A cell-permeant peptide corresponding to the cUBP domain of USP5 reverses inflammatory and neuropathic pain

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Abstract
Background: Cav3.2 T-type calcium currents in primary afferents are enhanced in various painful pathological conditions, whereas inhibiting Cav3.2 activity or expression offers a strategy for combating the development of pain hypersensitivity. We have shown that Cav3.2 channel surface density is strongly regulated by the ubiquitination machinery and we identified the deubiquitinase USP5 as a Cav3.2 channel interacting protein and regulator of its cell surface expression. We also reported that USP5 is upregulated in chronic pain conditions. Conversely, preventing its binding to the channel in vivo mediates analgesia in inflammatory and neuropathic pain models.

Results: To identify which USP5 domain is responsible for the interaction, we used a series of USP5-derived peptides corresponding to different regions in nUBP, cUBP, UBA1, and UBA2 domains to outcompete full length USP5. We identified a stretch of amino acid residues within the cUBP domain of USP5 as responsible for binding to Cav3.2 calcium channels. Based on this information, we generated a TAT-cUBP1-USP5 peptide that could disrupt the Cav3.2/USP5 interaction in vitro and tested its physiological effect in well-established models of persistent inflammatory pain (CFA test) and chronic mononeuropathy and polyneuropathy in mice (partial sciatic nerve injury and the (ob/ob) diabetic spontaneous neuropathy mice). Our results reveal that the TAT-cUBP1-USP5 peptide attenuated mechanical hyperalgesia induced by both Complete Freund’s Adjuvant and partial sciatic nerve injury, and thermal hyperalgesia in diabetic neuropathic animals. In contrast, Cav3.2 null mice were not affected by the peptide in the partial sciatic nerve injury model. Cav3.2 calcium channel levels in diabetic mice were reduced following the administration of the TAT-cUBP1-USP5 peptide.

Conclusions: Our findings reveal a crucial region in the cUBP domain of USP5 that is important for substrate recognition and binding to the III-IV linker of Cav3.2 channels. Targeting the interaction of this region with the Cav3.2 channel can be exploited as the basis for therapeutic intervention into inflammatory and neuropathic pain.

Keywords
T-type channels, Cav3.2, USP5, chronic pain, TAT-cUBP1-USP5 peptide, diabetic neuropathy, Complete Freund’s Adjuvant

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Introduction
Voltage-dependent Ca\(^{2+}\) channels provide a pathway for rapid influx of Ca\(^{2+}\) into excitable cells, thereby activating a number of downstream signaling pathways.\(^1\) The activity of T-type channels mediates several important physiological functions including burst firing in neurons, pacemaker activity in the heart, and secretion from endocrine tissues.\(^2\) The Cav3.2 T-type calcium channel isoform is prominently expressed in the afferent terminals in the spinal dorsal horn\(^3\) and dorsal root ganglia where it contributes importantly to the transmission and processing of pain signals.\(^4\) Upregulation of Cav3.2 channels in primary afferents caused by nerve injury and/or inflammation has been linked to pain hypersensitivity.\(^5\)–\(^8\) Recently, we identified key molecular signaling events that mediate injury induced upregulation of Cav3.2 in primary afferents and the spinal dorsal horn.\(^9\) We...
identified the deubiquitinase (DUB) USP5 as a key interacting partner of Cav3.2 channels. USP5 is a 835 residue multidomain enzyme that removes ubiquitin groups from ubiquitin-conjugated proteins, leading to reduced degradation of the target protein. Our work revealed that USP5 binds to the domain III-IV intracellular linker of the channel, thereby reducing Cav3.2 channel ubiquitination and enhancing Cav3.2 channel stability at the cell surface. We found that USP5 is also upregulated during peripheral inflammation and chronic nerve injury, thereby accounting for the injury induced increase of Cav3.2 channels. Blocking the interaction between USP5 and the channel resulted in analgesia.

Here, we describe the identification of a region in USP5 (cUBP, 206–244 amino acids) that is responsible for binding Cav3.2 calcium channels. We report the design of a TAT-cUBP1-USP5 peptide that is capable of disrupting the USP5/Cav3.2 interaction. We show that this peptide mediates analgesia in mouse models of peripheral inflammation, chronic neuropathy (PSNI), and diabetic neuropathy.

Materials and methods

Drugs and reagents

The following drugs were used in the study: Complete Freund’s Adjuvant (CFA) (Sigma Chemical Company, St. Louis, MO, USA) and TAT-cUBP1-USP5 (Genemed synthesis Inc., San Francisco, CA).

Affinity precipitation assays

Affinity purified recombinant human USP5 protein was solubilized in buffer containing 50 mM Tris pH 7.6, 150 mM NaCl, 0.2% Triton, 0.2% NP40, and protease inhibitors. A biotinylated Cav3.2 III-IV linker peptide, USP5 human recombinant protein, and nonbiotinylated USP5 peptides that correspond to different domains, i.e., nUBP, cUBP, UBA1, UBA2 (Genemed synthesis Inc., San Francisco, CA) were incubated with neutravidin beads for 2 h at 4°C. After washing with same buffer, bound USP5 was analyzed by SDS-PAGE and Western blot.

Western blots

Western blot analysis was performed using anti-actin mouse (Sigma), anti-Cav3.2 (H-300, Santa Cruz Biotechnologies, Inc.), and anti-USP5 (ProteinTech Group, Inc.) rabbit antibodies. Western blot quantification was performed using densitometry analysis (Quantity One-BioRad software). Student’s t-tests for unpaired data were performed to determine statistical significance.

Immunoprecipitation assays

Mouse dorsal horn tissue was lysed in RIPA buffer (in mM; 50 Tris, 100 NaCl, 1.0% (v/v) Triton X-100, 1.0% (v/v) NP-40, 10 EDTA + protease inhibitor cocktail, pH 7.5) and was used to immunoprecipitate Cav3.2 channels. 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 μl of Protein G/A beads (Piercenet) and 2 μg of anti-Cav3.2 (H-300, Santa Cruz Biotechnologies, Inc.) antibody overnight while tumbling at 4°C. Total inputs were taken from whole cell samples representing 4% of total protein and probed for actin. Immunoprecipitates were washed twice with (mM); 150 NaCl 50 Tris pH 7.5 buffer, beads were aspirated to dryness. Laemmli buffer was added and samples were incubated at 96°C for 7 min. Eluted samples were loaded on a 7.5% Tris-glycine gel and resolved using SDS-PAGE. Samples were transferred to 0.45 mm polyvinylidenedifluoride (PDVF) membranes (Millipore).

Animals

For this study, we used male adult mice (mus musculus, 20–28 g, 7–9 weeks old) C57BL/6J (wild-type) or Cav3.2 null (Cacna1h, background C57BL/6J) or yet the morbidly obese mice (ob/ob, background C57BL/6J, tested after development of neuropathic pain between 14 and 17 weeks old) and their respective age matching wild-type controls. All mice were purchased from the Jackson Laboratories and were kept at a maximum number of five per cage (30 x 20 x 15 cm) with access to water and food ad libitum and housed under controlled temperature of 23 ± 1°C under a 12-h light/dark cycles with lights on at 7 a.m. All experiments were performed between 10 a.m. and 3 p.m. always using different cohorts of mice for each experimental run. Experiments were performed after approval of an animal protocol by the Institutional Animal Care and Use Committee. Of note, best efforts were made to minimize animal suffering following the policies and recommendations of the International Association for the Study of Pain.

Diabetic neuropathic (ob/ob) mice and their age-matched wild-type controls (C57BL/6) were tested for thermal hyperalgesia using a plantar test device (Hargreaves) from UgoBasile. Due to the excessive body-weight of obese mice, measurements of mechanical hyperalgesia taken using the digital esthesiometer device were compromised. Upon their arrival at the age of 10 weeks, they were weighed and blood glucose levels were taken and analyzed every two weeks using tail blood samples and a One Touch-Verio glucometer (Johnson & Johnson, New Brunswick, NJ). The TAT-cUBP1-USP5 peptide did not modify blood glucose levels of diabetic animals.
Persistent inflammatory pain induced by CFA

With the intent to induce peripheral persistent inflammatory pain, we injected 20 μl of CFA subcutaneously (i.pl.) in the plantar surface of the right hindpaw of mice. Some animals received 20 μl of phosphate-buffered saline (PBS) in the ipsilateral paw in parallel, as control. Two days following i.pl. injections, CFA-injected mice were treated with the TAT-cUBP1-USP5 peptide (1–10 μg/i.t.) or vehicle (10 μl/i.t.). Their mechanical withdrawal thresholds were tested prior to CFA injection (baselines) immediately before treatment (Time 0) and at several time points after delivery of vehicle or TAT-cUBP1-USP5 peptide.

Chronic mononeuropathy induced by partial sciatic nerve injury

To produce neuropathic pain in mice, we performed a partial ligation of the sciatic nerve by tying the distal 1/3 to 1/2 of the dorsal portion of the nerve. In sham-operated mice, the sciatic nerve was exposed without any injury. All animals were anesthetized with isoflurane (4% induction, 2.5% maintenance). After ligation, the wound was sutured and covered with iodine solution. Mechanical withdrawal thresholds were taken before surgeries and considered as baselines. Fourteen days after surgeries, mechanical withdrawal thresholds were analyzed before treatment (Time 0) and at several time points after treatment of mice with TAT-cUBP1-USP5 peptide (1–10 μg/i.t.) or vehicle (10 μl/i.t.) while sham-operated animals received only vehicle (10 μl/i.t.).

Mechanical hyperalgesia measurements

Measurements of mechanical hyperalgesia were taken through the use of a digital Plantar Aesthesiometer (DPA, UgoBasile, Varese, Italy) according to previous work from our lab.9,12 Animals were placed individually inside enclosed testing chambers (10 cm × 10.0 cm × 13 cm, length × width × height) on top of a grid floor. Animals were kept in the experimental room in order to acclimate for a period of at least 90 min before any measurement was taken. The filament of the esthesiometer device was placed underneath the plantar surface of the ipsilateral hind paw of each mouse for each measurement and each paw was tested three times.

Thermal hyperalgesia

Diabetic neuropathic and nondiabetic/neuropathic controls were tested for thermal hyperalgesia by measuring the latency to withdrawal of right hind paws from an infra-red radiant heat (IR = 40) of a Plantar Test apparatus (Hargreaves, UgoBasile, Varese, Italy). Animals were kept individually in enclosed testing chambers (10 cm × 10 cm × 13 cm, length × width × height) placed on top of a grid floor. They were allowed to acclimate for at least 90 min before measurements started. The infra-red heat was positioned underneath the right hind paw and three measurements were taken for each mouse. The device was set at a cut-off time of 30 s to avoid tissue damage. Thermal hyperalgesia was analyzed was immediately prior to the treatments (Time 0) and at several time points after treatment.

Intrathecal drug treatment

Intrathecal injections of either the TAT-cUBP1-USP5 peptide or vehicle (control) were performed in conscious mice. After the dorsal fur of each mouse was shaved, mice were manually restrained with the spinal column arched then and a 30-gauge needle attached in a PE20 polyethylene tube connected to a 25-μl Hamilton micro-syringe (Hamilton, Birmingham, UK) was inserted into the subarachnoid space between the L4 and L5 vertebrae. Correct positioning of the needle tip was confirmed by a characteristic tail-flick response. Volumes of 10 μl were delivered over a period of 5 s.

Statistical analysis

For biochemical experiments, results are presented as mean ± S.E.M. and statistical significance was determined using Student’s t-test unless stated otherwise: *P < 0.05; **P < 0.01; ***P < 0.001; NS = statistically not different. For behavioral experiments, data are presented as means ± SEM and evaluated by one-way, two-way, or three-way analysis of variance (ANOVA) followed by a Tukey’s test. A value of P < 0.05 was considered to be significant (*P < 0.05; **P < 0.01; ***P < 0.001; NS = not different).

Results

Mapping the region in USP5 responsible for binding the Cav3.2 III-IV linker

Our initial finding on Cav3.2/USP5 interaction using a proteomic approach identified the III-IV intracellular linker of Cav3.2 calcium channels as the target site for ubiquitin conjugation and USP5 binding.9 The USP5 structural determinants responsible for Cav3.2 interaction have not been identified. Recently, the crystal structure of full length USP5 was resolved at a resolution of 3.49–2.80 Å.14 The general structure of USP5 consists of two zinc-finger ubiquitin-specific protease (ZnF-UBP) domains (nUBP and cUBP), a catalytic core, and two ubiquitin-associated domains (UBA1 or nUBA and UBA2 or cUBA) present in the carboxy-terminus14 Figure 1(a).

To determine which part of USP5 interacts with Cav3.2 channels, we designed short peptides (35–45
mers) corresponding to each of the major USP5 domains Figure 1(b), with each peptide containing at least one α-helix. We then performed in vitro affinity competition assays with a Cav3.2-III-IV-linker biotinylated peptide that binds USP5, a USP5 recombinant protein (long splice isoform) and different nonbiotinylated peptides corresponding to the various USP5 domains. We observed a significant displacement of recombinant USP5 from the Cav3.2 III-IV linker only with the cUBP peptide containing amino acid residues 206–244 of USP5, cUBP1 Figure 1(c). We observed a partial, albeit not statistically significant competition for the recombinant USP5 protein with a cUBP-derived peptide encompassing amino acids 247–281 (cUBP2; Figure 1(c) and (d)). Next, we asked if the cUBP1 peptide was able to interact with full length Cav3.2 channels from mouse brain lysates. As

![Figure 1](image-url). Mapped the USP5 interaction site where Cav3.2 binds via the III-IV linker: (a) USP5 crystal structure model, showing catalytic site (cyan), nUBP (yellow), cUBP (green), nUBA (pink), and cUBA (purple) domains. Taken from Avvakumov et al. (b) Peptide sequences corresponding to different USP5 domains. (c) In vitro affinity competition assays with a Cav3.2-III-IV-linker biotinylated peptide, a hUSP5 (long isoform) purified recombinant protein and nonbiotinylated peptides (ratio 1:1, Cav3.2 biotinylated to USP5 nonbiotinylated peptides) (shown in panel B) corresponding to different USP5 domains. n = 3–10 (d) Quantification of USP5 (full length protein) bound to Cav3.2-III-IV biotinylated peptide in the presence or absence of different USP5 domain peptides. (e) Binding of cUBP-USP5 (Biotin-NLWLNLTDGSILCGRRYFDGSGGNNHAVEHYRETG (cUBP; 206–244 amino acids)) peptide to full length Cav3.2 endogenous channels from mouse brain lysates, as seen by Western blot (n = 3). A control peptide was used (lane 1) for USP5-UBA1 domain (Biotin-GSLGFYGNEDFCSPHFSSDIS (UBA1; 635–658 amino acids)).
shown in Figure 1(e), this was indeed the case, whereas the UBA1-USP5 (635–658 a.a.) control peptide Figure 1(e) did not interact with the channels. Based on this information, we designed a permeable TAT-cUBP1-USP5 peptide Figure 2(a) for in vivo testing.

**Effect of a TAT-cUBP1-USP5 peptide on mechanical hyperalgesia induced by peripheral inflammation**

To ascertain the effects of the TAT-cUBP1-USP5 on pain arising from peripheral inflammation, we examined its action on mechanical hyperalgesia induced by injection of CFA into the right hindpaw. CFA triggers mechanical hyperalgesia that lasts for several days when compared with either the baseline responses or the non-inflamed group ($P < 0.001$). Intrathecal treatment of mice with the TAT-cUBP1-USP5 peptide (1–10 μg/i.t.) two days after CFA treatment produced rapidly developing (15 min after treatment), dose-dependent, and long-lasting anti-hyperalgesia effects that remained significant up to 3 h after delivery. In contrast, delivery of vehicle (PBS, 10 μl/i.t.) did not exert such effects Figure 2(b). We also performed a biochemical analysis of Cav3.2 protein levels in dorsal horn tissue isolated from CFA-injected mice that received intrathecal treatment with either the TAT-cUBP1-USP5 peptide, or with PBS. In two separate sets of experiments, each involving three mice in the saline and peptide groups, Cav3.2 channel protein levels (normalized to an actin control) were reduced by 35 ± 7% in TAT-cUBP1-USP5 peptide-treated mice compared to the PBS control group.

**Effect of the TAT-cUBP1-USP5 peptide on mechanical hyperalgesia produced by sciatic nerve injury**

To analyze whether the TAT-cUBP1-USP5 peptide inhibits mechanical hyperalgesia produced by peripheral neuropathy, we tested its effect two weeks after a partial sciatic nerve ligation procedure (PSNI). It is well established that sciatic nerve injury causes mechanical hyperalgesia that

![Figure 2. Anti-hyperalgesic effect of the TAT-cUBP1-USP5 peptide in the mouse model of peripheral inflammation: (a) TAT-cUBP1-USP5 peptide sequence. (b) Time-dependent analyses of mechanical hyperalgesia of CFA-injected mice treated with the TAT-cUBP1-USP5 peptide (1 and 10 μg/i.t.) or control vehicle (10 μl/i.t.), ($n = 5$–$7$). Peptides were delivered two days after CFA treatment. Hashtag symbols indicate statistical significance of CFA treatment relative to the PBS control ($P < 0.001$). Asterisks denote statistical significance relative to the CFA group.](image)
persists for several weeks. This is evident by comparing the baseline responses of nerve injured animals and the sham-operated group ($P < 0.001$) Figure 3(a). Treatment of neuropathic mice with the TAT-cUBP1-USP5 peptide (1–10 μg/i.t.) 14 days after nerve injury drastically reduced mechanical hyperalgesia at the highest concentration tested, and this effect remained significant up to 180 min after treatment Figure 3(a).

To test if the analgesic action of the TAT-cUBP1-USP5 peptide is indeed exclusively mediated by interfering with Cav3.2 channels, we repeated the sciatic nerve injury model in both Cav3.2 null mice and in wild-type animals in parallel. As a result of partial sciatic nerve injury (PSNI), Cav3.2 knockout mice developed mechanical hypersensitivity similar to that seen with WT mice Figure 3(b). The fact that Cav3.2-null mice develop neuropathic pain despite the absence of Cav3.2 channels is likely due to compensatory mechanisms. Importantly, Cav3.2-null mice were completely insensitive to the treatment with the TAT-cUBP1-USP5 peptide (10 μg/i.t.), while this peptide still produced pronounced analgesia in WT animals when tested in parallel Figure 3(b). These results demonstrate that the TAT-cUBP1-USP5 peptide mediates analgesia in vivo by modulating Cav3.2 channels.

**Effect of the TAT-cUBP1-USP5 peptide on neuropathic pain in diabetic mice**

We have previously reported that pain hypersensitivity in diabetic ob/ob mice can be reversed by blocking the interactions between USP5 and Cav3.2 by small organic molecule mimetics. To determine whether the TAT-cUBP1-USP5 peptide was similarly effective, we assessed thermal withdrawal threshold in ob/ob mice before and after delivery of the TAT-cUBP1-USP5 peptide. As shown in Figure 4(a), treatment of these diabetic neuropathic mice with the TAT-cUBP1-USP5 peptide (10 μg/i.t.), but not vehicle (PBS, 10 μl/i.t.), completely inhibited thermal hypersensitivity within 90 min of intrathecal delivery Figure 4(a). We also analyzed Cav3.2 expression levels from the dorsal horns of peptide treated ob/ob mice by immunoprecipitation Figure 4(b) and (c). As expected (and consistent with our analysis of CFA-treated animals), the TAT-cUBP1-USP5 peptide efficiently decreased Cav3.2 levels by 43% Figure 4(c), suggesting less stable channels due to a lack of binding to USP5. We could not examine mechanical hypersensitivity in these animals, because those morbidly obese mice are too heavy and slow for measurements using the digital esthesiometer device.

Altogether, our data indicate that disrupting the USP5/Cav3.2 interaction in vivo via a cell-permeant peptide corresponding to the cUBP domain reduces Cav3.2 channel expression in the dorsal horn, thereby mediating relief from diabetic pain.

**Discussion**

Here, we have identified a specific 35 amino acid region embedded in the cUBP domain of USP5 that is responsible for interacting with the III-IV linker region of Cav3.2 T-type calcium channels. Consistent with our observations, this region has been previously described as a potential site for substrate targeting and specificity. In contrast, this region does not appear to be important for substrate modification by the catalytic site because deletion of the cUBP (163–291) domain did not affect the hydrolysis rate of ubiquitin-AMC. Together with our previous identification of a short (~20 amino acid) stretch of residues in the domain.
III-IV linker of the Cav3.2 channel,\(^9\) we now have two complementary tools that allow us to disrupt USP5-Cav3.2 interactions both in vitro and in vivo.

We have recently reported that small organic molecules (from a compound library) that target the USP5-Cav3.2 channel interface can be effective analgesics in various rodent pain models.\(^{12}\) This was accomplished via a high throughput ELISA screen that involved binding of recombinant USP5 to immobilized Cav3.2 III-IV linker peptides.\(^{12}\) In this context, the USP5 structural information that is now in place can be used to devise an improved and more cost-effective screening assay for small molecule disruptors. Furthermore, we can now embark on molecular docking studies to obtain structural insights into the interaction between the two binding partners, and how small organic molecules may disrupt this process.

It is now well established that Cav3.2 channel is an important player in the development of pain hypersensitivity following inflammation or nerve injury.\(^{5-8}\) Inhibiting Cav3.2 channel activity either by siRNA depletions\(^4\) or direct inhibition\(^{12,15-18}\) mediates analgesia in various rodent models of inflammatory and neuropathic pain. Furthermore, T-type channel blockers are explored for treating pain in humans.\(^{19-21}\) Cav3.2 channels are also important for other physiological functions in both the nervous and the vascular systems\(^2\) and thus blocking these channels directly could potentially result in side effects. Furthermore, it is notoriously difficult to design blockers that discriminate among the three major T-type calcium channel isoforms.\(^{22}\) These issues can be circumvented by targeting the molecular process that results in aberrant upregulation of Cav3.2 channels. Indeed, uncoupling USP5 from Cav3.2 channels will spare the normal physiological channel function and only target those channels that are involved in pathophysiology. In this context, the use of a USP5 fragment as a competitive disruptor has the added advantage that it targets the channel rather than the interacting enzyme.

The two Tat peptides that correspond to sequences in the channel protein TAT-Cav3.2-III-IV\(^9\) and on USP5 (TAT-cUBP1-USP5) mediated remarkably similar physiological effects in mouse models of inflammatory and neuropathic pain. Both peptides acted rapidly (within 15 min of intrathecal delivery) and were effective in the CFA, PSNI, and diabetic neuropathy models, with the TAT-cUBP1-USP5 peptides perhaps showing greater efficacy than the TAT-Cav3.2-III-IV agent\(^9,12\) (in particular in the diabetic model). The fact that the USP derived peptide resulted in similar biological actions as our previously examined III-IV linker channel fragment indicates that the analgesic effects are indeed caused by the disruption of the complex, rather than global inactivation of the USP5 enzyme that could in principle have affected USP5 action on other targets. This is also supported by the observation that both peptides were ineffective in Cav3.2 null mice, thus further arguing against off-target effects of the peptides. Indeed, in our hands neither peptide mediated noticeable behavioral side effects, although it is important to acknowledge that we did not conduct an exhaustive battery of behavioral assessments.

In summary, we have extended our previous work on USP5-Cav3.2 channel interactions to identify a key structural USP5 domain that is responsible for the actions of USP5 on this channel isoform. The analgesic actions of the
TAT-cUBP1-USP5 further underscore the role of this interaction in the development of pain hypersensitivity.

Conclusions
The cUBP domain of USP5 is responsible for the interactions of USP5 with the Cav3.2 channel. Cell-permeant peptides mimicking this domain have analgesic properties by virtue of disrupting the USP5Cav3.2 complex. This knowledge will guide therapeutics development.

Author Contributions
GWZ, AGC, and VMG designed the experiments and wrote the manuscript; AGC performed molecular biology and biochemistry; LC contributed in tissue harvesting from animals for analysis; and VMG performed in vivo pain studies. GWZ supervised the study.

Declaration of Conflicting Interests
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