Multiple T-type Ca\textsuperscript{2+} current subtypes in electrophysiologically characterized hamster dorsal horn neurons: possible role in spinal sensory integration

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Ku W, Schneider SP. Multiple T-type Ca\textsuperscript{2+} current subtypes in electrophysiologically characterized hamster dorsal horn neurons: possible role in spinal sensory integration. J Neurophysiol 106: 2486–2498, 2011. First published July 27, 2011; doi:10.1152/jn.01083.2010.—Whole cell patch-clamp recordings were used to investigate the contribution of transient, low-threshold calcium currents (\(I_T\)) to firing properties of hamster spinal dorsal horn neurons. \(I_T\) was widely, though not uniformly, expressed by cells in Rexed’s laminae I–IV and correlated with the pattern of action potential discharge evoked under current-clamp conditions: \(I_T\) in neurons responding to constant membrane depolarization with one or two action potentials was nearly threefold larger than \(I_T\) in cells responding to the same activation with continuous firing. \(I_T\) was evoked by depolarizing voltage ramps exceeding 46 mV/s and increased with ramp slope (240±2.400 mV/s). Bath application of 200 \(\mu\)M Ni\textsuperscript{2+} depressed ramp-activated \(I_T\). Phasic firing recorded in current clamp could only be activated by membrane depolarizations exceeding ∼43–46 mV/s and was blocked by Ni\textsuperscript{2+} and mibebradil, suggesting \(I_T\) as an underlying mechanism. Two components of \(I_T\), “fast” and “slow,” were isolated based on a difference in time constant of inactivation (12 ms and 177 ms, respectively). The amplitude of the fast subtype depended on the slope of membrane depolarization and was twice as great in burst-firing cells than in cells having a tonic discharge. Post hoc single-cell RT-PCR analyses suggested that the fast component is associated with the Ca\textsubscript{v}3.1 channel subtype. \(I_T\) may enhance responses of phasic-firing dorsal horn neurons to rapid membrane depolarizations and contribute to an ability to discriminate between afferent sensory inputs that encode high- and low-frequency stimulus information.

single-cell RT-PCR; spinal cord; calcium channels; Ca\textsubscript{v}3 subunits; patch clamp

THE DORSAL HORN of the spinal cord is a structure of major importance for processing sensory information from the body. It receives synaptic terminations from primary sensory neurons that innervate a variety of tissues, including skin, muscle, tendons, joints, and viscera (Willis and Coggeshall 2004). In this region, diverse sensory information is integrated by local neural circuits, which are assembled from synaptic connections between multiple, physiologically distinct types of interneurons (Lu and Perl 2003, 2005; Santos et al. 2007; Schneider 2008) and then relayed to segmental reflex circuitry and ascending sensory pathways transmitted to the brain and brain stem.

Intrinsic electrophysiological properties of neurons can influence the integration of sensory information within dorsal horn circuits. At least three types of dorsal horn neurons have been identified in rodents based on discharge properties to current injection: tonic, phasic, and delayed firing (Graham et al. 2004; Hochman et al. 1997; Prescott and De Koninck 2002; Ruchsweyh and Sandkühler 2002; Schneider 2003; Walsh et al. 2009). Repetitive discharge of tonically firing cells has been reported to be regulated by a combination of voltage-gated Na\textsuperscript{+} and K\textsuperscript{+} channels and persistent Ca\textsuperscript{2+} currents (Melnick et al. 2004; Prescott and De Koninck 2005). Other evidence suggests that a transient, low-voltage-activated (T-type) Ca\textsuperscript{2+} conductance underlies the rapidly adapting discharge of phasic neurons (Russo and Hougsaard 1996).

T-type Ca\textsuperscript{2+} channels are widely expressed in the central nervous system (CNS) (McKay et al. 2006; Nilius et al. 2006; Talley et al. 1999), where they promote burst firing and intrinsic oscillatory activity, and participate in synaptically evoked Ca\textsuperscript{2+} influx (Huguenard 1996). A Ca\textsuperscript{2+} current mediated by T-type channels has also been reported in spinal dorsal horn neurons of rat and mouse (Huang 1989; Ryu and Randic 1990; Walsh et al. 2009) and also may contribute to rhythm generation by ventral horn interneurons (Wilson et al. 2005). Dorsal horn T-channels have received renewed focus because of evidence that they enable calcium-dependent long-term potentiation of nociceptive transmission mediating hyperalgesia (Heinke et al. 2004; Ikeda et al. 2003). Our interest in the contribution of T-type Ca\textsuperscript{2+} channels to spinal somatosensory processing was prompted by two observations. First, fast depolarizing current ramps activate a transient membrane depolarization and discharge in many phasic-firing dorsal horn neurons, with the amplitude of depolarization and firing frequency being graded with the ramp slope (Schneider 2003). Second, phasic cells in the dorsal horn region where mechanically sensitive cutaneous afferents terminate respond selectively to stimuli that produce rapid deformations in the skin (Schneider 2005), suggesting that this class of neurons may be part of specialized spinal circuits for encoding velocity information. A similar observation was made by Prescott and De Koninck (2002), who found that phasic cells in lamina I are driven by trains of high-frequency stimuli and argued that they act as coincidence detectors responding to simultaneous occurrence of separate afferent inputs. In thalamic neurons, T-type currents are selectively activated by fast voltage ramps (Crunelli et al. 1989) and generate large, transient Ca\textsuperscript{2+} depolarizations that contribute to oscillatory behavior (Gutierrez et al. 2001). Given the importance of T-type channels to rhythmic firing behavior in thalamic neurons, we considered the possibility that these channels also function in dorsal horn sensory integration to shape responsiveness of neurons to dynamic membrane depolarizations induced by primary sensory afferents.

Three pore-forming \(\alpha_3\)-subunits of T-type Ca\textsuperscript{2+} channels (Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3) have been cloned (Perez-Reyes...
MgCl₂, 11 EGTA, 10 HEPES, 2 Mg-ATP, and 0.1 Li-GTP (pH 7.3, 290–310 mosM). In some experiments, the Ba²⁺ lowered to 2 mM and Cd²⁺ was raised to 20 µM. 

METHODS

All protocols involving the use of five animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Slice preparation. Spinal cord tissue was obtained from 9- to 14-day-old Syrian hamsters of both sexes under urethane anesthesia (1.5 mg/g ip) followed by exsanguination as described previously (Schneider 2003, 2008). After removal of vertebræ and meninges, a block of lumbosacral spinal cord was isolated and glued to the stage of a vibrating microtome (Vibratome 3000, St. Louis, MO) and 300-µm-thick transverse slices were cut. Dissection and slicing were carried out at 4–8°C in a solution containing (mM) 179 sucrose, 2.5 KCl, 0.2 CaCl₂, 10 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 25 glucose (pH 7.35–7.45, 290–310 mosM) equilibrated with 95% O₂-5% CO₂.

For electrophysiological recording, slices were transferred to a chamber (volume 1.5 ml) mounted on a fixed-stage microscope (Olympus BX51WI) and continuously perfused at 5–6 ml/min with ACSF bubbled with 95% O₂-5% CO₂. Patch pipette recording electrodes (4–7 MΩ) were fabricated from borosilicate glass (N-51A, Drummond Scientific, Broomall, PA). The internal pipette solution contained (mM) 130 K-gluconate, 5 NaCl, 1 CaCl₂, 1 MgCl₂, 11 EGTA, 10 HEPES, 2 Mg-ATP, and 0.1 Li-GTP (pH 7.3, 280–285 mosM). For isolating T-type Ca²⁺ currents, we switched to a modified extracellular solution with the following constituents (mM): 107 NaCl, 3 BaCl₂, 2 CsCl, 1.3 MgCl₂, 26 NaHCO₃, 10 glucose, 20 TEA, 0.5 µM TTX, and 10 µM CdCl₂ (pH 7.35–7.45, 290–310 mosM). In some experiments, the Ba²⁺ concentration was lowered to 2 mM and Cd²⁺ was raised to 20 µM or 40 µM. All recordings took place within 4–5 h after tissue dissection and were performed at 27°C to promote slice viability (Schneider 2003).

Recording sites were first identified at low power (×10) relative to the translucent band corresponding to the substantia gelatinosa (lamina II) visible under transillumination. Individual neurons spanning a range of sizes were then targeted for patch-clamping using a ×40 water immersion objective and infrared differential contrast optics. Whole cell current and membrane voltage were recorded with an Axopatch 1D amplifier (MDS Analytical Technologies, Toronto, ON, Canada). All recordings were initiated in voltage-clamp mode after nulling the input offset voltage and minimizing capacitance transients. After whole cell recording was established, cell capacitance [25 ± 8 pF (SD) and series resistance [33 ± 9 MΩ (SD)] were stabilized. Signals were amplified (bandwidth 0–5 kHz) and saved to disk with a Digidata 1320A data acquisition system running pCLAMP software (MDS Analytical Technologies). Membrane current exceeding −0.05 nA at a holding potential of −60 mV indicated a poor recording condition, and these cells were excluded from analysis. Voltage-activated currents were low-pass filtered at 500 Hz (Gaussian). P/N leak subtraction was used to correct for passive membrane current. In most cases, currents are expressed as current density (pA/pF) by dividing by cell capacitance, as estimated by measuring the transient current in response to 5-mV hyperpolarizing current pulses and observing the value of the whole cell capacitance adjustment on the amplifier. Voltage commands and membrane potentials were corrected post hoc for a −14-mV liquid junction potential calculated with pCLAMP (MDS Analytical Technologies).

Single-cell RT-PCR. In some experiments, intracellular material was harvested by applying negative pressure to the electrode pipette. The pipette contents (~5–8 µl) were expelled into a PCR tube containing 5 µl of nuclelease-free water (Fisher Scientific, Pittsburgh, PA), 1 µl of dithiothreitol (DTT, 0.1 M; Invitrogen, Carlsbad, CA), 1 µl of RNase inhibitor (40 U/µl; Promega, Madison, WI), and 1 µl of random hexanucleotides (0.5 µg/µl; Promega) and stored at −80°C. Reverse transcription was carried out as previously reported (Han et al. 2005) with minor modification. The mixture was heated to 70°C for 10 min and quickly chilled on ice. After centrifugation, the solution was mixed with 4 µl of 5X First-Strand Buffer (mM: 250 Tris-Cl, 375 KCl, 15 MgCl₂; Invitrogen), 1 µl DTT (0.1 M; Invitrogen), 1 µl of RNase inhibitor (40 U/µl; Promega, Madison, WI), and 1 µl of random hexanucleotides (0.5 µg/µl; Promega) and stored at −80°C. Reverse transcription was carried out as previously reported (Han et al. 2005) with minor modification. The mixture was heated to 70°C for 10 min and quickly chilled on ice. After centrifugation, the solution was mixed with 4 µl of 5X First-Strand Buffer (mM: 250 Tris-Cl, 375 KCl, 15 MgCl₂; Invitrogen), 1 µl DTT (0.1 M; Invitrogen), 1 µl of RNase inhibitor (40 U/µl; Promega, Madison, WI), and 1 µl of SuperScript II reverse transcriptase (RT; Invitrogen). After 10-min incubation at room temperature, the solution was warmed to 42°C for 50 min for cDNA synthesis. The reaction was terminated by heating at 70°C for 15 min, and RNase H (2 U/µl; Invitrogen) was added to remove RNA from RNA-DNA hybrids. cDNA was stored at −20°C until being used in PCR. PCR conditions were optimized by using total hamster spinal cord RNA. The presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was examined to confirm successful cell harvesting and reverse transcription. For GAPDH-positive samples, a two-step PCR protocol was carried out to detect the three known Ca₃ subunit cDNAs. The first
amplification was performed on a TGradient thermocycler (Biomatra, Göttingen, Germany). The product was then diluted 10 times and used in subsequent real-time PCR with the Stratagene MX3000P QPCR system (Agilent Technologies, Santa Clara, CA). For the first-step of CaV3 PCR, one 50-µl reaction mixture was prepared for each sample and contained 25 µl of PCR Master Mix (Promega), 5 µl of single-cell cDNA template, and outer primers: CaV3 forward, 5'-CGCTCCACCCGGAAACGCGG-3'; CaV3 reverse, 5'-GCAAATGTTGATACAGTGTGGAGGA-3'. The reaction went through 40 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min. Second-step real-time PCR was performed with SYBR GreenER qPCR SuperMix (Invitrogen). Triple components (Fig. 1A), an initial rapidly decaying phase followed by a late sustained phase. Addition of 10 µM Cd2+ reduced the amplitude of the initial phase and almost completely abolished the late sustained current within 3 min of application (Fig. 1B). Increasing the concentration to 20 µM had no additional effect on the current, suggesting that 10 µM Cd2+ was sufficient to block high-voltage-activated Ca2+ channels. Therefore, subsequent experiments were performed with the lower Cd2+ concentration.

We next examined the voltage dependence of steady-state inactivation and activation of IT. Inactivation kinetics were analyzed by stepping the membrane potential to −44 mV every 20 s in 10-mV increments from 500-ms conditioning potentials of −134 mV to −54 mV. IT was largely inactivated at potentials positive to −74 mV, and this inactivation was removed as the membrane potential approached −114 mV. Averaged normalized currents (Imax) were plotted as a function of conditioning potential (n = 4) and fitted with the Boltzmann equation (Imax = I/I0(1 + exp(V - V1/2/k))} having a slope factor (k) of 12 mV and voltage for half-maximal current (V1/2) of −88 mV (Fig. 1C). Activation properties of IT were studied by delivering 200-ms depolarizing voltage pulses after a conditioning potential of −104 mV to −24 mV in 10-mV increments. IT appeared in the low-voltage range with an activation threshold near −74 mV and reached a maximum near −34 mV. Values for Imax were plotted against the membrane potential to generate an activation curve yielding k = 8 mV and V1/2 = −61 mV when fitted with the Boltzmann equation. Overall, activation and inactivation parameters were consistent with IT reported previously under similar conditions (Huang 1989; Huguenard and Prince 1992; Ryu and Randic 1990).

Having established that activation and inactivation characteristics of IT were similar in rat and hamster dorsal horn cells, we then investigated the sensitivity of the current to T-channel antagonists. As shown in Fig. 1D, 200 µM Ni2+ added to the bathing solution depressed IT by 52 ± 6% (n = 6). Reduction in IT was partially reversible, with recovery to 78 ± 10% of control after 4–16 min of washout. The IT antagonist mibebradil (5 µM) resulted in a 47% ± 4% (n = 3) reduction in IT amplitude after 6 to 10 min of perfusion (Fig. 1D2), and the effect persisted after drug washout. The results are consistent with previous pharmacological studies of T-current activation (Martin et al. 2000; Perez-Reyes 2003) but indicated that a portion of the transient inward Ca2+ current was resistant to these two T-channel antagonists.
the superficial and deep dorsal horn. Previous studies have suggested that burst firing of turtle dorsal horn neurons at a hyperpolarized membrane potential is associated with activation of a low-voltage-activated Ca\textsuperscript{2+} conductance (Russo and Houngsgaard 1996). Hamster dorsal horn neurons exhibit a range of spike frequency adaptation (Schneider 2003, 2005) and also include a type with a rapidly adapting burst discharge. Thus we examined \( I_T \) expression in a population of neurons characterized firing properties. Initial characterization of firing properties was performed in current-clamp mode in standard ACSF solution. Measurements of \( I_T \) were then made after switching to voltage clamp by depolarizing the membrane potential to \(-44\) mV from \(-104\) mV held for 500 ms and subtracting residual current activated from a conditioning potential of \(-54\) mV to minimize contribution from any unblocked high-voltage-activated Ca\textsuperscript{2+} current.

Cells were classified as tonic (\( n = 16/46, 35\% \)) or phasic (\( n = 26/46, 57\% \)) according to their firing behavior in response to depolarizing current pulses (Fig. 2, Table 1). [Analyses do not include four neurons that responded to the current pulse with a marked delay to the first action potential (not shown).] Tonic cells fired action potentials continuously during the period of depolarization (Fig. 2A, left). Phasic cells generated a transient, rapidly adapting discharge when activated by a constant level of depolarizing current (Fig. 2A, center and right). Phasic cells had a more hyperpolarized resting membrane potential and lower \( R_{\text{in}} \) than tonic cells (Table 1), consistent with previous observations (Schneider 2003). They displayed a fairly wide range of spike frequency adaptation and were further divided into two subgroups (Fig. 2A, Table 1). Short burst (SB) phasic cells responded to depolarizing current injection with only one or two action potentials superimposed on a transient membrane depolarization (Fig. 2A, arrowheads). The remaining phasic cells (non-SB) typically responded to depolarizing current with three or more action potentials at rapidly diminishing frequency, and the number of spikes increased with stimulus amplitude (Fig. 2A, right). When depolarized by ramp-hold current commands, tonic cells discharged primarily during the
steady-state depolarization and the discharge frequency was unrelated to the trajectory of the ramp phase (Fig. 2B, left). Typically, SB and non-SB-type phasic cells were activated only by the most rapid membrane depolarizations (Fig. 2B, right). The slope of membrane depolarizations needed to activate phasic cells was five times higher than those required for activating tonic-firing neurons (Table 1). The responses of SB cells to step and ramp-hold current injection were blocked by Ni\(^{2+}\) (Fig. 2C).

As expected, \(I_T\) was found in the majority of neurons in this sample (\(n = 43/46, 93\%)\) and showed considerable variation in amplitude [7 ± 6 pA/pF (SD); 1–36 pA/pF], consistent with the results of our initial voltage-clamp experiments. Figure 3A shows examples of \(I_T\) from tonic, SB-type phasic, and non-SB-type phasic cells, and data from all cells in the sample, expressed as current density, are summarized in Table 1 and Fig. 3B. Notably, \(I_T\) in SB-type phasic cells was almost threefold larger than that recorded from tonic-firing neurons. Thus \(I_T\) expression was greatest in dorsal horn neurons with signature rapidly adapting burst firing that are selectively activated by rapid membrane depolarizations.

Characterization of \(T\)-current components. Previous studies in thalamic neurons (Shcheglovitov et al. 2005; Tarasenko et
al. 1997; Zhuravleva et al. 1999, 2001) suggested that multiple channel subtypes may contribute to the net T-current in dorsal horn neurons when the high-voltage-activated Ca\textsuperscript{2+} current is blocked. We found that for the majority of cells (67%, 29/43), \(I_T\) could be dissected into two components based on time constants of decay (Fig. 4, A and B1). A rapidly inactivating component (“fast”) had a time constant (\(\tau_1\)) of 12.0 \(\pm\) 1.1 ms (\(n = 48\), Fig. 4C), and a slower component (“slow”) inactivated with a significantly longer time constant (\(\tau_2\)) of 177 \(\pm\) 12 ms (\(n = 32\); vs. \(\tau_1\), \(P < 0.0001\); Fig. 4C). The amplitudes of fast and slow currents were similar across cells (4.6 \(\pm\) 0.7 vs. 3.5 \(\pm\) 0.4 pA/pF, \(P = 0.2823\)). However, 10–90% rise time for the fast subtype was shorter than for the slow current (5.2 \(\pm\) 0.8 vs. 14.3 \(\pm\) 3.0 ms, \(P = 0.0167\)), suggesting a difference in activation kinetics. Two fast components, \(\tau_{1,1}\) and \(\tau_{1,2}\) (Fig. 4B2), having significantly different decay \(\tau(5.7 \pm 0.9\) vs. 21.1 \(\pm\) 3.9 ms, \(P = 0.0018\)) but similar current densities (3.2 \(\pm\) 0.5 vs. 3.4 \(\pm\) 0.5 pA/pF, \(P = 0.7374\)) could be distinguished in eight neurons (19%, 8/43). Therefore, the statistical sample size reflects the number of current components, rather than that of recorded neurons. Bath application of Ni\textsuperscript{2+} produced greater reduction of the fast current than the slow component (51 \(\pm\) 5% vs. 15 \(\pm\) 11%, \(P = 0.0144\); Fig. 4D). Furthermore, the fast current in SB-type cells was two- and threefold larger than in tonic and non-SB neurons, respectively, while the slow subtype was uniformly expressed across the different firing pattern groups (Table 2).

We next examined the voltage dependence of steady-state inactivation and activation of the fast and slow T-currents. Inactivation kinetics were assessed by stepping the membrane potential to \(-44\) mV every 20 s in 10-mV increments from conditioning potentials of \(-134\) mV to \(-84\) mV held for 500 ms. Activation properties were studied by delivering 200-ms depolarizing voltage steps in 10-mV increments from \(-64\) mV to \(-24\) mV after a conditioning potential of \(-104\) mV. Currents generated by voltage steps to values below \(-64\) mV were too small to be reliably analyzed by the stripping procedure, perhaps because of the small size of hamster dorsal horn neurons. Average normalized currents (\(I/I_{\text{max}}\)) were plotted as a function of conditioning potentials for inactivation or testing steps for activation (\(n = 3\), “fast”; \(n = 2\), “slow”) and fitted with the Boltzmann equation \(I/I_{\text{max}} = 1/[1 + \exp(V - V_{1/2}(\kappa))]\) (Fig. 5). The half-inactivation potentials for the fast and slow components were \(-96\) mV and \(-80\) mV, respectively, while the voltage required for half-maximal activation was \(-59\) mV for the fast subtype and \(-65\) mV for the slow subtype. The overlap region between the activation and inactivation curves (“window” current) is greater for the slow current component.

To find out what channel subtypes might contribute to \(I_T\) in hamster dorsal horn neurons, we performed single-cell RT-PCR (scRT-PCR) following patch-clamp recordings. Among 29 cells in which GAPDH mRNA was detected in the pipette aspirates, 6 were in L1/LII, 23 were from LIII–IV, and nearly all of them (93%, 27/29) evidenced \(I_T\) (\(7 \pm 1\) pA/pF). Ca\textsubscript{V}3 mRNAs were detected in 12 neurons (Table 3), all except 1 recorded from LIII–IV. The majority (64%, 7/11) had phasic firing patterns, with the remainder (35%, 4/11) being tonic fireers, not different from non-Ca\textsubscript{V}3-expressing neurons. Similarly, Ca\textsubscript{V}3-expressing cells evidenced \(I_T\) similar to their non-Ca\textsubscript{V}3-expressing counterparts (8 \(\pm\) 3 pA/pF vs. 6 \(\pm\) 1 pA/pF). Analyses showed a higher occurrence of the Ca\textsubscript{V}3.1 subunit (67%, 8/12) than Ca\textsubscript{V}3.2 (8%, 1/12) and Ca\textsubscript{V}3.3 (25%, 3/12). An example of a Ca\textsubscript{V}3.1 amplification plot resulting from a second-step real-time PCR, along with a voltage-clamp recording of \(I_T\) from the same cell, is shown in Fig. 6. In contrast to our electrophysiological analyses, there was no evidence from scRT-PCR that single neurons express multiple T-channel subtypes.

**Activation of T-type calcium currents in dorsal horn neurons by voltage ramps.** T-type calcium currents in dorsal horn neurons could have important functional consequences for spinal sensory processing. \(I_T\) in thalamic neurons exhibits a strong rate dependence; activation requires membrane depolarization exceeding 30 mV/s (Crunelli et al. 1989). Therefore, nonuniformity of \(I_T\) expression by dorsal horn neurons could contribute to their differential responses to rate of membrane depolarization and time-varying sensory stimuli (Schneider 2003, 2005). Hence, we investigated whether \(I_T\) in dorsal horn cells is related to the slope of membrane depolarization (dV/dt), similar to the characteristic reported for thalamic neurons (Crunelli et al. 1989; Gutierrez et al. 2001).

\(I_T\) activation dynamics were investigated by using voltage ramps to depolarize neurons to \(-44\) mV at different rates after a 500-ms hyperpolarizing prepulse to \(-104\) mV. As can be
seen in the example in Fig. 7A, the amplitude of \( I_T \) increased and time to peak decreased along with \( \frac{dV}{dt} \) between ramp slopes of 400 mV/s and 2,400 mV/s. Bath application of Ni\(^{2+}\)/H11001 depressed the ramp-activated currents (Fig. 7A, gray) in a manner similar to those activated by step depolarizations. Best-fit regression lines calculated from plots of \( I_T \) amplitude versus ramp slope for seven cells are shown in Fig. 7B. Both the \( x \)-intercept and slope varied widely within our sample. The threshold for \( I_T \) activation, as estimated from the \( x \)-intercept values, averaged 46 \( \pm \) 21 mV/s (2–138 mV/s). The sensitivity of \( I_T \) to ramp \( \frac{dV}{dt} \) (defined as slope of the regression lines, pA·s/mV) varied nearly fivefold (51–242 pA·s/mV) for the cells studied. Taken together, the data suggest that dorsal horn neurons are heterogeneous with respect to activation by voltage ramps. Four additional neurons were examined with a similar protocol ramping the membrane voltage from a conditioning potential of -114 mV to -104 mV to a potential of -34 mV, but with the Ba\(^{2+}\) concentration lowered to 2 mM Ba\(^{2+}\) and Cd\(^{2+}\) increased to 40 \( \mu \)M. The maximum amplitude, threshold, and rate sensitivity of \( I_T \) under these circumstances were not significantly different from data obtained with the standard conditions described above. Despite the cell-to-cell variability, regression analyses showed that 87 \( \pm \) 4% of the current amplitude (72–99%) was

### Table 2. Comparison of \( I_T \) subtypes in spinal dorsal horn neurons with different firing properties

<table>
<thead>
<tr>
<th></th>
<th>Phasic</th>
<th>Tonic (n = 14)</th>
<th>Short burst (n = 7)</th>
<th>Non-short burst (n = 16)</th>
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<tbody>
<tr>
<td><strong>“Fast” current, pA/pF</strong></td>
<td>4.2 ( \pm ) 0.9 (16)</td>
<td>9.0 ( \pm ) 2.7 (9)*†</td>
<td>3.0 ( \pm ) 0.3 (17)</td>
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<tr>
<td>( \tau_c ) ms</td>
<td>15.3 ( \pm ) 2.6 (16)</td>
<td>9.3 ( \pm ) 1.2 (9)</td>
<td>10.3 ( \pm ) 1.0 (17)</td>
<td></td>
</tr>
<tr>
<td><strong>“Slow” current, pA/pF</strong></td>
<td>3.8 ( \pm ) 0.9 (8)</td>
<td>3.3 ( \pm ) 0.8 (7)</td>
<td>3.6 ( \pm ) 0.8 (13)</td>
<td></td>
</tr>
<tr>
<td>( \tau_c ) ms</td>
<td>174 ( \pm ) 18 (8)</td>
<td>167 ( \pm ) 26 (7)</td>
<td>187 ( \pm ) 25 (13)</td>
<td></td>
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</table>

Data are given as means \( \pm \) SE. \( \tau_c \), time constant of fast component; \( \tau_c \), time constant of slow component. The number of measurements used to calculate each value is given in parentheses if different from the number of cells for the group. *Significantly different from tonic cells; †significantly different from non-short burst cells.
tion threshold estimated from the current amplitude was determined by regression analysis indicated that 90% of the fast (46 mV/s, significantly greater than the value observed for the and Randic 1990; Walsh et al. 2009). Our new findings point phasic-firing neurons to signal rapid membrane depolarization.

**DISCUSSION**

The present results supplement earlier studies showing that rodent dorsal horn neurons possess a low-threshold “T-type” Ca$^{2+}$ current (Huang 1989; Russo and Hounsgaard 1996; Ryu and Randic 1990; Walsh et al. 2009). Our new findings point out that, although $I_T$ appears to be prevalent among spinal LI–IV cells, expression is nonuniform, being highest in neurons responding to depolarizing current pulses with a rapidly adapting discharge of one or two action potentials. Here, we show that $I_T$ in the dorsal horn is dependent on the rate of membrane depolarization. In light of our observations that both $I_T$ and action potential discharges of phasic-firing dorsal horn neurons are activated by similar rates of mem-

**Fig. 5. Steady-state activation and inactivation of fast and slow T-currents in spinal dorsal horn neurons.** (A) and (B) for the fast (A) and slow (B) subtypes were fitted with the Boltzmann equation ($H_{max} = 1/[1 + \exp((V - V_{1/2})/k)]$). $V_{1/2}$ and $k$ derived from the fits are shown next to each curve. Error bars represent SE (n = 3, A and n = 2, B). $r = 0.99$ for all fits in A and B.

$\frac{d}{dt}$ $\left(\begin{array}{c} \text{fast} \\ \text{slow} \end{array} \right)$ $\left(\begin{array}{c} \text{activation} \\ \text{inactivation} \end{array} \right)$ $\text{rate}$ $\text{threshold}$ $\text{current}$ $\text{component}$ $\text{activation}$ $\text{inactivation}$ $\text{voltage}$ $\text{ramp}$ $\text{rate}$ $\text{dependence}$ $\text{amplitude}$ $\text{independent}$ $\text{dV}{/dt}$ $\text{fast}$ $\text{slow}$ $\text{amplitude}$ $\text{net}$ $\text{T}$-current (46 mV/s, significantly greater than the value observed for the

**Fig. 6. Detection of CaV3.1 subunit mRNA from a dorsal horn neuron with a “fast” $I_T$ component (see text).** Amplification plots of CaV3.1 subunit fragments were generated from a real-time PCR experiment. Each measurement was averaged from 3 replicate reactions. The fluorescence signal was normalized to an internal passive reference dye (dRn), and plots were base-lined by Strategene software. Amplified product from cell 080707-1 (●) and hamster spinal cord cDNA with (●, positive control 1) and without (●, positive control 2) a first-step CaV3 PCR amplification are shown. For a negative control, cDNA template was replaced with water (○). The threshold fluorescence value (indicated by dashed line) was determined by the software using an amplification-based algorithm, resulting in threshold cycles (C) of 23, 31, and 35 for positive control 1, amplified cell product, and positive control 2, respectively. Insert: the decay of $I_T$ (bottom) recorded from the same dorsal horn neuron can be fitted with a single exponential ($r = 20$ ms). The voltage command used to activate the current is shown above the trace.
brane depolarization and are blocked by T-type Ca\(^{2+}\) channel antagonists, \(I_T\) appears to contribute to selective excitation of these cells by rapid membrane depolarizations (Schneider 2003, 2005).

\(I_T\) expressed in hamster dorsal horn resembles a low-voltage-activated, transient Ca\(^{2+}\) current reported elsewhere in the CNS. Steady-state activation and inactivation kinetics are broadly similar to T-type Ca\(^{2+}\) currents in thalamus (Crunelli et al. 1989), hypothalamus (Niespodziany et al. 1999), suprachiasmatic nucleus (Kim et al. 2005), and cerebellum (Mouginot et al. 1997). As in these studies, our internal pipette solution contained the Ca\(^{2+}\) buffer EGTA. EGTA has been reported to slow the time course of recombinant CaV3.1 channel activation and inactivation but have no effect on the current density or voltage dependence of steady-state inactivation (Lacinová et al. 2006). Therefore, as with similar studies using a Ca\(^{2+}\) buffer in the internal recording solution, we cannot rule out possible effects of EGTA on T-channel function and on neuronal firing properties. We found that Ni\(^{2+}\) was an incomplete antagonist of \(I_T\) when applied at a concentration of 200 \(\mu\)M. This might be explained by an observation that 100 \(\mu\)M Ni\(^{2+}\) blocked only 30–50% of the current through CaV3.1 and CaV3.3 T-type currents recorded from cloned channels in oocytes and HEK-293 cells (Lee et al. 1999). We also found that mibefradil potency was lower than what has been reported in other studies (Kim et al. 2005; Lee et al. 2002; McDonough and Bean 1998; Viana et al. 1997). This may be due to the fact that mibefradil is a less effective T-channel antagonist when Ba\(^{2+}\) replaces Ca\(^{2+}\) as the charge carrier (Martin et al. 2000).

As discussed below, incomplete antagonism of \(I_T\) by Ni\(^{2+}\) and mibefradil may also indicate that other ionic conductances contributed to the inward current in our experiments.

Our experiments were performed on hamster spinal cord at an age when organization and electrical properties of dorsal horn neurons undergo developmental changes in rodents (Beal et al. 1988; Bicknell and Beal 1984; Walsh et al. 2009). For example, it has been reported that A-type potassium current in mouse spinal LI–II neurons decreases during the first 3 wk of

**Fig. 7. Activation of \(I_T\) in dorsal horn neurons by ramp depolarizations. A: \(I_T\) in a representative cell was activated by using voltage ramps (bottom) to depolarize the membrane potential to \(-44\) mV from a 500-ms conditioning potential of \(-104\) mV at different rates (indicated to left of each current trace). \(I_T\) before (control, black traces) and during (Ni\(^{2+}\), gray traces) presentation of 200 \(\mu\)M Ni\(^{2+}\) is shown. B: linear regression analysis between \(I_T\) amplitude and ramp slope is shown for 7 dorsal horn neurons recorded in media containing 2 or 3 mM Ba\(^{2+}\). Individual responses to ramp depolarizations for regression line \(a\) are shown in A. Inset: representative linear fit to data points for regression line \(b\). Solid lines denote tonic cells, and dashed lines indicate phasic cells (without regard to burst duration). The coefficients of determination \((r^2)\) from regression analyses (0.72–0.99) indicate that \(I_T\) amplitude and ramp slope are highly correlated. C: relationship between the amplitude of \(I_T\) current components and the ramp slope is described by a linear function for 3 dorsal horn neurons. C1: rate sensitivity of the fast component. C2: rate sensitivity of the slow component. The coefficient of determination \((r^2)\) from linear regression analysis indicates that the amplitude of the fast subtype and the slope are highly correlated, but the amplitude of the slow component does not parallel changes in ramp slope.
life (Walsh et al. 2009). Although the discharge properties of tonic and phasic firing neurons in 9- to 14-day-old hamsters were broadly similar to those recorded from animals at 3–4 wk of age (Schneider 2003), the extent to which dorsal horn T-type Ca\(^{2+}\) channel expression changes postnatally and how these changes influence discharge properties is not known. In the present study, we found that nearly all dorsal horn neurons expressed \(I_T\), with no difference between neurons recorded in the superficial and deep laminae. However, Walsh et al. (2009) reported that a \(T\)-current was present in only 25% of superficial dorsal horn neurons in mouse at ages comparable to the hamsters used our study. Therefore, it is possible that \(T\)-type current expression in dorsal horn neurons may exhibit species, as well as developmental, differences.

### Ionic basis of responses to time-varying membrane depolarizations

The activation of \(I_T\) in dorsal horn neurons by rapid ramp depolarizations resembles a property of T-type Ca\(^{2+}\) currents described in thalamic neurons (Crunelli et al. 1989), adrenal glomerulosa cells (Vármai et al. 1995), and olfactory receptor neurons (Kawai and Miyachi 2001). In particular, depolarizations exceeding 30 mV/s are required to activate an \(I_T\) in cat lateral geniculate neurons (Crunelli et al. 1989). In thalamic cells, voltage ramps of lower velocity appear to reverse the removal of T-channel inactivation by a prior hyperpolarization, so that there are fewer channels available for activation, resulting in diminishing current amplitude at slower membrane depolarizations (Crunelli et al. 1989; Gutierrez et al. 2001). It should be noted that cell-to-cell variability in the threshold for ramp activation for \(I_{hm}\) in hamster dorsal horn neurons (46 ± 21 mV/s) is greater than that reported by Crunelli et al. (1989) for thalamic cells (30 ± 2 mV/s). Furthermore, the sensitivity of dorsal horn neurons to rate of depolarization varied considerably between cells (Fig. 7B). These differences could reflect expression of T-channel subunits differing in activation kinetics (Chemin et al. 2002; Talley et al. 1999).

Several observations suggest that \(I_T\) contributes to an ability of phasic-firing dorsal horn neurons to selectively signal rapid membrane depolarizations and rapidly moving cutaneous stimuli (Schneider 2003, 2005). First, \(I_T\) activated when dV/dt exceeded ~46 mV/s and increased with the rate of depolarization. Second, phasic-firing cells expressed the highest \(I_T\) density in the dorsal horn. Third, Ni\(^{2+}\) blocked rate-sensitive activation of \(I_T\) under voltage-clamp conditions and also a transient depolarization with associated action potential discharge under current clamp. Although these findings support an involvement of \(I_T\) in shaping the ability of certain dorsal horn neurons to signal dynamic membrane depolarizations, the present experiments do not rule out the participation of other ionic mechanisms. For example, we found that the rate of membrane depolarization for activating SB and non-SB type phasic cells was significantly higher than for activating tonic cells. However, \(I_T\) was elevated only in SB cells, suggesting that another mechanism may underlie rate sensitivity in non-SB cells (Table 1). Safronov et al. (1997) described a slowly inactivating, TTX-sensitive Na\(^+\) current accounting for ~5–20% of total Na\(^+\) current in rat dorsal horn neurons that broadly resembles a persistent Na\(^+\) current \((I_{NAP})\) activated near ~65 mV (Magistretti et al. 2006; Magistretti and Alonso 1999; Parri and Crunelli 1998). Recent studies have also reported that \(I_{NAP}\) in spinal lamina I neurons and ventral horn motoneurons is activated by voltage ramps (Kuo et al. 2006; Prescott and De Koninck 2005). This current has a slower threshold (~10 mV/s) for ramp activation but could still contribute to neuronal excitation over the range of ramp depolarizations used in the present study.

### Expression of multiple T-channel subtypes in dorsal horn neurons

We used an indirect method in the present study to distinguish subtypes of T-currents in dorsal horn neurons based on differences in kinetics of inactivation. The results suggest that the net T-current is composed of “fast” and “slow” components, having decay time constants similar to two subtypes of low-voltage-activated Ca\(^{2+}\) currents reported for thalamic neurons (Shcheglovitov et al. 2005; Zhuravleva et al. 1999, 2001). The current densities for the dorsal horn subtypes (~3–4 pA/pF) are similar to neurons recorded in thalamic slices (Tarašenko et al. 1997), and the two subtypes appear to be present in approximately equal ratios in the spinal cord and thalamus. Besides kinetics, the two subtypes also appear to have other distinguishing features. First, the activation and inactivation curves for the slow component exhibit a larger overlap region (“window” current) than the fast subtype, similar to the slowly inactivating low-voltage Ca\(^{2+}\) current in isolated thalamic neurons (Zhuravleva et al. 2001). Second, the fast subtype was more effectively blocked by Ni\(^{2+}\) than the slow subtype, consistent with thalamic neurons recorded in transverse brain slices (Jokovic et al. 2005; Shcheglovitov et al. 2005). Third, and perhaps most interesting from a functional standpoint, we found that the amplitude of the fast current component was correlated to the slope of depolarizing voltage ramps, whereas the slow subtype appeared to be independent of depolarization rate over the testing range. Although rate sensitivity of the T-current subtypes was determined in only a few neurons, it is nonetheless tempting to speculate that the responses of phasic cells in the spinal dorsal horn to rapid membrane depolarizations as reported here and elsewhere (Schneider 2003, 2005) may partly arise from expression of the fast channel subtype. A result that bears mentioning is that the average threshold for activation of the fast current by voltage ramps was >10-fold higher that the activation threshold of \(I_T\). One possible explanation for this difference is that activation of the slow channels during ramp depolarizations might cause partial inactivation of the rate-sensitive fast channels, thereby shifting the activation threshold of the net \(I_T\) to slower voltage rates.

Our results showing that a subpopulation of spinal LI–IV neurons express Ca\(_{3.1}\) mRNA are consistent with a previous report of T-type channel transcripts in the spinal cord. The finding that Ca\(_{3.1}\) was the most abundant of the three known T-channel subtypes is in agreement with Talley et al. (1999), who reported relatively higher signal for \(\alpha_1G\) (Ca\(_{3.1}\) mRNA) than for \(\alpha_1H\) (Ca\(_{3.2}\) and \(\alpha_1L\) (Ca\(_{3.3}\)) mRNA in the rat spinal cord. We found that the proportion of dorsal horn neurons expressing Ca\(_{3.3}\) mRNA (44%) was much lower than the proportion of cells exhibiting a macroscopic T-current (>90%). In performing analyses only on samples in which GAPDH mRNA was detected, we hoped to reduce the impact of incompletely harvesting cellular mRNA in the patch pipette. Still, we may not have collected mRNA from dendrites, where some Ca\(_{3}\) gene products may function (Shcheglovitov et al. 2005; Tarašenko et al. 1997; Zhuravleva et al. 2001). We also took measures to limit recording time and protect the targeted
CaV3 transcripts from degradation. However, it is possible that our procedures were not optimal for the detection of small amounts of CaV3 mRNA. In fact, other studies using scRT-PCR have also reported that only a fraction of cells with functional neurotransmitter receptors express the corresponding mRNA (e.g., Férézou et al. 2006; Gallopin et al. 2005). It is therefore likely that the proportion of dorsal horn neurons expressing CaV3 isotypes is higher than what is indicated by our scRT-PCR results. Nonetheless, our results suggest that CaV3.1 is the most common subtype in the hamster dorsal horn and is expressed by neurons having a wide range of firing properties.

Determining the identity of CaV3 subunits that underlie kinetically distinct fast and slow T-currents in dorsal horn cells was beyond the scope of the present study. However, comparisons of the properties of endogenous T-current components with studies on recombinant T-current channel expression may offer clues about what molecular subtypes contribute to neuronal excitability in the dorsal horn. First, the decay τ we estimated for the slow current is similar to the inactivation τ reported for recombinant CaV3.3 subunits (Chemin et al. 2002; Klöckner et al. 1999; McRory et al. 2001). Second, decay τ of the fast current is in the range reported for inactivation of cloned CaV3.1 and CaV3.2 subunits (Chemin et al. 2002; McRory et al. 2001). Finally, our isolation of two fast currents having different decay τ also imply contributions from CaV3.1 and CaV3.2 subunits. These findings suggest that CaV3.1, and possibly CaV3.2, underlie the fast T-current in dorsal horn cells and that CaV3.3 contributes to the slow component, in general agreement with conclusions reached for thalamic neurons (Shcheglovitov et al. 2005).

Functional significance of dorsal horn T-current. Our results suggest a role for T-type Ca2+ channels in spinal sensory function besides spinal nociception (Heinke et al. 2004; Ikeda et al. 2003). \(I_T\) is largest in phasic-firing dorsal horn neurons that are selectively activated by a steep voltage trajectory. The dependence of \(I_T\) on high rates of membrane depolarization may boost responses to fast excitatory postsynaptic potentials (EPSPs), relative to slow EPSPs, generated by nociceptive and tactile inputs terminating in laminae I–II and III–IV, respectively. Almost all phasic cells in the dorsal horn are interneurons that form connections with neighboring neurons (Schneider 2003, 2008), suggesting that \(I_T\) contributes to signaling related to rapid stimulus movement or rate of change within local networks (Schneider 2003, 2005). Other mechanisms may selectively influence responses to sustained or slowly changing inputs. A persistent Na+ current (\(I_{NaP}\)) appears to enhance responses of spinal neurons to sustained excitatory inputs (Kuo et al. 2006). \(I_{NaP}\) is strongly expressed in tonic-firing dorsal horn cells and prolongs responses to transient inputs, leading to the proposal that it facilitates encoding of stimulus intensity (Prescott and De Koninck 2005). Thus differential expression of transient and persistent inward currents may contribute to processing of stimulus intensity and velocity information in functionally defined spinal circuits. T-channels may further tune responses to afferent inputs by modulating hyperpolarizing K+ currents. Calcium influx through T-channels modulates gating of small-conductance Ca2+-activated potassium (SK) channels (Cueni et al. 2009), which are strongly expressed in the dorsal horn (Mongan et al. 2005). Activation of SK channels decreases neuronal excitability by increasing firing adaptation to membrane depolarization (Stoker 2004) and may represent another means for sharpening dorsal horn neuronal responses to transient stimuli. Moreover, calcium entry from T-channels can also regulate the function of low-voltage-activated A-type K+ channels (Anderson et al. 2010). A-type K+ channels are also widely expressed in dorsal horn neurons (Grudt and Perl 2002; Hu and Gereau 2011; Huang et al. 2005; Walsh et al. 2009), suggesting that \(I_T\) may control firing frequency and timing by this mechanism as well. The influence of T-type channels on neuronal firing via modulation of K+ channel function would appear to be strongest for SB-type phasic cells, which express the largest \(I_T\). Taken as a whole, we believe our results expand a role for low-voltage, T-type Ca2+ channels in spinal sensory processing and suggest that these channels are particularly important for sharpening responses of dorsal horn neurons to afferent sensory stimuli that vary rapidly in the time domain.

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DISCLOSURES

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