l), denervated (control mice in which the saphenous and sciatic nerves were cut; j), low dose (k) and high dose (m) mice. Internal body temperature was measured in 30 min intervals in mice placed at 4°C for low dose (n) and high dose (o) diabetic mice (n = 4–5/group). Nerve fiber density images taken at 20x.

Scale bar = 50 μm . Sweat assay images taken at 9x to 20x. Scale bar = 100 μm . * = $p < 0.05$.
** = $p < 0.001$.

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Figure 3.

Tactile sensation is significantly reduced in low and high dose diabetic mice, while thermal nociception is unaltered. The Von Frey filament assay was used to measure tactile sensation. Tactile response threshold, which is the filament weight (g) at which a withdrawal response is achieved at least 50% of the time, is significantly increased in low dose (a) and high dose (b) diabetic mice compared to controls. The response ratio to a 1 g monofilament stimulus over 10 consecutive trials was also significantly reduced in low (c) and high (d) dose diabetic mice compared to controls. The hotplate test was used to measure thermal nociception in the hind paw. The withdrawal latency (s) was calculated as the time it took for the mouse to remove their paw from a hotplate set at 53.5°C. Low (e) and high (f) dose diabetic mice displayed no difference in withdrawal latency compared to control mice. n=10–13/group for each experiment. * = $p < 0.05$. ** = $p < 0.001$.

Figure 4.

Regeneration of sensory axons is impaired in low and high dose diabetic mice.

Representative images of SCG10-positive regenerating fibers in 20 μm thick sciatic nerve sections 48 h after a crush injury in control (a,c), low dose diabetic (b), and high dose diabetic (d) mice. The asterisk (*) in each image denotes the crush site. The dashed line in each image denotes the area of the nerve distal to the crush site where the percent area positively labeled for SCG10 is reduced by 50% compared to staining at the crush site. This was taken as the regeneration index with data represented as the number of μm distal to the crush site where SCG10 staining is reduced by 50% compared to the crush site. Axon regeneration, following a sciatic nerve crush, was impaired in low dose (e) and high dose (f) diabetic mice compared to non-diabetic control mice. To better illustrate the distance of the

regenerating sensory axons, a regenerative ratio was calculated every 0.5 mm distal to the injury site from 0.5 mm out to 3 mm. These data are represented as a ratio of the percent area stained for SCG10 at each distance to that at the crush site compared (**g,h**). Using the regenerative ratio by distance, low dose diabetic mice show a significant decrease in regeneration at 1mm and 1.5 mm distal to the crush site (**g**) and high dose diabetic mice demonstrate a deficit at 1.5mm compared to age-matched controls (**h**). Images taken at 10x. Scale bar=500 μ m. n=8/group. * = $p < 0.05$.

Figure 5.

Injury-induced mRNA expression of IL-6 and LIF are significantly reduced in the DRG and SCG in both low and high dose diabetic mice. Expression of *Lif* and *Il6* mRNA was determined in the SCG (a,b) and in the DRG (c,d) 6 h after axotomy for low dose (a,c) and high dose (b,d) diabetic mice. Relative expression was determined using the comparative Ct method (Ct) normalized to the housekeeping gene GAPDH. n=6–9/group. A magnetic bead multiplex array was used to quantify IL-6 and LIF protein levels in SCG and DRG 48 h after injury. IL-6 expression is displayed as picograms (pg) of IL-6 per μg of total protein for low dose (e) and high dose (f) diabetic mice. LIF expression is displayed as pg of LIF per μg of total protein for low dose (g) and high dose (h) diabetic mice. n=5/group. * = $p < 0.05$. ** = $p < 0.001$. # = $p < 0.05$ between injured and non-injured conditions.

Figure 6.

Activation of STAT3 is significantly impaired in the SCG and DRG of low and high dose diabetic mice. Cell counts were performed in the SCG (**a,b**) and DRG (**c,d**) for low dose (**a,c**) and high dose (**b,d**) diabetic mice. The percentage of HuD/C-positive neurons displaying positive staining for phosphorylated STAT3 in the nucleus was calculated. $n=5-7$ /group. * = $p < 0.05$ between control and diabetic groups. ** = $p < 0.001$ between control and diabetic groups.

Figure 7.

Injury-induced mRNA expression of regeneration-associated genes downstream of gp130 is significantly reduced in the DRG and SCG in both low and high dose diabetic mice. Expression of *Gap43*, *Vip*, *galanin*, *Pacp*, and *Cck* mRNA was determined in the SCG (a,b) and in the DRG (c,d) 48h after axotomy for low dose (a,c) and high dose (b,d) diabetic mice. Relative expression was determined using the comparative Ct method (ΔΔCt) normalized to the housekeeping gene GAPDH. n=5–7/group. * = $p < 0.05$ between control and diabetic groups. ** = $p < 0.001$ between control and diabetic groups. # = $p < 0.05$ between injured and non-injured conditions.