Selective inhibition of CaV3.2 channels reverses hyperexcitability of peripheral nociceptors and alleviates postsurgical pain

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Pain-sensing sensory neurons of the dorsal root ganglion (DRG) can become sensitized or hyperexcitable in response to surgically induced peripheral tissue injury. We investigated the potential role and molecular mechanisms of nociceptive ion channel dysregulation in acute pain conditions such as those resulting from skin and soft tissue incision. We used selective pharmacology, electrophysiology, and mouse genetics to link increased current densities arising from the CaV3.2 isoform of T-type calcium channels (T-channels) to nociceptive sensitization using a clinically relevant rodent model of skin and deep tissue incision. Furthermore, knockdown of the CaV3.2-targeting deubiquitinating enzyme USP5 or disruption of USP5 binding to CaV3.2 channels in peripheral nociceptors resulted in a robust antihyperalgesic effect in vivo and substantial T-current reduction in vitro. Our study provides mechanistic insight into the role of plasticity in CaV3.2 channel activity after surgical incision and identifies potential targets for perioperative pain that may greatly decrease the need for narcotics and potential for drug abuse.

INTRODUCTION

Although opioids are effective in treating the acute pain associated with surgical procedures, their use is associated with serious side effects, which include constipation, urinary retention, impaired cognitive function, respiratory depression, tolerance, and addiction (1, 2). More than 12 million people in the United States abused prescription opioids in 2010 alone (3), resulting in more overdose deaths than heroin and cocaine combined (4). The necessity to treat this acute type of pain is of paramount importance because its duration and intensity influence the recovery process after surgery and the onset of chronic postsurgical pain (5). Despite the use of several other groups of drugs as adjuvants (such as N-methyl-D-aspartate antagonists, gabapentinoids, acetaminophen, and α2-adrenergic agonists), there is a recognized need for developing therapeutic agents that improve outcomes of conventional treatment of postsurgical pain in clinical settings while avoiding dangerous side effects.

A growing body of evidence suggests an important role of the CaV3.2 isoform of T-type calcium channels (T-channels) in neuronal transmission of painful stimuli in both physiological and pathological states (6–10). Most of these studies are focused on chronic neuropathic pain; however, little is known about the potential role of T-channels in the development and maintenance of acute pain and hypersensitivity after surgery. Here, we used an experimental pain model described as plantar incision of the hind paw in rodents (11, 12). Rats subjected to this model exhibit both thermal and mechanical hypersensitivity with primary and secondary hyperalgesia (13), characteristic features of human postoperative pain. Our study revealed that the excitability of nociceptive neurons of dorsal root ganglia (DRGs) was increased in the immediate postsurgical period. This increase in excitability was due to enhanced T-channel current density because of deubiquitination of the channel. Furthermore, using both pharmacological and molecular tools, we showed that nociception was alleviated by selectively inhibiting CaV3.2 T-channels, thus establishing this channel as a promising therapeutic target for acute pain treatment after surgery.

RESULTS

Postsurgical hyperalgesia is associated with an increase in T-current density in acutely dissociated DRG rat sensory neurons

To determine the time course of the development of postsurgical pain, we performed paw incision surgeries on rats as previously described (11) and monitored them using evoked pain tests of thermal nociception (fig. S1A) and mechanical hypersensitivity (fig. S1B). Thermal paw withdrawal latencies (PWLs) and mechanical paw withdrawal responses (PWRs) were significantly reduced 6 and 5 days after incision, respectively. Almost complete recovery of paw responses to presurgery values was achieved around day 7 after surgery. This finding is consistent with previous reports of the development of thermal and mechanical postsurgical hyperalgesia in rodents (11, 14, 15). Because the most pronounced hyperalgesia was detected on days 1 and 2 after incision, subsequent in vitro and in vivo experiments were performed during that time frame (fig. S1, A and B, boxed area).

Although the role of T-currents in chronic pain states has been previously investigated (7), their role in acute postsurgical pain remains to be elucidated. Because voltage-dependent activation and inactivation can influence excitability of T-channel–expressing neurons, we first studied the effect of incision on these biophysical properties in DRG cells. We used sham-operated animals (which were exposed to anesthesia without surgery) and incised animals (which were exposed to anesthesia with surgery) on days 1 and 2 after the procedure. Recordings from small- to medium-size DRG neurons (less than 35 μm soma diameter) were made because they are likely nociceptors and show prominent T-currents (16, 17). To determine the expression of T-currents in DRG neurons, we set holding potentials (VH) to −90 mV and then depolarized neurons to test potentials (VT) from −75 to −30 mV in 5-mV increments; the resulting inward currents show a crisscrossing pattern typical of T-channels (Fig. 1A). From current-voltage (I–V) relationships,
sham and incised rats were used to generate T-current densities calculated from values (T-current densities measured in sensory neurons after incision were significantly increased twofold in the postincision group compared with the sham group over the range of test potentials (Fig. 1B). We also measured time-dependent inactivation (10 to 90% rise time) (Fig. 1C) and the time-dependent inactivation constant measured from I-V curves. Sham group: n = 14 cells, 10 rats. Postincision group: n = 20 cells, 10 rats. Interaction: F(3,96) = 2.36, P = 0.08; treatment: F(1,32) = 4.33, P = 0.04, two-way RM ANOVA. (E) Families of T-currents evoked in representative DRG neurons by steady-state inactivation protocols in sham (black traces) and incised (red traces) groups. (F) T-current densities from steady-state inactivation curves. Sham: n = 14 cells, 10 rats. Postincision group: n = 21 cells, 10 rats. Interaction: F(10,330) = 6.78, P < 0.0001; post hoc: *P = 0.026 at −110 mV and **P = 0.041 at −105 mV; treatment: F(1,33) = 4.15, P = 0.049, two-way RM ANOVA followed by Sidak’s post hoc test.

We found that average peak T-current densities were increased more than twofold in the postincision group compared with the sham group over the range of test potentials (Fig. 1B). We also measured time-dependent inactivation (10 to 90% rise time) (Fig. 1C) and the time-dependent inactivation constant in these cells (Fig. 1D). Although rise times between sham and postincision groups were not significantly different, time-dependent inactivation after surgery was increased about twofold, as compared to the sham group. To independently assess the effects of postoperative pain on T-current density, we used 3.5-s-long pulses at different conditioning potentials to evoke T-currents (Fig. 1E). T-current densities measured in sensory neurons after incision were significantly increased (about twofold) when compared to sham at the range of conditioning potentials (Fig. 1F). In contrast, the midpoint values (V50) for voltage-dependent steady-state activation (Fig. S2A; Eq. 1), steady-state inactivation curves (Fig. S2B; Eq. 2), and the deactivation time constant (Fig. S2C) did not differ between the postincision group and sham controls. Together, increased T-current densities may suggest that more T-channels were activated after incision compared to the sham group, perhaps due to their increased expression and/or due to the change in biophysical properties indicative of increased channel activity.

**Plantar skin incision increases high-frequency firing and excitability of DRG sensory neurons**

Next, we used a current clamp to monitor firing patterns of DRG neurons after surgery, and recorded firing frequencies of the first three APs from DRGs in three groups—sham, postincision, and postincision group incubated with TTA-P2. The average firing frequency of APs increased in DRGs from the postincision group by about 45% when compared to those from the sham group (Fig. 2E). Furthermore, in DRGs from the postincision group treated with TTA-P2, the average frequency of firing was significantly reduced compared to the postincision group and was not statistically different from the sham group (Fig. 2E). To make appropriate comparisons of excitability, we held the resting membrane potentials at similar values (Fig. S3A). Likewise, the input resistance was not significantly different between the sham and postincision groups (Fig. S3B). Hence, the increase in neuronal excitability in incised animals is not due to alterations in passive membrane properties.

Most of the T-channel–expressing DRG cells in our study likely belonged to a group of unmyelinated C-polymodal nociceptors sensitive to different noxious stimuli, including noxious heat (16, 17). Hence, we applied TTA-P2 intrathecaley in vivo before surgical incision in rats (Fig. 2F; preemptive application) and measured their response to noxious thermal (heat) stimuli. Applied in a single intrathecal dose of 215.5 μg as determined from experiments in healthy rats as the highest effective dose (Fig. S4, A to C), TTA-P2 significantly decreased nociceptive response to heat stimulus in incised rats compared to the vehicle-treated group during 5 days of postoperative follow-up (Fig. 2G). A group of animals that received TTA-P2 intrathecally immediately before surgery had a very fast onset of the reduction of heat nociception (2 hours after surgery) that persisted for the next 2 days, as compared to the vehicle-injected group. A significant antinociceptive effect of preemptive TTA-P2 was still noticeable on day 5, indicating that preemptive application of TTA-P2 successfully reduced thermal nociception; however, it did not increase the rate of recovery. Collectively, these data suggest that T-channels contribute to the increased excitability of nociceptive sensory neurons and transmission of noxious heat stimuli after incision.
Traces from single-spiking, multiple-spiking, and high-frequency firing patterns of DRG neurons in the postincision group and in the sham group. Sham group: $n = 28$ cells; Postincision group: $n = 29$ cells, 8 rats. Fisher’s exact test $P = 0.04$. (E) Bar graphs represent frequency of AP firing in the sham group (white bar), untreated postincision group (black bar), and postincision group incubated with TTA-P2 for 5 to 10 min (red bar). $n = 13$ cells from five rats in the sham group, 15 cells from seven rats in the untreated postincision group, and 8 cells from two rats in the postincision + TTA-P2 group. Unpaired t test; $P = 0.02$. The data are expressed as means ± SEM. (F) Experimental time course. I.t., intrathecal. (G) Thermal PWL. $n = 8$ rats per group. Interaction: $F(4,56) = 1.16; P = 0.3$; treatment: $F(1,14) = 17.61; **P < 0.001$, two-way RM ANOVA followed by Sidak’s post hoc test. The data are expressed as means ± SEM.

**Fig. 2. T-channels contribute to incision-induced hyperexcitability of sensory neurons.** (A) Representative traces of APs in a DRG neuron treated with a T-type channel blocker (TTA-P2) from the postincision group (current injection of 50 pA) before and after application of 3 μM TTA-P2. (B) Number of APs after incision before and after application of TTA-P2. $n = 8$ cells per group from 6 rats; each data point represents mean ± SEM. Interaction: $F(5,35) = 6.69, P < 0.001$; post hoc: **$P = 0.002$, ***$P < 0.001$, ***$P < 0.001$, and ***$P < 0.001$ for current injections 20, 30, 40, and 50 pA, respectively; treatment: $F(1,7) = 14.72, P = 0.006$, two-way RM ANOVA followed by Sidak’s post hoc. (C) Representative traces from single-spiking, multiple-spiking, and high-frequency-spiking DRG neurons. (D) The pie charts show the distribution of different types of firing patterns of DRG neurons in the postincision group and in the sham group. Sham group: $n = 28$ cells, 8 rats. Postincision group: $n = 29$ cells, 8 rats. Fisher’s exact test $P = 0.04$. (E) Bar graphs represent frequency of AP firing in the sham group (white bar), untreated postincision group (black bar), and postincision group incubated with TTA-P2 for 5 to 10 min (red bar). $n = 13$ cells from five rats in the sham group, 15 cells from seven rats in the untreated postincision group, and 8 cells from two rats in the postincision + TTA-P2 group. Unpaired t test; $P = 0.02$. The data are expressed as means ± SEM. (F) Experimental time course. I.t., intrathecal. (G) Thermal PWL. $n = 8$ rats per group. Interaction: $F(4,56) = 1.16; P = 0.3$; treatment: $F(1,14) = 17.61, **P < 0.001$, two-way RM ANOVA followed by Sidak’s post hoc test. The data are expressed as means ± SEM.

Repetitive intrathecal application of TTA-P2 in vivo alleviates postincision pain in rats

To further investigate the functional consequence of blockade of T-channels in vivo, we first applied TTA-P2 intrathecally in unoperated animals and recorded responses evoked by thermal and mechanical stimuli (fig. S4A). TTA-P2 substantially reduced thermal (fig. S4B) and mechanical (fig. S4C) responses in unoperated animals in a dose-dependent manner compared to the vehicle-treated group. To examine whether the antinociceptive effect of TTA-P2 could be due to its nonspecific effects such as sedation and/or motor weakness, we performed sensorimotor tests. The responses after treatment with TTA-P2 in these animals did not differ from those responses before injection in any of these tests. Animals spent the same amount of time on an inclined screen and an elevated platform before and after drug injection, and animals were able to walk the plank in two separate trials for 60 s before and after drug injection (fig. S4, D to F).

We next investigated the antinociceptive effect of TTA-P2 injected intrathecally at 2 hours and on days 1 and 2 after incision (Fig. 3A). We chose this particular dosing regimen because neuroaxial application of analgesics during the first 48 hours of a postoperative period is an established and safe practice in humans (19, 20). Either test compound or vehicle was applied in one group of animals. Although TTA-P2 did not affect heat nociception when applied 2 hours after incision (Fig. 3B), it significantly alleviated heat nociception when applied on days 1 and 2 after surgery (Fig. 3, C and D). The most prominent antinociceptive effect in heat nociception testing was achieved 90 min after injection, as measured 24 hours after the incision; after the third application 48 hours after incision, the antinociceptive effect was steadily present up to 90 min after injection. Repeated intrathecal application of TTA-P2 alleviated mechanical hypersensitivity at 2 hours and on days 1 and 2 after three consecutive applications, as measured up to 120 min after injection (Fig. 3, E to G).

Thus, our data indicate that selective and repeated blockade of T-channels leads to alleviation of thermal and mechanical hypersensitivity without apparent tolerance. The lack of effect of TTA-P2 on thermal nociception 2 hours after surgery could indicate that T-channels are not involved in the development, but are involved in the maintenance, of thermal hyperalgesia. In contrast, we found that TTA-P2 was effective in alleviating mechanical hypersensitivity as early as 2 hours after surgery, suggesting that T-channels likely play a role in both development and maintenance of mechanical hyperalgesia after incision.
Plantar skin incision increases the membrane fraction of CaV3.2 channels

To further elucidate whether there was an increased expression of T-channels that could explain increased current densities, we performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of all three T-type channel isoforms (CaV3.1, CaV3.2, and CaV3.3) after sham surgery or after incision on days 1 and 2 after surgery in L4 to L6 unilaterally harvested DRGs. As expected, qRT-PCR analysis of mRNA expression in sham rats revealed that the CaV3.2 isoform had the highest levels of mRNA compared to the two other isoforms, which is in accordance with in situ hybridization data (21). mRNA levels of the CaV3.2 isoform after incision were moderately decreased (by about 25%) compared to the sham group (Fig. 4A). To determine whether total CaV3.2 protein expression was changed, we performed Western blotting, which revealed that total protein levels of CaV3.2 in lumbar DRG tissues in the postinjury group were similar to that in the sham group (Fig. 4, B and C). Because both qRT-PCR and immunoblotting methods could not explain the increased T-current densities in DRG cells (Fig. 1, B and F), we hypothesized that the membrane fraction of the CaV3.2 channel in DRG cells may be increased after incision. Hence, we performed immunostaining of acutely dissociated sensory neurons (≤35 μm diameter) on day 2 after incision from ipsilateral and contralateral DRGs of incised rats using a CaV3.2-specific antibody and an antibody against cadherin, a structural membrane protein (Fig. 4, D and E). To determine whether there were changes in the membrane expression of the CaV3.2 channel after incision, we compared the fluorescence intensities of the CaV3.2 signal normalized to the pan-cadherin signal. The normalized CaV3.2 fluorescence was significantly increased (about twofold) in ipsilateral DRG cells from incised rats when compared to the contralateral DRG cells (Fig. 4D). These data suggest that the membrane fraction of CaV3.2 channels is increased in ipsilateral DRG sensory neurons after incision despite the unchanged total levels of the CaV3.2 protein (as suggested by our Western blot experiments). Hence, we reasoned that the increase in the membrane fraction of CaV3.2 channels after incision may contribute to the observed increase in T-current densities (Fig. 1, B and F) and hyperexcitability of DRG cells (Fig. 2, D and E).

Selective knockdown of deubiquitination of CaV3.2 channels in nociceptors prevents the development of mechanical hypersensitivity in mice after plantar skin incision

We have previously implicated the deubiquitinating enzyme ubiquitin-specific protease 5 (USP5) as a specific CaV3.2 interacting partner (22). Here, we investigated the role of CaV3.2 channel ubiquitination in the setting of surgical tissue injury using wild-type (WT) and CaV3.2 knockout (KO) mice. For these studies, we used intrathecal application of USP5-shRNA (short hairpin RNA) to knock down USP5 in mouse DRGs in vivo. The potency and specificity of this USP5-shRNA construct to reduce USP5 levels and mediate analgesia in inflammatory and neuropathic pain models were previously established (22).

Hyperalgesia in WT mice after surgical paw incision has a similar time course to that in WT rats with the maximal hyperalgesia at postoperative days 1 and 2 (23). Hence, we have limited our mechanistic studies in mice to days 1 and 2. Immunoblotting showed that USP5 protein levels are increased about threefold in ipsilateral DRG cells, as compared to the contralateral DRG cells in WT mice after incision (Fig. 5A). Next, we investigated the interaction between USP5 and CaV3.2 in vivo by measuring thresholds for PWRs to a punctate stimulus before surgery (baseline) and on day 1 after incision (Fig. 5A). As
expected, we found prominent mechanical hypersensitivity in WT mice that preemptively received vehicle treatments as indicated by decreased thresholds for PWRs (fig. S5B). In contrast, PWRs in contralateral, unoperated paws remained stable. To further confirm the selectivity of USP5 interaction with CaV3.2, we used CaV3.2 KO mice (fig. S5, C and D). After incision, mechanical sensitivity to a punctate stimulus was not significantly different between the untreated CaV3.2 KO group and the CaV3.2 KO group that preemptively received USP5-shRNA (fig. SSC). Furthermore, when we compared PWRs on day 1 after incision in two cohorts in ipsilateral paws, we noticed significantly less mechanical hypersensitivity in the KO group, as compared to the WT group (fig. S5D). These data suggest that CaV3.2 channels are required for the antihyperalgesic effect of USP5-shRNA and that CaV3.2 channels are important for the observed mechanical hyperalgesia induced by surgical paw incision in WT mice.

To confirm that the antinociceptive effect of USP5-shRNA appears as a consequence of the specific interaction of the CaV3.2 channel with USP5, we tested the effects of a selective tat peptide that corresponds to the intracellular domain III-IV linker region of the CaV3.2 channel, which is the binding site for USP5 (22). The specificity and selectivity of this reagent were confirmed previously in our study, which showed that a tat-CaV3.2-CT peptide (corresponding to the C terminus of CaV3.2) and a tat-free CaV3.2 III-IV linker peptide did not exhibit any effect in inflammatory and neuropathic pain models (22). Intrathecal injection of the tat III-IV peptide on day 2 after incision (Fig. 5B) relieved mechanical hypersensitivity, as evidenced by an increased threshold for PWRs in ipsilateral paws in WT mice (Fig. 5C), but not in CaV3.2 KO mice (Fig. 5D). When the data were expressed as the percentage of antihyperalgesic effect normalized to the preinjection baseline, the tat III-IV peptide almost completely reversed the response to mechanical stimuli 15 to 60 min after injection in WT mice (Fig. 5E), but not in KO mice (Fig. 5F). Comparison of the preinjection baseline values to mechanical stimulus revealed that the mechanical hypersensitivity in WT mice was significantly lower than that in KO mice, suggesting that the onset of recovery in KO began earlier than that in WT mice, further indicating a prominent role of CaV3.2 channels in this pain model (Fig. 5G). Time course of mechanical antihyperalgesia after intrathecal administration of the tat III-IV peptide in our experiments with paw incision corresponds well to its reported effects using other pain models in WT mice (22).

Overall, our findings support the idea that the development and maintenance of mechanical hypersensitivity after surgery are partially mediated by deubiquitination of the CaV3.2 protein, which likely leads to its increased membrane stability. Therefore, the CaV3.2 channel in peripheral nociceptors is required as a key mediator of analgesic effects of USP5-shRNA in WT mice after paw skin incision. However, the apparent lack of antinociceptive effect of USP5-shRNA and the tat III-IV peptide in CaV3.2 KO mice indicates that USP5-dependent deubiquitination is specific for CaV3.2 channels, making this interaction important for the development of postsurgical pain.

**Preventing the deubiquitination of CaV3.2 channels in nociceptors reduces T-current density and diminishes mechanical hypersensitivity after plantar skin incision in rats**

To test the effect of the disruption of CaV3.2 channels’ deubiquitination in vitro, we recorded T-currents in putative rat DRG nociceptors on days 1 and 2 after surgery from the sham group of rats, from rats that received intrathecal injections of USP5-shRNA 24 hours before plantar incision, and from untreated rats that received
only plantar incision. Representative traces from these recordings showed decreased T-current amplitudes recorded from animals that received USP5-shRNA, as compared to the untreated postincision group (Fig. 6A). Intrathecal applications of USP5-shRNA significantly decreased T-current densities when compared to cells from incised untreated rats (Fig. 6B). T-current density in the postincision group after knocking down USP5 did not significantly differ from that in the sham group (Fig. 6B). Further, we confirmed successful knockdown of USP5 by measuring thresholds for PWRs in incised rats that received USP5-shRNA intrathecally 24 hours before incision (Fig. 6, C and D). Thresholds for PWRs in ipsilateral paws after injection of USP5-shRNA were significantly higher as compared to the vehicle group at 2 hours and on days 1 and 2 after surgery, indicating reduced mechanical hypersensitivity in incised rats after treatment. In contrast, contralateral (unincised) paws compared to the ipsilateral paws in the same rats were unaffected by USP5-shRNA treatments (Fig. 6D). Overall, our data suggest that deubiquitination of CaV3.2 channels promotes an increase of the T-current densities in nociceptive DRG cells and implies a role for CaV3.2 in the development and maintenance of hyperalgesia after surgical tissue injury in rats and WT mice.

DISCUSSION

The present study demonstrated that T-channels played an important role in the development and maintenance of acute pain after surgery. Using a clinically relevant surgical incision model in rodents, we showed increased current density of T-channels in peripheral nociceptors. Our previous studies have shown an increase in current density of T-channels in peripheral nociceptors. Our previous studies have shown an increase in current density of the CaV3.2 isoform of T-currents and increased excitability of DRG sensory neurons in animals with painful diabetic neuropathy (10, 24, 25). In addition, successful alleviation of pain in several other chronic and visceral pain models has been achieved with blockers of T-channels (26–28), particularly the CaV3.2 subtype. We showed that T-channel inactivation was slower in sensory neurons after incision as compared to
the sham group, which could potentially implicate a contribution of high voltage-activated (HVA) voltage-gated calcium channels. However, slow deactivating tail currents are characteristic of T-currents and rule out a contribution from HVA calcium channels that typically display 10-fold faster deactivating currents (29).

We also examined potential changes in excitability of small sensory neurons in rats with postincisional hyperalgesia because both increased T-current densities and slower T-current inactivation could favor hyperexcitable states. We discovered that after incision, sensory neurons produced APs of higher frequency as compared to the sham group. In addition, the number of high-frequency APs was higher in the postincision group as compared to the sham group. We also showed that the direct application of the selective T-channel blocker TTA-P2 to dissociated DRG neurons markedly reduced AP frequency after incision. Similarly, we found that treating dissociated DRG neurons from the postincision group with TTA-P2 reduced AP frequency. However, the frequency of APs after preincubation with TTA-P2 was not statistically significantly different from AP firing frequency in sham animals. We chose to use dissociated DRG neurons to correlate electrophysiology with immunostaining experiments and to be able to discriminate cells based on their size more reliably than in whole tissue preparations. We acknowledge the possibility that isolated DRG neurons may not behave identically to those in intact tissue preparation. However, our in vitro data are consistent with our in vivo experiments. Preemptive intrathecal application of TTA-P2 in incised rats reduced thermal hypersensitivity as compared to the vehicle-injected group, indicating the importance of T-channels in the development of postsurgical nociception. Our results do not exclude the possibility that nociceptive channels such as TTX-resistant Na+ channels, which also show increased current density after incision, could work in concert with T-channels to contribute to increased excitability of DRG sensory neurons during the development and maintenance of postsurgical pain (30).

We have previously shown that intraperitoneal injection of TTA-P2 successfully alleviates inflammatory pain in mice and thermal hyperalgesia in neuropathic pain in rats (18). Furthermore, several studies have also confirmed the role of T-channels in various pain models, in which other T-channel blockers have been applied either systemically (31) or as an intraplantar local injection (32, 33). Our current study revealed a role of T-channels in an acute postincisional pain model associated with skin incision based on the ability of the selective T-channel blocker TTA-P2 to alleviate both heat and mechanical hyperalgesia.

TTA-P2 is a pan-selective T-channel blocker that also blocks the other two subtypes of T-channels (CaV3.1 and CaV3.3), not just the CaV3.2 isofom of the channel (34). Although the other two subtypes (CaV3.1 and CaV3.3) are not abundant in DRGs, intrathecal application of TTA-P2 could block CaV3.1, CaV3.2, and CaV3.3 channels when applied spinally because mRNAs encoding all three isoforms are found in pain-processing regions of the dorsal horn of the spinal cord (21). Nevertheless, our study establishes a role of CaV3.2 channels in postincisional hyperalgesia, whereas the possible role of other T-type isoforms remains to be studied.

Our results suggest that T-channels play a crucial role in the development and maintenance of mechanical hyperalgesia after incision, because TTA-P2 successfully alleviated mechanical hyperalgesia at 2 hours and on days 1 and 2 after surgery. On the other hand, the lack of immediate effect of TTA-P2 at 2 hours after incision in thermal nociception suggested that CaV3.2 channels are important for the maintenance but not for the development of thermal hyperalgesia after surgery. Similar findings have been reported in other studies with incisional pain models in rodents. For example, the GABA_A (γ-aminobutyric acid type A) receptor agonist muscimol has an antihyperalgesic effect on thermal stimulus on day 1 after surgery, but not on the day of the surgery, although it alleviates mechanical hyperalgesia on both days (35). Collectively, these results imply different mechanisms of modulation of thermal and mechanical hyperalgesia after surgery in at least two unrelated families of nociceptive ion channels. In addition, in different pain models, a potential analgesic compound could have an effect in one modality of evoked pain testing, but not another (36, 37). Finally, it appears that preemptive blocking of T-channels improves recovery when tested for thermal nociception. Together, the results from thermal and mechanical in vivo testing suggest that T-channels play an important role not only in maintenance (thermal and mechanical) but also in the development (mechanical) of postsurgical hyperalgesia after skin incision.

The expression of the CaV3.2 isoform of T-channels in DRGs is increased in different pain disorders such as painful diabetic neuropathy (10) and paclitaxel-induced painful neuropathy (38). Our qRT-PCR and Western blot analysis of DRG tissues did not support an increase at the mRNA or total protein levels of any of the three isoforms of T-channels after incision. However, our immunostaining experiments revealed an increase in membrane fraction of CaV3.2 channels in small-to-medium DRG sensory neurons, which supports our in vitro findings of increased T-current density and hyperexcitability of the nociceptive sensory neurons after incision. We proposed that the increase in the membrane abundance of CaV3.2 channels could be related to some other mechanism that contributed to the protein stability in the membrane, rather than an increase in channel production. The CaV3.2 channel is the most abundant isoform of T-channels at the soma of primary afferent fibers located in DRG, and as such is primarily responsible for subthreshold neuronal excitability (16, 17, 39). Increasing channel activity with reducing agents or glycosylation, independently of changes in CaV3.2 mRNA and protein expression, may induce potent sensitization of peripheral nociceptors (16, 17, 24, 25). In addition, deubiquitination of CaV3.2 channels induces hyperalgesia in neuropathic and inflammatory pain (22). This study used immuno-histochemistry to reveal that USP5, a ubiquitin protease, was coexpressed in the same classes of sensory neurons that express CaV3.2 channels such as myelinated (NF200-positive), unmyelinated (IB4-positive), and peptidergic DRG neurons (22). This coexpression agrees with our previous study that these three groups of sensory neurons from the DRG express abundant CaV3.2 channels (40). Furthermore, USP5 and CaV3.2 co-immunoprecipitate from DRG and spinal cord tissue (22). Our findings presented in this study revealed that the increased levels of USP5 after plantar skin incision could promote deubiquitination of the CaV3.2 channel. Our data are consistent with a mechanism in which postsurgical modulation of CaV3.2 channels contributes to the development of acute postoperative pain. This notion has been confirmed in behavioral experiments in which CaV3.2 KO mice exhibited reduced mechanical hypersensitivity after incision as compared to WT mice. Furthermore, the process of deubiquitination supports membrane stability of the channel, which in turn could also increase current density and trigger nociceptor hyperexcitability. Thus, silencing of USP5 could be a promising approach to reducing postincision hyperalgesia. The
specificity of interaction between USP5 and the CaV3.2 channel was confirmed by our experiments with the CaV3.2 KO mice. However, a global gene knockout approach can be limited by compensatory mechanisms, as has been shown for the nociceptor-specific TTX-resistant sodium channel NaV1.8 KO mice (41). Hence, we confirmed the role of CaV3.2 deubiquitination by blocking the binding of USP5 to the CaV3.2 channel with the tat III-IV peptide, which ameliorated hyperalgesia in WT mice after plantar skin incision similar to that observed after USP5 knockdown.

In conclusion, our study implicates T-channels in the development of postsurgical hyperalgesia after skin incision. We demonstrated that after plantar skin incision, increased current density and slower inactivation of these channels contribute to increased excitability of nociceptive DRG sensory neurons in vitro. In rats, a selective T-channel blocker effectively alleviated both heat and mechanical hyperalgesia after plantar incision, thus providing in vivo validation. Furthermore, we showed that the CaV3.2 channel isoform was particularly critical for postsurgical acute pain, because mechanical hyperalgesic responses were reduced in mice lacking this channel, as well as mice and rats with decreased expression of the deubiquitinating enzyme USP5, which stabilizes CaV3.2 channels in the membrane. Therefore, our data suggest that the CaV3.2 isoform of T-channels could be considered a potential drug target for treating postsurgical pain.

MATERIALS AND METHODS
Study design
The study objective was to investigate whether T-channels are involved in mechanical and thermal nociception in vivo after plantar skin incision using adult rats and mice of both sexes (8 to 10 weeks of age). In addition, in vitro studies with whole DRG tissues were used for studies of mRNA and CaV3.2 protein expression. Finally, acutely dissociated DRG neurons were used for immunostaining and electrophysiology experiments for analysis of current waveforms and AP firing properties.

All efforts were made to minimize the number of animals to obtain reliable scientific data. In all our experiments, sample size was chosen using standard algorithms (42) with reference to our previously published data. For each experiment, animals were randomized in experimental groups to generate biological replicates. Rats and mice were litter-matched, age-matched, and gender-matched to keep the treatment groups as similar as possible. Except for the duration of in vitro studies in which animals were euthanized at different time points, all studies were predetermined to last 7 days from the incision. To assure stable recording conditions for the measurements of mechanical and thermal sensitivities, we determined baseline values on both paws 2 days before surgery. After surgery, baseline paw responses to mechanical stimulus were measured at 2 hours and on days 1 and 2 after incision. Mechanical hypersensitivity to a punctate stimulus after plantar incision in rats with selective knockdown of USP5 and in the vehicle group. n = 7 to 8 rats per group. Two hours after incision: *P = 0.014, 1 day after incision: **P < 0.001, 2 days after incision: **P = 0.004, two-way RM ANOVA followed by Sidak’s post hoc test. Each data point represents the average thresholds for PWR ± SEM.
guidelines. All animals were maintained on a 12-hour light-dark cycle with food and water ad libitum. All experiments involving rats used adult Sprague-Dawley (Envigo) female rats weighing 200 to 240 g. We also used WT C57BL/6 and CACNA1H (Caᵥ_3.2) global KO mice, both males and females (8 to 10 weeks of age).

**Incisional pain model**

The acute postsurgical pain model has been described previously (11, 23). In brief, animals were anesthetized with isoflurane (2 to 3%), and the plantar surface of the right paw was incised longitudinally. The underlying plantaris muscle was elevated and incised, after which the skin was closed with two sutures. Each animal was allowed to recover individually in a cage, and all experiments were initiated as early as 2 hours after incision.

**Intrathecal injections**

To study the antinociceptive effects of spinalally applied drugs, intrathecal injections were performed. After anesthetizing animals with isoflurane (2 to 3%), the back of each animal was shaved to expose the injection site in the L₄ to L₆ region of the spinal column. A 28- to 30-gauge needle was used for acute intrathecal injection. After inserting the needle into the L₄ to L₆ lumbar region, the experimental compound or vehicle was delivered intrathecally (50 µl in rats and 10 µl in mice), and the animal was left to recover before initiating experiments.

**Drugs and treatments**

A racemic mixture of the pan-selective T-channel blocker, TTA-P2 was purchased from Alomone Labs and was dissolved as suspension in 15% 2-hydroxypropyl-β-cyclodextrin solution in pH-balanced saline (to avoid tissue irritation) for all behavioral experiments. 2-Hydroxypropyl-β-cyclodextrin (45% solution) was purchased from Santa Cruz Biotechnology and diluted to 15% using pH-balanced saline. USP5-shRNA-cyclodextrin (45% solution) was purchased from Santa Cruz Biotechnology and diluted to 15% using pH-balanced saline. USP5-shRNA-cyclodextrin solution at room temperature through 2 hours after incision.

**Acute dissociation of DRG neurons**

DRG neurons from adolescent rats were prepared as we previously described (43). In brief, animals were first deeply anesthetized with 5% isoflurane, after which decapillation was performed, and unilateral L₄ to L₆ DRGs were removed and immediately placed in ice-cold Tyrode’s solution, which contained 140 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 10 mM glucose, and 10 mM Hapes, adjusted to pH 7.4 with NaOH. After harvesting, the tissue was transferred into Tyrode’s solution containing collagenase H (Sigma-Aldrich) and dispase II (Roche) and incubated for 50 min at 35°C. Dissociated single neuronal cell bodies were obtained by triturating in Tyrode’s solution at room temperature through fire-polished pipettes of progressively reduced sizes. After trituration, cells were plated onto uncoated glass coverslips, placed in a culture dish, and perfused with external solution. All in vitro experiments were done at room temperature. Only small- to medium-sized cells (<35 µm soma diameter) were used in recordings, because we have found that T-channels are abundant in these cells, which are putative nociceptors (16, 17, 43).

**Electrophysiology**

The external solution for voltage-clamp experiments measuring T-currents contained 152 mM tetraethylammonium (TEA)–Cl, 2 mM CaCl₂, and 10 mM Hapes, with TEA-OH, which was used for adjustment of pH to 7.4. To study well-isolated T-currents in acutely isolated DRG neurons, we used only fluoride-based internal solution to ensure HVA Ca²⁺ current rundown (43). The internal solution contained 135 mM tetrabutylammonium-OH, 40 mM HEPES, 10 mM EGTA, and 2 mM MgCl₂, adjusted to pH 7.2 with hydrogen fluoride. Internal solution for current-clamp recordings contained the following: 130 mM potassium-D-glucurate, 5 mM EGTA, 4 mM NaCl, 0.5 mM CaCl₂, 10 mM HEPES, 2 mM Mg adenosine 5′-triphosphate, and 0.5 mM tris guanosine 5′-triphosphate (pH 7.2). All current-clamp recordings were performed in Tyrode’s external solution. Series resistance (Rₛ) and membrane capacitance (Cₓ) were recorded directly from the amplifier after electronic subtraction of the capacitative transients. Current densities were calculated by dividing current amplitudes with cell capacitance. Spike-firing properties of DRG sensory neurons were characterized by injecting a family of depolarizing current pulses of 500-ms duration in 10-pA incremental steps through the recording pipette. Resting membrane potential was measured at the beginning of each recording and was not corrected for the liquid junction potential, which was around 10 mV in our experiments. The membrane input resistance was calculated by dividing the end amplitude of steady-state hyperpolarizing voltage deflection by the injected current.

I-V curves were generated by voltage steps from holding potentials (Vₛ) of –90 mV to test potentials (Vₜ) from –75 to –30 mV in incremental steps of 5 mV. Steady-state inactivation curves were generated by voltage steps from a Vₛ of –90 mV to conditioning prepulse potentials from –110 to –50 mV in incremental steps of 5 mV and then to a Vₛ of –30 mV. To study the possible effects of skin incision on the voltage dependence of deactivation kinetics, tail currents were recorded over a sufficiently negative range of membrane potentials (for example, –160 to –60 mV in 10-mV increments) after an 8-ms-long depolarizing step to –30 mV (43). The voltage dependences of activation and steady-state inactivation were expressed with single Boltzmann distributions:

\[ G(V) = G_{max}/(1 + \exp[-(V - V_{50})/k]) \]  \( (1) \)

\[ I(V) = I_{max}/(1 + \exp[(V - V_{50})/k]) \]  \( (2) \)

**Thermal noiception testing**

For assessment of thermal (heat) nociception threshold, an apparatus based on the Hargreaves method was used (custom-created at the University of California, San Diego University Anesthesia Research and Development Group, La Jolla, CA). Briefly, the system consists of a radiant heat source mounted on a movable holder positioned underneath a glass surface on which animals are placed in enclosed plastic chambers. After 15 min of acclimation, a radiant heat source is positioned directly underneath a plantar surface of hind paws to deliver a thermal stimulus. When the animal withdraws the paw, a photocell detects interruption of a light-beam reflection, and the automatic timer shuts off, measuring the animal’s PWL. Each paw was tested three times, and the average value of PWLs was used in further analysis. To prevent thermal injury, the light beam was automatically discontinued at 20 s if the rat failed to withdraw its paw.
Mechanical sensitivity
To test mechanical sensitivity in animals, we used the electronic Von Frey apparatus (Ugo Basile), which consists of one single rigid probe that exerts pressure in a range from 0 to 50 g. Animals were placed in plastic enclosures on a wire mesh stand to habituate for 15 min. After habituation, a probe was applied to the plantar surface of the paw through the mesh floor of the stand, and constant force was applied to the mid-plantar area of the paw. As soon as the immediate brisk paw withdrawal appeared as a response to a punctate stimulus, the apparatus displayed a force in grams that represents a threshold for PWR. Each paw was tested three times, and the average value of threshold PWRs was used in further analysis. Any other voluntary movement of animals was not considered as a response.

Assessment of sensorimotor abilities
To eliminate the potential of the highest applied dose of the tested drug to exert motor impairment, we tested unoperated rats in the following behavioral experiments (44). For inclined-plane experiments, a rat was placed in the middle of a wire mesh (eight squares in 10 cm) tilted at a 60° angle. The animal was placed with her head down, and how long the animal can stay without falling down was measured. A cutoff value of 120 s was assumed as the maximum time the animal can stay on the inclined mesh. For elevated-platform experiments, a rat was placed on a platform (7.6 cm × 15.2 cm) 61 cm above the ground, and the animal was timed for how long it can remain there. A mean value was calculated from two trials with a maximum of 120 s (test cutoff value). For ledge experiments, a rat was timed for how long it stayed on a 3-cm-wide plank. Means for each animal were calculated over two trials, with a maximum of 60 s per trial. The highest dose of TTA-P2 that exerted an analgesic effect in healthy animals was tested 30 min after intrathecal injection.

Quantitative real-time PCR
To study changes in expression of mRNAs encoding T-channel isoforms, we used tissue from dissociated lumbar DRGs (50 to 70 mg of tissue per sample). RNA was isolated using the RNeasy Microarray Tissue Mini Kit with QiAzol (Qiagen), and quantitative real-time PCR was performed on a BioRad iCycler, with the RT2 First Strand Kit with QiAzol (Qiagen), and quantitative real-time PCR analysis and no template controls were included in each run. Primers for all T-channel subtypes were purchased from Qiagen (CACNA1G NM_031601 catalog number: PPR52633A-200; CACNA1H NM_153814 catalog number: PPR59378A; CACNA1I NM_020084 catalog number: PPR50245A-200). We used cyclophilin as an internal standard (Qiagen; Ppid NM_001004279 catalog number: PPR59729A). qRT-PCR data were analyzed as previously described (24) using the cycle threshold method.

Western blot analysis
To measure the protein abundance of CaV3.2 channels, we harvested L4 to L6 DRGs of rats that were previously deeply anesthetized with 5% isoflurane and decapitated. For incised animals, ipsilateral DRGs were collected 24 and 48 hours after incision (for the sham-operated group, both ipsilateral and contralateral DRGs were collected). The DRGs of two animals were pooled into one sample for the sham-operated group, and the DRGs of three incised animals were pooled into one sample, giving a total of four samples in each group. Tissue was homogenized with a pestle in 7 (v/v) volumes of modified radioimmunoprecipitation assay (RIPA) buffer (pH 7.4) containing 150 mM NaCl, 25 mM tris, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors (#8866; Thermo Fisher Scientific). Samples were exposed to three freeze-thaw cycles, sonicated (Branson), and centrifuged (30 min; 14,000 rpm). Supernatants were placed in a clean tube, assayed for total protein (bicinchoninic acid assay; Life Sciences), and stored at −80°C until use. Samples (20 μl per lane) were prepared for electrophoresis by incubating with 2× sample buffer for 1 hour at 24°C with gentle shaking. Proteins were loaded on 4 to 20% tris-glycine polyacrylamide gradient gels (Bio-Rad) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were blocked for 1 hour in 5% nonfat milk in TBST buffer [150 mM NaCl, 50 mM tris, and 0.1% Tween-20 (pH 7.5)] at room temperature and incubated overnight (4°C) with a primary antibody [anti-CaV3.2 (1:3,000), Alomone Labs; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:15,000), Millipore] diluted in 2.5% milk in TBST. The PVDF membrane was subsequently washed and incubated with a horseradish peroxidase–conjugated secondary antibody (1:15,000, Santa Cruz Biototechnology) diluted in TBST (1 hour at room temperature). Washing was repeated and the blot was developed using enhanced chemiluminescent substrate (Super Signal West Femto; Thermo Fisher Scientific). Emitted light was detected with a G:BOX chemiluminescence image analyzer (Chemix XR5; Syngene) and analyzed densitometrically using the computerized image analysis program ImageQuant 5.0 (GE Healthcare; Life Sciences). Signal intensity for CaV3.2 was normalized to GAPDH, which was used as a loading control.

To measure the protein abundance of USP5, contralateral and ipsilateral L4 to L6 DRGs were harvested from incised WT mice. For immunoprecipitation assays, incised mouse ipsilateral and contralateral L4 to L6 DRGs were lysed in RIPA buffer [50 mM tris, 100 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) NP-40, 10 mM EDTA + protease inhibitor cocktail (pH 7.5)]. Tissue lysates were used to immunoprecipitate USP5 with a specific USP5 polyclonal antibody (ProteinTech Group Inc.). Lysates were prepared by sonicating samples at 60% pulse for 10 s twice and by centrifugation at 13,000 rpm for 15 min at 4°C. Supernatants were transferred to new tubes, and solubilized proteins were incubated with 50 μl of protein G/ A beads (Pierce) and 1 μg of USP5 polyclonal antibody overnight while tumbling at 4°C. Inputs, representing 7% of total lysate, were probed for α-tubulin with a specific mouse antibody as loading controls. USP5 immunoprecipitates were washed twice with 500 mM NaCl and 50 mM tris (pH 7.5) buffer, and beads were aspirated to dryness. Laemmli buffer was added, and samples were incubated at 96°C for 10 min. Eluted samples and inputs were loaded on 10% tris-glycine gels resolved using SDS–polyacrylamide gel electrophoresis. Samples were transferred to 0.45-mm PVDF membranes (Millipore) by dry transfer with I-Blot apparatus (Invitrogen).

Western blot assays were performed using mouse anti–α-tubulin (1:2000; Abcam) and rabbit anti-USP5 (1:500; ProteinTech Group Inc.) antibodies. Quantification was performed using densitometry analysis (Quantity One Bio-Rad software).

Immunohistochemistry
The experiments were performed in a blinded manner, as previously described (40, 45). The L4 to L6 DRGs of rats were harvested, and the cells were dissociated for electrophysiology experiments. Cells were plated onto glass coverslips and left in a cell oven for 1 to 3 hours at 37°C, and the wells were then flooded with 4% paraformaldehyde and 0.1 M phosphate buffer for 10 min at 4°C. Cells were then rinsed with 0.01 M phosphate-buffered saline (PBS) for 3 × 5 min at room temperature. Cells were then simultaneously permeabilized, and non-specific binding was blocked using 0.1% Triton X-100 and 0.01 M PBS.
supplemented with 5% donkey serum for 30 min at room temperature. Cells were rinsed with 0.01 M PBS for 3 × 5 min at room temperature. Cells were then incubated in primary antibody (1:500, anti-Cav3.2 ACC-025, Alomone Labs) in 0.01 M PBS overnight at 2° to 8°C. The specificity of this antibody has been previously validated (46). After rinsing with 0.01 M PBS for 3 × 5 min at room temperature, cells were then left overnight to incubate with the second primary antibody (anti-pan-cadherin, 1:200 ab22744, Abcam). After rinsing with 0.01 M PBS for 3 × 5 min at room temperature, and then subsequently incubated with appropriate secondary antibody (1:2000, Alexa Fluor 488 A27034, Invitrogen) for 2 hours at room temperature, then rinsed again with 0.01 M PBS for 3 × 5 min at room temperature, and subsequently incubated with appropriate secondary antibody (1:2000, Alexa Fluor 568 A11004, Invitrogen) for 2 hours at room temperature. Cell plates were mounted using fluorescent medium with DAPI (Vectorshied). All pictures were taken with an Olympus FV1000 FCS/RCIS confocal microscope.

Image analysis was performed with ImageJ. As a measurement of cell surface expression for both Cav3.2 and pan-cadherin, fluorescence intensities were obtained by subtracting the intracellular fraction from the total cell fluorescence for each channel. For each individual cell, the relative level of expression of Cav3.2 was obtained by normalizing the raw fluorescence intensity to the intensity of pan-cadherin. The specificity of used antibodies was confirmed in control experiments by omitting the primary antibodies.

**Data analysis and statistics**

All data from electrophysiological experiments are presented as means ± SEM. GraphPad Prism and SigmaPlot were used to analyze the data. Statistical significance was determined using either two-way RM ANOVA followed by Sidak’s post hoc test, Fisher’s exact test, or unpaired t test as appropriate. P < 0.05 was considered to be statistically significant.

Data from biochemical experiments are expressed as means ± SEM and analyzed with either one-way ANOVA followed by Tukey post hoc test, Mann-Whitney test, or unpaired t test. Behavioral data were expressed as means ± SEM and evaluated by two-way RM ANOVA using fluorescent medium with DAPI (Vectorshied). All pictures were taken with an Olympus FV1000 FCS/RCIS confocal microscope.

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Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

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Selective inhibition of CaV3.2 channels reverses hyperexcitability of peripheral nociceptors and alleviates postsurgical pain

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Preventing postsurgical pain

Opiate abuse has necessitated finding alternative therapeutic targets to alleviate postsurgical pain. The Ca\(^{2+}\) channel CaV3.2 has been implicated in inflammatory and neuropathic pain. Joksimovic et al. now show that postsurgical pain also triggers CaV3.2 channel activity by increasing its stability at the surface of pain-sensing neurons. In rodents, pharmacologically inhibiting CaV3.2 or preventing the deubiquitinating enzyme USP5 from stabilizing the channel reversed postoperative hypersensitivity to mechanical and heat stimuli, pain syndromes that are also typical in patients after surgery. Targeting CaV3.2 or the ability of USP5 to bind to the channel may provide a strategy for relieving postsurgical pain that lacks the addiction potential of opiates.

SUPPLEMENTARY MATERIALS
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