Nerve Growth Factor Mediates Mechanical Allodynia in a Mouse Model of Type 2 Diabetes

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Abstract

We reported previously that the C57BLKS db/db (db/db) mouse develops the neuropathy of type 2 diabetes. In the current study, we demonstrate that the db/db mouse develops transient mechanical allodynia at the early stage of diabetes. We hypothesize that nerve growth factor (NGF) mediates the development of mechanical allodynia in the db/db mouse. NGF, substance P (SP), and calcitonin gene related peptide (CGRP) gene expression was upregulated in the dorsal root ganglion (DRG) of db/db mice during the period of mechanical allodynia. In parallel, there were increased numbers of small- to medium-sized NGF-immunopositive DRG neurons in db/db mice in comparison with the control db+ mice. These neurons also expressed SP, suggesting that they mediated nociception. The NGF expression in the hind paw skin was also increased in the period of mechanical allodynia. This peripheral NGF upregulation was associated with increased numbers of SP-positive intraepidermal nerve fibers (IENF). This upregulation of NGF coincided with enhanced tropomyosin-related kinase (Trk) A receptor phosphorylation in DRG. Finally, an antibody against NGF significantly inhibited mechanical allodynia and SP expression in db/db mice. The current findings provide evidence that inhibition of NGF action is a potential strategy for treating painful diabetic neuropathy.

Key words: Diabetes; Neuropathy; Nerve Growth Factor; Pain; Substance P; Dorsal Root Ganglion; Db/db mice
Introduction

Painful diabetic neuropathy (PDN) is a common complication of both type 1 and type 2 diabetes. PDN is an early manifestation of diabetic neuropathy and frequently precedes the diagnosis of diabetes (1, 2). Several recent studies have suggested that nearly one-third of the patients with impaired glucose tolerance (pre-diabetes) seek medical attention for a pain syndrome identical to PDN (3). While PDN is a persistent symptom in epidemiological studies of patients with type 2 diabetes, it is less common in type 1 diabetes (4-7); while estimates vary, approximately half of all patients with diabetic neuropathy and type 2 diabetes experience PDN, usually at the onset of their disease.

Patients with PDN experience allodynia and hyperalgesia; allodynia occurs when normally non-painful stimuli become painful, whereas hyperalgesia is increased sensitivity to normally painful stimuli. PDN is a major factor in decreased quality of life for patients with diabetes (8, 9). Over a period of time that could last several years, PDN subsides and the disabling pain is replaced by a complete loss of sensation, leading to the numb, insensate diabetic foot (2, 10).

Nociceptive dorsal root ganglial (DRG) neurons, which supply Aδ and C fibers, can be divided into peptidergic and nonpeptidergic groups. The peptidergic DRG neurons express neuropeptides, including substance P (SP) and calcitonin gene related peptide (CGRP) to mediate nociception. The development of peptidergic DRG neurons requires nerve growth factor (NGF). Nerve growth factor (NGF) belongs to the neurotrophin family of growth factors that regulate the development and survival of neurons in the central and peripheral nervous systems (11). Members of the neurotrophin family share homology, but individual neurotrophins mediate specific actions by binding to corresponding receptors.
known as tropomyosin-related kinases (Trks) (11-13). Trk A is the high-affinity receptor for NGF and is expressed on petidergic DRG neurons. NGF is produced and released from target tissues, binds to receptors on the Aδ and C nerve fibers, and is transported in a retrograde manner to DRG neurons (14). These Trk A-expressing, NGF-responsive peptidergic DRG neurons are the key generators of neuropathic pain (11, 12) and are affected early in the course of diabetes (11). In addition to Trk A, the low-affinity p75 neurotrophin receptor (p75) can also contribute to nociception by enhancing Trk A actions (15). In contrast, the non-peptidergic nociceptive neurons respond to glial cell-derived neurotrophic factor (GDNF) and express RET receptors (16, 17). In addition, these neurons have high affinity to isolectin B4 (IB4). It is unclear if there are distinct roles for the peptidergic and nonpeptidergic neurons to mediate various modalities of nociception.

Two of the most extensively studied neuropeptides are SP and CRRP. SP is a tachykinin neuropeptide that mediates nociception and is used as a marker for pain in animal models (18, 19). Under normal conditions, SP is expressed only in small- to medium-sized TRK A-positive DRG neurons (18). During painful conditions, SP is upregulated in these DRG neurons and released to the Lamina I and the outer layer of Lamina II of the spinal cord dorsal horn to activate secondary sensory neurons (18, 20). CGRP is also upregulated under painful conditions in similar population of TRK A-positive sensory neurons (21).

NGF is a major factor in enhancing the expression of SP and CGRP (22). Exogenous exposure to high levels of NGF, both in vivo and in vitro, increases the intracellular content and release of SP and CGRP (23-26). Based on previous reports, we hypothesize that NGF mediates PDN via upregulation of SP and CGRP. Development of
neuropathic pain following diabetes may be accompanied by alterations in the level or
distribution of these neuropeptides (27). Understanding the role that NGF plays in
nociceptive peptide expression would set the stage for uncovering the mechanisms that
underly the induction and modulation of neuropathic pain in type 2 diabetes.

To study PDN in type 2 diabetes, we use the C57BLKS db/db (db/db) mouse,
which carries a leptin receptor null mutation and is a well-characterized animal model of
type 2 diabetes (28, 29). The db/db mouse develops features of type 2 diabetes, including
obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia. The db/db mouse develops
diabetic phenotypes at 4-5 wk of age and significant diabetic neuropathy at 24 wk of age
(30). In the current study, we first determine the presence of mechanical allodynia in the
db/db mouse at 8-12 wk of age. We then use this window of increased nociception to study
the roles of NGF and Trk A in PDN of type 2 diabetes. We hypothesize that enhanced
NGF-mediated Trk A activation during the period of mechanical allodynia increases the
expression of SP in DRG neurons and serves as a molecular mechanism for PDN in type 2
diabetes.
Materials and Methods

Animals

Male C57BLKS db/db (stock number 000662) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The homozygous ($\text{Lepr}^{\text{db}}/\text{Lepr}^{\text{db}}$, or db/db) mice were used as a model of type 2 diabetes, while heterozygous mice ($\text{Lepr}^{\text{db}}/+$, 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.amdcc.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.

Blood Chemistry

The onset of diabetes was confirmed by measuring fasting blood glucose levels. One drop of tail blood was analyzed using a standard glucometer (One Touch Profile, LIFESCAN, Inc., Milpitas, CA) beginning at 4 wk of age and repeated weekly to document the progression of diabetes. Glycosylated hemoglobin (HbA1c) was measured using the Helena Laboratories Test Kit Glyco-Tek Affinity Column Method. Insulin was measured using a rat/mouse insulin ELISA Kit (Linco Research, St. Charles MO, #EZRMI-13K) according to the manufacturer's protocol.

Mechanical Allodynia:

Mechanical allodynia was assessed by two methods:
1. Up-down method:

The threshold for a non-noxious mechanical stimulus was assessed using von Frey filaments. The animals were placed in a Plexiglas cage with mesh flooring and allowed to acclimate for 1 h. A logarithmic series of calibrated monofilaments (von Frey hairs; Stoelting, Wood Dale, IL) with bending forces from 1 to 4 g were applied to the midplantar surface of the hind paw and pressed to the point of bending. Brisk withdrawal of the stimulated paw was recorded as a positive response. Testing began with the 1 g filament, followed by larger filaments if no response was observed, using the up–down method (31), with a 10 min interval to allow the animals to recover between tests. Although all responses were noted, counting of the critical 6 data points did not begin until the response threshold was first crossed. The resulting pattern of the 6 positive and negative responses was tabulated, and 50% gram threshold was calculated using the formula described previously (32). Mechanical allodynia was determined by a significant decrease in mechanical threshold compared to that of a db+ mouse of the same age.

2. Percentage of response using a single von Frey filament: The experimenter applied a 1.4-g von Frey filament to the surface of the hind paw until the filament was just bent. This stimulus was repeated 5 times at a frequency of about 1 stimulus/s. Each trial was repeated 4 times at approximately 3-min intervals on each hind paw. The paw withdrawal frequency was then calculated using the formula: (# of paw withdrawals/20 stimuli) x 100 = % response frequency (33).

Real time RT-PCR
Total RNA was extracted from L4-6 DRG using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Six DRG (bilateral L4-6) were used for each animal and total of 4 animals were used per condition. Reverse transcription was performed using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Briefly, 5X iScript Reaction Mix, 1 μl iScript Reverse Transcriptase and total RNA template were added to a final volume of 20 μl. Reaction conditions were 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. PCR was performed as described previously (34) using the primer sequences: NGF sense 5'-CCAAGGACGCAGCCTTTCTAT-3'; NGF antisense 5'-CTCCGGTGAGCTTCTGGGA-3'; SP sense 5'-ATGGCCAGATCTCTCACAAAAG-3'; SP antisense: 5'-AAGATGAATAGATAGTGCGTTCAGG-3'; BDNF sense 5'-AGGCCAACTGAAGCAGTATTTC-3'; BDNF antisense 5'-AGGCCAACTGAAGCAGTATTTC-3'; GAPDH sense 5'-TCCATGACAACTTTGGCATCGTGG-3' and GAPDH antisense 5'-GTTGCTGTTGAAGTCACAGGAGAC-3'.

All real-time PCR reactions were carried out in 96-well PCR plates sealed with iCycler Optical Sealing Tape (BioRad). The PCR reactions contained 1X SYBR Green iCycler iQ mixture (BioRad), 0.2 μM of each forward and reverse primer, and cDNA preparation to 25 μl total volume. The PCR amplification profile was 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 30 sec, followed by 72°C for 5 min. The mRNA expression levels of the genes were tested, and amplification and fluorescence detection were performed using iCycler iQ Real-time Detection System (BioRad). At the end of the PCR, melting curves were obtained.
from 46 subsequent temperature increments by measuring fluorescence every 10 s with +0.5°C/step increments and beginning at 72°C. The quality of PCR products was determined by melting curve analysis. The fluorescence threshold value was calculated by the iCycler iQ system software, and the levels were normalized to values obtained for GAPDH. A non-template control (NTC) was run with every assay.

Immunoprecipitation and Immunoblots

Following deep anesthesia, L4-6 DRG were dissected from 4 mice per condition (db/db and db+) and homogenized in ice-cold T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) containing protease inhibitors (1 μM sodium orthovanadate and 1 μM sodium fluoride; Sigma Life Science, St. Louis, MO). Lysates were sonicated for 5 sec, centrifuged and processed for protein concentration using Dc Protein Assay Reagents (BioRad). Two hundred μg of protein were mixed with antibody against total Trk A (2 μg/ml, rabbit polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA) and incubated overnight at 4°C. The samples were then precipitated by incubation with protein A/G agarose (Santa Cruz) and rinsed 3 times before the precipitates were boiled in 2X sample buffer, separated on a SDS-PAGE gel, and transferred to a PVDF membrane. Membranes were blocked and incubated overnight at 4°C with a goat polyclonal antibody against phosphorylated Trk A at tyrosine 496 (pTrk, 1:1000, Santa Cruz) or total Trk A (1:1000, Santa Cruz) for measuring the levels of phosphorylated or total protein correspondingly.. Membranes were then rinsed and incubated with HRP-conjugated secondary antibodies for 1 h at 25°C and processed with chemiluminescence substrate (Pierce) before being exposed to Hyperfilm (Amersham, Piscataway, NJ). Densitometry
was performed using Image J software, and the results were normalized against Trk A densities from the same sample.

Immunohistochemistry

Four mice from each group (db+ and db/db) were deeply anesthetized and perfused with 2% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2, 0.1M). L4-L6 DRG were dissected and post-fixed by immersion in 2% paraformaldehyde overnight at 4°C, then rinsed in graded sucrose solutions (5-30% in PBS), embedded in mounting media (OCT), and flash-frozen in liquid nitrogen. Tissue sections (10 μm) were cut and mounted onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80°C until ready for use. For immunolocalization, tissue sections were thawed on a warming plate (55°C for 10 min), hydrated with PBS, and blocked in 0.1% TX100 and 5% non-fat dry milk in PBS. Sections were then incubated at room temperature for 16-24 h with antibody against NGF (rabbit polyclonal, 1:500, Santa Cruz), and/or SP (rat polyclonal, 1:500, Abcam Inc., Cambridge, MA), pTrk (1:100, Santa Cruz), or Trk A (1:500, Santa Cruz). Sections were then rinsed 3 times in PBS and incubated with secondary antiserum conjugated with different fluorophores (AlexaFluor 488, 594, or 647, Invitrogen, Carlsbad, CA). For Isolectin B-4 labeling studies, the sections were incubated with AlexaFluor 594 labeled Griffonia simplicifolia isolecitn B-4 (1:500, Invitrogen) in PBS with 5% milk for 1 h. Sections were rinsed and mounted in ProLong® Gold antifade reagent (Invitrogen). To ensure specificity, sections were incubated with primary or secondary antisera alone to confirm there were no nonspecific immunoreactions. Fluorescent signals were examined using an Olympus FluoView 500 laser scanning confocal microscope.
The percentage of immunopositive cells was analyzed by counting the number of immunopositive neurons and multiplying by 100 / number of total number of neurons. The cell size distribution studies were performed on the same image. A total of 6 DRG were measured in each animal. Images of DRG sections were captured with a Nikon camera (Nikon Microphot-FXA), and the number of immunoreactive neuronal profiles was counted in a blinded fashion. Every tenth section was picked from a series of consecutive DRG sections (10 μm), and three to four sections were counted for each DRG and expressed as the percentage of total neuronal profile measured by neuronal specific enolase (NSE, 1:1000, Millipore, Billerica, MA) immunohistochemistry (35). To ensure specificity, sections were incubated with primary antisera alone, secondary antisera alone, or primary antisera preabsorbed with available antigens including NGF (500 ng/ml, Harlan, Indianapolis, IN) or SP (500 ng/ml, Cayman Chemical Co., Ann Arbor, MI). No significant nonspecific immunolabeling was detected in either control condition.

Intraepidermal nerve fiber (IENF) measurement

Prior to perfusion, hind foot pads were collected from the plantar surface of the hind paw, immersed overnight at 4°C in Zamboni's fixative (2% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer), rinsed in graded sucrose solutions (5-30% in PBS), cryoembedded in mounting media (OCT), sectioned (30 μm) and processed for pan-axonal marker (PGP9.5) (1:2000, Millipore) and SP immunohistochemistry (36). Fluorescent images were collected on an Olympus FluoView 500 confocal microscope using a 60 × 1.2 water immersion objective at a resolution of 800 × 600 pixels. The optical section thickness was 0.5 μm. Forty images per stack were flattened using the MetaMorph (version 6.14)
The data were presented as the number of fibers per linear mm of epidermis (37) or relative fold change to db+ mice at the same age. For specific cell type studies, antibodies for cell markers of macrophages (CD68, rat polyclonal, 1:1000, Abcam), fibroblasts (type 1 collagen, goat polyclonal, 1:1000, Abcam), mast cells (mast cell tryptase, goat polyclonal, 1:1000, Santa Cruz), and T cells (CD4 and CD8, rat monoclonal, 1:100, Santa Cruz) were used in conjunction with a rabbit anti-NGF antibody to identify the cell types of NGF immunopositive cells in the dermis.

Anti-NGF treatment

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 once weekly at the beginning of 6 and 7 wk of age for 2 wk (38). Mechanical thresholds were measured at 8 wk of age, and tissues, including hind foot pads and L4-6 DRG, 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 one-way ANOVA followed by a post hoc Tukey’s multiple comparison test. A p value of less than 0.05 was considered statistically significant.
Results

Db/db mouse develops features of type 2 diabetes

Serum levels of fasting glucose, glycosylated hemoglobin (HbA1c), insulin, and body weight were measured in db/db and db+ mice from 5 to 21 wk of age to monitor the development of type 2 diabetes. As demonstrated in Fig 1, there were significantly higher levels of fasting glucose (Fig 1A), HbA1c (Fig. 1B), insulin (Fig. 1C), and increased body weight (Fig. 1D) in db/db mice than in db+ mice. The fasting glucose level reached 400 mg/dl at 5 wk and was persistently elevated through 21 wk. HbA1c levels progressively increased from 5 wk through 21 wk, indicating a chronic state of hyperglycemia (Fig. 1B). Fasting insulin levels were also significantly elevated at 5 wk, persisting to 21 wk (Fig. 1C), which, in combination with hyperglycemia, suggested that the db/db mice were insulin-resistant, a key feature of type 2 diabetes. In addition to the biochemical markers of type 2 diabetes, the body weight was also significantly higher in db/db mice beginning at 5 wk and thereafter in the period tested (Fig. 1D). By 21 wk, the body weights of db/db mice were almost twice that of db+ of the same age. Collectively, these data indicate that db/db mice develop a condition similar to type 2 diabetes that begins at 5 wk of age and persists through 21 wk of age.

Db/db mouse develops mechanical allodynia at the early stage of diabetes

Given that the db/db mice exhibit hallmark signs of type 2 diabetes, we hypothesized they would exhibit behaviors indicative of PDN. To test this hypothesis, we measured mechanical allodynia in db+ and db/db mice from 5-21 wk of age. Mechanical allodynia was measured with von Frey filaments, using the up-down method (31); the 50% gram thresholds were calculated as described (32). As demonstrated in Fig. 2, the
nociceptive threshold was significantly lower for db/db mice beginning at 8 wk of age, suggesting increased sensitivity to mechanical stimuli or mechanical allodynia (Fig. 2A). The mechanical allodynia continued until 16 wk, when the difference between db+ and db/db mice became statistically insignificant. At 21 wk of age, the mechanical pain threshold increased in db/db mice, indicating the presence of sensory neuropathy. Similar results were obtained using the method described by Bennett et al., which uses a single von Frey filament (33). A significantly increased percentage of response to a 1.4 gm von Frey filament was detected in db/db mice at 8-12 wk of age, compared to db+ controls (Fig. 2B). The results suggest the presence of mechanical allodynia during 8-12 wk of age in db/db mice.

Increased NGF, SP, and CGRP gene expression during the period of mechanical allodynia

We determined whether the gene expression of NGF, BDNF, SP, and CGRP was altered during the period of mechanical allodynia. The gene expression of NGF (Fig. 3A), SP (Fig. 3B), BDNF (Fig. 3C), and CGRP (Fig. 3D) was measured by real time RT-PCR using mRNA from L4-6 DRG of db+ and db/db at 4-16 wk of age. Increased NGF gene expression was detected in db/db mice, compared to db+ mice of the same age, at 5 and 8 wk of age (Fig. 3A). At 12 wk, the NGF gene expression returned to the control levels (Fig. 3A). At 16 wk, NGF expression decreased to below the control levels (Fig. 3A). The gene expression of SP became elevated at 8 wk, peaked at 12 wk, and returned to the levels of db+ at 16 wk (Fig. 3B). Unlike NGF, BDNF expression was not significantly affected in DRG of db/db mice compared to db+ mice of the same age until 16 wk of age when its level dropped below the control level (Fig. 3C). Similar to SP, CGRP gene expression was upregulated during the period of mechanical allodynia but at a later time
point (11wk, Fig. 3D). The chronological order of initial NGF and subsequent SP and CGRP expression suggests that NGF could be responsible for increased SP and CGRP expression during the period of mechanical allodynia in db/db mice. However, the temporal pattern of SP expression is more closely related to the course of mechanical allodynia (compare Fig. 2 and 3).

**Increased NGF- and SP-positive DRG neurons during the period of mechanical allodynia**

Based on the results from the behavior and gene expression studies, we next examined the NGF and SP protein expression during the period of mechanical allodynia. As a first step, we used a polyclonal anti-NGF antibody to examine the expression of NGF in L4-6 DRG during the periods of PDN. The same DRG sections were double-labeled with an antibody against SP to identify nociceptive neurons. In 8-week-old db+ mice, NGF immunoreactivity was detected mostly in large-sized (Fig. 4A, arrowheads), but not small-to medium-sized DRG neurons (Fig. 4A, arrows). The NGF-positive DRG neurons did not express SP, which was mostly expressed in small- to medium-sized neurons mediating nociception (Fig. 4B, arrows). A clear distinction of NGF- and SP-positive neurons can be seen in the merged images in db+ mice (Fig. 4C). In contrast, both small- to medium-sized and large-sized DRG neurons in db/db mice expressed NGF at 8 wk of age (Fig. 4D, arrows and arrowheads). The majority of NGF-positive DRG neurons also expressed SP, as demonstrated in the merged images (Fig. 4F, arrows and arrowhead).

The percentages of NGF- and SP-positive neurons were quantified. At 4 wk of age, before the development of diabetes, there was no difference in the percentages of NGF- and SP-positive neurons between db+ and db/db mice (Fig. 5A, C). However, the percentage of NGF-positive neurons in db/db mice increased 3-fold over db+ mice at 8 wk
of age (Fig. 5A). This upregulation of NGF expression diminished by 12 wk of age. At 8 wk of age, most NGF-positive neurons in db+ mice were large-sized (> 30 μm in diameter) (Fig. 5B). The majority of NGF-positive neurons in db/db mice were small- and medium-sized neurons (< 30 μm in diameter) (Fig. 5B). Similarly, the percentage of SP-positive neurons increased at 8 and 12 wk, corresponding to the pattern of SP gene expression (Fig. 5C). Although the majority of SP-positive neurons were small- to medium-sized (< 30 μm in diameter) in both db+ and db/db mice (Fig. 5C), there were large-sized DRG neurons expressed SP in db/db mice (Fig. 5C). We also studied the percentages of GDNF-responsive neurons, as demonstrated by IB4 labeling at 8 wk of age. There was no statistical difference between the percentages of IB4-labeled DRG neurons in db+ and db/db mice (Fig. 5E). In both groups of mice, IB4-labeled DRG neurons were small- to medium-sized (Fig. 5F).

*Increased NGF expression in hind paw skin during the period of mechanical allodynia*

NGF is not only expressed locally in DRG neurons, but is also transported in a retrograde manner along nerves from the peripheral tissues. We next examined the NGF expression in the hind paw skin, a target tissue of L4-6 DRG. NGF immunohistochemistry was performed using sections of hind paws from db+ and db/db mice at the age of 8 wk; there was no significant NGF expression in the hind paw skin of db+ mice (Fig. 6A). In contrast, NGF immunoreactivity was detected strongly in dermal cells (Fig. 6B and 6C, arrows) in db/db mice. These cells were determined to be fibroblasts, macrophages, mast cells, and T cells using specific cell markers (data not shown). The intraepidermal nerve fibers (IENF) were also positive for NGF (Fig. 6B and 6C, arrowheads) in db/db mice.
Increased SP-positive intraepidermal nerve fibers during mechanical allodynia

An increased SP level in the skin enhances nociception (18). With the evidence of increased SP expression in DRG neurons, we hypothesize there would also be increased numbers of SP-positive IENF to mediate mechanical allodynia in db/db mice. We used immunohistochemistry to examine the number of SP-positive nerve fibers in hind paw skin during the period of mechanical allodynia. PGP 9.5 immunochemistry was also performed to determine if there was a change in total IENF densities. At 8 wk of age, SP-positive IENF were significantly increased in db/db, compared to db+ mice (compare Fig. 7A, C, E). In contrast, the total PGP 9.5-positive IENF densities were not different between db+ and db/db mice (compare Fig. 7B, D, and F). The SP fiber density plateaued at 11 wk of age and declined by 16 wk of age (Fig. 7E). The time course of increased SP IENF density is consistent with the period of mechanical allodynia in db/db mice. In comparison, there was no difference in the numbers of PGP9.5 positive IENF between db+ and db/db mice across all tested age groups (Fig. 7F).

Trk A phosphorylation during the period of mechanical allodynia

Most NGF actions are mediated by the Trk A receptor. Therefore, we further examined Trk A phosphorylation during the period of NGF upregulation and mechanical allodynia. Trk A phosphorylation at tyr-496 was tested using Trk A immunoprecipitation, followed by pTrk immunoblotting procedures (Fig. 8A). A significant increase of Trk A phosphorylation in L 4-6 DRG of db/db mice was detected at the age of 5-12 wk (Fig. 8B). Since the pTrk antibody is not completely specific for Trk A, double immunofluorescence studies with antibodies against pTrk and Trk A were performed to localize activated Trk A. There was no significant pTrk immunoreactivity detected in the DRG of db+ mice at 8 wk of
age (Fig. 8C). Trk A immunoreactivity was mostly detected in small- and medium-sized (Fig. 8D, arrows) and also some large-sized neurons (Fig. 8D, arrowhead). In the DRG of db/db mice of the same age, pTrk was detected in both large-sized (Fig. 8F, arrowheads) and small- to medium-sized DRG neurons (Fig. 8F, arrows). In the same section, Trk A immunoreactivity was detected mostly in small- to medium-sized (Fig. 8G arrows) and also some large-sized neurons (Fig. 8G, arrowheads). The merged pictures demonstrate colocalization of pTrk and Trk A immunoreactivity in these neurons (Fig. 8H, yellow).

**Anti-NGF decreases mechanical allodynia in db/db mouse**

To determine whether the enhanced NGF expression is responsible for the development of mechanical allodynia in db/db mice, we treated both db+ and db/db mice with control IgG or anti-NGF 10 mg/kg intraperitoneally once weekly at the ages of 6 and 7 wk, followed by behavior tests for mechanical allodynia and tissue collection at 8 wk of age. The mechanical threshold was measured, using both the up-down method (Fig. 9A) and the percentage of response method using a 1.4 g von Frey filament (Fig. 9B). As demonstrated in Fig. 9, mechanical allodynia in db/db mice at 8 wk of age was reversed by anti-NGF treatment, but not by IgG treatment, using both measurement methods. Anti-NGF had no effect on the mechanical thresholds of db+ mice (Fig. 9).

**Anti-NGF decreases SP expression in DRG of db/db mouse during mechanical allodynia**

We further examined the effects of anti-NGF treatment on enhanced SP expression in DRG neurons and IENF of hind paw skin at 8 wk of age. The enhanced SP gene expression in the DRG of db/db mice was decreased by anti-NGF treatment as
measured by RT-PCR. Meanwhile, the anti-NGF treatment did not affect the SP gene expression in db+ mice (Fig. 10A). The elevation of NGF gene expression in the DRG of db/db mice was significantly decreased by anti-NGF (Fig. 10B). Interestingly, anti-NGF treatment slightly promoted NGF gene expression in db+ mice (Fig. 10B). The percentage of SP-positive DRG neurons was significantly lower in anti-NGF-treated db/db mice than the IgG-treated db/db mice (Fig. 10C). In contrast, anti-NGF did not affect SP expression in db+ mice (Fig. 10C). In the hind paw skin, anti-NGF also significantly decreased the number of SP-positive, but not PGP9.5-positive, IENF in db/db mice (Fig. 10D).

Discussion

The current study uses a mouse model of type 2 diabetes, the db/db mouse, to study the molecular mechanisms underlying PDN of type 2 diabetes (29). We report that the db/db mouse develops mechanical allodynia as a feature of PDN at the early stage of diabetes. The mechanical allodynia is transient (between 8-12 wk of age) but correlates with the elevation of NGF and SP expression. We hypothesize that NGF-induced SP elevation is a key mechanism for the development of mechanical allodynia in this animal model.

In the current study, we detected the presence of mechanical allodynia at the early stage of diabetes in db/db mice. These findings conflict with a report from Wright et al., who detect hypoalgesia in these animals (39). They demonstrate that db/db mice develop higher mechanical thresholds starting at 4 wk of age by using a single von Frey (1g) filament and at 9 wk of age by using an aesthesiometer. Together, we believe there are two major reasons why the data from these two studies differ: 1) Wright et al. use both
male and female mice. Sex is a well-known factor that affects the results of pain behavior studies. Female steroid hormones have been reported to modulate NGF receptor and affect CGRP expression (40). In female animals, estrogen controls PKC epsilon-dependent mechanical hyperalgesia through direct action on nociceptive neurons (41). Thus, using mixed-gender mice could alter the results of pain behavior studies (42, 43). In Wright’s study, the ratio of female/male mice used in each pain behavior testing was not clarified. We believe our data are more reliable by using all male mice. 2) The methodology for their behavior testing is different from ours. We used the up-down method, which is widely used in the published pain studies (31). In contrast, Wright et al used a plantar aesthesiometer for measuring the mechanical thresholds. Although both methods have been used to measure mechanical thresholds, it is difficult to directly compare the results from the up-down method to data generated from a plantar aesthesiometer. While Wright et al did not detect a change in PGP9.5-positive IENF between db+ and db/db mice to correlate with their behavior findings, the current study provides evidence not only from behavioral testing but also from anatomical and biochemical analysis to indicate the presence of mechanical allodynia and its underlying mechanisms. Consistent with our results, other animal models of type 2 diabetes, including BBZDR/Wor rats (44), Zucker fatty rats (45), and ob/ob mice (46), exhibit similar patterns of pain behaviors.

The current study detected a transient increase in NGF expression in the DRG neurons and hind paw skin in db/db mice. This phenomenon coincides with the development of mechanical allodynia, suggesting that NGF could play an important role in mediating pain in type 2 diabetes. In animal models of nerve and spinal cord injury, NGF is expressed locally in the DRG neurons and is considered as an important contributor to
post-injury pain (11, 47, 48). Although NGF expression has also been reported in the satellite cells of DRG after nerve injury (49, 50), we did not detect glial NGF immunoreactivity in db/db mice. In the STZ model of type 1 diabetes, NGF gene expression in the sciatic nerve is upregulated 1.65-fold (51), and higher NGF levels are detected in the superior mesenteric and celiac ganglia (52). Elevated NGF levels could be involved in the early mechanical allodynia and thermal hyperalgesia reported in these animals (37). The mechanism for increased NGF expression in diabetes is unclear. Although the upregulation of NGF expression in DRG after axonal injury may be a compensatory local synthesis for the disruption of NGF supply from target tissues (50), we did not detect obvious axonal damage by electromyography and nerve conduction studies (unpublished data) or loss of PGP9.5-positive IENF at 8-16 wk of age (Fig. 7) to support this speculation. Here we postulate that increased NGF expression could be an initial protective mechanism of tissues in response to a variety of metabolic stressors in type 2 diabetes, including hyperglycemia (53), impaired insulin signaling (54), hyperlipidemia (55), excessive oxidative stress (56), enhanced cytokine expression (57) or by a combination of one or more of these conditions. Future studies focused on correcting each metabolic stressor will help elucidate the mechanism of elevated NGF expression in PDN.

Our findings suggest increased NGF expression in skin could also contribute to mechanical allodynia. Using specific cell markers, we determined that dermal fibroblasts, macrophages, mast cells, and T cells are strongly positive for NGF. These cell type are well-known in the literature to express NGF in a variety of conditions (58). These findings could be the results of two potential mechanisms: (1) Increased levels of peripheral NGF from the dermal cells could be taken up by Trk A-positive nerve fibers in the skin and
delivered in a retrograde manner to DRG neurons (14); (2) NGF secreted by peripheral nerve endings could trigger neurogenic inflammation by attracting inflammatory mediators, including mast cells and macrophages (59). We speculate that this retrograde transport of peripherally produced NGF could contribute to PDN by maintaining increased neuropeptide expression and mechanical allodynia while the NGF gene expression diminished in the DRG. Other evidence to support our hypothesis includes: [1] the db/db mouse has normal fast and slow axonal transport during the period of mechanical allodynia (60); [2] subcutaneous NGF administration enhances SP expression in DRG neurons (61). Further studies using subcutaneously administered labeled NGF to the hind paws followed by sciatic nerve ligation will help establish the role of axonal transport and dermal NGF production in the allodynia response.

Our findings also reveal decreased NGF and BDNF expression following the period of mechanical alldynia during the development of sensory loss in the db/db mouse. In the STZ model of type 1 diabetic neuropathy, NGF levels decrease as diabetes progress, a result of impaired axonal transport and/or DRG neuronal damage (62, 63). The long-term loss of NGF neurotrophism is considered a major cause of sensory loss in the diabetic neuropathy of type 1 diabetes (62). In late-stage type 1 diabetes, NGF treatment improves hypoalgesia after the development of sensory neuropathy in a type 1 diabetic model (64). At this stage of diabetic neuropathy, NGF treatment also enhances SP expression in DRG neurons and restores the myelinated nerve fiber morphology (65). We detected similar findings in db/db mice after 12 weeks of age, indicating that NGF exhaustion in DRG could be an important factor for the development of peripheral neuropathy of type 2 diabetes. In support of our results, Walwyn and colleagues delivered the NGF gene via a herpes
simplex viral vector to DRG and delayed the development of hypoalgesia in a leptin receptor mutant mouse (66). Collectively, our findings, along with other reports in the literature, suggest an important role of NGF to modulate nociception in diabetic neuropathy.

Our results demonstrate an increased number of SP-positive DRG neurons in the db/db mice during the period of mechanical allodynia. The increased number of SP immunopositive neurons could mediate enhanced pain behaviors in db/db mice by activating the post-synaptic NK1 receptors on secondary sensory neurons in the spinal cord dorsal horn. In the BBZDR/Wor rat, a model of type 2 diabetes, there are no significant differences in SP expression in DRG at 8 months of age, near the end of the period of pain behaviors (44). Unfortunately, there are no measurements at earlier stages of PDN in that study to compare to our findings. Many studies have suggested that SP levels change in PDN of type 1 diabetes. SP is upregulated in spinal dialysates in the STZ model of PDN (27). Intraperitoneal injection of RP 67580, a NK1 receptor antagonist, fails to interfere with PDN in STZ-treated mice (67). However, intrathecal RP 67580 is able to reverse the mechanical allodynia and thermal hyperalgesia in STZ rats, suggesting that SP actions during PDN occur in the central nervous system (68). These reports support our hypothesis that increased SP expression in DRG neurons could result in elevated levels of SP being released in the spinal cord dorsal horn to mediate mechanical allodynia.

Here, we show for the first time that Trk A phosphorylation is significantly increased during the period of PDN, and report that NGF is elevated in small- to medium-sized DRG neurons during PDN. The small- to medium-sized DRG neurons express Trk A and mediate elevated NGF action during PDN. It is well recognized that the DRG neurons supply the small fibers that are first affected in diabetic neuropathy (1). In these DRG
neurons, we report a concordance between elevated NGF and Trk A phosphorylation, suggesting that these neurons are responding to NGF. The elevated SP expression was also detected in the same populations of neurons, but later in the period of PDN, suggesting that activation of NGF/Trk A signaling events precedes and may lead to increased SP gene expression. NGF has been reported to enhance SP expression directly via both Trk A and p75 receptors. In cultured DRG neurons, NGF enhances SP expression (26). Both K-252a, a Trk A inhibitor, and a Trk A antibody block NGF effects on SP expression. These findings provide evidence to support a novel therapeutic strategy to treat PDN by inhibition of Trk A activation. The role of p75 in PDN is likely to enhance Trk A actions (15). However, future studies are needed to determine whether p75 is a suitable therapeutic target for PDN.

Our data indicated that anti-NGF significantly decreased mechanical allodynia and SP expression in DRG neurons and IENF. The efficacy of this antibody is reported in a nerve injury model (38). However, while 100 mg/kg anti-NGF is needed for blocking mechanical allodynia in a mouse chronic constriction injury (CCI) model, we discovered that 10 mg/kg of anti-NGF is sufficient to inhibit the mechanical allodynia in our model. In contrast to way in which only small- to medium- sized DRG are injured in our model, the CCI model creates advanced denervation and demyelination, affecting axons of all calibers within the injured nerve. It is thus consistent that a lower dose of anti-NGF is adequate to neutralize NGF-mediated pain behaviors in our model. The current findings suggest the degree of NGF-dependent mechanical allodynia varies among different pain models, as described by Wild et al. (38).
Since decreased NGF neurotrophism is a mechanism for sensory loss at a later stage of diabetic neuropathy (62), anti-NGF treatment could inhibit the mechanical allodynia by decreasing the number of DRG neurons and IENF and induce sensory neuropathy. This concern is answered by our findings that anti-NGF had no effect on the number of PGP9.5-positive IENF in either control or db/db mice. Our results indicate that anti-NGF specifically decreased SP-positive DRG neurons and IENF but did not initiate sensory neuropathy. In addition, we did not detect decreased numbers of DRG neurons after anti-NGF treatment (data not shown). Our current findings suggest a transient antagonism against NGF actions during the period of pain behaviors could be a reasonable mechanistic approach for treating PDN of type 2 diabetes. In contrast, long-term anti-NGF treatment would result in several potential problems. For example, anti-NGF treatment could neutralize the trophic effects of endogenous NGF on axonal regeneration and worsen peripheral neuropathy. In addition, anti-NGF treatment could induce immunological responses against NGF-positive neurons or peripheral nerve fibers. In support of our speculation, Sena et al. reported that high levels of auto-antibodies against NGF are detected in patients with leprosy-induced chronic neuritis (69). Finally, anti-NGF treatment could lower the levels of endogenous NGF in cerebrospinal fluid and worsen dementia in patients with Alzheimer’s disease, a common condition associates with type 2 diabetes (70, 71).

Collectively, our studies suggest that during the early stage of diabetes-induced nerve injury, NGF expression is elevated and mediates SP expression that underlies the development of transient mechanical allodynia. The current report indicates increased NGF expression in both hind paw skin and DRG neurons during this period of mechanical
allodynia. The enhancement of NGF expression is associated with phosphorylation of Trk A and increased SP expression; this NGF-induced SP elevation could be an important mechanism for the development of mechanical allodynia in type 2 diabetes.

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Figure legends:

Fig. 1. Blood Chemistry Studies. Measurements of (A) fasting blood glucose, (B) glycosylated hemoglobin (HbA1c), (C) fasting serum insulin and (D) body weight from db+ and db/db mice 5 - 21 wk of age. Data are mean ± SEM from 4 animals of each group (*, p < 0.05, compared with db+ of the same age).

Fig. 2. Mechanical Allodynia in db/db mice. Mechanical thresholds of db+ and db/db mice from age 5, 8, 12, 16 and 21 wk of age were assessed by a set of von Frey filaments. A: Fifty percent g thresholds were measured using the up-down method. Significantly lower mechanical thresholds were detected in db/db mice at 8 and 12 wk of age in comparison with db+ mice of the same age. At 16 wk of age, the mechanical alldynia diminished. Higher mechanical thresholds were detected in db/db mice at 21 wk of age. B: Mechanical sensitivity was tested by a 1.4 g von Frey filament with repetitive stimulation. Increased percentages of response were detected at 8 and 12 wk of age. Data are mean ± SEM from 4 animals of each group (*, p < 0.05; **, p< 0.01, compared with db+ of the same age).

Fig. 3. Increased NGF and SP gene expression in db/db mice during the period of mechanical allodynia. NGF (A) and SP (B) gene expression was measured by real time RT-PCR. A: NGF gene expression was upregulated at 5 and 8 wk of age in L 4-6 DRG of db/db mice. This upregulation of NGF expression diminished by 12 wk of age, and NGF gene expression fell below the control level at 16 wk of age. B: SP gene expression was enhanced at 8 and 12 wk of age in db/db mice. C: BDNF expression was not affected
during the period of mechanical allodynia but decreased at 16 wk of age in db/db mice. D: Increased CGRP gene expression was detected in db/db mice at 11 wk of age. Data are normalized to that of db+ mice of the same age as mean ± SEM from 4 animals of each group (*, p < 0.05, compared with db+ of the same age).

Fig. 4. Increased numbers of NGF- and SP-positive DRG neurons in db/db mice.

Representative confocal microscopic pictures of double immunofluorescent studies for NGF (A, db+; D, db/db: green), SP (B, db+; E, db/db: red), or merged (C, db+; F, db/db) in L 4-6 DRG at 8 wk of age. A: NGF immunoreactivity was detected in mostly large-sized (arrowhead) but not small- to medium-sized (arrows) DRG neurons in db+ mice. B: SP immunoreactivity was only in small- to medium-sized (arrows) but not large-sized (arrowhead) neurons in db+ mice. C: Merged picture did not demonstrate colocalization of NGF/SP in db+ mice. D: NGF expression was detected in large-sized (arrowhead) and also small- to medium-sized (arrows) DRG neurons in db/db mice. D: An increased number of SP-positive neurons with large- (arrowhead) and small- to medium-sized (arrows) neurons was detected in db/db mice. F: Merged picture demonstrates most SP-positive DRG neurons also expressed NGF in db/db mice (yellow, arrows and arrowhead). Bar = 50 μm.

Fig. 5. Quantification and cell-size distribution of NGF- and SP-positive DRG neurons. A: The percentages of NGF immunopositive neurons were significantly higher at 8 wk of age in db/db mice. B: Most of the increased NGF positive neurons are small- (< 20 μm in diameter) and medium-sized (20 – 30 μm in diameter). C: The percentages of SP-positive
neurons also increased at 8 wk of age and remained significantly higher in db/db than db+ mice at 12 wk of age. D: Compared to db+ mice, most of the increased SP expression was localized to small- to medium-sized DRG neurons. However, some large-sized neurons also were SP-positive in db/db but not the control mice. E: The percentages of IB4-positive and GDNF-responsive neurons were not different between db+ and db/db mice at 8 wk of age. F: Most IB4-positive DRG neurons were small- to medium-sized. Data are mean ± SEM from 4 animals of each group (*, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with db+ of the same age).

Fig. 6. Increased NGF expression in the hind paw skin of db/db mice. A: No significant NGF immunoreaction was detected in the hind paw skin of db+ mice. B: NGF immunoreactivity increases in the hind paw skin of db/db mice. C: High-power magnification of the window in B. The majority of NGF immunoreaction was detected in dermal cells (B and C: arrows). In addition, IENF are also positive for NGF in db/db mice (B and C: arrowheads). Bars = 100 μm.

Fig. 7. Increased SP-, but not PGP9.5-, positive IENF in db/db mice. Representative SP (A, C: red) and PGP9.5 (B, D: green) double immunofluorescence studies were performed in the hind paw skin of db+ (A, B) and db/db (C, D) mice at 8 wk of age. The SP-immunopositive IENF were labeled with asterisks (A, C), and PGP-positive IENF were labeled with white dots (B, D). Bar = 100 μm. E: The densities of SP-positive IENF from 4-16 wk of age were quantified. There was a significant increase of SP-positive IENF in db/db mice compared to db+ mice at 8 and 11 wk of age (E). In contrast, there was no
significant change of PGP9.5-positive IENF between db+ and db/db mice (F). Data are mean ± SEM from 4 animals of each group (*, p < 0.05; **, p < 0.01 compared with db+ of the same age).

Fig. 8. Phosphorylation of Trk A in L4-6 DRG of db/db mice during the period of mechanical allodynia. A: Representative result of pTrk immunoblotting following Trk A immunoprecipitation (IP). Increased Trk A phosphorylation was detected in the L4-6 DRG of 8-wk-old db/db mice, compared to that of the db+ mice at the same age. Trk A immunoblots demonstrated no difference in Trk A expression between db+ and db/db mice. B: Densitometry analysis of Trk A phosphorylation in L4-6 DRG from 4-12 wk of age. Increased Trk A phosphorylation in db/db mice was detected at 5, 8, 10 and 12 wk of age. Data are mean ± SEM from 4 animals of each group (*, p < 0.05, compared with db+ of the same age). C, D, E: pTrk (C), Trk A (D) and merged (E) immunohistochemistry on L4-6 DRG of db+ mice at 8 wk of age. There was no pTrk immunoreactivity detected. F, G, H: pTrk (F), Trk A (G) and merged (H) immunohistochemistry on L4-6 DRG of db/db mice at 8 wk of age. In db/db mice, pTrk immunoreactivity was detected in small- to medium- sized (arrows) and also large-sized (arrowheads) DRG neurons (F, red). The pTrk immunoreaction colocalized with that of Trk A (G, green), as demonstrated by the merged picture (H, yellow). Bar = 50 μm.

Fig. 9. The effects of anti-NGF on mechanical allodynia in db/db mice. Anti-NGF or control IgG 10 mg/kg was administered intraperitoneally at 6 and 7 wk of age. Mechanical thresholds were assessed by the up-down method (A) and percentage of response to a 1.4
g von Frey filament (B) at 8 wk of age. A: Anti-NGF, but not IgG, treatment significantly reversed mechanical allodynia in db/db mice. In contrast, anti-NGF did not affect the mechanical thresholds of db+ mice. B: Anti-NGF significantly decreased the percentages of response to a 1.4 g von Frey filament in db/db mice but not db+ mice. Data are mean ± SEM from 6 animals of each group. (**, p < 0.01)

Fig. 10. The effects of anti-NGF on gene and protein expression in L 4-6 DRG and IENF of hind paw skin. A: Anti-NGF significantly lowered the enhanced SP gene expression in db/db mice at 8 wk of age. B: Anti-NGF did not affect the NGF gene expression in db/db mice at 8 wk of age. There was a slight increase of NGF expression in db+ mice after anti-NGF treatment. C: Anti-NGF significantly decreased the percentages of SP-positive neurons in L4-6 DRG of db/db mice at 8 wk of age. Anti-NGF treatment had no effect on the percentages of SP-positive neurons in DRG of db+ mice. D: Anti-NGF decreased SP-positive IENF in db/db mice at 8 wk of age. In contrast, the PGP9.5-positive IENF was not affected by anti-NGF treatment. Data are mean ± SEM from 6 animals of each group (*, p < 0.05; ***, p< 0.001).