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Identification of interleukin-1 beta as a key mediator in the upregulation of Cav3.2–USP5 interactions in the pain pathway

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Abstract

We recently reported that nerve injury or peripheral inflammation triggers an upregulation of the deubiquitinase, USP5 in mouse dorsal root ganglion and spinal dorsal horn. This leads to dysregulated ubiquitination of Cav3.2 T-type calcium channels, thus increasing Cav3.2 channel plasma membrane expression and nociceptive signaling in the primary afferent pain pathway. This phenomenon could be recapitulated by noninvasive, optogenetic activation of transient receptor potential vanilloid-1–expressing nociceptors, indicating that neuronal activity is a key player in this process. Given the relevance of the pro-inflammatory cytokine interleukin-1 beta in many forms of pathological pain, we hypothesized that interleukin-1 beta may be a critical cofactor required to drive upregulation of interactions between USP5 and Cav3.2 channels. Here, we report that gene expression, as well as protein levels for interleukin-1 beta and the endogenous interleukin-1 receptor-1 antagonist, IL-1Ra are unaltered following conditioning stimulation of optogenetically targeted cutaneous nociceptors, indicating that neuronal activity is not a driver of interleukin-1 beta signaling. In contrast, co-immunoprecipitation experiments revealed that intrathecal administration of interleukin-1 beta in wild-type mice led to an increase in the interaction between USP5 and Cav3.2 in the spinal dorsal horn. Moreover, disruption of the interaction between USP5 and Cav3.2 with TAT peptides suppressed acute nocifensive responses produced by interleukin-1 beta, which was similar to that achieved by elimination of T-type channel activity with the channel blockers, mibefradil, or TTA-A2. Finally, this upregulation could be maintained in dorsal root ganglion neuron cultures exposed overnight to interleukin-1 beta, while the copresence of interleukin-1 receptor antagonist or the dampening of neuronal cell activity with tetrodotoxin attenuated this response. Altogether, our findings identify interleukin-1 beta as an upstream trigger for the upregulation of interactions between USP5 and Cav3.2 channels in the pain pathway, presumably by triggering increased firing activity in afferent fibers.

Keywords

Interleukin-1 beta, pain, Cav3.2, T-type channels, USP5, spinal cord, dorsal root ganglion, optogenetics

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Introduction

T-type calcium channels are critical for the detection and delivery of nociceptive information from the periphery to central nervous system. Cav3.2 is the predominant isoform found in sensory neurons,[1] and its expression is increased in a wide variety of pathological pain states.[2–5] This leads to increases in nociceptor cell excitability in the dorsal root ganglion (DRG)[4] and increased excitatory neurotransmission in the superficial layers of

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the spinal dorsal horn.[6] Furthermore, genetic ablation of Cav3.2 channels,[7] their knock down,[8] or their pharmacological inhibition[9] increases pain threshold, and therefore, these ion channels serve as attractive targets for the development of future pain therapeutics. We recently described an interaction between Cav3.2 channels and the deubiquitinase, USP5,[10] whose upregulation after injury spares Cav3.2 channels from proteasomal degradation, giving rise to ongoing pain that remains exquisitely sensitive to this interaction. Since this process is relevant to both inflammatory and neuropathic pain states,[10,11] we initially proposed that neural activity may be a common trigger.[12] This idea was supported by observations that activity stemming from optogenetically targeted cutaneous nociceptors alone is sufficient to upregulate USP5 expression, and therefore to increase interactions between USP5 and Cav3.2.[12] However, this activity-dependent phenomenon is not self-sustaining, instead promoting only a transient pain state that cannot account for the upregulation observed after injury.

Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine known be released in response to injury and to have elevated levels in numerous persistent/chronic pain states.[13–16] Consistent with the relevance of IL-1β to chronic pain, interference of IL-1β binding to its biologically active receptor, interleukin-1 receptor-I (IL-1RI), with interleukin-1 receptor antagonist (IL-1Ra) alleviates neuropathic pain.[17] Furthermore, nociceptive sensory neurons express IL-1RI,[18,19] allowing IL-1β to produce direct[19] and enduring increases in cell excitability[20] through actions on key voltage-gated ion channel mechanisms.[19,21,22] Surprisingly, the effect of IL-1β on Cav3.2 T-type channels in the context of pain remains elusive. In the present study, we test the hypothesis that IL-1β activity can trigger USP5-mediated Cav3.2 channel plasticity to induce pain.

Materials and methods

Animals

All testing procedures were performed as per protocols approved by the Institutional Animal Care and Use Committee, and all efforts were made to minimize animal suffering in accordance with policies and recommendations established by the International Association for the Study of Pain. C57BL/6 (wild-type) mice, as well as homozygous B6.129-Trpv1tm1(cre) Bbm/J (TRPV1cre, Stock No: 017769) and B6.Cg-Gt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J (Ai32, Stock No: 012569) mice were purchased from The Jackson Laboratory. As described previously,[12] mice that express channelrhodopsin-2 (ChR2) fused with enhanced yellow fluorescent protein (EYFP) in transient receptor potential vanilloid-1 (TRPV1)-lineage neurons (TRPV1-ChR2-EYFP) were generated by crossing the TRPV1cre driver mouse line with Ai32 conditional allele mice. All mouse colonies were maintained through in-house breeding programs. Male mice, aged 6 to 12 weeks old were used in all experiments. Animals were housed at a maximum of five per cage ($30 \times 20 \times 15$ cm) with ad libitum access to food and water. They were kept in controlled temperature of 23 ± 1°C on a 12 h light/dark cycle (lights on at 7:00 a.m.). Experiments were performed between 10 a.m. and 4 p.m. With the exception of experiments involving intrathecal (i.t.) injections, different cohorts of mice were used for each test, except when stated.

Optogenetic stimulation

TRPV1-ChR2-EYFP mice were lightly anesthetized (1%–2% isoflurane) and kept on a heating pad, while an optic cannula (diameter: 400 μm; Thorlabs) coupled to a laser (Laser Glow) delivered blue light (wavelength: 473 nm, irradiance: 10 mW/cm^2) at a rate of 10 Hz for 10 min to the plantar surface of the right hind paw.

Tissue harvest

As described previously,[10,12] TRPV1-ChR2-EYFP or wild-type mice were deeply anesthetized with isoflurane (1 ml liquid in 1000 cm^3 chamber) and, immediately following decapitation, a laminectomy was performed to remove lumbar-level DRG or spinal cord.

Real time quantitative RT-PCR measurements

Immediately after conditioning optogenetic stimulation, TRPV1-ChR2-EYFP mice were killed by decapitation. DRGs (L4–L6) were rapidly dissected at 0°C to 4°C and immediately flash frozen to be stored at −80°C. Total RNA was extracted using the NucleoSpin RNA II extraction kit (Macherey-Nagel, USA) and quantified using a NanoDrop. First-stranded cDNA synthesis (from 660 ng total RNA per 20 μl of reaction mixture) was carried out using high-capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies). PCR amplification, in triplicate for each sample, was performed using Eppendorf Mastercycler® ep realplex (Eppendorf) and TaqMan® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems, Life Technologies, USA) for target genes: IL1-Ra (assay ID: Mm00446186_m1) and IL-1β (Mm00434228_m1). Semiquantitative determinations were made with reference to the reporter genes encoding glyceraldehyde 3-phosphate dehydrogenase (Mm99999915_g1) and Beta-Actin (Mm00607939_s1). The polymerase activation step at 95°C for 15 min was followed by 40 cycles

of 15 s at 95°C and 60 s at 60°C. The validity of the results was checked by running appropriate negative controls (replacement of cDNA by water for PCR amplification; omission of reverse transcriptase for cDNA synthesis). Specific mRNA levels were calculated after normalizing to GaDPH and ACTB mRNA in each sample, using the 2(-Delta Delta C(T)) method.[23] Data are presented as relative mRNA units compared to control values.

Intrathecal drug treatments

Wild-type mice had dorsal hair shaved prior to all experiments that involved i.t. injections. Adopting the approach described by Hylden and Wilcox,[24] animals were manually restrained, with the spinal column arched, and a 30-gauge needle attached to a PE20 polyethylene tube that coupled to a 25-µl Hamilton microsyringe (Hamilton) was inserted into the subdural space between the L4 and L5 vertebrae. Correct i.t. positioning of the needle tip was confirmed by a characteristic tail-flick response. A 10-µl volume of vehicle control (PBS) or IL-1β (0.01 pg/µl in PBS; Peprotech) combined with either PBS, TAT-3.2-III-IV (1 µg/µl in PBS) or mibefradil (1 µg/µl in PBS; Sigma Aldrich) was then delivered over a period of 5 to 10 s. In a similar set of experiments, i.t. IL-1β was combined with either 2-(4-Cycloprop ylphenyl)-N-[(1 R)-1-[5-(2,2,2-trifluoroethoxy)pyridin-2-yl]ethyl]acetamide (TTA-A2; 0.05 µg/µl; Abcam) or DMSO (4.4% in PBS; Sigma Aldrich)—the manufacturer recommended vehicle for TTA-A2. Following all injections, mice were placed in testing arenas for subsequent examination of nocifensive responses.

Nocifensive responses

Analogous to Paszcuk et al.,[25] wild-type mice were observed individually for 15 min following i.t. injection. A nocifensive response is defined as a single head movement directed at the flanks or hind limbs, resulting in contact of the animal's snout with these regions which typically results in biting. The cumulative amount of time that animal spent responding in this manner was timed with a chronometer and considered indicative of nociception. The dose of IL-1β and time of evaluation were determined in preliminary work, where the lowest dose needed to achieve a clear increase in nocifensive behavior was selected (data not shown). Intrathecal doses of TAT-3.2-III-IV and mibefradil have been determined previously.[10]

Biochemistry

Co-immunoprecipitation (co-IP) experiments followed by Western blots of the spinal dorsal horn were performed 30 min following i.t. injections, while those involving DRG cell cultures were conducted after overnight exposure to assigned treatments. All tissues were derived from wild-type mice. Both DRG cells in culture and dorsal horn tissue were lysed in modified RIPA buffer (in mM; 50 Tris, 100 NaCl, 0.2% (v/v) Triton X-100, 0.2% (v/v) NP-40, 10 EDTA + protease inhibitor cocktail, pH 7.5). Tissue lysates were used to immunoprecipitate Cav3.2 channels with a specific Cav3.2 polyclonal antibody (1 µg per immunoprecipitation; Santa Cruz Biotechnologies, Inc.). Lysates were prepared by sonicating samples at 60% pulse for 10 s and by centrifugation at 13,000 r/min for 15 min at 4°C. Supernatants were transferred to new tubes and solubilized proteins were incubated with 50 ml of Protein G/A beads (Piercenet) and 2 µg of anti-Cav3.2 (Santa Cruz Biotechnologies, Inc.) antibody overnight while tumbling at 4°C. Cav3.2 immunoprecipitates were washed twice with modified RIPA buffer and beads were aspirated to dryness. Laemmli buffer was added, and samples were incubated at 96°C for 10 min. Eluted samples were loaded on a 7.5% Tris-glycine gel or 10% Tris-glycine gel for inputs and resolved using SDS-PAGE. Samples were transferred to 0.45 mm polyvinylidenedifluoride membranes (Millipore) by dry-transfer with i-blot (Invitrogen). Membranes were probed for USP5 with a specific rabbit polyclonal antibody (1:500; ProteinTech Group, Inc.). Inputs, representing 4% of total lysate, were probed for actin (Sigma), as loading controls. Densitometry analysis was performed using ImageJ (rsb.info.nih.gov/ij).

DRG cell culture establishment and drug treatment

DRGs from lumbar and thoracic spinal segments were harvested and collected in an ice-cold HBSS (Invitrogen) dissection solution, containing 0.25% HEPES (Sigma Aldrich) and adjusted to 310 mOsm with sorbitol (pH 7.2). DRGs were cut into two to three pieces and then incubated at 37°C in DMEM (Invitrogen), containing 0.4% collagenase type I (Invitrogen), 3% papain (Worthington), and 0.5 µM EDTA for 25 min, followed by 4 µg/ml DNase (Worthington) for another 10 min. DRGs were washed with culture medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen)) and then dissociated by five to seven passages through a fire-polished Pasteur pipette. Cells were plated onto 35-mm tissue culture dishes (Corning), pretreated with 10 µg/ml poly-D-lysine and 1 µg/ml laminin (both from Sigma), and kept at 37°C in 5% CO_2 incubator.

After recovery of cell cultures in serum-based medium (~24 h), efforts were taken to minimize ambient levels of IL-1β through approaches established previously,[20]

involving medium exchange with a serum free, defined medium (1:3 F12:DMEM supplemented with 1% N-2 supplement (both from Gibco) and 1% penicillin/streptomycin (Invitrogen)). After 1 h, overnight (~24 h; 37°C in 5% CO_2 incubator) treatments commenced, where defined medium cell cultures were treated with vehicle control (0.1% BSA (Sigma Aldrich) in PBS), or IL-1β (10 ng/ml; Peprotech) in the absence or presence of IL-1Ra (100 ng/ml; R&D Systems) or tetrodotoxin (1 μM; Tocris Bioscience).

Statistical analysis

All data values are presented as means ± SEM for n experiments. For real-time RT-qPCR data, significance was determined with a two-tailed, paired-sample t test. Results from i.t. studies were evaluated by one-way analysis of variance, followed by a Tukey test, or a two-sample t test. All cell culture data were assessed with a two-sample t test, and $p < 0.05$ was considered significant ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). All data met the assumption of a normal distribution in the Kolmogorov-Smirnov test for normality. Paired data sets in Figure 1(a) were assumed to have similar variance, while a Levene's variance test verified that the independent data set in Figure 1(f) had no significant differences in variance.

Results and discussion

We first examined the possibility that IL-1β plays a downstream role in activity-dependent T-type plasticity. Under the same experimental conditions as our previous work,[12] IL-1β and IL-1Ra gene expression in the ipsilateral DRG is not altered in transgenic mice immediately following transcutaneous optogenetic stimulation of transient receptor potential vanilloid-1 (TRPV1)-expressing nociceptors (Figure 1(a)). A similar finding for IL-1Ra is revealed at the protein level in the foot pad of sensitized animals with ELISA at one hour post-stimulation, whereas IL-1β levels could not be detected (data not shown). Here, it is important to note that successful optostimulation of these animals was verified by confirming behavioral sensitization to mechanical stimuli as described by us previously.[12] Taken together, activity in nociceptors and the induction of a heightened state of pain appears to take place in the absence of a pro-inflammatory IL-1β-mediated response, which fits with the broader observation that optogenetic stimulation of nociceptors is not associated with a putative neurogenic inflammatory response.[12,26] This appears to contrast findings from an i.t. microdialysis study, where spinal IL-1β protein levels increased in response to c-fiber strength electrical stimulation of the sciatic nerve.[27] However, this difference may be related to variations in stimulation protocol, site of stimulation, and perhaps most importantly, the degree of invasiveness.

We next turned our attention to directly examine the effect of IL-1β on USP5-mediated T-type plasticity in the pain pathway. In wild-type mice that received a single i.t. injection of IL-1β (0.1 pg), co-immunoprecipation (co-IP) experiments demonstrated that IL-1β increases the interaction between USP5 and Cav3.2 in the spinal dorsal horn, relative to vehicle control (PBS) mice (Figure 1(b) and (c)). Inclusion of TAT-3.2-III-IV peptide (a peptide corresponding to the USP5 interaction domain on Cav3.2 channels, delivered at 10 μg, i.t.) attenuated this interaction as expected (Figure 1(c)). Intrathecal administration of IL-1β is well known to produce a nocifensive response in mice that lasts approximately 15 min and is characterized by excessive biting of the flanks and hind paws.[25,28] Consistent with our biochemical findings, the TAT-3.2-III-IV peptide (10 μg, i.t.) in combination with IL-1β (0.1 pg, i.t.) suppressed nocifensive responses by approximately 40%, which was to a similar degree as direct block of the T-type channel when TAT-3.2-III-IV peptide was substituted for either mibefradil (10 μg, i.t.) or the more selective blocker, TTA-A2 (5 μg, i.t.) in the cocktail (Figure 1(d)).

While the above findings support the hypothesis that IL-1β drives upregulation of T-type channels via USP5 to induce pain, these effects were studied under acute conditions (<1 h) that yield transient pain (~15 min) and may be secondary to interactions between IL-1β and glial cells, which are known to express IL-1RI.[29,30] To study neuroinflammatory interaction more directly, while reflecting sustained release after physical injury, we used DRG neuron cultures to evaluate the level of USP5 bound to Cav3.2 in response to overnight (~24 h) exposure to IL-1β. Defined medium was used to keep ambient levels of IL-1β in cultures low[20] and therefore establish a clear response to elevated IL-1β (10 ng/ml). Under these conditions and relative to vehicle control (0.1% BSA in PBS), IL-1β produced a clear increase in the level of interaction between USP5 and Cav3.2 channels in co-IP experiments (Figure 1(e) and (f)), which is mediated by IL-1RI, as this effect was almost completely attenuated in the presence of IL-1Ra (100 ng/ml). Altogether, these findings indicate that IL-1β is capable of supporting persistent/chronic pain through USP5-mediated T-type plasticity in the pain pathway.

In this study, we demonstrated that IL-1β induces pain through a process that spares Cav3.2 T-type channels from ubiquitin-mediated degradation, thereby allowing them to accumulate in neuronal cell membranes and facilitate pain signaling. Interestingly, this form of T-type channel upregulation appears to extend beyond the duration of pain-related responses elicited by i.t. IL-1β—at least 30 min versus approximately 15 min. One explanation may be that IL-1β causes acute pain,

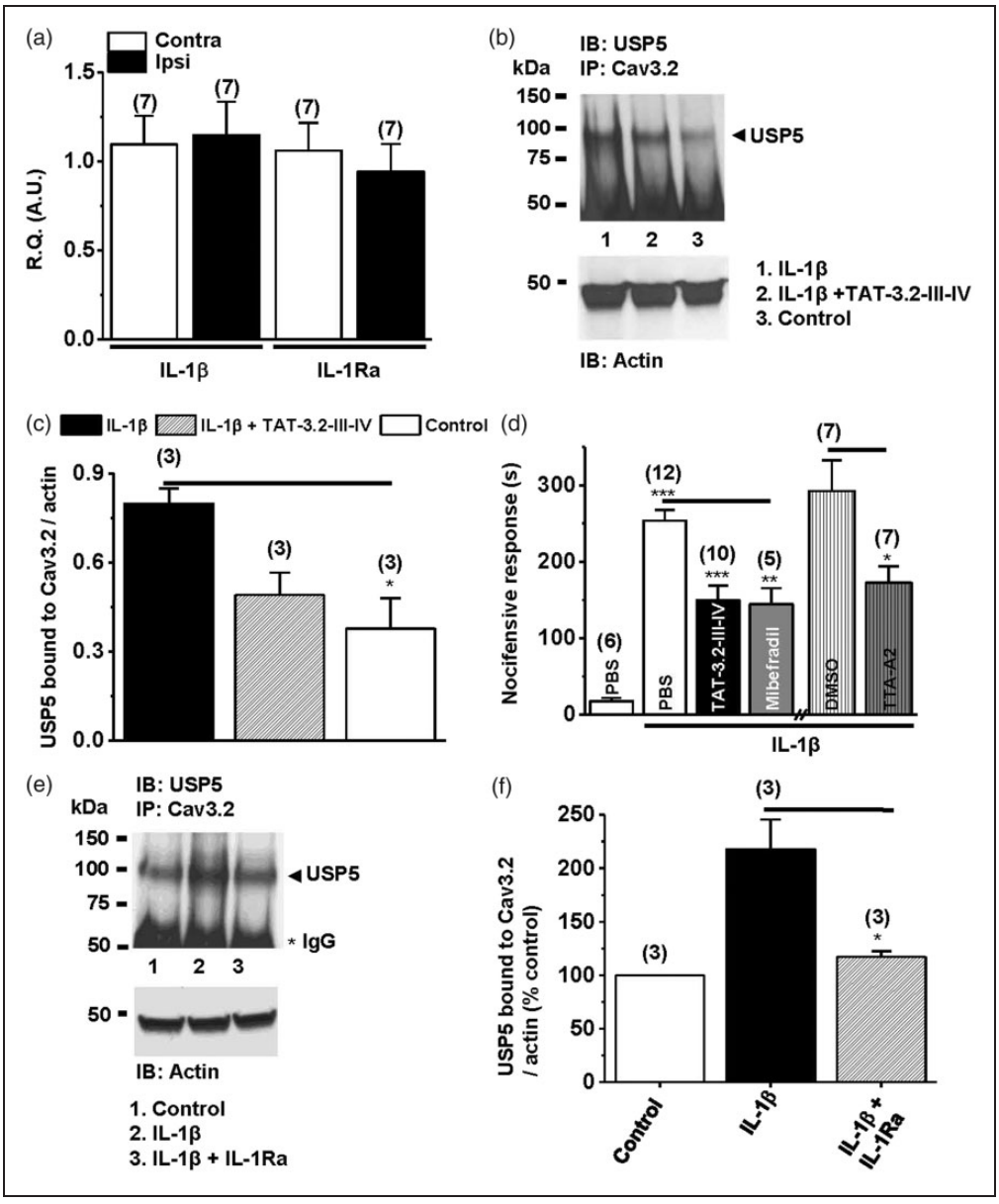

Figure 1. IL-1β induces and maintains upregulation of Cav3.2-USP5 interactions in spinal and peripheral components of the pain pathway. (a) Quantification of gene expression in DRG for IL-1β (left bars) and IL-1Ra (right bars) with real-time RT-qPCR immediately following conditioning via transcutaneous stimulation of nociceptors with blue light (wavelength: 473 nm, irradiance: 10 mW/cm^2, rate: 10 Hz, duration: 10 min) in the right hind paw of transgenic mice that express channelrhodopsin-2 (ChR2) in TRPV1—lineage neurons. Data ($n = 7$ for each group) are expressed as the ratio of specified mRNA over GaPDH mRNA (R.Q. (A.U.)). Contra, contralateral; Ipsi, ipsilateral. (b) Representative Co-IP of Cav3.2 and USP5 from the spinal dorsal horn of wild-type mice that received a single intrathecal (i.t.) injection of IL-1β (0.1 pg) in the absence or presence of TAT-3.2-III-IV peptide (10 μg, i.t.) or vehicle control (PBS), 30 min prior to tissue harvest. (c) Quantification of co-IP experiments in (b), normalized to actin ($n = 3$ for each group). (d) Cumulative duration of nocifensive responses over a 15-min period, following intrathecal administration in wild-type mice of vehicle control (PBS; $n = 6$; left of axis break) or IL-1β (0.1 pg; left of axis break) in combination with PBS ($n = 12$), TAT-3.2-III-IV peptide (10 μg, i.t.; $n = 10$) or mibefradil (10 μg, i.t.; $n = 5$). Right of axis break, IL-1β (0.1 pg; i.t.) in combination with vehicle control (4.4% DMSO; $n = 7$) or TTA-A2 (5 μg, i.t.; $n = 7$). (e) Representative Co-IP of Cav3.2 and USP5 from DRG cultures exposed overnight (~24 h) to vehicle control (0.1% BSA in PBS) or IL-1β (10 ng/ml) in the absence or presence of IL-1Ra (100 ng/ml). (f) Quantification of co-IP experiments in (e), normalized to actin and expressed as a percent of control ($n = 3$ for each group). All data represent means ± SEM. Values in parentheses indicate sample size (n). Statistical analyses were performed by two-tailed paired-sample t test in (a), one-way analysis of variance, followed by a Tukey test in (c) and (d, left of axis break), or two-sample t test in (d, right of axis break) and (f); $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Ipsi, ipsilateral; contra, contralateral; stim, stimulation; i.t., intrathecal.

which may engage descending projections that dampen nocifensive responses, even though USP5 expression may still be elevated.

Our results parallel findings in experimental models of neuropathic and inflammatory pain,[10,11] and therefore, it is tempting to speculate that IL-1β is a major driver of this process in persistent/chronic pain states. In support of such a mechanism, we discovered that, by sustaining elevated levels of IL-1β in DRG cultures, interactions between USP5 and Cav3.2 can be maintained. However, we cannot dismiss the possibility that numerous other inflammatory mediators released in response to injury are just as relevant. It is interesting that in a preliminary in vitro screen of potential mediators, which included brain-derived neurotrophic factor, nerve growth factor, and tumor necrosis factor-alpha, none were as effective as IL-1β at inducing interactions between USP5 and Cav3.2 (data not shown).

IL-1β increases neuronal excitability[20,31] and this may in turn be the trigger of the induction mechanism. Our in vitro work points to a direct interaction between IL-1β and sensory neurons that express IL-1RI, as observed previously.[31] Given that activity alone is sufficient to trigger this process, IL-1β may therefore play an upstream role, where sustained release can continue to feed this process. In support of this idea, the combination of a single i.t. injection of IL-1β with optogenetic stimulation does not enhance or extend mechanical sensitization beyond that achieved with conditioning stimulation of nociceptors alone (data not shown). Moreover and consistent with an effect of IL-1β that is mediated by neuronal activity, tetrodotoxin co-treatment (1 μM; overnight) in DRG cell cultures attenuated IL-1β-induced USP5 protein expression by $61.2 \pm 2.8\%$ ($n = 2$ cultures).

Although we focused on direct actions in sensory neurons, our study leaves open the possibility that elevated spinal levels of IL-1β can have direct actions on spinal dorsal horn neurons that facilitate pain signaling. This is important to consider moving forward, given that spinal dorsal horn neurons also express IL-1RI[32] and respond to IL-1β,[33,34] some of which may overlap with Cav3.2 T-type channel enriched interneuron subpopulations.[35]

The effect of IL-1β on T-type channels is poorly understood. By identifying a mechanism that upregulates T-type channels, the current study builds on existing work that identifies effects on numerous other voltage-gated ion channels.[21,22,31] Indeed, further delineation of this process in future work will be critical to better understanding neuro-inflammatory interactions that give rise to ongoing pathological pain.

Authors' Contributions

PLS conceptualized the study, wrote the manuscript, and established all DRG cell cultures. AGC performed co-IPs. VMG performed all behavioral experiments and i.t. injections. SM conducted real-time RT-qPCR experiments. LC harvested all tissues for co-IPs. IAS completed work with ELISA. GWZ directed and supported the study and edited the manuscript.

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Declaration of Conflicting Interests

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