Diabetic neuropathy is a common form of peripheral neuropathy, yet the mechanisms responsible for pain in this disease are poorly understood. Alterations in the expression and function of voltage-gated tetrodotoxin-resistant (TTX-R) sodium channels have been implicated in animal models of neuropathic pain, including models of diabetic neuropathy. We investigated the expression and function of TTX-sensitive (TTX-S) and TTX-R sodium channels in dorsal root ganglion (DRG) neurons and the responses to thermal hyperalgesia and mechanical allodynia in streptozotocin-treated rats between 4–8 weeks after onset of diabetes. Diabetic rats demonstrated a significant reduction in the threshold for escape from innocuous mechanical pressure (allodynia) and a reduction in the latency to withdrawal from a noxious thermal stimulus (hyperalgesia). Both TTX-S and TTX-R sodium currents increased significantly in small DRG neurons isolated from diabetic rats. The voltage-dependent activation and steady-state inactivation curves for these currents were shifted negatively. TTX-S currents induced by fast or slow voltage ramps increased markedly in neurons from diabetic rats. Immunoblots and immunofluorescence staining demonstrated significant increases in the expression of Na\textsubscript{v}1.3 (TTX-S) and Na\textsubscript{v}1.7 (TTX-S) and decreases in the expression of Na\textsubscript{v}1.6 (TTX-S) and Na\textsubscript{v}1.8 (TTX-R) in diabetic rats. The level of serine/threonine phosphorylation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 increased in response to diabetes. In addition, increased tyrosine phosphorylation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7 was observed in DRGs from diabetic rats. These results suggest that both TTX-S and TTX-R sodium channels play important roles and that differential phosphorylation of sodium channels involving both serine/threonine and tyrosine sites contributes to painful diabetic neuropathy.

Diabetes mellitus is one of the most common chronic medical problems, affecting over 100 million people worldwide (1). Diabetic patients frequently exhibit one or more types of stimuli-evoked pain, including increased responsiveness to noxious stimuli (hyperalgesia) as well as hyper-responsiveness to normally innocuous stimuli (allodynia). The underlying mechanisms of persistent pain in diabetic patients remain poorly understood. In animal models of diabetes, hyperalgesia to nonnoxious thermal stimulation as well as tactile allodynia have been observed (2–4). The streptozotocin (STZ)-induced diabetic rat model demonstrates many of the abnormalities observed in humans (5). Treatment with insulin prevents development or reverses many of the abnormalities observed in early painful diabetic neuropathy (6, 7).

In diabetic rats with hyperalgesia, dorsal root ganglion (DRG) neurons display increased frequency of action potential generation in response to sustained suprathreshold mechanical stimulation (3, 4, 8–10) and increased spontaneous activity (11). Both effects are thought to contribute to the sensation of pain. Voltage-gated sodium channels generate and propagate action potentials in excitable cells. Based on differential sensitivity to tetrodotoxin (TTX), sodium currents in DRG neurons are classified into TTX-sensitive (TTX-S) and TTX-resistant (TTX-R) components (12–14). At least two TTX-S sodium channel α-subunits, Na\textsubscript{v}1.6, and Na\textsubscript{v}1.7, are constitutively expressed in the peripheral nervous system (15). In addition, Na\textsubscript{v}1.3, a TTX-S sodium channel that is normally expressed during embryonic development, is up-regulated in the peripheral nervous system following nerve injury (16). Two TTX-R sodium channels, Na\textsubscript{v}1.8 (17) and Na\textsubscript{v}1.9 (18, 19), have been identified in DRG neurons and changes in their expression levels have been implicated in painful diabetic neuropathy (20–23). TTX-S sodium channels in brain are composed of a pore-forming α-subunit and one or two auxiliary β-subunits (24–27). The subunit composition of TTX-R sodium channels, however, is not clear. Increased TTX-R sodium current (21), but decreased expression levels of Na\textsubscript{v}1.8 mRNA and protein have been reported in models of diabetic neuropathy (28). However, a systematic analysis of the relative contributions of TTX-S and TTX-R sodium channels, including their phosphorylation status, has not been performed in animal models with documented painful diabetic neuropathy.

In the present study, we investigated the expression and functional properties of TTX-S and TTX-R sodium channels in acutely dissociated small to medium sized (nociceptive) DRG neurons isolated from diabetic rats with documented painful neuropathy. We demonstrate that TTX-S and TTX-R sodium currents increased significantly and the voltage-dependent activation and steady-state inactivation curves were negatively shifted in these DRG somas. TTX-S currents induced by both
fast and slow voltage ramps increased significantly in diabetic neurons. The protein expression levels of Na\textsubscript{v}1.3 and Na\textsubscript{v}1.7 increased in DRG homogenates from diabetic animals. In contrast, the protein expression levels of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 decreased in DRG homogenates. Interestingly, serine/threonine phosphorylation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 and tyrosine phosphorylation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7 increased in neurons from diabetic rats. We propose that Na\textsubscript{v}1.8 phosphorylation may, in part, be responsible for the increased TTX-R current observed in diabetic neurons. Using immunofluorescence staining, we observed that the expression of Na\textsubscript{v}1.7 increased while the expression of Na\textsubscript{v}1.8 decreased in small C-fiber neurons of diabetic rats compared with controls, in agreement with our Western blot results. These data support the abnormal function of noncelective fibers observed in early painful diabetic neuropathy involves both TTX-S and TTX-R sodium channels.

**EXPERIMENTAL PROCEDURES**

All experiments were approved by the University of Michigan Committee on Use and Care of Animals according to National Institutes of Health guidelines.

**Animal Model**—Male Sprague-Dawley (Harlan, Indianapolis, IN) rats weighing 150–200 g were fasted overnight to maximize the effectiveness of STZ treatment. Diabetes mellitus was induced by a single injection of STZ (Sigma) at a dose of 45 mg/kg body weight in a citrate buffer (pH 4.5). Age-matched rats in the control group received injections of saline vehicle. STZ-injected animals were given 10% sucrose buffer (pH 4.5). Age-matched rats in the control group received injections of saline vehicle. STZ-injected animals were given 10% sucrose buffer (pH 4.5). Tail vein blood glucose levels were measured 48 h after injection and the onset of the diabetic condition was defined as glucose levels greater than 300 mg/dl. Animals were euthanized for study 4 to 8 weeks after induction of diabetes. Our previous studies with this model indicated that rats with diabetes for 4–8 weeks demonstrate a variety of functional abnormalities including delayed nerve conduction velocity, increased calcium influx, impaired inhibitory G protein function, and activation of the apoptosis cascade in acutely dissociated DRG neurons that were reversible after 2 weeks of insulin-mediated euglycemia (6, 7).

**Behavioral Tests**—Prior to electrophysiological studies, a subset of STZ-treated diabetic rats and age-matched healthy controls were evaluated for changes in sensory perception as described below. All animals were acclimated for 1 week prior to testing. During this period the animals were handled extensively and habituated to the behavioral testing procedures.

**Mechanical Allodynia**—To quantify mechanical sensitivity of the foot, brisk foot withdrawal in response to a normally innocuous mechanical stimulus was measured as described previously (30). Response to the mechanical stimulus was measured with a calibrated electronic von Frey pressure algometer (Somedic Sales AB). This system consists of a hand-held electronic von Frey probe with a circular probe tip of 0.5 mm in diameter. The algometer is connected to a computerized data collection system, allowing on-line display of the applied force as well as rate of stimulus application. The rat was placed in a hanging cage with a metal mesh floor and acclimated for 10 min. A 0.5-mm diameter von Frey probe was manually applied to the plantar surface of the hind foot with a pressure increasing by ∼0.05 Newtons/s and the pressure at which a paw withdrawal occurred was recorded. For each hind paw, the procedure was repeated 5 times and the average pressure to produce a withdrawal computed. Successive stimuli were applied to the hind foot at ∼30-s intervals. A significant decrease in the pressure necessary to elicit a brisk foot withdrawal in response to this mechanical stimulus was interpreted as mechanical allodynia.

**Thermal Hyperalgesia (Hargreaves Test)**—To quantify thermal sensitivity, rats were placed in a clear a Plexiglas chamber (10 cm × 20 cm × 20 cm) and an elevated floor (20 cm) given 5–10 min to habituate. The glass floor was maintained at 31 ± 1°C. A radiant heat source delivered a thermal stimulus to the plantar surface of each hind foot. The latency to foot withdrawal (escape) served as the behavioral measure of thermal nociception. If the foot was not withdrawn within 20 s, the stimulus was automatically terminated and a new stimulus was applied. Each foot was tested to between 2 and 4 stimulations of either foot to avoid peripheral sensitization effects. The mean withdrawal latency for each foot was computed by averaging the 5 measurements. As compared with the baseline (control) latency, a significant decrease in the latency of foot withdrawal in response to the thermal stimulus was interpreted as indicating the presence of thermal hypealgesia (31).

**Cell Preparation**—DRGs were isolated from acutely dissociated thoracic and lumbar regions of the spinal column, and neurons were prepared according to the methods described previously (32). Briefly, ganglia were digested with 0.3% collagenase ( Worthington Type 2) in a minimal essential medium (MEM, Invitrogen) supplemented with 16.5 μM NaF, and 28.2 μM glucose (MEM) for 50 min and then 0.1% trypsin (type 1, Sigma) for 10 min. The digested DRGs were centrifuged in 2% bovine serum albumin in M-MEM at 4°C for 5 min and washed twice with M-MEM solution. After titration in M-MEM with additional 10% fetal bovine serum (Invitrogen), DRG neurons were plated on 35-mm sterile culture dishes coated with calf collagen. Isolated neurons were incubated in 5% CO\textsubscript{2} for 2–7 h prior to plating.

**Whole Cell Patch Clamp Recording**—Sodium currents were recorded in the whole cell patch clamp configuration using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at room temperature (20–23°C). Electrodes (1–2 MΩ) were pulled from standard wall glass pipettes (G150F-4, Warner Instrument) using a horizontal puller P-87 (Sutter Instrument) and filled with (in mM): 100 CsCl, 30 tetraethylammonium-Cl, 5 NaCl, 2 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 3 EGTA, 10 HEPES, 2 Mg-ATP, 1 Li-GTP; pH was adjusted to 7.2 by Tris base and osmolarity was adjusted to 285 mOsm. Pipette solutions were filtered at 0.2 μm immediately before use. The bath solution used to record currents contained (in mM): 35 NaCl, 30 tetraethylammonium-Cl, 1 MgCl\textsubscript{2}, 0.05 CaCl\textsubscript{2}, 10 HEPES, and 11 glucose. The pH was adjusted to 7.4 and osmolarity was 325 mOsm. Reduced extracellular Na\textsuperscript{+} was required to reduce the magnitude of sodium currents to improve the fidelity of the voltage clamp (33). Under these recording conditions, the estimated Nernst reversal potential for Na\textsuperscript{+} was +53 mV. In some experiments, action potentials were recorded in the current clamp mode using methods described previously (34). TTX-R sodium currents were isolated from TTX-S currents by adding TTX (200 nM, Sigma) to the bath solution. The TTX stock solution (20 μM) was prepared with distilled water and stored at −20°C. Working TTX solutions were made with extracellular solution on the day of experiments.

After formation of a gigaseal (1–5 GΩ) and compensation of pipette capacitance with amplifier circuitry, whole cell access was established. The pipette potential was zeroed before seal formation. Membrane resistance, series resistance, and capacitance were determined from current transients elicited by 5-mV depolarizing steps from a holding potential of −80 mV, via the membrane test application of pClamp software (v8.2, Axon Instruments).

**Data Analysis**—Activation and steady-state inactivation data were fitted with a Boltzmann equation of the form: \( G = G_{\text{max}}/[1 + \exp(V_m - V_{1/2})/k] \). Here \( G \) equals to \( I/(V_m - V_{\text{rev}}) \), where \( V_m \) is the potential at which current is evoked, and \( V_{\text{rev}} \) is the reversal potential for the current. \( G_{\text{max}} \) is the maximum conductance, \( V_{1/2} \) is the potential of half activation or inactivation, and \( k \) is the slope factor. All data were expressed as means ± S.E. Statistical analyses were performed using the Student's t test.

**Immunofluorescence Labeling of Perineurial and Sodium Channels**— DRGs from thoracic and lumbar regions of the spinal column were quickly removed, postfixed for 2–3 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), and cryoprotected in 10% sucrose in 0.1 M PB buffer for 24 h at 4°C. Transverse sections (10 μm thick) were cut on a cryostat and mounted serially onto Superfrost/plus microscope slides (Fisher Scientific).

For immunofluorescence labeling of sodium channels, the sections were first washed with 0.1% PB, permeabilized with 0.3% Triton X-100.
in 0.1 M PB (PBST) for 2 h at room temperature, and blocked with 10% normal goat serum in PBST (PBSTG) for at least 4 h. The sections were then incubated with anti-sodium channel antibodies and monoclonal anti-peripherin (1:150, Chemicon) antibody in PBSTG for 24 h at 4 °C. Antibodies for sodium channels used were anti-rabbit Na\(_1\).6 (1:200, Sigma), anti-rabbit Na\(_1\).7 (1:100, Sigma), or anti-rabbit Na\(_1\).8 (1:100) from Dr. S. R. Levinson. After three washes with PBST, the sections were incubated with secondary antibodies Alexa Fluor 488 (goat anti-mouse IgG) and Alexa Fluor 594 (goat anti-rabbit IgG) from Molecular Probes (Eugene, OR) for 2 h at room temperature. The sections were then washed, mounted with anti-fade fluorescence mounting medium and stored at 4 °C. All images were captured with a Zeiss Axiosplan microscope with a CCD digital camera and processed with Adobe Photoshop 7.

**Immunoprecipitation and Western Blotting**—DRGs in the lumbar and thoracic regions of the spinal column from diabetic and control rats were removed and homogenized in ice-cold lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EGTA, 50 mM NaF, 1.5 mM MgCl\(_2\), 10% v/v glycerol, 1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO\(_4\), and "Complete" protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). Aliquots of DRG homogenates containing equal amounts of total protein were mixed with the anti-phosphoserine (Chemicon), anti-phosphothreonine (Chemicon), or anti-phosphotyrosine (Sigma) specific antibodies at a 1:40 dilution. The mixtures were incubated and rotated in Eppendorf tubes in the presence of 50 mM NaF and protease inhibitors for 4–14 h at 4 °C. Protein G-agarose beads (Roche Diagnostics) were then added to the samples and incubated overnight at 4 °C. After washing three times with ice-cold lysis buffer, the protein G beads were pelleted and mixed with 2× SDS sample buffer. Proteins were separated on 4–15% gradient Tris-HCl gels and transblotted onto polyvinylidene difluoride membranes (Amersham Biosciences). In some experiments, crude DRG homogenates were loaded on gels for Western blot analysis. The membrane blots were blocked with 10% nonfat dry milk for 2 h at room temperature, and incubated with primary antibodies: anti-Nav1.3 (1:200, Sigma), anti-Nav1.6 (1:200), anti-Nav1.7 (1:100), or anti-Nav1.8 (1:100) overnight at 4 °C. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences) for 2 h at room temperature and developed using the Supersignal West Pico chemiluminescence kit (Pierce). The corresponding bands were scanned at 1200 dpi and semiquantified with Image Quanti-Scan software (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**Short Term Diabetic Rats Develop Mechanical Allodynia and Thermal Hyperalgesia**—Two days after injection of STZ, 70% of the rats developed high levels of blood glucose (mean level = 460 ± 22 mg/dl), whereas untreated rats had normal levels (mean level = 85 ± 6 mg/dl). The elevated level of blood glucose in STZ-injected rats was maintained during the entire experimental period. These results are very similar to our previous studies (32). In the present study, mechanical allodynia was determined by measuring the paw withdrawal threshold in response to application of a von Frey probe (Fig. 1A). Diabetic rats showed a significant decrease in the pressure required to elicit paw withdrawal as compared with their pre-diabetic baseline responses (p < 0.05, n = 6). The paw pressure withdrawal threshold for diabetic rats began to decrease 1 week following STZ-injection, but this change in responsiveness to mechanical stimuli did not reach significance until 4 weeks after the induction of diabetes. Thermal hyperalgesia was determined by measuring the withdrawal latency to a radiant heat stimulus applied to the hind foot. Fig. 1B illustrates the decrease in the withdrawal latency to thermal stimulation in hind paws of diabetic rats. As compared with pre-diabetic baseline responses, diabetic rats began to exhibit a significant reduction in the temperature required to elicit a hind paw withdrawal 4 weeks after STZ injection (p < 0.01, n = 6). Behavioral signs of both mechanical allodynia and thermal hyperalgesia persisted for up to 8 weeks after the onset of diabetes, the maximum duration of our observations. We found no significant differences between baseline and post-injection responses to either mechanical or thermal stimuli of similarly

**Role of TTX-S and TTX-R Sodium Channels in Diabetic Rats**

**TTX-S and Total Sodium Currents**

**Diabetic Neuropathy Is Associated with Increased Amplitude and Altered Properties of TTX-R Sodium Current**—To record TTX-R sodium currents (I\(_{\text{Na}}\)), small-sized DRG neurons (soma diameter < 25 μm, cell capacitance < 35 pF) were isolated and voltage-clamped at −80 mV in the presence of 200 nM TTX in the bath solution. The average resting membrane potential was −60.3 ± 0.3 mV in DRG neurons from control rats and −52.7 ± 0.7 mV in diabetic rats (n = 110 for each group). The difference between these two groups was highly significant (p < 0.0001). The average whole cell capacitance was 22.3 ± 1.2 pF in the control group and 23.8 ± 2.1 pF in the diabetic group. Fig. 2A shows original current traces of TTX-R I\(_{\text{Na}}\) in DRG neurons prepared from a diabetic rat 6 weeks after the onset of diabetes compared with an age-matched control. The amplitude of outward I\(_{\text{Na}}\) was significantly larger in neurons from diabetic rats compared with controls (Fig. 2B). The mean peak current density in diabetic neurons was 39.2 ± 1.5 pA/pF (n = 12) compared with 27.8 ± 2.3 pA/pF in control neurons (n = 15) and the difference between these two values was significant (p < 0.01). As shown in Fig. 2C, the current-voltage relationship calculated for DRG neurons isolated from diabetic rats was shifted ~8 mV in the hyperpolarizing direction compared with that calculated for controls. Peak current density was elicited by depolarizing control neurons to +0 mV or by depolarizing diabetic neurons to ~10 mV. The kinetics of activation was determined by curve fitting the rising phase of the conductance using a single exponential function Boltzmann equation (Fig. 2D). The midpoint of the voltage-dependence of activation was −14.8 ± 0.4 mV in cells from diabetic rats. This value was significantly more negative than the value obtained for control neurons (−8.5 ± 0.3 mV, p < 0.001). The voltage dependence of steady-state inactivation was best fit to a modified Boltzmann function: (I\(_{\text{Na}}\)/I\(_{\text{Na max}}\)) = [1 + exp(V − V\(_{1/2}\))/k]−1, where V\(_{1/2}\) is the midpoint of steady-state inactivation and k is the slope factor. The midpoints of steady-state inactivation were −29.1 ± 0.3 mV in neurons from diabetic rats and −23.8 ± 0.3 mV in control neurons. The difference between these values was significant (p < 0.05). Thus, the steady-state inactivation of TTX-R currents was also negatively shifted in small-sized DRG neurons from diabetic rats compared with controls.

**TTX Neurons from Diabetic Rats Demonstrate Increases in TTX-S and Total Sodium Currents**—To elicit total I\(_{\text{Na}}\), small-
sized DRG neurons were depolarized by a pre-pulse to −120 mV for 50 ms and then stepped to potentials ranging from −50 mV to +50 mV in 5-mV increments every 10 s. Fig. 3A demonstrates the current-voltage relationships for the total I_{Na} of DRG neurons isolated from control and diabetic rats. The peak current density of total I_{Na} was −59.7 ± 8.0 nA/m² in cells from control rats and −73.4 ± 5.0 nA/m² in diabetic rats (p < 0.05, n = 11 for each cell type). Maximal currents for diabetic neurons were measured at −10 mV compared with −5 mV for control neurons. We also observed increased action potential amplitudes in DRG neurons isolated from diabetic rats compared with controls. In current clamp whole cell configuration, the membrane potential was not adjusted, and the action potential was elicited by delivery of 1.5 nA current for 0.5 ms to the patched cells through the amplifier, leaving most of the action potential free of the effect of injected current (36). The majority of the action potentials recorded displayed a characteristic shoulder on the falling phase, indicating the activation of non-nociceptive neurons corresponding to C-type fibers, as previously described (37, 38). The mean amplitude of the action potential was significantly larger in diabetic neurons (130.3 ± 1.3 mV) compared with controls (115.3 ± 0.9 mV) (p < 0.05, n = 8 for each cell type). The 10–90% rise time of the action potential was significantly shorter in diabetic neurons (0.27 ± 0.01 ms) than in control cells (0.58 ± 0.05 ms), as demonstrated in Fig. 3B (p < 0.001, n = 9 for each cell type). These results suggest that diabetic DRG neurons are more sensitive to action potential stimulation than control cells under the same conditions. Fig. 4A shows original traces of total and TTX-R I_{Na}, evoked by voltage step to 0 mV. The TTX-S current was obtained by digitally subtracting the currents recorded in the absence of TTX. The rising phase of the TTX-S current precedes the peak of TTX-R current, suggesting that the activation of TTX-S current is more rapid. The current-voltage relationship for TTX-S I_{Na} in small-sized DRG neurons from control and diabetic rats is shown in Fig. 4B. The peak current density for TTX-S I_{Na} was −35.1 ± 3.1 nA/m² in cells from diabetic rats, which is significantly more negative than that for control neurons (−25.5 ± 2.7 mV, p < 0.05).

The kinetics of activation of TTX-S I_{Na} were calculated and fit with a single Boltzmann equation as shown in Fig. 4C. The midpoint of voltage-dependent activation for TTX-S I_{Na} was −12.8 ± 0.4 mV in cells from control rats and −16.1 ± 0.1 mV in cells from diabetic rats. The difference between these values was significant (p < 0.05). The slope factors for the TTX-S I_{Na} activation curves were of 2.7 ± 0.1 for diabetic neurons and 5.4 ± 0.3 for control neurons, and the difference between these values was significant (n = 8; p < 0.01).

Increased Slow and Fast Ramp Currents in DRG Neurons from Diabetic Rats—Because threshold ramp currents might influence the excitability of DRG neurons, we also examined

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**Fig. 2.** Changes in amplitude and properties of TTX-R I_{Na} in small-sized DRG neurons from diabetic rats. A, typical traces of TTX-R I_{Na} conducted in voltage-clamp mode in control (top) and diabetic (bottom) rats. Cells were voltage-clamped at −80 mV, and currents were elicited by stepping voltage from −50 mV in the presence of 200 nM TTX. B, single traces of TTX-R I_{Na} elicited by depolarizing cells to 0 mV from the holding potential in neurons from control and diabetic rats. This I-V curve shifted leftward in DRG cells from diabetic (H11002) compared with control (H11006) rats. Cells were voltage-clamped at −80 mV, and currents were elicited by stepping voltage from −50 mV to +45 mV in 5-mV increments every 10 s. Fig. 3A demonstrates the current-voltage relationships for the total I_{Na} of DRG neurons isolated from control and diabetic rats. The peak current density of total I_{Na} was −59.7 ± 8.0 nA/m² in cells from control rats and −73.4 ± 5.0 nA/m² in diabetic rats (p < 0.05, n = 11 for each cell type). Maximal currents for diabetic neurons were measured at −10 mV compared with −5 mV for control neurons. We also observed increased action potential amplitudes in DRG neurons isolated from diabetic rats compared with controls. In current clamp whole cell configuration, the membrane potential was not adjusted, and the action potential was elicited by delivery of 1.5 nA current for 0.5 ms to the patched cells through the amplifier, leaving most of the action potential free of the effect of injected current (36). The majority of the action potentials recorded displayed a characteristic shoulder on the falling phase, indicating the activation of non-nociceptive neurons corresponding to C-type fibers, as previously described (37, 38). The mean amplitude of the action potential was significantly larger in diabetic neurons (130.3 ± 1.3 mV) compared with controls (115.3 ± 0.9 mV) (p < 0.05, n = 8 for each cell type). The 10–90% rise time of the action potential was significantly shorter in diabetic neurons (0.27 ± 0.01 ms) than in control cells (0.58 ± 0.05 ms), as demonstrated in Fig. 3B (p < 0.001, n = 9 for each cell type). These results suggest that diabetic DRG neurons are more sensitive to action potential stimulation than control cells under the same conditions. Fig. 4A shows original traces of total and TTX-R I_{Na}, evoked by voltage step to 0 mV. The TTX-S current was obtained by digitally subtracting the currents recorded after applying 200 nM TTX from the currents recorded in the absence of TTX. The rising phase of the TTX-S current precedes the peak of TTX-R current, suggesting that the activation of TTX-S current is more rapid. The current-voltage relationship for TTX-S I_{Na} in small-sized DRG neurons from control and diabetic rats is shown in Fig. 4B. The peak current density for TTX-S I_{Na} was −35.1 ± 3.1 nA/m² in cells from diabetic rats, which is significantly more negative than that for control neurons (−25.5 ± 2.7 mV, p < 0.05).

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sodium currents in small-sized DRG neurons elicited in response to a slow voltage ramp (~0.23 mV/ms) depolarization from −120 mV to +40 mV over a 695 ms time course. To increase $I_{\text{Na}}$, the maximum current density of $I_{\text{Na}}$ in DRG neurons from diabetic rats was ~130% larger in neurons from diabetic rats compared with the controls (Fig. 5A). When the peak ramp currents were normalized to cell capacitance, the mean current density was −21.6 ± 3.0 pA/pF whereas it was −6.5 ± 1.3 pA/pF in control neurons ($p < 0.01$, $n = 13$ for each cell type).

To elicit the fast ramp currents, DRG neurons were depolarized to +40 mV from −120 mV at a rate of 2.3 mV/ms. As shown in Fig. 5B, the fast ramp current elicited in diabetic neurons was larger than the control. The mean peak current density elicited by fast voltage ramp was −24.9 ± 5.3 pA/pF in controls and −42.6 ± 5.9 pA/pF in diabetic neurons (Fig. 5C). We also examined the levels of resurgent current in diabetic versus control neurons (39). After a conditioning step to +30 mV for 50 ms to promote inactivation, hyperpolarizing pulses ranging from −50 to 0 mV were used to assay resurgent current. Fig. 5D shows the original traces recorded in neurons depolarized to +30 mV followed by a hyperpolarizing pulse to −40 mV. Large sized DRG neurons elicited a resurgent current using this protocol; however, there were no significant differences in this current observed between diabetic and control neurons. Resurgent currents were barely measurable in small sized DRG neurons from either control or diabetic rats.

Diabetic Neuropathy Is Associated with Differential Changes in Sodium Channel Expression in DRG Neurons—To test whether the observed increases in TTX-S and TTX-R $I_{\text{Na}}$ in diabetic neurons could be due to altered sodium channel expression, we extracted crude homogenates of DRGs and analyzed the expression of sodium channels by Western blot. Our results show that the expression levels of TTX-S and TTX-R in DRGs of diabetic rats (Fig. 6). In agreement with previous studies (28), we found that the expression of Na,1.8 significantly decreased (40% less) in diabetic DRGs compared with controls ($p < 0.05$, $n = 5$). In contrast to previous studies (28), we measured that the expression of Na,1.6 also decreased in diabetic DRGs (38%, $n = 5$, Fig. 6, A and B). The expression levels of Na,1.3 and Na,1.7 increased significantly in DRGs from diabetic rats compared with controls ($p < 0.05$, $n = 4$ for each group). These increases were ~82 and 45% of Na,1.3 and Na,1.7, respectively, in DRGs from diabetic rats as compared with the controls (Fig. 6B).

Immunolocalization of Sodium Channels in DRG Neurons—As shown in the Fig. 7A, we observed that Na,1.6 was...
expressed primarily in small and medium-sized DRG neurons in both control and diabetic rats. We found that Na$_v$1.6 immunoreactivity was similar in control and in diabetic DRGs. As expected, the 38% decrease in total Na$_v$1.6 protein expression observed in Fig. 6 was not detectable using immunofluorescence. Of the total DRG neurons, 63.6% were immunoreactive for Na$_v$1.6 in control rats and 59.7% in diabetic rats (Table I). This difference was not significant ($p > 0.05$). The distribution of Na$_v$1.6 expression among small and large diameter neurons also did not appear to change in diabetes. For example, 44.7% versus 44.8% of the neurons that were Na$_v$1.6-positive were also positive for peripherin (a marker of unmyelinated C-fibers) (35, 41) in control and diabetic DRGs, respectively.

Na$_v$1.7 is highly expressed in small DRG neurons (42, 43), has slow repriming kinetics, and a slow onset of closed-state inactivation (44). In agreement with our Western blot results (Fig. 6), we found that the level of Na$_v$1.7 immunoreactivity increased in small and medium sized DRG neurons from diabetic rats compared with controls (Fig. 7B). 55.6 ± 2.4% of total DRG neurons were immunoreactive for Na$_v$1.7 in control rats compared with 65.6 ± 1.1% in diabetic rats (Table I), representing an increase of ~18% ($p < 0.01$). The percentage of the neurons that stained positively for both Na$_v$1.7 and peripherin was 24.1 ± 1.3% in control rats and 28.2 ± 1.4% in diabetic rats. While this difference was significant ($p < 0.05$) (Table I), it also indicates that the greatest increase in Na$_v$1.7 expression was in non-C-fiber neurons. These data are the first demonstration of changes in Na$_v$1.7 expression in painful diabetic neuropathy.

Na$_v$1.8 is expressed primarily in small- and medium-sized DRGs and has been implicated in the transmission of neuropathic pain (45, 46). As demonstrated in Fig. 7C, the level of Na$_v$1.8 staining decreased in DRGs from diabetic rats compared with controls, in agreement with our Western blot results (Fig. 6) and with the results of previous studies (28). The majority of peripherin-positive neurons also expressed Na$_v$1.8 in control animals. Based on the total neurons counted (Table I), -77.6 ± 3.0% of control neurons were immunopositive for Na$_v$1.8 in control rats. This value dropped significantly (20%), to 62.6 ± 2.1% in diabetic rats ($p < 0.01$). Neurons that stained positively for both Na$_v$1.8 and peripherin were 37.2 ± 4.3% of the total in control rats and 29.5 ± 0.4% of the total in diabetic rats ($p > 0.05$) (Table I). Thus, both the overall levels of Na$_v$1.8 protein expression and the number of neurons expressing Na$_v$1.8 decreased with the onset of diabetic neuropathy despite increased TTX-R current.

**Enhanced Phosphorylation of Both TTX-S and TTX-R Sodium Channels in DRG Neurons from Diabetic Rats**—Phosphorylation of TTX-R sodium channels results in increased sodium current and this may play a role in nociceptive processing (26, 45, 47). To test whether nociceptive signaling in diabetic neuropathy also involves changes in sodium channel phosphorylation, we immunoprecipitated solubilized DRG homogenates with anti-phosphoserine-, anti-phosphothreonine-, or anti-phosphotyrosine-specific antibodies, and then analyzed the immunoprecipitated proteins by Western blot using anti-sodium channel antibodies. We also performed reciprocal immunoprecipitation experiments using the antibodies in the reverse order. The results of both sets of experiments were indistinguishable and thus the data were pooled. As demonstrated in Fig. 8A, an increased level of anti-phosphoserine immunoreactivity (~60%) for Na$_v$1.8 protein was measured in DRG homogenates from diabetic rats compared with controls. Na$_v$1.8 anti-phos-
The protein expression of Na\textsuperscript{v}1.3 and Na\textsuperscript{v}1.7 were found to be significantly increased (p < 0.05) in DRGs from diabetic rats compared with controls (n = 4, each group), whereas the total protein of Na\textsuperscript{v}1.6 decreased significantly as assessed by both Western blot analysis of DRG homogenates and immunocytochemical analysis of peripherin-positive DRG neurons. Our data suggest that TTX-R sodium currents have been implicated in chronic pain, including diabetic neuropathy. We believe that phosphorylation status, in diabetic neuropathy. Our data demonstrate that both TTX-S and TTX-R sodium currents increased in small (nociceptive) DRG neurons from diabetic rats and that these increases in current correlated with the development of hyperalgesia and allodynia in early diabetes. The expression levels of TTX-S and TTX-R channel subtypes were differentially affected in diabetic rats. Expression of Na\textsuperscript{v}1.8 decreased significantly as assessed by both Western blot analysis of DRG homogenates and immunocytochemical analysis of peripherin-positive DRG neurons. Our data suggest that the development of diabetes in this model does not involve alterations in the percentage of C-type neurons that express Na\textsuperscript{v}1.8, but does involve decreases in the level of Na\textsuperscript{v}1.8 expression in individual C-type neurons compared with healthy controls. In contrast to Na\textsuperscript{v}1.8 expression, we observed a robust increase in the expression of Na\textsuperscript{v}1.7 that paralleled the development of hyperalgesia and allodynia. We observed an increase in the number of Na\textsuperscript{v}1.7-positive neurons in the DRG, an increase in the strength of the Na\textsuperscript{v}1.7 immune signal in individual neurons, and an increase in Na\textsuperscript{v}1.7 protein expression in Western blot analysis of DRG homogenates. These results support our hypothesis that both TTX-S and TTX-R sodium channels play important roles in the development of early painful diabetic neuropathy.

We observed a significant increase in TTX-R sodium current, decreased protein expression, and increased phosphorylation of Na\textsuperscript{v}1.8 in DRG neurons isolated from diabetic rats. These data, together with the results from other groups (21, 28), support a role for Na\textsuperscript{v}1.8 channel in painful diabetic neuropathy. The level of serine/threonine phosphorylation of Na\textsuperscript{v}1.8 increased at DRGs from diabetic rats, strengthening the evidence that phosphorylation of sodium channels is involved in modifying channel properties in pain-related animal models and showing for the first time that sodium channel phosphorylation contributes to painful diabetic neuropathy. The observed phosphorylation in our diabetic model may be the cause of the increase in TTX-R current observed in diabetic rats since cAMP-dependent phosphorylation of Na\textsuperscript{v}1.8 increased TTX-R currents in both oocytes and transfected COS-7 cells (48, 49). We propose that phosphorylation of Na\textsuperscript{v}1.8, possibly at sites on the DI-DII linker domain, alters the voltage-dependent activation and inactivation or open probability of the channel but not the incorporation of additional channels into the plasma membrane, and consequently increases the TTX-R current. In our experiments, based on the inactivation phase of the TTX-R current, 80–90% of the TTX-R current observed in DRG neurons can be attributed to Na\textsuperscript{v}1.8 because Na\textsuperscript{v}1.9 shows much slower inactivation kinetics compared with Na\textsuperscript{v}1.8. The role of Na\textsuperscript{v}1.9 remains to be examined in diabetic neuropathy although a recent study shows that knockdown of this channel produced no effects on thermal hyperalgesia or tactile hypersensitivity in the neuropathic rat (50).

Other studies support the interpretation that TTX-R channels are not the only subtypes that are involved in painful diabetic neuropathy. A recent study reports that TTX application to injured nerve only partially reverses neuropathic pain (51). In Na\textsuperscript{v}1.8-null mutant mice, nerve injury elicits thermal hyperalgesia and tactile hypersensitivity after day 3, suggesting that neuropathic pain is developed and maintained despite the absence of Na\textsuperscript{v}1.8 (46, 52). Taken together, these results suggest that TTX-S channels also contribute to neuropathic...
pain. We observed that TTX-S current contributes 40–50% of the total current in small DRG neurons from both control and diabetic rats. In diabetic rats, the TTX-S currents increased significantly and the midpoint voltage of activation shifted modestly in the hyperpolarizing direction. Since the contribution of TTX-S currents to the threshold and upstroke of nociceptor action potentials is likely to be sensitive to small changes in the resting potential (36), the increased amplitude and negative shift of the activation of TTX-S current (see Fig. 4) could result in a larger contribution to the electrogenesis of action potentials in cells with a depolarizing resting potential near the \(-52\) mV observed in diabetic rats. The faster activation kinetics of TTX-S currents in diabetic rats are also predicted to allow channels to inactivate more rapidly, thereby maintaining higher excitability.

Among the TTX-S channels, Na\(_{\text{v}1.7}\) displays slow activation and inactivation kinetics and slow repriming kinetics (44). In diabetic rats, the increased protein expression of Na\(_{\text{v}1.7}\), particularly in peripherin-positive (C-fiber) neurons, may lower the threshold in these neurons (34) that previously expressed low levels of Na\(_{\text{v}1.7}\). Consequently, nociceptive C-fiber neurons may generate more robust responses to slowly depolarizing inputs in diabetic rats compared with healthy controls because of the increased slowly inactivating ramp currents (Fig. 7). The increased expression of Na\(_{\text{v}1.7}\) in diabetic rats may also increase the duration of the action potential and decrease the conduction velocity (43). Previous studies showed that phosphorylation of Na\(_{\text{v}1.7}\) decreased current amplitude in oocytes either via a protein kinase A (PKA)-mediated pathway without altering voltage sensitivity and gating kinetics or via a protein kinase C (PKC)-dependent pathway that is accompanied by a depolarizing shift in the steady-state activation (48). In adrenal chromaffin cells, activation of PKC down-regulated Na\(_{\text{v}1.7}\) via either promoting endocytic internalization of Na\(_{\text{v}1.7}\) channels or destabilizing the mRNA (55). In contrast to these results, we observed increased Na\(_{\text{v}1.7}\) protein, currents and increased phosphorylation of Na\(_{\text{v}1.7}\) at Ser/Thr residues and significantly at Tyr residues in DRGs isolated from diabetic rats. We propose that modulation of Na\(_{\text{v}1.7}\) through phosphorylation may affect channel function and expression differently in DRG neurons in vivo compared with adrenal chromaffin cells or oocytes. For example, forskolin decreased Na\(_{\text{v}1.7}\) currents at low concentrations but failed to alter Na\(_{\text{v}1.7}\) currents at high concentrations, suggesting a biphasic effect of forskolin on

![Double immunofluorescence staining of DRG neurons with C-type fiber marker peripherin (green) and sodium channel (red) antibodies. The neurons of double immunoreactivity positive are shown in yellow. Left panel, control; right panel, diabetic. A, decreased expression of TTX-S Na\(_{\text{v}1.6}\) in DRG neurons from diabetic rats (right panel) compared with the control (left panel). B, increased protein expression TTX-S Na\(_{\text{v}1.7}\) was detected in DRG neurons from diabetic (right panel) rats compared with the control (left panel). Both the percentage of Na\(_{\text{v}1.7}\)-positive and the percentage of neurons double positive for Na\(_{\text{v}1.7}\) and peripherin were increased significantly for DRGs from diabetic rats \((p < 0.05)\). C, protein expression of TTX-R Na\(_{\text{v}1.8}\) decreased in DRG neurons from diabetic rats. The percentage of DRG neurons positive for both peripherin (green) and Na\(_{\text{v}1.8}\) (red) decreased in DRG neurons from diabetic rats. Scale bar, 50 \(\mu\)m.](image)

<table>
<thead>
<tr>
<th>Table I</th>
<th>The proportion of neurons that are immunoreactive-positive for sodium channel (\alpha)-subunits and peripherin in DRGs from control and diabetic rats</th>
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<tbody>
<tr>
<td>Cell subtype</td>
<td>Na(_{\text{v}1.6})</td>
</tr>
<tr>
<td></td>
<td>All</td>
</tr>
<tr>
<td>Control</td>
<td>63.6 ± 1.9</td>
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<tr>
<td>Diabetic</td>
<td>59.7 ± 4.7</td>
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\(a\) Represents significant difference between control and diabetic rats \((p < 0.01)\).

\(b\) Represents significant difference between control and diabetic rats \((p < 0.05)\).
channel function (48). Our data also raise the possibility that tyrosine kinase-mediated pathways modulate Na\textsubscript{v}1.7 in addition to PKA and PKC. It has been reported that tyrosine phosphorylation produced by stimulating receptor tyrosine kinases inhibits sodium channel currents in PC12 cells (54) and dephosphorylation provides opposite regulation of sodium channel function (40). However, this inhibition of tyrosine phosphorylation may be tissue-specific since inhibition of protein tyrosine kinase inhibits fast sodium currents in rabbit cardiac myocytes (53). Therefore, the role of tyrosine phosphorylation in regulating individual sodium channel subtype, i.e. Na\textsubscript{v}1.7 in diabetic neuropathy, remains to be investigated.

For Na\textsubscript{v}1.6, our data indicate that it is highly expressed in both small and large DRG neurons (Fig. 7). The overall expression of Na\textsubscript{v}1.6 channel decreased in homogenates of diabetic DRG neurons but there was no difference in either the percentage of neurons expressing Na\textsubscript{v}1.6 or in its expression in C-fibers as assessed by immunocytochemistry. We observed phosphorylation of Na\textsubscript{v}1.6 at Tyr residues and the level of tyrosine phosphorylation increased in DRGs isolated from diabetic rats. Tyrosine phosphorylation of cerebellar Purkinje cell Na\textsubscript{v}1.6 channels has been implicated in the maintenance of resurgent current (29, 39). However, we did not detect resurgent currents in small DRG neurons from either control or diabetic rats despite this phosphorylation, suggesting that this effect may be cell-specific.

In conclusion, this study demonstrates that the function of TTX-S and TTX-R sodium channels are increased in early diabetic neuropathy and, therefore, may contribute to the pathophysiology of painful diabetic neuropathy. These enhanced sodium currents are correlated with increased phosphorylation at both serine/threonine and tyrosine sites. A new generation of specific inhibitors or blockers for TTX-S sodium channels would help to elucidate the contribution of different subtypes of TTX-S channels to the development of thermal hyperalgesia and mechanical allodynia in painful diabetic neuropathy, setting the foundation for improved targeted therapeutic interventions.
REFERENCES