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

Hsinlin T. Cheng, MD, PhD, Jacqueline R. Dauch, BA, John M. Hayes, BA, Yu Hong, MS, and Eva L. Feldman, MD, PhD

Abstract
C57BLKS db/db (db/db) mice develop a neuropathy with features of human type 2 diabetic neuropathy. Here, we demonstrate that these mice develop transient mechanical allodynia at the early stage of diabetes. We hypothesized that nerve growth factor (NGF), which enhances the expression of key mediators of nociception (i.e., substance P [SP] and calcitonin gene-related peptide), contributes to the development of mechanical allodynia in these mice. We found that NGF, SP, and calcitonin gene-related peptide gene expression is upregulated in the dorsal root ganglion (DRG) of db/db mice before or during the period that they develop mechanical allodynia. There were more small- to medium-sized NGF-immunopositive DRG neurons in db/db mice than in control db+ mice; these neurons also expressed SP, consistent with its role in nociception. Nerve growth factor expression in the hind paw skin was also increased in a variety of dermal cell types and nerve fibers, suggesting the contribution of a peripheral source of NGF to mechanical allodynia. The upregulation of NGF coincided with enhanced tropomyosin-related kinase A receptor phosphorylation in the DRG. Finally, an antibody against NGF inhibited mechanical allodynia and decreased the numbers of SP-positive DRG neurons in db/db mice. These results suggest that inhibition of NGF action is a potential strategy for treating painful diabetic neuropathy.

Key Words: Db/db mice, Diabetic neuropathy, Dorsal root ganglion, Nerve growth factor, Pain, Substance P, Trk receptors.

INTRODUCTION
Painful diabetic neuropathy (PDN) is common in type 1 and type 2 diabetes and is an early manifestation of diabetic neuropathy that frequently precedes the clinical diagnosis of diabetes (1, 2). Nearly one third of patients with impaired glucose tolerance (i.e., prediabetes) seek medical attention for a pain syndrome identical to PDN (3). Estimates vary, but approximately half of all patients with diabetic neuropathy and type 2 diabetes experience PDN, usually at the disease onset. Painful diabetic neuropathy is a persistent symptom in patients with type 2 diabetes, but it is less frequently persistent in type 1 diabetes (4–7). Patients with PDN experience allodynia, that is, when normally nonpainful stimuli become painful, and hyperalgesia, that is, an increased sensitivity to normally painful stimuli. Painful diabetic neuropathy is a major factor in decreased quality of life for patients with diabetes (8, 9); it often subsides over time, sometimes several years. The disabling pain is replaced by a complete loss of sensation, leading to the numbness and insensitivity characteristic of the diabetic foot (2, 10).

Nociceptive dorsal root ganglion (DRG) neurons, which supply Aδ and C fibers, can be divided into peptidergic and nonpeptidergic groups. Peptidergic DRG neurons express neuropeptides, including substance P (SP) and calcitonin gene-related peptide (CGRP), which mediate nociception. The development of these neurons requires nerve growth factor (NGF), a member of 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). Tropomyosin-related kinase A is the high-affinity receptor for NGF and is expressed on peptidergic DRG neurons. Nerve growth factor is produced and released from target tissues; it 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 and are affected early in the course of diabetes (11, 12). 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, nonpeptidergic nociceptive neurons respond to glial cell-derived neurotrophic factor and express RET receptors (16, 17). These neurons have a high affinity to isolecitin B4 (IB4). It is unclear whether there are distinct roles for the peptidergic and nonpeptidergic neurons in the mediation of the various nociception modalities.

Substance P and CGRP are 2 of the most extensively studied neuropeptides that mediate nociception; SP is a tachykinin neuropeptide that 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 neurons and is released to the lamina I and outer layer of lamina II of the spinal cord dorsal horn to activate secondary sensory neurons (18, 20). The CGRP is also upregulated under painful conditions in a similar population of Trk A–positive sensory neurons (21).

Nerve growth factor is a major factor that enhances SP and CGRP expression (22). Exogenous exposure to high levels of NGF increases the intracellular content and release of SP and CGRP both in vitro and in vivo (23–26). Because the development of neuropathic pain after diabetes may be accompanied by alterations in the level or distribution of these neuropeptides (27), NGF may mediate PDN via upregulation of SP and CGRP. Understanding the role that NGF plays in nociceptive peptide expression would set the stage for uncovering the mechanisms underlying the induction and modulation of neuropathic pain in type 2 diabetes.

The C57BLKS db/db (db/db) mouse carries a leptin receptor null mutation and is a well-characterized animal model of type 2 diabetes (28, 29). These mice develop obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia. Diabetic phenotypes are present at 4 to 5 weeks of age, and diabetic neuropathy is evident at 24 weeks of age (30). Here, we first determined the presence of mechanical allodynia in the db/db mouse at 8 to 12 weeks of age. We then used this window of increased nociception to study the roles of NGF and Trk A in PDN. We hypothesize that enhanced NGF-mediated Trk A activation during mechanical allodynia increases the expression of SP in DRG neurons and is a molecular mechanism for PDN in type 2 diabetes.

**MATERIALS AND METHODS**

**Animals**

Male C57BLKS db/db (stock no. 000662) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Homozygous (Leprdb/Leprdb [db/db]) mice were used as a model of type 2 diabetes; heterozygous mice (Leprdb/+ [db+]) served as nondiabetic controls. Analyses and procedures were performed in compliance with protocols established by the Animal Models of Diabetic Complications Consortium (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. Beginning at 4 weeks of age, 1 drop of tail blood was analyzed using a standard glucometer (One Touch Profile, LIFESCAN, Inc, Milpitas, CA); this was repeated weekly to document the progression of diabetes. Glycosylated hemoglobin (HbA1c) level was measured using the Touch Profile, LIFESCAN, Inc, Milpitas, CA). Briefly, 5× 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 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. Polymerase chain reaction (PCR) was performed as previously described (34) using the primer sequences: NGF sense 5′-CC AAGGACGCAGCTTTCTAT-3′, NGF antisense 5′-CTC CGGTGACTTCTGTTGAA-3′, SP sense 5′-ATGGCCAG ATCTCTCACA AAAAG-3′, SP antisense 5′-AAGATGGAAT AGATA GTGCCTGTCAGG-3′, brain-derived neurotrophic factor (BDNF) sense 5′-AGGCCA ACTGCA AAGCTT GAT-3′, BDNF antisense 5′-CCGA ACTGCA ATGGTTGCA GACTCT GC-3′, CGRP sense 5′-TGGTTCAGCTCAGA GGTCGGC-3′, CGRP antisense 5′-tctgatagtctcagac-3′, glyceraldehyde 3-phosphate dehydrogenase sense 5′-TCCAGC AACTTTGG CATGTCGG-3′, and glyceraldehyde 3-phosphate dehydrogenase antisense 5′-GTGC GTGGTGAAGTCACAGGAC-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 1× SYBR Green iCycler iQ mixture (BioRad), 0.2 μmol/L of each forward and reverse primer, and cDNA preparation to 25 μL total volume. The PCR amplification profile was 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 30 seconds, followed by 72°C for 5 minutes. 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 seconds with +0.5°C per step increment 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 glyceraldehyde 3-phosphate dehydrogenase. A nontemplate control was run with every assay.

**Immunoprecipitation and Immunoblots**

After deep anesthesia, L4 to L6 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 μmol/L sodium orthovanadate and 1 μmol/L sodium fluoride; Sigma Life Science, St Louis, MO). Lysates were sonicated for 5 seconds and centrifuged and processed for protein concentration using DC Protein Assay Reagents (BioRad). Aliquots (200 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 Biotechnology) and rinsed 3 times before the precipitates were boiled in 2× sample buffer, separated on a sodium dodecyl sulfate-polyacrylamide electrophoresis gel and transferred to a polyvinylidene fluoride membrane. Membranes were blocked and incubated overnight at 4°C with a goat polyclonal antibody.

**FIGURE 1.** Blood chemistry and body weight analyses. Measurements of fasting blood glucose (A), glycosylated hemoglobin (HbA1c) level (B), fasting serum insulin (C), and body weight (D) of db+ and db/db mice at 5 to 21 weeks of age. Data are mean ± SEM from 4 mice per group (*p < 0.05 compared with db+ mice of the same age).
against phosphorylated Trk A at tyrosine 496 (pTrk, 1:1000; Santa Cruz Biotechnology) or total Trk A (1:1000; Santa Cruz Biotechnology) for measuring the levels of phosphorylated or total protein, respectively. Membranes were then rinsed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at 25°C and processed with chemiluminescence substrate (Pierce) before being exposed to Hyperfilm (Amersham, Piscataway, NJ). Densitometry was performed using ImageJ software, and the results were normalized against Trk A densities from the same sample.

**Immunohistochemistry**

The db+ and db/db mice (n = 4 per group) were deeply anesthetized and perfused with 2% paraformaldehyde in PBS (pH 7.2, 0.1 mol/L). L4 to L6 DRG were dissected and postfixed by immersion in 2% paraformaldehyde overnight at 4°C, rinsed in graded sucrose solutions (5%–30% in PBS), embedded in mounting media (OCT), and flash-frozen in liquid nitrogen. Before perfusion, hind foot pads were collected from the plantar surface of the hind paw, immersed overnight at 4°C in Zamboni fixative (2% paraformaldehyde, 0.2% picric acid in 0.1 mol/L phosphate buffer), rinsed in graded sucrose solutions (5%–30% in PBS), and cryosectioned in mounting media (OCT). Tissue sections (10 μm) were cut and mounted onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at −80°C. For immunolocalization, tissue sections were thawed on a warming plate (55°C for 10 minutes), hydrated with PBS, and blocked in 0.1% TX100 and 5% nonfat dry milk in PBS. Sections were then incubated at room temperature for 16 to 24 hours with antibody against NGF (rabbit polyclonal, 1:500; Santa Cruz Biotechnology), and/or SP (rat polyclonal, 1:500; Abcam, Inc, Cambridge, MA), pTrk (1:100; Santa Cruz Biotechnology), or Trk A (1:500; Santa Cruz Biotechnology). For specific cell-type studies in the foot pads, antibodies for cell markers of macrophages (CD68, rat polyclonal, 1:1000; Abcam, Inc), fibroblasts (type 1 collagen, goat polyclonal, 1:1000; Abcam, Inc), mast cells (mast cell tryptase, goat polyclonal, 1:1000; Santa Cruz Biotechnology), and T cells (CD4 and CD8, rat monoclonal, 1:100; Santa Cruz Biotechnology) were used in conjunction with a rabbit anti-NGF antibody to identify the cell types of NGF-immunopositive cells in the dermis. 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 IB4 labeling studies, the sections were incubated with AlexaFluor 594-labeled *Griffonia simplicifolia* IB4 (1:500; Invitrogen) in PBS with 5% milk for 1 hour. Sections were rinsed and mounted in ProLong Gold antifade reagent (Invitrogen). To ensure specificity, sections were incubated with primary or secondary antiserum alone to confirm there were no nonspecific immunoreactions. Fluorescent signals were examined using an Olympus FluoView 500 laser scanning confocal microscope.

Percentages of immunopositive cells were determined by counting the number of immunopositive neurons and multiplying by 100 divided by the 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 mouse. 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 10th section was picked from a series of consecutive DRG sections (10 μm), and 3 to 4 sections were counted for each DRG and expressed as the percentage of total neuronal profile measured by neuron-specific enolase (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.

![FIGURE 2. Mechanical allodynia in db/db mice. (A) Mechanical thresholds of db+ and db/db mice at ages 5, 6, 8, 12, 16, and 21 weeks were assessed by a set of von Frey filaments; 50% g thresholds were measured using the up-down method. Significantly lower mechanical thresholds were detected in db/db mice at 6 to 12 weeks of age compared with db+ mice of the same age. Mechanical allodynia peaked at 8 weeks of age (1-way analysis of variance, p < 0.05 compared with 6- and 12-week data); mechanical allodynia diminished at 16 weeks of age. Higher mechanical thresholds were detected in db/db mice at 21 weeks of age. (B) Mechanical sensitivity was tested by a 1.4-g von Frey filament with repetitive stimulation in mice with 5, 6, 8, 12, 16, and 21 weeks of age. Increased percentages of response were detected at 6 to 12 weeks of age. Data are mean ± SEM from 4 mice per group (*p < 0.05, **p < 0.01 compared with db+ of the same age).](image-url)
**Anti-NGF Treatment**

To inhibit NGF action during allodynia, we administered anti-NGF (10 mg/kg, mouse monoclonal antibody clone AS21; Exalpha Biologicals, Maynard, MA) or control IgG intraperitoneally once weekly beginning at 6 and 7 weeks of age for 2 weeks (36). Mechanical thresholds were measured at 8 weeks of age, and L4 to L6 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 U test. Statistical comparisons between different age groups were made by 1-way analysis of variance followed by a post hoc Tukey multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

**RESULTS**

**Db/db Mouse Develops Features of Type 2 Diabetes**

Serum levels of fasting glucose, HbA1c, and insulin, and body weight were measured in db/db and db+ mice from 5 to 21 weeks of age to monitor the development of type 2 diabetes. There were significantly higher levels of fasting glucose, HbA1c, and insulin, and increased body weight in db/db mice compared to db+ mice.

**FIGURE 3.** Increased nerve growth factor (NGF) and substance P (SP) gene expression measured by real-time polymerase chain reaction in db/db mice during mechanical allodynia. (A) Nerve growth factor gene expression was upregulated at 5 and 8 weeks of age in L4 to L6 DRG of db/db mice. This upregulation of NGF expression diminished by 12 weeks of age, and NGF gene expression fell below the control level at 16 weeks of age. (B) Substance P gene expression was enhanced at 8 and 12 weeks of age in db/db mice. (C) Brain-derived neurotrophic factor (BDNF) expression was not affected during mechanical allodynia but decreased at 16 weeks and 21 weeks of age in db/db mice. (D) Increased calcitonin gene-related peptide (CGRP) gene expression was detected in db/db mice at 11 weeks of age. Data are normalized to that of db+ mice of the same age as mean ± SEM from 4 mice per group (*$p < 0.05$, **$p < 0.001$ compared with db+ of the same age).
compared with db+ mice (Fig. 1). The fasting glucose level reached 400 mg/dL at 5 weeks and was persistently elevated through 21 weeks. Levels of HbA1c progressively increased from 5 weeks through 21 weeks, indicating a chronic state of hyperglycemia (Fig. 1B). Fasting insulin levels were also significantly elevated at 5 weeks, persisting to 21 weeks (Fig. 1C), which, in combination with hyperglycemia, suggests that the db/db mice were insulin resistant. In addition, the body weight was also significantly higher in db/db mice beginning at 5 weeks and thereafter in the period tested (Fig. 1D). By 21 weeks, the weights of db/db mice were almost twice those 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 weeks of age and persists through 21 weeks of age.

Db/db Mouse Develops Mechanical Allodynia at the Early Stage of Diabetes

Because db/db mice exhibit hallmark signs of type 2 diabetes, we hypothesized that they would exhibit behaviors indicative of PDN. The nociceptive threshold was significantly lower for db/db mice beginning at 6 weeks of age, suggesting increased sensitivity to mechanical stimuli or mechanical allodynia (Fig. 2A). The mechanical allodynia peaked at 8 weeks of age and continued until 16 weeks when the difference between db+ and db/db mice became insignificant. At 21 weeks of age, the mechanical pain threshold increased in db/db mice, indicating the presence of sensory neuropathy. Similarly, a significantly increased percentage of response to a 1.4-g von Frey filament was detected in db/db mice at 6 to 12 weeks of age compared with db+ controls (Fig. 2B). These results suggest the presence of mechanical allodynia during 6 to 12 weeks of age in db/db mice.

Increased Gene Expression at Early Stages of Diabetes

Increased NGF gene expression was detected in db/db mice compared with db+ mice of the same age at 5 and 8 weeks of age (Fig. 4). The percentages of NGF-immunopositive neurons were significantly higher at 8 weeks of age in db/db mice. The percentages of SP-positive neurons also increased at 8 weeks of age and remained significantly higher in db/db than in db+ mice at 12 weeks of age. Compared with db+ mice, most of the increased SP expression was localized to small- to medium-sized DRG neurons, but some large neurons are also SP positive in db/db but not in db+ mice. The percentages of isolectin B4 (IB4)-labeled (glial cell-derived neurotrophic factor-responsive) neurons were not different between db+ and db/db mice at 8 weeks of age. Most IB4-labeled DRG neurons were small to medium sized. Data are mean ± SEM from 4 mice per group (*p < 0.05, **p < 0.01, ***p < 0.001 compared with db+ of the same age).
8 weeks of age. At 12 weeks, the NGF gene expression returned to the control levels, and at 16 weeks, NGF expression decreased to below the control levels (Fig. 3A). The gene expression of SP became elevated at 8 weeks, peaked at 12 weeks, and returned to the levels of db+ at 16 weeks (Fig. 3B). Unlike NGF, BDNF expression was not significantly affected in DRG of db/db mice compared with db+ mice of the same age until 16 weeks and 21 weeks of
age when its level dropped below the control level (Fig. 3C). Similar to SP, CGRP gene expression was upregulated during mechanical allodynia but at a later time point (11 weeks; 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 mechanical allodynia in db/db mice. The temporal pattern of SP expression, however, is more closely related to the course of mechanical allodynia (Figs. 2 and 3).

Increased NGF- and SP-Positive Neurons During Mechanical Allodynia

In 8-week-old db+ mice, NGF immunoreactivity was detected mostly in large (Fig. 4A, arrowheads) but not small- to medium-sized DRG neurons (Fig. 4A, arrows). Nerve growth factor-positive DRG neurons did not express SP, which was mostly expressed in small- to medium-sized neurons that mediate nociception (Fig. 4B, arrows). A clear distinction of NGF- and SP-positive neurons was evident in the merged images (Fig. 4C). In contrast, both small- to medium-sized and large DRG neurons in db/db mice expressed NGF at 8 weeks of age (Fig. 4D, arrows and arrowheads). Most NGF-positive DRG neurons also expressed SP (Fig. 4F, arrows and arrowhead).

At 8 weeks 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 (Figs. 5A, C). The percentages of NGF-positive neurons in db/db mice increased 3-fold over db+ mice at 8 weeks of age (Fig. 5A). This upregulation of NGF expression diminished by 12 weeks of age. At 8 weeks, most NGF-positive neurons in db+ mice were large (>30 μm in diameter) (Fig. 5B). Most 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 weeks, corresponding to the pattern of SP gene expression (Fig. 5C). Although most SP-positive neurons were small- to medium-sized in both db+ and db/db mice (Fig. 5C), there were large neurons that did express SP in db/db mice (Fig. 5C).

At 8 weeks of age, there was no statistical difference between the percentages of IB4-labeled DRG neurons in db+ and db/db mice (Fig. 5E). The IB4-labeled DRG neurons were small to medium sized in both groups (Fig. 5F).

**FIGURE 6.** Increased nerve growth factor (NGF) expression in the hind paw skin of db/db mice. (A) No significant NGF immunoreactivity was detected in the hind paw skin of db+ mice. (B) Nerve growth factor immunoreactivity increases in the hind paw skin of db/db mice. (C) High-power magnification of the box in (B). Most NGF immunoreactivity is in dermal cells ([B, C] arrows). Intraepidermal nerve fibers are also positive for NGF in db/db mice ([B, C] arrowheads). Scale bars = 100 μm.
Increased NGF Immunoreactivity in Hind Paw Skin During Mechanical Allodynia

Because 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 immunoreactivity in the hind paw skin, a target tissue of L4 to L6 DRG. Nerve growth factor immunohistochemistry was performed on sections of hind paws of the mice at the age of 8 weeks, with no significant NGF expression detected in the hind paw skin of db+ mice (Fig. 6A). In contrast, NGF immunoreactivity was detected strongly in dermal cells (Figs. 6B, C, 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). Intraepidermal nerve fibers were also positive for NGF (Figs. 6B, C, arrowheads) in db/db mice.

**FIGURE 7.** Phosphorylation of tropomyosin-related kinase A (Trk A) in L4 to L6 dorsal root ganglia (DRG) of db/db mice during mechanical allodynia. (A) Representative pTrk immunoblot after Trk A immunoprecipitation (IP). There is increased Trk A phosphorylation in the L4 to L6 DRG of 8-week-old db/db mice compared with that of db+ mice of the same age. Tropomyosin-related kinase A immunoblots demonstrated no difference in Trk A expression between db+ and db/db mice. (B) Densitometry analysis of Trk A phosphorylation in L4 to L6 DRG from 4 to 12 weeks of age. Increased Trk A phosphorylation in db/db mice was detected at 5, 8, 10, and 12 weeks of age. Data are mean ± SEM from 4 mice per group (*p < 0.05 compared with db+ of the same age). (C, D, E) The pTrk (C), Trk A (D), and merged (E) immunohistochemistry (IHC) on L4 to L6 DRG of db+ mice at 8 weeks of age. No pTrk immunoreactivity was detected. (F, G, H) The pTrk (F), Trk A (G), and merged (H) IHC on L4 to L6 DRG of db/db mice at 8 weeks of age. The pTrk immunoreactivity in db/db mice was detected in small- to medium-sized (arrows) and also large (arrowheads) DRG neurons ([F] red). The pTrk immunoreactivity colocalized with that of Trk A ([G] green) in the merged image ([H] yellow). Scale bar = 50 μm.
**Trk A Phosphorylation During Mechanical Allodynia**

Because most NGF actions are mediated by the Trk A receptor, we examined Trk A phosphorylation during NGF upregulation and mechanical allodynia. Tropomyosin-related kinase A phosphorylation at tyr-496 was tested using Trk A immunoprecipitation, followed by pTrk immunoblotting procedures (Fig. 7A). There was a significant increase of Trk A phosphorylation in L4 to L6 DRG of db/db mice detected at ages 5 to 12 weeks (Fig. 7B). Because 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 weeks of age (Fig. 7C). Tropomyosin-related kinase A immunoreactivity was mostly detected in small- and medium-sized (Fig. 7D, arrows) and also some large neurons (Fig. 7D, arrowhead). In the DRG of db/db mice of the same age, pTrk was detected in both large (Fig. 7F, arrowheads) and small- to medium-sized DRG neurons (Fig. 7F, arrows). In the same section, Trk A immunoreactivity was detected mostly in small- to medium-sized (Fig. 7G arrows) and also some large-sized neurons (Fig. 7G, arrowheads). Merged images demonstrate colocalization of pTrk and Trk A immunoreactivity in these neurons (Fig. 7H, yellow).

**Anti-NGF Treatment Decreases Mechanical Allodynia in db/db Mouse**

The mechanical threshold in anti-NGF and control-treated db/db and db+ mice was measured using both the up-down method (Fig. 8A) and the percentage of response method using a 1.4-g von Frey filament (Fig. 8B). Mechanical allodynia in db/db mice at 8 weeks 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. 8).

**Anti-NGF Decreases DRG Neuron SP Expression in db/db Mice During Mechanical Allodynia**

The enhanced SP gene expression in the DRG of db/db mice at 8 weeks of age was decreased by anti-NGF treatment, as measured by reverse transcription–PCR, whereas treatment did not affect SP gene expression in db+ mice (Fig. 9A). Interestingly, anti-NGF treatment slightly promoted NGF gene expression in db+ mice but did not significantly affect that of db/db mice (Fig. 9B). 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. 9C). In contrast, anti-NGF did not affect SP expression in db+ mice (Fig. 9C).

**DISCUSSION**

We used a mouse model to study the molecular mechanisms underlying PDN of type 2 diabetes. We found that db/db mice develop transient (i.e. between 6 and 12 weeks of age) mechanical allodynia that correlates with elevation of NGF and SP expression as a feature of PDN at the early stage of diabetes. We hypothesize that NGF-induced SP elevation is a key mechanism for the development of mechanical allodynia in this model.

Our findings conflict with a report from Wright et al (37), who detected hypoalgesia in these animals. They demonstrate that db/db mice develop higher mechanical thresholds starting at 4 weeks of age by using a single von Frey (1 g) filament and at 9 weeks of age by using an esthesiometer. We believe that there are 2 major reasons why the data from these studies differ. First, Wright et al (37) use both male and female mice. Female steroid hormones have been reported to modulate NGF receptors and affect CGRP expression (38). In that study, the ratio of female-male mice used in each pain behavior testing was not presented, whereas our data were all collected in male mice. Second, the methodology for behavior testing differs in the 2 studies. We used the up-down method (31),
whereas Wright et al (37) used a plantar esthesiometer for measuring mechanical thresholds. Although both methods are valid, it is difficult to directly compare the results from the up-down method with data generated from a plantar esthesiometer. Our study provides evidence not only from behavioral testing, but also from biochemical analysis that indicates the presence of mechanical allodynia and its underlying mechanisms. Consistent with our results, other animal models of type 2 diabetes, including BBZDR/Wor rats (39), Zucker fatty rats (40), and ob/ob mice (41), exhibit similar pain behavior patterns. Although both methods are valid, it is difficult to directly compare the results from the up-down method with data generated from a plantar esthesiometer. Our study provides evidence not only from behavioral testing, but also from biochemical analysis that indicates the presence of mechanical allodynia and its underlying mechanisms. Consistent with our results, other animal models of type 2 diabetes, including BBZDR/Wor rats (39), Zucker fatty rats (40), and ob/ob mice (41), exhibit similar pain behavior patterns. Although there are variations for the clinical course of PDN in patients with type 2 diabetes, our findings suggest that db/db mice could model a subgroup of patients who develop mechanical allodynia before the development of evident sensory neuropathy.

We detected a transient increase in NGF immunoreactivity in the DRG neurons and hind paw skin in db/db mice. This phenomenon coincides with the development of mechanical allodynia and suggests 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 to be an important contributor to postinjury pain (11, 42, 43). Although NGF expression has also been reported in the satellite cells of DRG after nerve injury (44, 45), we did not detect glial NGF immunoreactivity. In the streptozotocin (STZ) model of type 1 diabetes, NGF gene expression in the sciatic nerve is upregulated 1.65-fold (46), and higher NGF levels are detected in the superior mesenteric and celiac ganglia (47). Elevated NGF levels could be involved in the early mechanical allodynia and thermal hyperalgesia reported in these animals (48). 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 (45), we did not detect obvious axonal

**FIGURE 9.** Effects of anti-nerve growth factor (NGF) on gene and protein expression in L4 to L6 DRG and intraepidermal nerve fibers of hind paw skin. (A) Anti-NGF significantly lowered the enhanced substance P (SP) gene expression in db/db mice at 8 weeks of age. (B) Anti-NGF did not affect the NGF gene expression in db/db mice at 8 weeks 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 DRG of db+/db mice. Data are mean ± SEM from 6 mice per group (*p < 0.05, ***p < 0.001). RT-PCR, reverse transcription-polymerase chain reaction.
damage by electromyography and nerve conduction studies (unpublished data) or loss of PGP9.5-positive intraepidermal nerve fibers at 8 to 16 weeks of age (Fig. 7) to support this speculation. We postulate that increased NGF expression could be an initial protective mechanism of tissues in response to a variety of metabolic stressors, including hyperglycemia (49), impaired insulin signaling (50), hyperlipidemia (51), excessive oxidative stress (52), enhanced cytokine expression (53), or by a combination of these conditions.

Our findings suggest that increased NGF expression in skin could also contribute to mechanical allodynia. We determined that dermal fibroblasts, macrophages, mast cells, and T cells are strongly positive for NGF; these cell types are known to express NGF in a variety of conditions (54). These findings could be the result of 2 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); and 2) NGF secreted by peripheral nerve endings could trigger neurogenic inflammation by attracting inflammatory mediators, including mast cells and macrophages (55). We speculate that retrograde transport of peripherally produced NGF could contribute to PDN by maintaining increased neuropeptide expression and mechanical allodynia as the NGF gene expression diminished in the DRG. Other evidences supporting this interpretation are that the db/db mouse has normal fast and slow axonal transport during mechanical allodynia (56) and that subcutaneous NGF administration enhances SP expression in DRG neurons (57). 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.

We also found decreased NGF and BDNF expression after the period of mechanical allodynia when db/db mice develop sensory loss. In the STZ model of type 1 diabetic neuropathy, NGF levels decrease as diabetes progresses, a result of impaired axonal transport and/or DRG neuronal damage (58, 59). The long-term loss of NGF neurotropism is considered to be a major cause of sensory loss in this diabetic neuropathy (58). In the late stage of a type 1 diabetes model, NGF treatment improves hypoalgesia after the development of sensory neuropathy (60). At this stage, NGF treatment also enhances SP expression in DRG neurons and restores the myelinated nerve fiber morphology (61). We made similar observations 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 this, Walwyn et al (62) delivered the NGF gene via a herpes simplex viral vector to DRG and delayed the development of hypoalgesia in a leptin receptor mutant mouse. Our findings, along with other reports in the literature, suggest an important role of NGF for modulating nociception in diabetic neuropathy.

Increased numbers of SP-immunopositive neurons could mediate enhanced pain behaviors in db/db mice by activating the postsynaptic neurokinin 1 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 (39). Unfortunately, there are no measurements at earlier stages of PDN in that study to compare with our present findings, but many studies have suggested that SP levels change in PDN of type 1 diabetes. Intraperitoneal injection of RP 67580, a neurokinin 1 receptor antagonist, fails to interfere with PDN in STZ-treated mice (63). Intrathecal RP 67580, however, reverses mechanical allodynia and thermal hyperalgesia in STZ rats, suggesting that SP actions during PDN occur in the central nervous system (64). These reports support our contention 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. In addition to SP, several NGF-dependent nociceptive molecules have also been reported in PDN of type 1 diabetic models, including P2X receptors (65, 66) and transient receptor potential receptors (67), but the roles of these molecules in PDN of type 2 diabetes are still unclear.

The expression of SP (a marker for small- to medium-sized neurons that provide Aδ and C fibers) in large DRG neurons (which provide Aβ fibers in db/db mice indicates neuronal plasticity after painful insults and has been described in other models of nerve injury (19, 68). The same findings are supported by Noguchi et al (69), who demonstrated SP expression in large DRG neurons after axotomy. The NF200-positive large DRG neurons supply Aβ fibers to mediate proprioception but do not mediate nociception under normal conditions. The involvement of Aβ fibers in hyperalgesia was also demonstrated in STZ-treated mice (70), but this mechanism is not a Trk A–dependent event because most large DRG neurons do not express Trk A. Alternative mechanisms for SP expression in large DRG neurons are proposed by Minichiello et al (71), who report that SP expression is detected in large neurons expressing Trk B or Trk C in Trk A–knockout mice, indicating that BDNF or neurotrophin-3 could contribute to an elevated SP level in large-sized DRG neurons.

We show for the first time that Trk A phosphorylation is significantly increased before and during PDN and report that NGF is elevated in small- to medium-sized DRG neurons during PDN. Because DRG neurons supply the small fibers that are first affected in diabetic neuropathy (1), the concordance between elevated NGF and Trk A phosphorylation in DRG neurons suggests that they are responding to NGF. The elevated SP expression was also detected in the same populations of neurons, although later suggesting that activation of NGF/Trk A signaling events precedes and may lead to increased SP gene expression. Indeed, NGF has been reported to directly enhance SP expression via both Trk A and p75 receptors. In cultured DRG neurons, NGF enhances SP expression (26), and both K-252a, a Trk A inhibitor, and a Trk A antibody block NGF effects on SP expression. These findings suggest 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), but it is not known whether p75 is a suitable therapeutic target for PDN.

We found increased NGF/Trk A signaling at 5 to 12 weeks of age but enhanced SP expression and mechanical...
allodynia at 6 to 12 weeks of age. This time lag suggests 2 possibilities. First, although elevated NGF gene expression occurs early, NGF protein levels might not be sufficient to increase SP gene expression until a later stage. Second, there are multiple downstream mechanisms that may mediate NGF-induced SP expression. The potential mediators include mitogen-activated protein kinases (72), protein kinase C, and/or calcium-calmodulin–dependent protein kinase II (73).

Our data also indicate that anti-NGF antibody significantly decreased mechanical allodynia and SP expression in DRG neurons and intraepidermal nerve fibers. The efficacy of this antibody has been reported in a nerve injury model (36), but whereas 100 mg/kg anti-NGF is needed for blocking mechanical allodynia in a mouse chronic constriction injury model, 10 mg/kg of anti-NGF was sufficient to inhibit the mechanical allodynia in our model. In contrast to the way in which only small- to medium-sized DRG are injured in our model, the chronic constriction injury model creates advanced denervation and demyelination, affecting axons of all calibers within the injured nerve. Thus, it is not surprising that a lower dose of anti-NGF is adequate to neutralize NGF-mediated pain behaviors in our model. The current findings suggest that the degree of NGF-dependent mechanical allodynia varies among different pain models, as described by Wild et al (36).

Because decreased NGF neurotropism is a mechanism for sensory loss at a later stage of diabetic neuropathy (58), anti-NGF treatment could inhibit mechanical allodynia by decreasing the number of DRG neurons to induce sensory neuropathy. We did not observe evidence of sensory neuropathy, however. Our findings suggest that 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 likely not be feasible. 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 this speculation, Sena et al (74) reported that high levels of autoantibodies against NGF are detected in patients with leprosy-induced chronic neuritis. Finally, anti-NGF treatment could lower the levels of endogenous NGF in cerebrospinal fluid and worsen dementia in patients with Alzheimer disease, a common condition associated with type 2 diabetes (75, 76).

In summary, our studies suggest that during the early stage of diabetes-induced nerve injury, NGF expression is elevated in both hind paw skin and DRG neurons and mediates SP expression that underlies the development of transient mechanical allodynia in an animal model of PDN. 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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