Cellular/Molecular

Presynaptic $Ca_V3.2$ Channels Regulate Excitatory Neurotransmission in Nociceptive Dorsal Horn Neurons

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It is generally accepted that presynaptic transmitter release is mainly regulated by subtypes of neuronal high-voltage-activated Ca^{2+} channels. Here for the first time, we examined the role of T-type Ca^{2+} channels (T-channels) in synaptic transmission in the dorsal horn (DH) of the spinal cord using patch-clamp recordings from acute spinal cord preparations from both rat and mouse. We found that selective pharmacological antagonism of T-channels inhibited spontaneous synaptic release of glutamate in superficial laminae I-II of the DH, while GABA release was spared. We found similar effect in identified nociceptive projection neurons of lamina I of the DH, but not in inhibitory DH interneurons. In comparison, antagonism of T-channels did not affect excitatory transmission in deeper non-nociceptive DH laminae. Furthermore, we used isoform-specific agents, knock-out mice and immunohistochemistry to specifically implicate presynaptic $Ca_V3.2$ channels. We also used an animal model of painful diabetic neuropathy to demonstrate that blocking T-channels in superficial DH neurons suppressed spontaneous excitatory synaptic transmission in diabetic rats in greater degree than in healthy age-matched animals. These studies provide previously unknown information regarding the role of presynaptic T-channels in nociceptive signaling in the spinal cord.

Introduction

The synapses formed between primary afferent fibers that carry nociceptive information and second-order neurons of the superficial laminae (lamina I and II) of the dorsal horn (DH) of the spinal cord (Bennett, 1998) are critical components in the pain-processing pathway and are regulated by both presynaptic and postsynaptic inputs. Central sensitization results from plasticity of neurons of the CNS and underlies many pain disorders including chronic neuropathic pain. DH neurons exhibit an increased synaptic efficacy, increased membrane excitability, or reduced inhibition during sensitization (Latremoliere and Woolf, 2009). Plasticity of glutamatergic receptors in pain pathways has been recognized as an important factor contributing to sensitization (Campbell and Meyer, 2006), however changes in voltage-gated ion channels that control excitatory and inhibitory synaptic transmission may contribute as well. Several subtypes of voltage-gated calcium channels (VGCCs) are expressed in neurons in the pain pathway and are crucial not only in shaping action potentials, but in controlling cellular excitability and synaptic transmission in these cells. Neuronal VGCCs are heteromeric complexes in the plasma membrane and are divided in two major subclasses based on the potentials at which they gate ion currents. High-voltage-activated (HVA) channels require strong membrane depolarization for activation while low-voltage-activated (LVA or T) channels activate with small membrane depolarization. Recent studies have suggested an important role for T-channels in peripheral sensitization of pain responses (for review, see Jevtovic-Todorovic and Todorovic, 2006; Todorovic and Jevtovic-Todorovic, 2011). Furthermore, the role of T-channels in DH in neuropathic pain responses has been suggested with *in vivo* electrophysiological (Matthews and Dickenson, 2001) and behavioral studies (Maeda et al., 2009) where intrathecal administrations of T-channel blockers rapidly reduced neural excitability and pain responses, respectively. While these studies suggest influence of T-channels on sensory neurotransmission, the contribution of either presynaptic or postsynaptic T-channels to sensory processing in spinal DH neurons is not well studied mostly because of the lack of selective pharmacological agents. Most previous studies examining T-channels in the DH have used less specific blockers such as ethosuximide (Matthews and Dickenson, 2001), nickel and mibefradil (Maeda et al., 2009) to determine channel function, which has left the precise role that T-channels play in sensory transmission in the DH largely unknown. Importantly, at least one study demonstrated that spontaneous glutamate release in laminae I-II of DH is largely calcium-dependent (Bao et al., 1998). Thus, we hypothesize that $Ca_V3.2$ T-channels may have a novel and relatively unique role in supporting spontaneous synaptic neurotransmitter release in pain pathway. To address this issue we took advan-

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tage of the recently discovered selective and potent antagonist of T-channels TTA-P2 (Shipe et al., 2008; Choe et al., 2011). We used patch-clamp recordings from acute spinal cord preparations of rats and mice and immunohistochemistry to provide evidence for a novel presynaptic role of $Ca_V3.2$ T-channels in spontaneous excitatory synaptic transmission in the DH of the spinal cord.

Materials and Methods

Preparation. Spinal cord slices from Sprague Dawley rats 7–35 d old were prepared as described previously (Li and Perl, 1994). Briefly, rats of either sex were deeply anesthetized using isoflurane and the lumbar region of the spinal column was quickly removed, euthanizing the animal. Rats and mice were housed in a local animal facility in accordance with protocols approved by the University of Virginia Animal Use and Care Committee. We adhered to the guidelines in the National Institute of Health Guide for the Care and Use of Laboratory Animals. The spinal cord was then removed and placed in ice-cold sucrose solution which contained the following (in mM): 50 sucrose, 92 NaCl, 15 D-glucose anhydrous, 26 $NaHCO_3$, 5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, and 7 $MgSO_4$ equilibrated with 95% O_2 and 5% CO_2. After removing dorsal roots and dura, the spinal cord was embedded in 2% agarose for slicing. Horizontal slices, 300 μm thick were then sectioned from the spinal cord using a vibratome (TDK-1000, Ted Pella Instruments). The slices were incubated for a minimum of 30 min in constantly oxygenated artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 20 D-glucose anhydrous, 26 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$ at 37° before being used for electrophysiological recordings. Slices were then placed in a recording chamber (1 ml bath volume, RC-24N, Warner Instruments) that was steadily superfused at a rate of 2 ml/min. Slices were maintained in recording chamber at room temperature on room air, where they remained viable for $\geq$1 h.

Electrophysiology. Lamina I and II in the superficial layers of the spinal dorsal horn were recognized as translucent bands using a light microscope. Once this region was identified, recording electrodes with resistances of 5–10 MΩ were used to perform whole-cell recordings. Neurons were considered to be from deep lamina when present in slices taken from a depth of 600 μm from the dorsal surface of the spinal cord. Individual dorsal horn neurons were visually identified by videomicroscopy (high-performance CCD camera, Cohu) combined with a BX51WI microscope with oblique illumination (Olympus). For recording miniature EPSCs (mEPSCs), the external solution consisted of ACSF supplemented with 1 μM tetrodotoxin (TTX) to block spontaneous action potential (AP) generation and 20 μM picrotoxin, a $GABA_A$ antagonist, to block miniature IPSCs (mIPSCs). The internal solution for these experiments contained the following (in mM): 130 K^+-D-gluconate, 5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 11 EGTA, 10 HEPES, and 4 NaATP, ph 7.3. For recording of mIPSCs, the external solution consisted of ACSF containing 5 mM KCl, supplemented with 1 μM TTX (Tocris Bioscience) and 5 μM NBQX (Tocris Bioscience), an AMPA receptor antagonist, to block mEPSCs. The pipette solution for mIPSC recordings contained the following (in mM): 130 KCl, 4 NaCl, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, 2 MgATP, and 0.5 Tris-GTP, pH 7.3. Recordings are determined to be from neurons by the appearance of large capacitative transients after rupture of gigaohm seals and presence of spontaneous synaptic potentials. Neurons were held at −70 mV for all experiments. In all experiments measurements were made from one neuron per slice.

Recordings were made using a MultiClamp 700B patch-clamp amplifier (Molecular Devices) at a sampling rate of 10 kHZ using a low-pass Bessel filter of 1 kHz. Data were acquired and analyzed using pClamp10 software. The frequency, amplitude, and time to decay of spontaneous postsynaptic events were analyzed using the MiniAnalysis Program by Synaptosoft. For generating all datasets, a minimum of 200 events were analyzed.

Drugs. All agents were added directly to the ACSF in the recording chamber. TTA-P2 (3, 5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydropyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide) (Merck) was made up as a 250 μM stock in dimethyl sulfoxide (DMSO) and diluted freshly for experiments so that the final concentration was <0.04%. In most experiments stock solution of a particular drug was applied to the bath directly with a pipette, giving a calculated nominal concentration of it in the bath as indicated in the results. However, since drug diffusion in the intact spinal cord slice preparation is compromised due to a decline in drug concentration along the length of the bath and diffusion through the tissues, actual concentrations of drugs are likely to be much lower than those reported. Picrotoxin was obtained from Sigma. 3βOH was a kind gift from Dr. Doug Covey (Washington University, St. Louis, Missouri).

Retrograde labeling of spino-parabrachial neurons. Stereotaxic injections were performed on Sprague Dawley rats 4 d old as described previously (Ikeda et al., 2003). Briefly, rats were anesthetized with isoflurane and placed in the stereotaxic apparatus. A small cut was made in the scalp and a hole was drilled into the skull above the target brain area. A 500 nl Hamilton syringe was then used to inject 80 nl of [1, 1′-Dioctadecyl-3, 3, 3′-tetramethylindocarbocyanine iodide] (DiI) (Invitrogen) (2.5%) into the right lateral parabrachial area. The head wound was then closed using Gluture skin adhesive (Abbott Labs) and animals were monitored daily until use. After a 3–5 d survival period, spinal cord slices were prepared as described above. mEPSC recordings were then made from fluorescently labeled projection neurons using standard appropriate fluorescence filter.

Induction of painful diabetic neuropathy (PDN). Male and female Sprague Dawley rats 3–4 weeks old were used for these experiments. We used a well established model to induce PDN as we reported previously (Jagodic et al., 2007; Messinger et al., 2009). We intraperitoneally (i.p.) injected freshly dissolved streptozocin in sodium citrate buffer at pH 5–6 at a dose of 200 mg/kg. After injection of STZ blood glucose levels were measured weekly using a blood glucometer (Accu-Check Active, Roche Diagnostics). Rats having a blood glucose reading >400 mg/dl were considered hyperglycemic and were included in the experimental group. Rats of the same age that did not receive STZ-injections were used as controls.

Immunohistochemistry. Spinal cord slices were prepared as above except cut transversely and to a thickness of 50 μm. After the slicing procedure, slices were rinsed 2 times with phosphate-buffed saline (PBS) and placed in 4% paraformaldehyde (PFA) for 1 h as a fixative. After fixing, slices were rinsed 3 times in PBS and then placed in 5% donkey serum prepared in 0.1% Triton-X/PBS for 1 h at room temperature (RT). Samples were then washed 3 times in PBS. Following the last wash, samples were incubated with the appropriate concentration of primary antibody for the protein of interest. We obtained new anti-$Ca_V3.2$ rabbit polyclonal antibody raised against epitope corresponding to amino acids 581–595 of rat intracellular loop connecting D1 and D2 transmembrane domains of $Ca_V3.2$ (Sigma-Aldrich). For $Ca_V3.2$, primary antibody (Sigma-Aldrich) was used at a concentration of 1:250 in PBS; for isolectin B4, an antibody to lectin from *Bandeiraea simplicifolia* conjugated to fluorescein isothiocyanate (FITC) (Sigma) was used at a 1:500 dilution. A mouse monoclonal anti-calcitonin gene-related peptide (CGRP) antibody (Sigma) was also used at a dilution of 1:500. Appropriate secondary antibodies conjugated to either AlexaFluor488 or AlexaFluor546 (Invitrogen) were used at a dilution of 1:5000.

$Ca_V3.2$ (−/−) mice. Experiments were performed using $Ca_V3.2$ knock-out (KO) mice or age-matched wild-type (WT) B6/C57 mice as we described previously (Joksovic et al., 2006). Mice ages 7–14 d old were used and DH spinal cord slices were prepared as described above.

Results

Using whole-cell, patch-clamp recordings from horizontal slices that contain the DH of the spinal cord we observed that neurons from lamina I and II of the DH exhibit spontaneously occurring, small inward currents in the absence of stimulation (Fig. 1*A*). These events are variable in amplitude when the neuron is held at −70 mV and are almost completely abolished in the presence of the AMPA-receptor antagonist, NBQX (Fig. 1*A*, lower trace) confirming that they are spontaneous miniature excitatory synaptic currents (mEPSCs). For all mEPSCs recordings,

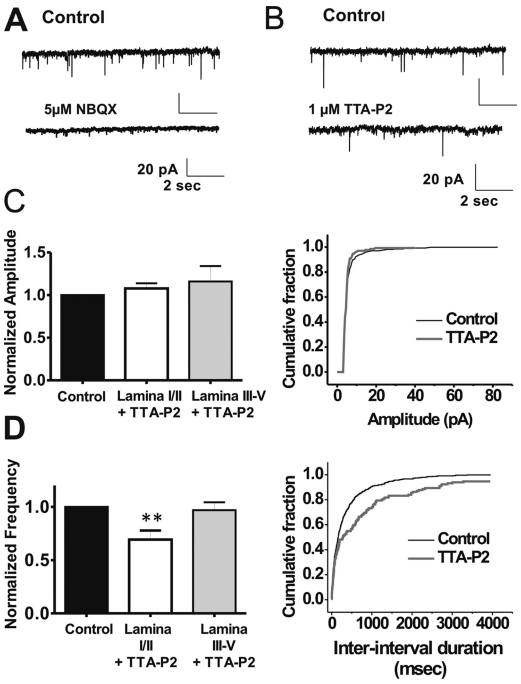

Figure 1. Effects of TTA-P2 on spontaneous mEPSCs from neurons in the superficial lamina of the DH of the spinal cord. Voltage-clamp, whole-cell recording from a lamina I neuron in a horizontal spinal cord slice held at −70 mV. ***A***, Recording of mEPSC activity from a single lamina I neuron in ACSF alone (Control, top) and after bath application of 5 μM NBQX, an AMPA-receptor antagonist (bottom). ***B***, Recording of mEPSC activity from a single representative neuron in ACSF (Control, top) and after bath application of 1 μM TTA-P2 (bottom). ***C***, Pooled data showing normalized mEPSC amplitude under control conditions (filled bar) and with TTA-P2 present in lamina I and II (open bar, $n = 10$) and in deeper lamina (III-V) of the DH (gray bar, $n = 8$). Error bars indicate SEM. Cumulative distributions of event amplitudes resulting from one representative experiment (same cell as ***B***) in the superficial lamina (right). ***D***, Pooled data showing normalized mEPSC frequency under control conditions (4.03 ± 1.09 Hz, 2.45 ± 0.34 Hz) (filled bar) and in the presence of TTA-P2 in lamina I/II neurons (2.40 ± 0.64 Hz) (open bar) and deep lamina neurons (2.38 ± 0.38 Hz) (gray bar). Error bars indicate SEM. **, significantly different ($p < 0.01$) from control. Cumulative distributions of event frequencies resulting from one representative experiment in the superficial lamina, same cell as ***B*** (right).

1 μM tetrodotoxin (TTX) and 20 μM picrotoxin were included in the extracellular solution to block spontaneous action potential (AP) generation and inhibitory $GABA_A$-mediated currents, respectively.

The role of subtypes of VGCCs that control Ca^{2+} entry into presynaptic nerve terminals during spontaneous synaptic transmission (mEPSCs, mIPSCs) in the spinal cord remains unclear. For our studies we first examined mEPSCs in the presence of the novel, piperidine derivative 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydropyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2). This compound has been shown to be a potent and selective blocker of both recombinant (IC_{50} 20–100 nM) (Shipe et al., 2008) and native T-channels (IC_{50} 100 nM) (Dreyfus et al., 2010; Choe et al., 2011). We recorded mEPSCs from the same neuron before (control) and after addition of 1 μM TTA-P2 to the bath solution. For all recordings, 1 μM tetrodotoxin (TTX) and 20 μM picrotoxin were included in the extracellular solution to block spontaneous action potential (AP) generation and inhibitory currents, respectively. In the presence of TTA-P2, there is a significant decrease (~34%) in the frequency of mEPSCs (Fig. 1*B*,*D*), however no significant change in amplitude (Fig. 1*B*,*C*) or decay time (data not shown) of mEPSCs was observed ($n = 10$). In the presence of vehicle alone (DMSO <0.04%) no changes in mEPSC frequency, amplitude, or decay time were observed ($n = 4$, data not shown). For all studies, average recording time of mEPSCs was ~10 min and no significant rundown was observed during this time. To further test the idea that T-channels may contribute to mEPSCs in these cells, we used a novel neuroactive steroid, 3βOH. Our previous studies established that 3βOH (3β, 5β,17β)-3-hydroxyandrostane-17-carbonitrile is a voltage-dependent and selective blocker of T-channels in acutely dissociated DRG cells (IC_{50} 0.7 μM) (Todorovic et al., 2004) and at these concentrations selectively inhibits burst firing in these cells (Nelson et al., 2005). We found that 1 μM 3βOH, similarly to TTA-P2, reduced the frequency (29.8 ± 7.6%, $n = 6$, $p < 0.001$) but not the amplitudes (12.7 ± 13.4%, $n = 6$, $p > 0.05$) of mEPSCs in superficial laminae of DH (data not shown). It is generally accepted that alterations in frequency of mEPSCs with any agent targeting an ion channel indicate that the particular channel may play a presynaptic role, while changes in amplitude or decay time of mEPSCs would suggest a postsynaptic role of the channel. Thus, these data strongly suggest that presynaptic T-channels may play a crucial role in excitatory synaptic transmission in the superficial lamina of the DH of the spinal cord. Primary afferent nociceptive fibers from the periphery often terminate on neurons in lamina I and II of the DH of the spinal cord making these neurons critical in the pain-processing pathway. On the other hand, neurons in the deeper lamina of the DH mainly receive input from mechanosensitive, non-nociceptive sensory fibers. To determine whether the effect of T-channels on mEPSCs was specific to putative nociceptive neurons, we also performed the experiment with TTA-P2 on neurons from deeper lamina (lamina III-V) of the DH. In contrast to its effect in superficial laminae, we found that 1 μM TTA-P2 in these neurons did not change mEPSC amplitude (Fig. 1*C*), frequency (Fig. 1*D*), or decay time (data not shown).

Table 1. Contribution of HVA channels to mEPSCs in the superficial and deep laminae of the DH of the spinal cord

Agents (number of cells)	mEPSC (frequency)	mEPSC (amplitude)
Superficial laminae		
Agent		
500 nM SNX-482 (8)	−24.5% (±9.6), *$p < 0.05$	+9.6% (±13.7), NS
1 μM ω-conotoxin GVIA (7)	−16.1% (±12.1), NS	−2.8% (±5.8), NS
10 μM nifedipine (5)	−6.0% (±11.6), NS	−31% (±10.6), *$p < 0.05$
1 μM agatoxin IVA (7)	+6.7% (±18.1), NS	−32.9% (±9.1), *$p < 0.05$
Deeper laminae		
500 nM SNX-482 (7)	+26.0% (±14.4), NS	+0.1% (±12.6), NS
1 μM ω-conotoxin GVIA (6)	−37.3% (±8.7), **$p < 0.01$	−7.0% (±5.1), NS
10 μM nifedipine (6)	+8.3% (±20.1), NS	−10.0% (±8.3), NS
1 μM agatoxin IVA (6)	−45.2% (±16.3), *$p < 0.05$	−20.7 (±8.2), NS

NS, Not significant.

Furthermore, we examined whether subtypes of HVA calcium channels differentially affect mEPSCs in superficial and deeper laminas of DH. Using specific pharmacological agents for $Ca_V2.3$ R-type channels (SNX-482), L-type channels (nifedipine), N-type channels (ω-conotoxin GVIA) and P/Q-type channels (agatoxin IVA) we determined that among HVA channels only R-type channels significantly contribute to spontaneous excitatory synaptic transmission in superficial laminas while N-type and P/Q-type greatly support transmission in deeper laminae of DH (Table 1). These data suggest that different subtypes of voltage-gated Ca^{2+} channels contribute to spontaneous vesicular release in nociceptive and non-nociceptive-processing regions of DH.

The idea that T-channels in lamina I and II DH neurons play a role in excitatory synaptic transmission also raises the possibil-

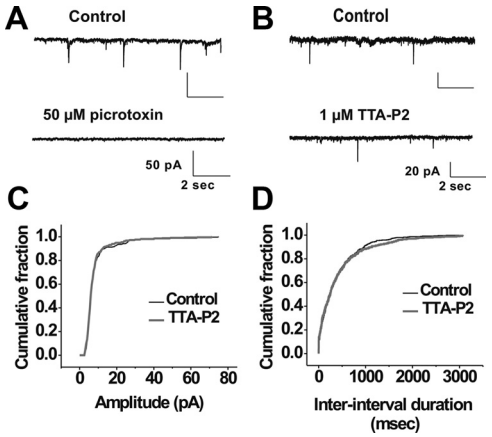

Figure 2. TTA-P2 does not affect spontaneous mIPSCs in neurons from the superficial lamina of the DH. Voltage-clamp, whole-cell recording from a lamina I neuron in a horizontal spinal cord slice held at −70 mV. ***A***, Recording of mIPSC activity from a single lamina I neuron in ACSF alone (Control, top) and after bath application of 50 μM pictrotoxin, a $GABA_A$ and glycine receptor antagonist (bottom). ***B***, Recording of mIPSC activity from a single neuron in ACSF (Control, top) and after bath application of 1 μM TTA-P2 (bottom). ***C***, Cumulative distributions of the amplitudes of events resulting from one representative experiment (same cell as ***B***). ***D***, Cumulative distributions of the frequencies of mIPSCs resulting from one representative experiment (same cell as ***B***).

ity that they may be important in inhibitory synaptic transmission of these neurons as well. Previous studies have found that while neurons in the superficial lamina of the DH express functional $GABA_A$ and glycine receptors the occurrence of mIPSCs may be scarce (Yoshimura and Jessell, 1990). To ameliorate this, we recorded spontaneous inhibitory synaptic transmission using an ACSF solution that contained 5 mM K^+ to depolarize presynaptic terminals and increase the frequency of events. For comparison, we first recorded mEPSCs under these conditions and observed that in the presence of 5 mM K^+ there was still a significant decrease in mEPSC frequency in the presence of TTA-P2 (~43%) while amplitude and decay time remained unchanged (data not shown, $n = 4$). All mIPSC recordings were also performed in presence of 1 μM TTX and 5 μM NBQX to eliminate AP generation and mEPSCs, respectively. As shown in Figure 2, spontaneous, inhibitory synaptic currents were recorded from lamina I and II DH neurons held at −70 mV in the whole-cell configuration. We verified that these currents were indeed inhibitory by applying 50 μM picrotoxin to block $GABA_A$ currents (Fig. 2*A*, bottom trace). After recording mIPSCs for 3 min, 1 μM TTA-P2 was applied to the cell and mIPSCs were recorded for another 3 min (Fig. 2*B*) ($n = 6$). In the presence of TTA-P2 there was no significant change in mIPSC amplitude (6.5 ± 0.77 pA control, 6.75 ± 0.86 pA with TTA-P2) (Fig. 2*C*), frequency (1.4 ± 0.52 Hz control, 0.88 ± 0.06 Hz with TTA-P2) (Fig. 2*D*), or decay time (data not shown). These results suggest that while presynaptic T-channels are important in spontaneous excitatory synaptic transmission in the superficial DH, they do not play a significant role in spontaneous inhibitory transmission in the same cells.

Furthermore, our results with TTA-P2 and 3βOH strongly suggest that the main presynaptic role of T-channels in sensory transmission in the spinal cord may be specific to neurons that receive nociceptive input. However, it is not clear whether presynaptic T-channels are specifically located on projections neuron or interneurons of superficial DH laminas. Lamina I and II of the DH of the spinal cord are made up of projection neurons as well as interneurons, and previous studies have determined that

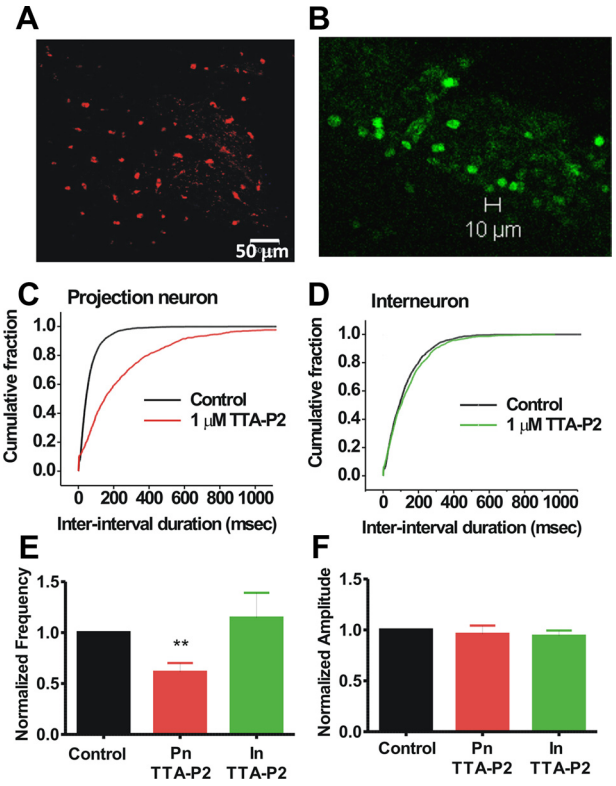

Figure 3. TTA-P2 has a presynaptic effect on excitatory transmission in nociceptive lamina I projection neurons but has no effect on mEPSCs from fluorescently labeled spinal cord DH inhibitory interneurons. ***A***, Confocal image of horizontal spinal cord slice, showing labeled (red color) lamina I projection neurons 4 d after stereotaxic injection of the retrograde neuronal label DiI (80 nl, 2.5%) into the lateral parabrachial nucleus. ***B***, Confocal image of horizontal spinal cord slice of the DH region showing YFP-labeled inhibitory interneurons (green color). ***C***, ***D***, Cumulative distributions of mEPSC frequencies from one representative experiment from a projection neuron (***C***) and one representative experiment from an interneuron (***D***). Note that TTA-P2 had a strong inhibitory effect in a projection neuron, while it had very little effect on an interneuron. ***E***, Pooled, normalized data showing mEPSC frequency before (6.04 ± 0.67 Hz, 1.85 ± 0.59 Hz) (filled bar) and after addition of TTA-P2 in Di-I labeled projection neurons (Pn) (3.75 ± 0.67 Hz, red bar) and in YFP-labeled interneurons (In) (green bar, 1.90 ± 0.58 Hz). **, significantly different ($p < 0.01$) from control. ***F***, Pooled data showing normalized mEPSC amplitude before (solid black bar) and after TTA-P2 addition in Di-I labeled projection neurons (Pn) (red bar, $n = 6$) and YFP-labeled interneurons (In) (green bar, $n = 5$). Error bars indicate SEM.

somatic T-channels are located largely on projection neurons of lamina I (Ikeda et al., 2003). Projection neurons from lamina I are responsible for forwarding nociceptive signals to different regions of the brain as part of the ascending pain pathway. Approximately 80% of lamina I neurons project to the parabrachial area (PB) of the brainstem while the remaining 20% project to the periaqueductal gray (PAG) region (Todd et al., 2000). Both of these regions are important in pain processing so next we examined the role of T-channels in synaptic transmission specifically in projection neurons from lamina I of the DH. For these experiments, lamina I projection neurons were identified by injecting the retrograde label DiI (80 nl, 2.5%) into the PB using a stereotaxic apparatus. Three to five days after injection, lamina I projection neurons could be readily identified as fluorescently labeled cells in horizontal slices of the spinal cord (Figure 3*A*). We then performed the same experiment as described in Figure 1 recording mEPSCs from only fluorescently labeled projection neurons. In these neurons, we again saw a significant decrease in frequency of mEPSCs (~39%) (Fig. 3*C*,*E*), while amplitudes of

mEPSCs were not affected with TTA-P2 (Fig. 3*F*) ($n = 6$). These results again demonstrate that presynaptic T-channels play an important role in synaptic transmission in the pain processing regions of DH, specifically in synaptic transmission of nociceptive projection neurons. We also wanted to determine whether presynaptic T-channels are important in synaptic transmission of inhibitory spinal DH interneurons. To do this we performed experiments using transgenic rats that coexpress Venus, a derivative of yellow fluorescent protein with the vesicular GABA transporter (VGAT-Venus rat) (Uematsu et al., 2008). We recorded mEPSCs from lamina I and II DH neurons from the transgenic rats before and after application of TTA-P2 as stated previously. As shown in Figure 3*B*, many fluorescently labeled VGAT interneurons were present in the spinal DH superficial lamina from our slice preparations. In the presence of TTA-P2, there was no significant change in amplitude (Fig. 3*F*), frequency (Fig. 3*D*,*E*), or decay time (data not shown) of mEPSCs recorded from the fluorescently labeled interneurons ($n = 5$). These results demonstrate that while T-channels are important in synaptic transmission of projection neurons that are part of the pain pathway, they do not play a significant role in synaptic transmission of DH inhibitory interneurons.

LVA Ca^{2+} channels, also referred to as transient or T-channels, are encoded by the Ca_v3 family of genes, with at least 3 isoforms ($Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$) being expressed in the CNS (Perez-Reyes, 2003). TTA-P2 is potent and selective blocker of all three isoforms of T-channels (Shipe et al., 2008). Similarly, 3βOH inhibits with similar potency multiple isoforms of T-channels (Todorovic et al., 2004; Joksovic et al., 2007). Thus the specific T-channel isoform that is involved in presynaptic regulation of synaptic transmission in the DH of the spinal cord remains unknown. Bao et al. (1998) have shown that the frequency of spontaneous EPSCs in the DH is reduced in the presence of mibefradil and nickel. Nickel preferentially inhibits $Ca_v3.2$ relative to $Ca_v3.1$ and $Ca_v3.3$ T-channel isoforms (Perez-Reyes, 2003) which suggests that $Ca_v3.2$ may be the T-channel subtype responsible for presynaptically modulating spontaneous synaptic transmission in lamina I and II. However, nickel within the same concentration range inhibits R-type $Ca_v2.3$ as well (Zamponi et al., 1996). To determine whether $Ca_v3.2$ is the primary T-channel subtype that contributes to DH synaptic transmission, mEPSCs were recorded in the presence of 100 μM $NiCl_2$ before and after addition of 1 μM TTA-P2. As shown in Figure 4*A*, inhibiting $Ca_v3.2$ T-channels first with $NiCl_2$ prevents TTA-P2 from having any significant effect on mEPSC frequency, suggesting that the presynaptic $Ca_v3.2$ subtype is important in DH synaptic transmission ($n = 7$). Previous studies from our lab have also shown that several endogenous reducing agents at physiological concentrations selectively modulate $Ca_v3.2$ T-currents (Todorovic et al., 2001; Nelson et al., 2005; Joksovic et al., 2006). Ascorbic acid (ascorbate, vitamin C) is a redox agent that is ubiquitously expressed in neurons (Rice, 2000) and has been shown to selectively and potently albeit partially (60–70%) inhibit $Ca_v3.2$ versus $Ca_v3.1$ and $Ca_v3.3$ in both native thalamic and sensory neurons, as well as recombinant cells (Nelson et al., 2007). As shown in Figure 4*A*, mEPSC frequency (gray bar) is significantly reduced (~22%, $n = 5$) in the presence of 300 μM ascorbic acid, while mEPSC amplitude (Fig. 4*B*, gray bar) is unaffected. These results further suggest that $Ca_v3.2$ is the primary presynaptic T-channel subtype involved in spontaneous synaptic transmission in the superficial laminas of the DH.

To validate that presynaptic $Ca_v3.2$ T-channels are important in regulating spontaneous synaptic transmission in the DH, we

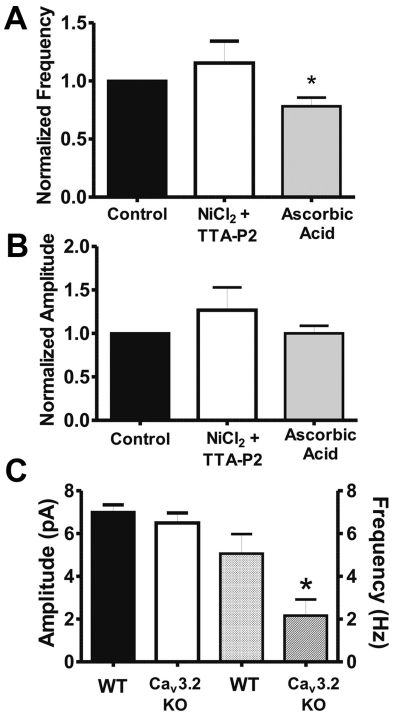

Figure 4. The $Ca_V3.2$ T-channel subtype plays a presynaptic role in spontaneous synaptic transmission in neurons from the superficial lamina of the DH. ***A***, Pooled data from DH neurons showing normalized mEPSC frequency under control conditions (2.10 ± 0.50 Hz, 2.62 ± 0.94 Hz) (filled bar), after addition of 1 μM TTA-P2 with $NiCl_2$ present (2.20 ± 0.50 Hz) (open bar), and after addition of 300 μM ascorbic acid (2.26 ± 0.92 Hz) (gray bar). Error bars indicate SEM. *, significantly different ($p < 0.05$) from control. ***B***, Pooled data from DH neurons showing normalized mEPSC amplitude under control conditions (filled bar), after addition of 1 μM TTA-P2 with $NiCl_2$ present (open bar) and after addition of 300 μM ascorbic acid (gray bar). Error bars indicate SEM. ***C***, Pooled data from 6 wild-type (filled bar) and 6 $Ca_v3.2$ KO (open bar) DH neurons showing mEPSC amplitude (solid bars) and frequency (patterned bars). Error bars indicate SEM. *, significantly different ($p < 0.05$) from wild-type mouse.

next examined mEPSC frequency, amplitude, and decay time in spinal cord DH slices from mice that have a homozygous deletion of the α1H ($Ca_V3.2$) gene. The average frequency of mEPSCs recorded from lamina I and II of the spinal DH from wild-type mice was similar to the average frequency of mEPSCs recorded from age-matched control rats (5.06 ± 0.92 Hz and 4.03 ± 1.09 Hz, respectively). On the other hand, when we recorded mEPSCs from spinal cord DH neurons of $Ca_v3.2$ knock-out (KO) mice there was a significant decrease (~42%, 2.16 ± 0.76, $n = 6$) in mEPSC frequency when compared with age-matched wild-type (WT) mice (Fig. 4*C*, patterned bars). There was no significant difference in mEPSC amplitude (Fig. 4*C*, solid bars) or decay time (data not shown) between WT and KO mice confirming the importance of $Ca_v3.2$ T-channels in presynaptic regulation of spontaneous synaptic transmission in the superficial lamina of the spinal DH. Furthermore, while 1 μM TTA-P2 reduced mEPSC frequency in WT mice ($n = 4$), it had no effect on mEPSC frequency in DH neurons from KO mice ($n = 2$) (data not shown) further demonstrating that presynaptic $Ca_v3.2$ T-channels are contributing to synaptic transmission in these cells.

While the mRNA distribution of the 3 subtypes of T-channels has been examined in the spinal cord, previous studies have not been able to examine T-channel protein distribution in this tissue largely due to paucity of subtype-specific antibodies (Talley et al., 1999). Next, we used in Figure 5 a new commercially available,

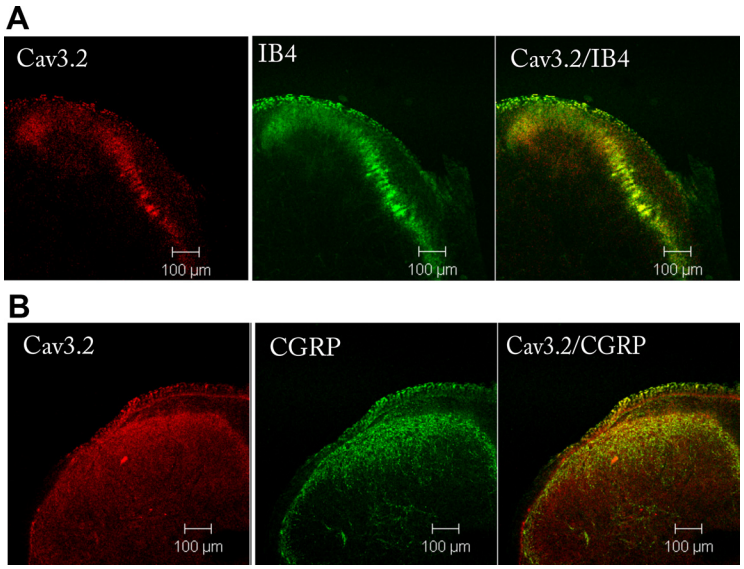

Figure 5. The $Ca_V3.2$ T-channel subtype is expressed in the DH of the spinal cord and colocalizes with presynaptic markers IB_4 and CGRP. ***A***, Confocal images of 50 μm transverse spinal cord slices with antibodies for $Ca_V3.2$ (red color, left) and IB_4 (green color, middle). Panel on the right shows colocalization of $Ca_V3.2$ and IB_4 immunoreactivity (yellow color). ***B***, Confocal images of 50 μm transverse spinal cord slices stained with antibodies for $Ca_V3.2$ (red color, left), CGRP (green color, middle). Right panel shows colocalization of $Ca_V3.2$ and CGRP immunoreactivity (yellow color).

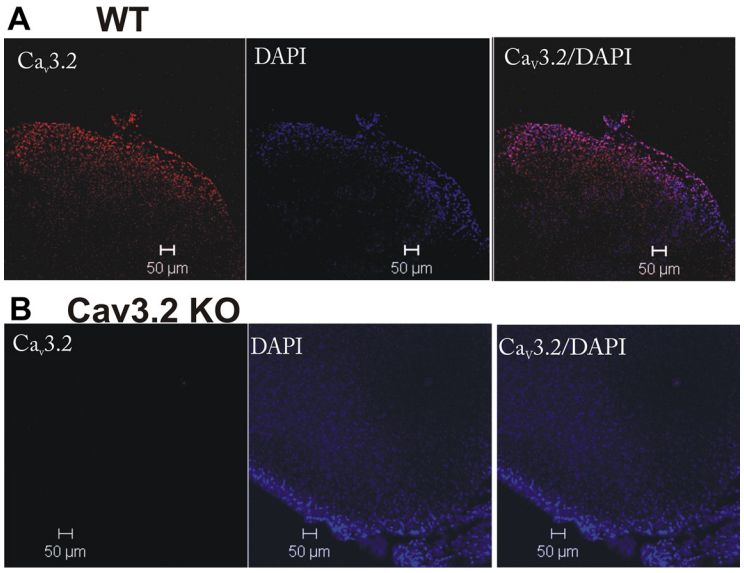

Figure 6. Cav3.2 immunoreactivity is not detected in the DH of the spinal cord from $Ca_V3.2$ knock-out (KO) mice. ***A***, Single plane, 10× confocal image of 50 μm transverse spinal cord slice from a wild-type mouse with antibody for $Ca_V3.2$ (red color, left), and DAPI (blue color, middle). Right panel shows overlap. ***B***, Single plane, 10× confocal image of 50 μm transverse spinal cord slice from a Cav3.2 knock-out mouse with antibody for $Ca_V3.2$ (red color, left), and DAPI (blue color, middle). Right panel shows overlap.

polyclonal, $Ca_V3.2$-specific antibody generated in rabbit (Sigma-Aldrich) to examine the protein expression pattern of this T-channel subtype in the DH of the spinal cord (red). To facilitate our study of $Ca_V3.2$ expression in the spinal cord DH we used the presynaptic, nonpeptidergic nerve terminal marker isolectin B4 (Fig. 5*A*) and a presynaptic, peptidergic nerve terminal marker calcitonin-gene related peptide (CGRP) (Fig. 5*B*). As expected from previous molecular studies (Talley et al., 1999), $Ca_V3.2$ antibody stained somas of many neurons in superficial DH laminae. After determining the expression pattern of $Ca_V3.2$ (Fig. 5*A*,*B* left, red), we next wanted to determine the possible presynaptic localization of this T-channel subtype. In Figure 5*A* (middle), we used an anti-isolectin B_4 antibody (IB_4, green) as a marker of presynaptic, nonpeptidergic nerve terminals that synapse in the superficial laminae of the DH. We found that at least some $Ca_V3.2$ fluorescence does colocalize with IB_4 (Fig. 5*A*, right, yellow puncta) in the superficial laminae of the DH. To quantify the colocalization of $Ca_V3.2$ at nonpeptidergic presynaptic terminals we used the ImageJ colocalization plugin and determined that an average of 7.8% of IB_4-positive areas also contained the $Ca_V3.2$ signal ($n = 4$). We also examined the expression of the commonly used presynaptic, peptidergic nerve terminal marker calcitonin-gene related peptide (CGRP) (Fig. 5*B*, middle, green). Again we observed significant colocalization of $Ca_V3.2$ fluorescence with this presynaptic marker in the most superficial lamina of the DH (Fig. 5*B*, right, yellow) with ~12.3% of CGRP-positive areas also containing $Ca_V3.2$ ($n = 4$). In control experiments with primary or secondary antibodies alone no immunofluorescent signal was detected suggesting our results are not due to nonspecific binding of antibodies or auto-fluorescence. In Figure 6, we also tested specificity of $Ca_V3.2$ antibody by staining spinal cord tissue from WT and $Ca_V3.2$ KO mice. We found that strong $Ca_V3.2$-specific staining was present only in tissues from WT mice (Fig. 6*A*) while we detected only background staining in tissues from KO mice (Fig. 6*B*) ($n = 3$). Together these immunohistochemical results confirm our electrophysiological results and provide further evidence for a novel presynaptic role of the T-channel subtype $Ca_V3.2$ in spontaneous synaptic transmission in the DH of the spinal cord.

For practical reasons, all of our preceding recordings were performed in young (7–14 d old) naive animals. However, we wished to examine the potential role of presynaptic $Ca_V3.2$ channels in animal models of neuropathic pain using adult (27–35 d old) rats. Our previous studies have established that amplitudes of DRG $Ca_V3.2$ currents are increased >2-fold in animal models of painful diabetic neuropathy (Jagodic et al., 2007; Latham et al., 2009; Messinger et al., 2009). Furthermore, another previous study has suggested that in diabetic STZ-treated animals which experience neuropathic pain mEPSC frequency in spinal cord DH neurons is significantly increased (Wang et al., 2007). Thus we asked whether the effect of TTA-P2 on mEPSCs is altered in diabetic animals using STZ-injected rats with a blood glucose reading of >400 mg/dl. We found that STZ-injected animals had approximately threefold higher mEPSC frequencies (5.38 ± 0.58 Hz, $n = 6$) when compared with control animals (2.15 ± 0.19 Hz, $n = 7$, $p < 0.001$). In contrast, there was only a modest decrease in the amplitudes of mEPSCs in these cells in

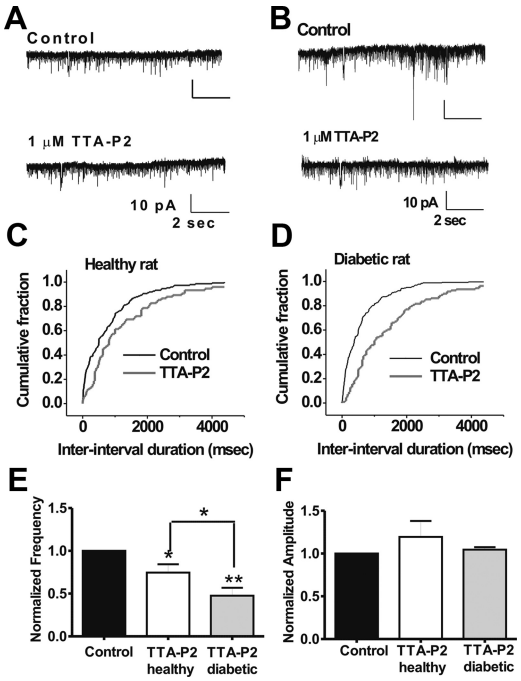

Figure 7. TTA-P2 has a greater inhibitory effect on excitatory synaptic transmission in the rats with STZ-model of painful diabetic neuropathy than in healthy rats. ***A***, Recordings of mEPSC activity from a single lamina I neuron from a healthy rat in ACSF alone (Control, top) and after bath application of 1 μM TTA-P2 (bottom). ***B***, Recordings of mEPSC activity from a single lamina I neuron from an STZ-injected diabetic rat in ACSF alone (Control, top) and after bath application of 1 μM TTA-P2 (bottom). ***C***, ***D***, Cumulative distributions of mEPSC frequencies from representative experiments with a healthy rat (***C***) and one representative experiment with a diabetic rat (***D***) where controls are indicated with black solid lines and the effects of TTA-P2 are indicated with gray solid lines (same cells as depicted on ***A*** and ***B*** of this figure). Note that TTA-P2 had greater inhibitory effect in diabetic rat than in healthy one. ***E***, Pooled data showing normalized mEPSC frequency in control conditions (2.15 $\pm$ 0.19 Hz and 5.38 $\pm$ 0.58 Hz) (filled bar), with 1 μM TTA-P2 in healthy animals (1.50 $\pm$ 0.17 Hz) (open bar) and in diabetic animals with 1 μM TTA-P2 (2.38 $\pm$ 0.44 Hz) (gray bar). Error bars indicate SEM. *, significantly different ($p < 0.05$) from control, **, significantly different ($p < 0.01$) from control. ***F***, Pooled data showing normalized mEPSC amplitude in control conditions (filled bar), healthy animals with 1 μM TTA-P2 (open bar), and in diabetic animals with 1 μM TTA-P2 (gray bar). Error bars indicate SEM.

diabetic rats (9.1 $\pm$ 0.5 pA) when compared with healthy ones (11.3 $\pm$ 0.6, $p < 0.05$). Importantly, diabetic rats had an increase in charge transfer of mEPSCs (56.08 $\pm$ 7.3 pAms, $n = 7$) when compared with healthy rats (36.57 $\pm$ 4.5 pAms, $n = 6$, $p < 0.05$). To determine whether the increase in frequency was due to presynaptic T-channel activity, we compared the effect of 1 μM TTA-P2 in healthy and diabetic animals. In healthy animals, again we observed a significant decrease in mEPSC frequency (~25.5%,) with TTA-P2 (Figure 7*A*,*C*,*E*) but no effect of TTA-P2 on mEPSC amplitude (Fig. 7*F*) or mEPSC decay (data not shown) ($n = 4$). Furthermore, in diabetic animals we observed an even greater inhibitory effect of TTA-P2 on mEPSC frequency (~52.4%) (Fig. 7*B*,*D*,*E*) and no significant effect on the amplitudes of mEPSCs (Fig. 7*F*) ($n = 5$). These results suggest that presynaptic T-channels contribute to the increase in mEPSC frequency seen in animal models of diabetic neuropathic pain and could be useful new targets for therapeutic agents.

Discussion

The central processes of most nociceptive sensory neurons terminate in superficial layers of the DH (laminae I and II) of the spinal cord, an important pain processing and integration region. Previous studies have documented that T-channels are expressed in somas of superficially located rat spinal DH neurons, including projections neurons. These channels may be important for activity-dependent long-term potentiation of synaptic strength between nociceptive afferents and lamina I DH neurons (Ikeda et al., 2003, 2006). However, the possible functional role of presynaptic T-channels in DH neurons has not been well studied. Thus, in this study we focused on studies of spontaneous mEPSCs. Our results show that specific pharmacological inhibition of $Ca_V3.2$ T-channels reduces mEPSC frequency in nociceptive projection neurons from the superficial laminae of the DH while mIPSCs are not affected. This finding indicates that activation of presynaptic $Ca_V3.2$ channels may result in selective increase of excitatory neurotransmitter release at the nociceptive spinal synapse and consequently fine tuning of postsynaptic excitability. This is important since laminae I and II are the central termination sites for the majority of DRG nociceptors and increase in neuronal excitability potentially could contribute to the induction of central sensitization (Latremoliere and Woolf, 2009). While one previous study has suggested a role for T-channels in the presynaptic regulation of mEPSCs in unidentified DH neurons of the laminae I-II of the spinal cord, these studies were performed using the less specific calcium channel antagonists Ni^{2+} and mibefradil (Bao et al., 1998). At the concentrations used in this study (100 μM Ni^{2+} and 5 μM mibefradil) both of these antagonists will inhibit both T- and R-type VGCCs (Randall and Tsien, 1997; Perez-Reyes, 2003). Indeed our results with SNX-482 demonstrate that R-type channels also contribute to excitatory synaptic transmission in superficial laminae of DH. Therefore work with more selective T-channel and R-channel blockers was needed to demonstrate a conclusive role of these channels in spontaneous synaptic transmission in the DH. Our results suggesting the role of presynaptic $Ca_V2.3$ R-type channels in nociceptive transmission in DH are novel but unsurprising given that all HVA Ca^{2+} channels subtypes have been involved in fast synaptic transmission in various CNS neurons (Catterall and Few, 2008). Furthermore, mice lacking the $Ca_V2.3$ gene exhibit altered pain phenotype (Saegusa et al., 2000). We have recently shown that potency of TTA-P2 in inhibiting $Ca_V2.3$ currents is approximately two orders of magnitude lower than for inhibition of native $Ca_V3.2$ T-currents in sensory neurons (Choe et al., 2011). In addition to the T-channel selective agents like TTA-P2 and 3βOH, our notion that $Ca_V3.2$ channel support excitatory transmission in DH neurons is fostered by the use of $Ca_V3.2$ selective agents like ascorbic acid and use of $Ca_V3.2$ KO mouse. Thus, both pharmacological and genetic tools identify the novel role of $Ca_V3.2$ channels in supporting neurotransmission in nociceptive DH neurons. $Ca_V3.2$ T-currents are thought to have a unique function in neuronal excitability by providing low-threshold calcium spikes (LTCSs) that function to lower threshold for spike firing in various central and peripheral neurons (for review, see Perez-Reyes, 2003; Khosravani and Zamponi, 2006). In contrast to the established role of T-currents in neuronal excitability in central and peripheral neurons, a possible role of these channels in fast synaptic transmission in CNS neurons is not well studied. Recent pharmacological, genetic and *in vivo* knockdown studies strongly implicate $Ca_V3.2$ T-channels in nociception (Todorovic et al., 2001; Bourinet et al., 2005; Nelson et al., 2005; Choi et al., 2007; Latham et al., 2009; Messinger et al., 2009). However, most previous studies have been focused on the involvement of $Ca_V3.2$ in sensitization of peripheral pain responses. Here, we demonstrate the important role of these channels in controlling excitatory spontaneous transmission in nociceptive neurons of superficial laminae of DH. Hence, $Ca_V3.2$ channels expressed in peripheral and central

endings of sensory neurons may work in concert to support nociceptive signaling and consequently facilitate peripheral and central sensitization, respectively.

Our conclusions from patch-clamp experiments that $Ca_V3.2$ channels presynaptically modulate excitatory transmission in DH neurons are supported by immunohistological data using new $Ca_V3.2$ antibody and two commonly used markers of nociceptive fibers like CGRP and IB_4. Small C-type fiber nociceptors can be subdivided on the basis of histological markers (Snider and McMahon, 1998; Stucky and Lewin, 1999). One major group expresses proinflammatory peptides such as substance P and calcitonin-gene-related peptide (CGRP), and project to the most superficial layers of the spinal cord dorsal horn (e.g., lamina I and the outer zone of lamina II). A second group does not express substance P or CGRP, but can be identified by the presence of specific enzymes (e.g., fluoride-resistant acid phosphatase) or binding sites for the isolectin B_4 (IB_4). These nonpeptidergic C fibers project to the slightly deeper inner lamina II of the spinal cord dorsal horn. Importantly, we found that $Ca_V3.2$-specific antibody colocalized with both IB_4 and CGRP-positive presynaptic fibers (Fig. 5). Interestingly, histological differences may suggest distinct functional roles for nociceptors. While both classes of nociceptors are believed to respond to noxious chemical, thermal and mechanical stimuli, studies suggest that these two nociceptor cell types may contribute differentially to the generation and maintenance of pain. For example, peptidergic neurons appear to be the major effectors of neurogenic inflammation, releasing proinflammatory peptides such as substance P from their peripheral terminals in response to activation. On the contrary, genetic studies with knock-out mice suggest that IB_4-positive, nonpeptidergic nociceptors are more involved in chronic neuropathic pain resulting from nerve injury (reviewed by Snider and McMahon, 1998). However, here we provide the first evidence of the importance of presynaptic $Ca_v3.2$ channels in controlling neurotransmission in the pain pathway. Thus, presynaptic facilitation of excitatory transmission by $Ca_V3.2$ channels may participate in both inflammatory and neuropathic pain processes. Indeed, results of our experiments with STZ-injected diabetic rats (Fig. 7) strongly support the idea that presynaptic $Ca_V3.2$ channels in DH neurons can at least contribute to the neuropathic pain disorders. Further experiments are needed to support the notion that presynaptic $Ca_V3.2$ channels in nociceptive DH neurons may also contribute to inflammatory pain as well.

Several recent studies strongly suggest that there exists differential regulation of spontaneous and evoked neurotransmitter release in CNS neurons (for review, see Ramirez and Kavalali, 2011). Our data support this view since we found that spontaneous transmitter release in nociceptive DH neurons is largely supported by $Ca_V3.2$ T-type and $Ca_V2.3$ R-type channels, and not with canonical presynaptic VGCCs channels like N-type and P/Q types that support evoked excitatory neurotransmission in these cells (Bao et al., 1998; Heinke et al., 2004; Rycroft et al., 2007; Motin and Adams, 2008; for review, see Zamponi et al., 2009). In contrast, we found that mEPSCs in DH neurons in deeper nonnociceptive laminae are mostly supported by N-type and P/Q subtypes of HVA channels (Table 1). Precise relationship between $Ca_V2.3$ and $Ca_V3.2$ channels in modulating nociceptive transmission in DH neurons is not known but it is possible that $Ca_V3.2$ channels play a permissive role. It has been shown that T-currents have a small fraction of channels that are active at physiological membrane potentials contributing to "window current" that can tonically depolarize neurons (Perez-Reyes, 2003; Nelson et al., 2005). Thus, we propose that presynaptic $Ca_V3.2$ channels may be involved in setting a relatively depolarized resting membrane potential that could in turn allow $Ca_V2.3$ channels to drive secretion in nociceptive DH neurons. Alternatively, both channels could directly contribute to vesicular release of glutamate.

Toward this end, recent studies have suggested that presynaptic T-channels are important in controlling spontaneous vesicular release in other synapses in CNS such as enthorinal cortex pyramidal neurons (Huang et al., 2011) and hippocampal CA1 neurons (Tang et al., 2011). It is well known that HVA channels facilitate evoked Ca^{2+} entry into the presynaptic nerve terminal and that this Ca^{2+} entry is then coupled to synaptic exocytosis due to the interaction of SNARE proteins with the channels (Llinas et al., 1992; Jarvis and Zamponi, 2001). While T-channels lack a similar SNARE binding site, it has recently been shown that the SNARE protein, syntaxin-1A forms a complex with $Ca_v3.2$ T channels in central neurons (Weiss et al., 2012). While this complex could facilitate low-threshold synaptic exocytosis, the precise molecular mechanisms responsible for the role of presynaptic $Ca_v3.2$ T-channels in spontaneous synaptic transmission in the spinal DH still need to be elucidated. Furthermore, there is current dogma in the field that T-channels in nociceptive sensory neurons are important strictly for controlling cellular excitability. The data presented here provide much needed information for understanding the actions of presynaptic $Ca_V3.2$ channels on sensory neurons in control of excitatory transmission in nociceptive DH neurons. Thus, drugs that modulate function of $Ca_V3.2$ channel may affect both peripheral and central sensitization of neurons in pain pathway.

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