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Early loss of peptidergic intraepidermal nerve fibers in an STZ-induced mouse model of insensate diabetic neuropathy

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Introduction

Pain is mediated by small-diameter, unmyelinated or lightly-myelinated nociceptive neurons responding to mechanical, thermal, and chemical stimuli. Two largely non-overlapping classes of C-fiber nociceptors have been identified and characterized based on their trophic factor requirements, biochemical phenotypes, and terminations (7,23,32,33,42). Peptidergic nociceptors express CGRP and substance P, neuropeptides classically considered important in pain transmission, and respond to nerve growth factor (NGF) via the expression of TrkA. Nonpeptidergic nociceptors are sensitive to glial cell line-derived neurotrophic factor (GDNF) and are recognized by their binding of the *Griffonia simplicifolia* lectin IB-4. In addition, marked topographical segregation between these 2 nociceptive subpopulations exists peripherally via distinct peripheral terminations in the epidermal strata (55); centrally with segregated spinal termination zones in the dorsal horn (31); and at higher neural levels by engaging separate ascending pathways of pain circuitry (7). Finally, these 2 nociceptive subpopulations also differ in their electrophysiological properties (44) and neurochemical expression (6,11,19). Collectively, these differences argue for the existence of separate but parallel pain pathways originating from nonpeptidergic and peptidergic nociceptive neurons (7,22,55).

The functional relevance of these parallel pain pathways has been surprisingly difficult to establish. Behavioral studies using selective knockdown approaches (48,49) have not clearly revealed modality-specific differences, although recent studies suggest that discriminative versus affective components of pain are differentially transmitted by the peptidergic and nonpeptidergic pathways, respectively (7,54). Only recently have studies begun to address how these populations are associated with various pain states induced by disease or injury (20,29).

Neuropathy is a principle chronic complication of diabetes and is characterized, in part, by the distal degeneration of peripheral axons. The sensory axon degeneration is strongly associated with a range of positive and/or negative sensory complications (9,10,14,24,28,36,43,50). Our studies in a type 1 model of diabetes demonstrate that diabetic C57BL/6 mice develop progressive, insensate neuropathy reflected by reduced behavioral responses to noxious stimuli concomitant with reductions in cutaneous innervation and spinal activation (12,13,14,24). The

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loss of cutaneous innervation in human patients and rodent models of diabetes is now consistently observed (12,13,21,25,35,40), but it is unknown whether nociceptive subpopulations contribute equally to this loss. In the current study, experiments were carried out in genetically modified mice that express green fluorescent protein (GFP) only in nonpeptidergic cutaneous afferents (55). The goal was to determine the extent to which peptidergic and nonpeptidergic axons were affected during the progressive loss of epidermal axons, and how their respective loss contributes to the gradual decline in behavioral responses. Our results demonstrate that peptidergic axons are preferentially lost in the mouse epidermis during the emergence of behavioral deficits, suggesting that these two populations are differentially damaged by diabetes and that behavioral symptoms may be more closely tied to damage within the peptidergic population.

Materials and Methods

MrgD Mice

MrgD is a member of the Mas-related G protein-coupled receptor family, and its expression identifies a distinct neuronal subpopulation (55). The MrgD mouse line was generated (by D. Anderson, California Institute of Technology) using a construct that replaced amino acids 20 to 315 of the 321 amino acid MrgD coding region with an in-frame fusion of farnesylated enhanced green fluorescent protein (GFP), so that GFP is expressed in MrgD-expressing neurons (55). Wildtype (MrgD^{+/+}) mice have two full copies of the MrgD gene, while MrgD^{+/-} mice are heterozygous for the GFP gene-deleting allele expressed from the MrgD promoter (see Ref. 55 for more details). Zylka et al. (55) reported that GFP⁺ neurons in MrgD^{+/-} mice peripherally project exclusively to skin, terminating as free nerve endings in the epidermis, and do not innervate any other body tissue examined. By coimmunostaining for a cocktail of antibodies including CGRP, Zylka et al. (55) also demonstrated that virtually all epidermal fibers can be accounted for by either MrgD or CGRP, and that MrgD neurons “likely constitute all nonpeptidergic innervation of the epidermis.” MrgD neurons in MrgD^{+/-} and ^{-/-} mice are similar in GFP expression and projection profiles (55).

Heterozygous MrgD mice (MrgD^{+/-}) mice backcrossed to C57BL/6 mice for 5 generations were kindly provided by D. Anderson (California Institute of Technology). Upon receipt, backcrossing of MrgD^{+/-} mice to C57BL/6 mice (Charles River, Wilmington, MA) continued; all mice used in the following experiments were backcrossed for a total of 10 to 13 generations. Mice were housed 2 to 4 per cage on a 12:12-hour light/dark cycle under pathogen-free conditions with free access to water and mouse chow (Harlan Teklad 8604, 4% kcal derived from fat). MrgD^{+/+}, ^{+/-}, and ^{-/-} mice were identified by polymerase chain reaction (PCR) on DNA harvested from tail snips using the following PCR primers: 5'-CTGCTCATAGTCAACATTTCTGC-3'; 5'-CATGAGATGCTCTATCCATTGGG-3'; 5'-GGAGAAACAGCTAAAGTGCG-3'. PCR reaction products were run on an agarose gel, and MrgD^{+/+} mice were identified by the presence of a single band corresponding to 960 base pairs (bp); MrgD^{+/-} mice by the presence of bands at 960 bp and 516 bp; and MrgD^{-/-} mice by a single 516 bp band. All animal use was in accordance with NIH guidelines and conformed to the principles specified by the University of Kansas Medical Center Animal Care and Use Protocol.

STZ-Induced Diabetes

Diabetes was induced in 8-wk old male MrgD^{+/+} and MrgD^{+/-} mice following baseline behavioral testing. Mice were injected with a single intraperitoneal injection of streptozotocin (180 mg/kg body weight; Sigma, St. Louis, MO) dissolved in 10 mmol/L sodium citrate buffer, pH 4.5. Littermate controls received sodium citrate buffer alone. Food was removed from all animal cages for 5 hours prior to and 4 hours following STZ injection. Animal weight and

blood glucose levels (using glucose diagnostic reagents; Sigma) were measured 2 weeks after STZ injection and every other week thereafter. Mice were included in the diabetic group if their whole blood glucose level, tested by tail vein sampling for intermediate measures and sampling from the decapitation pool for the terminal measure, was > 16.0 mmol/L at every measure. Insulin was not provided at any time. Mice were euthanized following behavioral testing at either 4 or 8 weeks post-STZ.

Quantification of Intraepidermal Nerve Fiber (IENF) Density

Tissue Collection—Mice were anesthetized with Avertin (1/25% v/v tribromoethanol, 2.5% tert-amyl alcohol; 200 μ L/10 g body weight) to areflexia. The entire plantar surface of the left hind paws, including all volar pads, were dissected, immersion-fixed for 1 hour in Zamboni's fixative (3% paraformaldehyde, 15% picric acid in 0.1 M phosphate buffered saline [PBS], pH 7.4) at 4°C, rinsed in PBS overnight, then placed in fresh PBS every morning for three days. Tissue was cryoprotected in 30% sucrose at 4°C for 24 hours, then frozen in Tissue Tek (Sakura, Torrance, CA) and stored at -80°C until sectioning. Longitudinal sections (along the rostral-caudal axis) were cut on a cryostat at 30 μ m. Sections were mounted on Superfrost Plus slides (Fisher Scientific, Chicago, IL) and stored at -20°C until immunostaining.

Immunohistochemistry—After thawing 5 min. at room temperature, slide-mounted tissue was encircled with a hydrophobic barrier using a Pap Pen (Research Products International, Mt. Prospect, IL) and treated with a blocking solution (0.5% porcine gelatin, 1.5% normal goat serum, 0.5% Triton-X in Superblock Buffer [Pierce, Rockford, IL]) for 1 hr. at room temperature. The primary antibody (rabbit anti-PGP9.5; 1:400; Ultracclone, Isle of Wight, UK) was diluted in blocking solution and incubated with sections overnight at 4°C. For visualization, sections were washed 3 $\times$ 5 min. with PBS followed by incubation for 1 hr. with a fluorochrome-conjugated secondary antibody (goat anti-rabbit Alexa 555; 1:2000; Molecular Probes, Eugene, OR) diluted in PBS. Following three washes with PBS, slides were coverslipped and stored at 4°C until viewing.

IENF Quantification—IENF density was quantified according to recently published European Federation of Neurological Societies guidelines (26). Four randomly chosen tissue sections separated by at least 60 μ m were quantified from each animal. For each section, four 40 $\times$ frames of view were randomly chosen for quantification and included regions all along the rostral-caudal axis so that every part of the footpad was represented in the final analysis. Sections were counted live while viewing using a Nikon Eclipse 80i fluorescent microscope. Only single IENFs crossing the dermal-epidermal junction were counted, excluding secondary branching and nerve fragments not crossing the dermal-epidermal junction. PGP9.5+/GFP+ fibers were considered nonpeptidergic, while PGP9.5+/GFP- fibers were considered peptidergic. PGP9.5+/GFP+ and PGP9.5+/GFP- fiber counts were summed to yield the total number of fibers. For each group (total, PGP9.5+/GFP+, and PGP9.5+/GFP-), the number of fibers counted in the frame of view was divided by the length of the dermal-epidermal junction in the frame of view (measured in NIH Image J) to arrive at the IENF density measure (number of fibers/mm). IENF density in 4 frames of view was averaged to yield the IENF density per 30 μ m section, 4 sections were averaged to yield IENF density per animal, and group means were calculated. All data are reported as group mean $\pm$ standard error of the mean (SEM).

To generate the high-resolution images in figures 1 and 3, 10 μ m footpad sections were cut and immunostained as above. Images were acquired in 0.5 μ m steps using a Nikon Digital Eclipse C1si confocal microscope equipped with an HeNe laser for the excitation and detection of the Alexa Fluor 555 and an Argon laser for the excitation and detection of GFP fluorescence. Images were adjusted for brightness and contrast using NIH Image J or Adobe Photoshop.

Behavioral Assessments of Cutaneous Sensitivity

Mechanical—Behavioral responses to a Von Frey monofilament (1.4 g) were assessed at baseline (8 weeks of age prior to STZ injection) and either 4 or 8 weeks post-STZ. Mice underwent a training session on the day prior to the first day of testing. On testing days, mice were placed in individual clear plastic cages (11 × 5 × 3.5 cm) on a wire mesh screen elevated 55 cm above the table and allowed to acclimate for 45 min. The combined mean percent withdrawal of three applications to each foot on two consecutive testing days was calculated for each animal, and group means were calculated. Figure data is reported as group means.

Thermal—Thermal testing was performed using a thermal analgesiometer (UARDG, Dept. of Anest., Univ. Cal. San Diego, La Jolla, CA) on the same days as, but immediately following, mechanical testing. Mice were placed in individual clear plastic compartments (9 × 9 × 14 cm) atop a glass surface and allowed to acclimate for 1 hr. A radiant heat source (4.0 V) 40 mm from the glass floor was applied to the mid-plantar surface of 1 hind paw for a maximum of 20 seconds, and the number of seconds until paw withdrawal was recorded as the latency. Latencies were recorded three times on alternating hind paws, with at least 10 minutes separating applications to the same hind paw. The mean withdrawal latency of 6 total applications on two consecutive testing days was calculated for each animal, and group means were calculated. Figure data is reported as group means.

Chemogenic—To assess chemogenic nociception, the formalin test was performed prior to sacrifice on the day following the final mechanical and thermal testing day. After a 1 hr. habituation to individual observation chambers (27 × 18 × 21 cm), mice were injected with 20 µL of 5% formalin (in saline, pH 7.0) subcutaneously into the dorsal surface of the right hind paw using a 1 CC insulin syringe and a 28-gauge needle. The amount of time devoted to the injected foot (licking and biting) was recorded in two 10-minute windows during the acute (Phase 1; 0-10 min. post-injection) and inflammatory (Phase 2; 40-50 min. post-injection) phases of the formalin test. Figure data is reported as group means.

Statistical Analysis

To test whether the MrgD-GFP transgene affected the development of diabetes or the progression of diabetes-induced behavioral deficits, comparisons were made between nondiabetic and diabetic MrgD^{+/+} and MrgD^{+/-} mice at each time point using repeated measures (RM) analysis of variance (ANOVA) (weight, blood glucose) and two-way ANOVA (Von Frey, thermal, formalin Phase 1 and 2) followed by post-hoc analysis using Fisher's protected least significant differences (PLSD). Fiber subtypes in nondiabetic mice were compared between 4 and 8 weeks post-vehicle using Student's T-test. All other comparisons between MrgD^{+/-} nondiabetic and diabetic mice at the 4 and 8 week time points were performed using RM ANOVA (weight, blood glucose) and two-way ANOVA (IENF density, Von Frey, thermal, formalin Phase 1 and 2) followed by Fisher's PLSD. For correlations, populations were first examined for normal distribution using the Kolmogorov-Smirnov Normality Test. Populations with normal distributions were compared using the Pearson Product Moment Correlation; those that failed the normality test were compared using the Spearman Rank Order Correlation. Simple linear regression analyses were also performed to examine relationships between behavioral measures and innervation densities. All significance levels were set to 0.05. All data reported as mean $\pm$ SEM.

Results

Wild-type vs. Heterozygous MrgD Mice

Although MrgD^{+/-} mice were first reported to have no obvious behavioral or phenotypic abnormalities (55), the MrgD allele has not been extensively studied with regard to its effect

on animal behavior or the progression of nerve complications. Therefore, throughout these studies, MrgD^{+/+} and MrgD^{+/-} littermates were tested alongside each other for each measure. There were no statistical differences between MrgD^{+/-} and MrgD^{+/+} mice from the same experimental group and time point on any of the following measures: terminal weight, terminal glucose, baseline mechanical sensitivity, final mechanical sensitivity, thermal responses, and responses during the formalin test (data not shown), indicating neither the transgene nor MrgD heterozygosity affect baseline behavioral measures, the development of diabetes, or the progression of diabetes-induced behavioral deficits.

IENTF density in nondiabetic mice

Because virtually all epidermal fibers are either MrgD⁺ or CGRP⁺ (55), using PGP9.5 immunostaining in conjunction with GFP expression allows for the extrapolation of peptidergic fibers. Thus, PGP⁺/GFP⁺ fibers were considered nonpeptidergic, while PGP⁺/GFP⁻ fibers were considered peptidergic. Similar to Zylka et al. (55), we observed distinct morphologies among PGP⁺/GFP⁺ and PGP⁺/GFP⁻ fiber terminations in nondiabetic MrgD^{+/-} mice (Figure 1). PGP⁺/GFP⁺ fibers predominantly coursed straight through underlying strata, then exhibited a tortuous, meandering pattern prior to terminating among keratinocytes of the stratum granulosum (Figure 1B). In contrast, PGP⁺/GFP⁻ fibers coursed directly and terminated in the stratum basalis or spinosum (Figure 1C). Occasional intertwining of PGP⁺/GFP⁺ and PGP⁺/GFP⁻ fibers was observed.

Quantitation of the total number of epidermal fibers (PGP⁺/GFP⁺ and PGP⁺/GFP⁻ fibers) revealed an average of 51.7 $\pm$ 1.86 and 57.4 $\pm$ 2.91 fibers/mm at 4 and 8 weeks post vehicle, respectively (12 and 16 weeks of age; Figure 2). When divided into peptidergic and nonpeptidergic subtypes, the nonpeptidergic (PGP⁺/GFP⁺) fibers represented 74.1% of the total fiber population (38.4 $\pm$ 1.36 fibers/mm) at 4 weeks post-vehicle. In contrast, PGP⁺/GFP⁻ presumptive peptidergic fibers constituted only 25.9% of the total (13.4 $\pm$ 1.32 fibers/mm). The proportions were similar 8 weeks post-vehicle, with 75.5% nonpeptidergic (43.4 $\pm$ 2.30 fibers/mm) and 24.5% peptidergic (14.1 $\pm$ 1.18 fibers/mm; Figure 2). No significant differences were found between 4 and 8 weeks in the number of fibers/mm, either in total or in subpopulations.

STZ-Induced Diabetes

Typical of this murine model and type 1 diabetes, STZ-injected mice exhibited polydipsia, polyuria, and weight loss. Nondiabetic mice increased in weight by 9.8% and 16.8% over the course of the study, whereas diabetic mice lost 12.9% and 7.4% of their body weight (4 and 8 week post-STZ, respectively; Tables 1 and 2). Blood glucose levels of diabetic mice were significantly higher than nondiabetic mice at all time points examined (Tables 1 and 2).

Progressive loss of IENTF density in diabetic mice

As a likely consequence of chronic hyperglycemia, the dermis and epidermis in diabetic mice often appeared less compact and the dermal/epidermal junction basement membrane appeared less precise in diabetic mice. In addition, PGP⁺ fibers in diabetic mice often appeared thinner and shorter compared to fibers in nondiabetic mouse skin. Moreover, long fibers extending to the superficial epidermis were generally less prevalent in diabetic skin (Figure 3A, B, C, D).

At 4 weeks post-STZ, the total number of PGP⁺ fibers/mm (representing all epidermal fibers) was 51.7 $\pm$ 1.86 and 47.8 $\pm$ 4.08 in skin from nondiabetic and diabetic mice, respectively, representing a nonsignificant 7.5% loss of overall epidermal innervation in diabetic mice (p = 0.3810; Figure 3A, C, 4A). Evaluation of both axonal subtypes revealed that at this early time point, nonpeptidergic fibers did not decrease in diabetic (39.9 $\pm$ 4.31 fibers/mm) compared to nondiabetic (38.4 $\pm$ 1.36 fibers/mm) mice (p = 0.7291; Figure 3A, C, 4B). In contrast,

peptidergic fibers were decreased by 40.6% in skin from diabetic (7.9 $\pm$ 1.00 fibers/mm) compared to nondiabetic mice (13.4 $\pm$ 1.32 fibers/mm; $p = 0.0045$; Figure 3A, C, 4C).

At 8 weeks post-STZ, a significant loss in total epidermal innervation was evident in diabetic mice, with a 36.5% decrease in the total number of PGP+ fibers/mm in diabetic (36.5 $\pm$ 2.75) compared to nondiabetic mice (57.4 $\pm$ 2.91; $p < 0.0001$; Figure 3B, D, 4A). In contrast to 4 weeks, however, both fiber subpopulations contributed to the overall loss at this later time point. Nonpeptidergic innervation was significantly decreased from 43.4 $\pm$ 2.30 to 28.6 $\pm$ 2.36 PGP+/GFP+ fibers/mm ($p = 0.0003$; Figure 3B, D, 4B), a 34.1% decrease in diabetic compared to nondiabetic mice. The reduction in peptidergic innervation was similar to levels observed at 4 weeks post-STZ. At 8 weeks post-STZ, peptidergic fibers were reduced by 43.8% in diabetic mice, from 14.1 $\pm$ 1.18 PGP+/GFP- fibers/mm in nondiabetic mice to 7.9 $\pm$ 1.00 in diabetic mice ($p = 0.0011$; Figure 3B, D, 4C).

Overall, only PGP+/GFP- peptidergic fibers were lost 4 weeks post-STZ. This loss was not mirrored by PGP+/GFP+ fibers, which remained stable at this time point, nor was it reflected in a loss of total innervation. PGP+/GFP+ fibers were not decreased until 8 weeks post-STZ, at which time a loss in overall epidermal innervation was evident.

Diabetes-induced deficits in behavioral responses to stimuli

Baseline Mechanical Sensitivity—To establish baseline mechanical sensitivity, heterozygous mice from all groups were tested prior to STZ-injection (at 8 weeks of age) for withdrawal responses to a 1.4 g Von Frey monofilament. There were no effects of either time point ($p = 0.1843$) or experimental group ($p = 0.3105$) on the average percent withdrawal of 6 applications to the hind paw footpads, indicating all groups had comparable mechanical sensitivity at 8 weeks of age upon entering the study (data not shown).

Reduction in Cutaneous Sensitivity Following Diabetes Induction

Mechanical: Consistent with previous studies using this model (14,53), diabetic mice displayed reduced percent paw withdrawals to monofilament application compared to nondiabetic mice at both 4 weeks and 8 weeks post-STZ (Figure 5A). At 4 weeks post-STZ, diabetic mice exhibited a withdrawal response an average of 34.7% $\pm$ 6.23 of the time, significantly less than nondiabetic mice (62.6% $\pm$ 4.90; $p = 0.0021$). Similarly, at 8 weeks post-STZ, the percent withdrawal response was 62.7% $\pm$ 4.60 in nondiabetic and 45.2% $\pm$ 5.04 in diabetic mice ($p = 0.0198$). Importantly, two-way ANOVA revealed a significant effect of experimental group (nondiabetic vs. diabetic; $p = 0.0001$) but not time point (4 vs. 8 weeks post-STZ; $p = 0.3239$), indicating the behavioral deficits at 4 weeks did not worsen with the diabetes progression. At 4 weeks post-STZ, percent withdrawal was not significantly correlated with the number of PGP+/GFP+ nonpeptidergic fibers/mm ($p = 0.157$; Figure 5B). By contrast, correlation with PGP+/GFP- peptidergic fibers was moderately positive ($p = 0.0251$; Figure 5C). By 8 weeks post-STZ, both PGP+/GFP+ ($p = 0.0009$) and PGP+/GFP- ($p = 0.0013$) fibers were strongly positively correlated with percent withdrawal (data not shown).

Thermal: Diabetic mice also demonstrated an increased latency in the time to withdrawal from a radiant heat source supplied by a thermal analgesimeter (Hargreaves apparatus) at both 4 and 8 weeks post-STZ (Figure 6A). The latencies of nondiabetic mice were 9.0 s $\pm$ 0.50 and 9.4 s $\pm$ 0.62 at 4 and 8 weeks post-STZ, respectively. Diabetic mice latencies were longer compared to nondiabetic mice, both at 4 weeks post-STZ (13.8 s $\pm$ 0.89; $p = 0.0001$) and 8 weeks post-STZ (15.4 s $\pm$ 1.01; $p < 0.0001$). Similar to measures of mechanical sensitivity, there was no effect of time point on thermal latencies ($p = 0.2257$), suggesting the early loss of thermal sensitivity did not progress with time from 4 to 8 weeks post-STZ. At 4 weeks post-STZ, thermal latencies were negatively correlated with PGP+/GFP- ($p = 0.0267$), but not PGP

+/-GFP+ ($p = 0.5200$), innervation (Figure 6B, C). By 8 weeks post-STZ, thermal latencies showed a strong, negative correlation with both fiber types (PGP+/-GFP+: $p = 0.0005$; PGP+/-GFP-: $p = 0.0083$; data not shown).

Formalin: The time devoted to the formalin-injected hind paw was recorded during the acute (Phase 1; Figure 7) and inflammatory (Phase 2; Figure 8) stages of the formalin test. Similar to mechanical and thermal hyposensitivity, early reductions in chemogenic sensitivity were apparent at 4 weeks and then sustained at 8 weeks post-STZ in both phases of the formalin test.

During Phase 1, behavioral responses of diabetic mice were significantly reduced compared to nondiabetic mice at 4 ($p = 0.0301$) and 8 ($p = 0.0016$) weeks post-STZ. Nondiabetic mice devoted 142.5 ± 33.20 and 177.5 ± 27.28 seconds to the injected foot at 4 and 8 weeks, respectively, and diabetic mice 64.3 ± 14.38 and 64.3 ± 9.29 (Figure 7A). There was no statistical effect of time point. Phase 1 behavioral responses were significantly correlated with PGP+/-GFP- ($p = 0.0171$; Figure 7C) innervation at 4 weeks post-STZ, but not with PGP+/-GFP+ ($p = 0.1870$) innervation (Figure 7B). Correlations with both fiber subtypes were significant at 8 weeks post-STZ (PGP+/-GFP+: $p = 0.0056$; PGP+/-GFP-: $p = 0.0190$; data not shown).

Behavioral responses during Phase 2 were decreased in diabetic (58.3 ± 23.60 , 46.4 ± 18.79) compared to nondiabetic (146.4 ± 28.58 , 160.6 ± 23.70) mice at both 4 ($p = 0.0288$) and 8 ($p = 0.0017$) weeks post-STZ, respectively (Figure 8A). Again, there was no statistical effect of time point on Phase 2 behavioral responses to formalin ($p = 0.9617$), suggesting stability of the behavioral changes following their appearance 4 weeks after diabetes induction. Interestingly, at the 4 week time point, Phase 2 behavioral responses were negatively correlated with PGP+/-GFP+ fibers ($p = 0.0427$; Figure 8B). In contrast, a strong positive correlation was seen with PGP+/-GFP- fibers ($p = 0.0052$; Figure 8C). Both correlations were strong and in the positive direction 8 weeks post-STZ (PGP+/-GFP+: $p = 0.0002$; PGP+/-GFP-: $p = 0.0072$; data not shown).

In summary, behavioral deficits in mechanical, thermal, and chemogenic sensitivity emerged at 4 weeks post-STZ and did not worsen by 8 weeks post-STZ. At the 4 week time point, PGP+/-GFP- peptidergic innervation was a better predictor of behavioral responses than PGP+/-GFP+ + nonpeptidergic innervation.

Discussion

MrgD+/- mice provide a new tool for assessing subpopulation-specific changes in epidermal nerve fiber composition in diabetes and other settings of peripheral nerve damage. The relative exclusivity among MrgD-GFP- and CGRP-expressing DRG neurons (55) allow for the evaluation of C-fiber subtypes at the level of the free nerve ending. The approach used here involving GFP transgene expression in conjunction with PGP9.5 immunostaining, compared to alternative techniques using immunostaining for multiple neuropeptide markers, avoids confounds associated with diabetes-induced neuropeptide modulation (1,8,38,46) or phenotypic switching (3,20,51). Our findings of a 75:25% distribution of nonpeptidergic to peptidergic fibers differ slightly from previous reports (55), perhaps due to extensive back-crossing onto a pure C57BL/6 background. Nonetheless, the anatomical differences in morphologies and termination zones between these fiber subtypes, first reported by Zylka and colleagues (55) and repeated here, suggest truly distinct populations and support investigations into their functional roles.

Previous studies addressing the functionality of nociceptor subtypes have used a variety of approaches including selective knock-down (48,49) or mice null mutant for subpopulation-

specific genes (27,45). Here, we evaluated axonal loss in an insensate dying back neuropathy with regard to nociceptor subtypes and changes in cutaneous sensitivity. The current study is unique because it demonstrates a dichotomy in the loss of nociceptor subpopulations resulting from a progressive disease state. Our results revealed a preferential decline of peptidergic fibers early in the disease progression that mirrored the temporal appearance of reduced nociceptive sensitivity, suggesting the peptidergic subpopulation may have increased sensitivity to stresses on peripheral axons. Whether this sensitivity is indicative of an overall vulnerability of peptidergic neurons, a hallmark of diabetic neuropathy, or a phenomenon specific to Type 1 diabetes in C57BL/6 mice is still unclear. Regardless, we have shown that a selective loss is possible. How a differential loss of fiber subtypes affects sensory function remains to be determined.

Our previous work with this model consistently reveals the emergence of behavioral deficits around 4 weeks following diabetes induction (14,24,53). In this study, the behavioral deficits we observed at 4 weeks were concomitant with the reduction in peptidergic innervation, suggesting the loss of peptidergic fibers may be important in the loss of sensitivity. Furthermore, correlations between behavioral responses and peptidergic innervation at this time point were moderate to strong. Behavioral deficits, once present, were maintained as the disease progressed and did not worsen during periods in which nonpeptidergic axons were degenerating, suggesting reduced nonpeptidergic epidermal innervation does not contribute significantly to changes in withdrawal-related cutaneous sensitivity of the animal. In addition, it should be noted that damage to low-threshold mechanoreceptive A-fibers is also occurring in the dermis of these mice (Christianson et al. 2007). It is plausible to suggest that a combination of C and A-fiber dysfunction contributes to the mechanical insensitivity that initiates in STZ-induced C57BL/6 mice after 4 weeks.

The idea that small reductions in the peptidergic axonal number can manifest as significant changes in nociceptive behaviors is supported by our own previous studies in this same model in which NGF was delivered intrathecally to diabetic mice (12,14). In these studies, deficits in mechanical and formalin sensitivity were improved by 2 weeks of NGF administration; however, NGF did not statistically increase total PGP-positive axonal innervation of the skin. Based on our current observations that the NGF-responsive population comprises such a small percent of the total skin innervation, it is not surprising that NGF did not change the overall innervation because any subpopulation-specific increases would be masked by the large numbers of nonpeptidergic axons.

Likewise, in the current study, peptidergic fibers were significantly reduced 4 weeks following diabetes induction, yet total epidermal innervation was unchanged. Again, this is likely because changes in this population only subtly change total numbers. However, this does not preclude the fact that these small changes may be functionally relevant. Previous studies in rodents have reported an onset of behavioral deficits prior to quantifiable intraepidermal fiber loss (4,52). In addition, early symptomatic changes in human patients with impaired glucose tolerance suggests that neural complications in human patients can develop prior to years of quantifiable diabetes-induced cutaneous loss (41). Our results suggest a subpopulation-specific loss masked in the total could account for sensory threshold changes and should be considered in future studies. In addition to overt fiber loss, it is likely that metabolic damage leading to dysfunction of intact fibers could be contributing to the behavioral deficits observed here. Such dysfunction could include electrophysiological, anatomical, and neurochemical changes. It is possible that in a diabetes-induced setting of metabolic disturbances, the loss of a small number of fibers becomes functionally relevant.

The striking distinctions between nonpeptidergic and peptidergic DRG neurons suggest they also have functional differences or may communicate divergent information. For example,

nonpeptidergic neurons have longer-duration action potentials and may lead to more reliable transmitter release from terminals (44). Nociceptor subtypes may also differ in their expression of sodium channels, particularly NaV1.8 and NaV1.9 (18,19). Moreover, nonpeptidergic neurons may be more responsive to ATP via P2X3 expression (6,42), but peptidergic neurons are the primary responders to protons and capsaicin (17). Although ablation of nonpeptidergic neurons results in transient decreases in thermal and mechanical sensitivity (49), peptidergic neurons display greater heat sensitivity (44). In addition, substance-P neuronal ablation significantly attenuates escape responses from nociceptive thermal stimuli (48) and reduces mechanical and thermal hypersensitivity induced by capsaicin, formalin, or nerve injury (30, 34). Finally, nonpeptidergic and peptidergic neurons differ in their vulnerability to and recovery from spinal nerve ligation (20) and dorsal rhizotomy (5). Perhaps most revealing was the finding that the central neural circuit originated by nonpeptidergic DRG neurons projects to brain regions (globus pallidus, amygdala, ventromedial nucleus of the hypothalamus, and bed nucleus of the stria terminalis) associated with motivational/affective components of pain, rather than regions accessed by circuits originating with peptidergic neurons and associated with sensory/discriminative aspects of pain (7,54). Our findings that behavioral symptoms are closely related to the losses in peptidergic epidermal fibers support the notion that peptidergic input is largely responsible for the discrimination of pain sensation, with nonpeptidergic input serving only to modulate the principal information provided by the peptidergic.

A selective subtype loss disrupting the overall balance of peripheral input into the dorsal horn could have significant implications for settings of pain in addition to the insensate model explored here. Several putative mechanisms to address include the anatomical rearrangement of central projections in the dorsal horn, the hyperexcitability of remaining peripheral fibers of the affected subpopulation as a compensation, or the sensitization of the remaining, unaffected, subpopulation. As an example, uninjured afferents have been implicated in mechanical hyperalgesia in neuropathic settings via the upregulation of neuropeptides, neurotrophic factors, and signal transduction proteins (37). Although the model studied here results in cutaneous hypoalgesia and the selective early loss of peptidergic fibers, it could be that the nature of the disease or injury (neuropathic vs. inflammatory) or the mechanism of damage influence the outcome (painful vs. insensate) of this imbalance. Investigating whether similar imbalances in peripheral input exist in painful models of diabetic neuropathy or in other disease states will be important in future studies.

Dramatic differences have been reported in the ability of peptidergic and nonpeptidergic DRG neurons to regenerate centrally following dorsal rhizotomy, with peptidergic afferents vigorously sprouting into denervated segments compared to poor sprouting by nonpeptidergic afferents (5). The suggestion that peptidergic afferents are “primed” to better respond during nerve injury, perhaps via higher constitutive GAP43 expression (39,47), is consistent with a model wherein peptidergic inputs provide the primary, discriminative sensory input. That plasticity may allow them to respond to a changing peripheral environment and maintain precise sensory information. A greater plasticity by peptidergic neurons could explain our previous data demonstrating differences in the amount of damage to peptidergic and nonpeptidergic dorsal horn terminals in the STZ-diabetes model (2). The persistence of peptidergic compared to nonpeptidergic central projections could actually reflect sprouting of these afferents in response to the diabetes-induced stressors to which the peptidergic subpopulation appears more vulnerable. Compensatory mechanisms inducing this sprouting response could include target derived neurotrophic support, since increased levels of NGF mRNA has been reported in the footpad skin of diabetic mice (12) and in the lateral calf muscles of diabetic human patients (16). Experiments to preclude this central overcompensation may prevent the development of chronic pain and shed light on mechanisms that lead to painful versus insensate neuropathy in diabetic patients.

As a consequence of the growing prevalence of diabetes, symptoms associated with peripheral nerve damage are increasing but remain difficult to treat. In order to develop more efficacious therapeutics, we still require a better understanding of the spectrum of mechanisms underlying these conditions. Our results suggest that differential vulnerability and loss of nociceptor subtypes may play a role in the phenotype of neural symptoms that develop in patients with peripheral nerve damage. Our results add a new layer to the growing differences between peptidergic and nonpeptidergic C-fibers and reveal that these subtypes differ in their sensitivity to metabolic and oxidative stress associated with diabetes. The approaches presented here should lead to improved studies of fiber-type changes in neuropathic and inflammatory pain as well as sensory loss.

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Figure 1. PGP+/GFP+ (nonpeptidergic) and PGP+/GFP- (peptidergic) fibers are present in nondiabetic MrgD+/- mice

IENFs in hind paw footpad skin from nondiabetic 12-week old MrgD+/- mice. (A) GFP+ fibers representing nonpeptidergic IENFs. (B) IENFs immunostained for PGP9.5, representing all epidermal fibers and including both subpopulations. (C) Merged view. Arrows indicate the location of PGP+/GFP- presumptive peptidergic IENFs (red).

Figure 2. Fiber subtypes and total number of fibers/mm is similar at 12 and 16 weeks of age in nondiabetic mice

Proportions of fiber subtypes in nondiabetic MrgD^{+/-} mice at 4 and 8 weeks post-vehicle (12 and 16 weeks of age, respectively). At 4 weeks post-vehicle, the composition of epidermal fibers was 74.1% nonpeptidergic (38.4 ± 1.36 fibers/mm) and 25.9% peptidergic (13.4 ± 1.32 fibers/mm). Similarly, at 8 weeks post-vehicle, 75.5% of epidermal fibers were nonpeptidergic (43.4 ± 2.30 fibers/mm) and 24.5% were peptidergic (14.1 ± 1.18 fibers/mm). Data plotted as mean ± SEM.

Figure 3. IENFs are reduced in diabetic mice

Merged images of PGP immunostaining (red) and GFP expression (green) in hind paw footpad skin from nondiabetic and diabetic MrgD^{+/-} mice. (A) PGP⁺/GFP⁺ and PGP⁺/GFP⁻ IENFs in nondiabetic mice 4 weeks post-STZ. (B) PGP⁺/GFP⁺ and PGP⁺/GFP⁻ IENFs in nondiabetic mice 8 weeks post-STZ. (C) PGP⁺/GFP⁺ and PGP⁺/GFP⁻ IENFs in diabetic mice 4 weeks post-STZ. PGP⁺/GFP⁻ fibers are reduced in diabetic mice at this time point. (D) PGP⁺/GFP⁺ and PGP⁺/GFP⁻ IENFs in diabetic mice 8 weeks post-STZ. Both PGP⁺/GFP⁻ and PGP⁺/GFP⁺ fibers are reduced in diabetic mice at this time point.

Figure 4. Loss of IENFs is subpopulation-specific early in the progression of diabetes

Quantification of total PGP+ (A), nonpeptidergic PGP+/GFP+ (B), and peptidergic PGP+/GFP- (C) IENFs in hind paw footpad skin from nondiabetic and diabetic MrgD^{+/-} mice at 4 and 8 weeks post-STZ. (A) There were no significant differences in the total number of epidermal PGP+ fibers at 4 weeks post-STZ; however, at 8 weeks the total number of fibers/mm was reduced in diabetic mice. (B) Nonpeptidergic PGP+/GFP+ fibers remained unchanged 4 weeks post-STZ, but were reduced 8 weeks post-STZ. (C) The number of peptidergic PGP+/GFP- fibers/mm was reduced at the early 4 week post-STZ time point. This reduction was maintained at 8 weeks post-STZ. Data plotted as mean $\pm$ SEM.

* $p < 0.05$ vs. nondiabetic mice of the same time point

** $p < 0.0001$ vs. nondiabetic mice of the same time point

Figure 5. Behavioral responses to mechanical stimuli were suppressed in diabetic animals at 4 weeks post-STZ and correlated with peptidergic innervation

(A) Behavioral responses to mechanical stimuli in nondiabetic and diabetic MrgD^{+/-} mice 4 and 8 weeks post-STZ. The average percent withdrawal from a 1.4 g monofilament was reduced at 4 weeks of diabetes; this reduction was maintained and did not worsen by 8 weeks post-STZ. Data plotted as mean $\pm$ SEM. (B) Mean percent withdrawal did not correlate with the number of PGP⁺/GFP⁺ epidermal fibers. (C) Correlation with the number of PGP⁺/GFP⁻ fibers revealed a moderate, positive relationship. r = Pearson product moment correlation coefficient. b = slope of the linear regression line. The solid line represents the linear regression line and

dotted lines represent 95% confidence intervals. Filled circles represent average values for individual animals.

* $p < 0.05$

Figure 6. Behavioral responses to noxious thermal stimuli were delayed in diabetic animals 4 weeks post-STZ and correlated with peptidergic innervation

(A) The latency to withdrawal from a thermal heat source was longer in diabetic compared to nondiabetic MrgD^{+/-} animals 4 weeks post-STZ and to a similar degree 8 weeks post-STZ. Data plotted as mean $\pm$ SEM. (B) Paw withdrawal latencies did not show a significant correlation with PGP⁺/GFP⁺ innervation. (C) Paw withdrawal latencies were moderately, negatively correlated with PGP⁺/GFP⁻ innervation. r = Pearson product moment correlation coefficient. b = slope of the linear regression line. The solid line represents the linear regression line and dotted lines represent 95% confidence intervals. Filled circles represent average values for individual animals.

* $p < 0.05$

Figure 7. Phase 1 behavioral responses during the formalin test were suppressed in diabetic animals beginning 4 weeks post-STZ and were correlated with peptidergic innervation

(A) At 4 weeks post-STZ, the amount of time devoted to the injected foot was decreased during the first 10 minutes of the formalin test in diabetic compared to nondiabetic MrgD^{+/-} mice. This reduction was maintained 8 weeks post-STZ but did not worsen. Data plotted as mean $\pm$ SEM. (B) Phase 1 behavioral responses were not correlated with PGP⁺/GFP⁺ innervation. (C) A strong positive correlation was observed between Phase 1 responses and the number of PGP⁺/GFP⁻ fibers. r = Spearman rank order correlation coefficient. b = slope of the linear regression line. The solid line represents the linear regression line and dotted lines represent 95% confidence intervals. Filled circles represent average values for individual animals.

* $p < 0.05$

Figure 8. Phase 2 formalin responses were reduced in diabetic animals 4 weeks post-STZ and were correlated with both peptidergic and nonpeptidergic innervation

(A) At 4 weeks post-STZ, diabetic mice exhibited reduced behavioral responses during Phase 2 of the formalin test. This reduction was maintained 8 weeks post-STZ. Data plotted as mean $\pm$ SEM. (B) Interestingly, responses during Phase 2 of the formalin test were negatively correlated with PGP+/GFP+ innervation. (C) A strong positive correlation between Phase 2 responses and the number of PGP+/GFP- fibers was observed. r = Pearson product moment correlation coefficient. b = slope of the linear regression line. The solid line represents the linear regression line and dotted lines represent 95% confidence intervals. Filled circles represent average values for individual animals.

* $p < 0.05$

Table 1
Weight and Blood Glucose Levels: 4 weeks post-STZ

	Week 0	Week 2	Week 4
Nondiabetic			
Weight	24.0 +/- 0.71	23.6 +/- 0.65	26.4 +/- 0.79
Glucose	NM	6.6 +/- 0.20	6.8 +/- 0.39
Diabetic			
Weight	23.6 +/- 0.73	19.1 [*] +/- 0.72	20.6 [*] +/- 1.12
Glucose	NM	23.9 ^{**} +/- 1.42	24.7 ^{**} +/- 1.52

Weight and blood glucose levels of nondiabetic and diabetic MrgD^{+/-} mice from the 4-week time point at weeks 0, 2, and 4 post-STZ.

NOTE. Weight is in grams; glucose levels are mmol/L. Data represented as means +/- standard error of mean.

NM = not measured

*
p < 0.05 vs nondiabetic mice

**
p < 0.0001 vs nondiabetic mice

Table 2

Weight and Blood Glucose Levels: 8 weeks post-STZ

	Week 0	Week 2	Week 4	Week 6	Week 8
Nondiabetic					
Weight	23.8 +/- 0.57	24.5 +/- 0.64	24.5 +/- 0.65	26.4 +/- 0.76	27.8 +/- 1.01
Glucose	NM	6.1 +/- 0.26	6.6 +/- 0.20	6.8 +/- 0.46	7.6 +/- 0.55
Diabetic					
Weight	22.7 +/- 0.47	19.0 ^{**} +/- 0.90	19.8 [*] +/- 1.22	19.1 ^{**} +/- 1.21	21.0 [*] +/- 1.27
Glucose	NM	22.9 ^{**} +/- 1.14	19.8 ^{**} +/- 0.96	22.3 ^{**} +/- 0.81	24.7 ^{**} +/- 1.25

Weight and blood glucose levels of nondiabetic and diabetic MrgD +/- mice from the 8-week time point at weeks 0, 2, 4, 6, and 8 post-STZ.

NOTE. Weight is in grams; glucose levels are mmol/L. Data represented as means +/- standard error of mean.

NM = not measured

* p < 0.05 vs nondiabetic mice.

** p < 0.0001 vs nondiabetic mice