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# Efficacy of (*S*)-lacosamide in preclinical models of cephalic pain

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**Abstract**

Migraine is one of the world's most common neurological disorders. Current acute migraine treatments have suboptimal efficacy, and new therapeutic options are needed. Approaches targeting calcitonin gene related peptide (CGRP) signaling are clinically effective, but small molecule antagonists have not been advanced because of toxicity. In this study, we explored the axonal growth/specification collapsin response mediator protein 2 (CRMP2) as a novel "druggable" target for inhibiting CGRP release and for potential relevance for treatment of migraine pain. Collapsin response mediator protein 2 has been demonstrated to regulate N-type voltage-gated $Ca^{2+}$ channel activity and $Ca^{2+}$-dependent CGRP release in sensory neurons. The coexpression of CRMP2 with N-type voltage-gated $Ca^{2+}$ channel and CGRP in trigeminal ganglia (TGs) sensory neurons suggested the possibility of a novel approach to regulate CGRP release in the trigeminal system. Screening protocols surprisingly revealed that (*S*)-lacosamide ((*S*)-LCM), an inactive analog of the clinically approved small molecule antiepileptic drug (*R*)-lacosamide (Vimpat), inhibited CRMP2 phosphorylation by cyclin-dependent kinase 5 in rat TG slices and decreased depolarization-evoked $Ca^{2+}$ influx in TG cells in culture. (*S*)-LCM significantly blocked capsaicin-evoked CGRP release from dural nerve terminals in the rat in ex vivo cranial cup preparation. Additionally, cephalic and extracephalic cutaneous allodynia induced in rats by activation of dural nociceptors with a cocktail of inflammatory mediators, was inhibited by oral administration of (*S*)-LCM. The confirmation of CRMP2 as an upstream mediator of CGRP release, together with the brain penetrance of this molecule suggests (*S*)-LCM as a potential therapy for acute migraine.

**Keywords:** CaV2.2, CRMP2, Phosphorylation, Cdk5, (*S*)-Lacosamide, CGRP, Calcium imaging, Trigeminal ganglia, Cutaneous allodynia, Migraine, Headache-related pain

## 1. Introduction

Migraine affects approximately 11% to 13% of adults in the United States and is a prevalent and under-diagnosed disorder that severely impacts quality of life of afflicted individuals, and presents an enormous economic cost to society.[48] Therapies for migraine may be acute or preventative. Currently available acute therapies include nonsteroidal anti-inflammatory drugs, triptans (eg, serotonin receptor [5-HT1B/1D] agonists), and opioids.[4] These treatments are often inadequate. Less than one-half of patients taking oral triptans report being pain free at 2 hours, and up to one-third report headache recurrence within 24 hours.[30,56] The response rate for nonsteroidal anti-inflammatory drugs is similar.[41,60,68] The recognition that blood levels of calcitonin gene related peptide (CGRP) are elevated during migraine attack[35,62] has led to a groundswell of research, targeting CGRP and its cognate receptor. Clinical investigations demonstrated that small molecule CGRP receptor antagonists are efficacious against migraine[37,54]; however, development of this therapeutic class remains uncertain because of safety issues. New therapeutic options for acute migraine treatment are urgently needed.

The N-type voltage-gated calcium channel (CaV2.2) has been clinically validated as a mechanism for treatment of pain, by the efficacy of ziconotide, a peptide limited to intrathecal delivery because of severe supraspinal side-effects.[49,66] N-type voltage-gated calcium channel activity controls neuropeptide release,[47] and more specifically CGRP, in the peripheral nervous system[8,44,57] including trigeminal ganglia (TGs)[1,2] and dura mater.[2] Additionally, CaV2.2 is a major determinant of nociceptive signaling from the dura to the trigeminal nucleus caudalis,[23,64] thus placing the channel in a critical position to contribute to

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headache-related pain transmission. Collectively, these observations suggest that approaches that could modulate CaV2.2 activity with diminished side effects are attractive for novel therapies including migraine.

We have shown that CaV2.2 activity is dependent on the axonal collapsin response mediator protein 2 (CRMP2)[8,9,15] and its cyclin-dependent kinase 5 (Cdk5)-phosphorylated form.[10,50,51] Inhibiting CRMP2/CaV2.2 interaction[8] reduced capsaicin-evoked CGRP release from dorsal root ganglia (DRGs) sensory neurons and capsaicin-induced meningeal blood flow, possibly by actions on sensory neurons related to headache.[8,61] Recently, increased CRMP2 expression was reported in trigeminal branches from patients with chronic migraine (>1.5-fold enrichment over healthy controls).[36] On this basis, we hypothesized that targeting CRMP2 phosphorylation in TG neurons, could be a promising strategy for curbing CaV2.2 activity and inhibiting dural CGRP release, contributing to prevention of cephalic pain. A screening protocol of >50 novel molecules unexpectedly identified the small molecule (*S*)-lacosamide ((*S*)-LCM)[51] as a novel inhibitor of CRMP2 interactions with CaV2.2. Here, we tested the possible causal relation between (*S*)-LCM–mediated inhibition of CRMP2 phosphorylation by Cdk5 in TG neurons. Our studies show that (*S*)-LCM reduces calcium influx in TG neurons, diminishes evoked CGRP release in dural afferents, and after oral administration, reverses cephalic and extra-cephalic cutaneous allodynia (CA), observed after direct activation of rat dural nociceptors. These studies confirm CRMP2 as a critical regulator of CGRP in the trigeminal system and identify (*S*)-LCM as a novel potential therapeutic for the acute treatment of migraine.

## 2. Methods

### 2.1. Animals

Pathogen-free, adult male Sprague–Dawley rats (150-200 g; Envigo [Indianapolis, IN]) were housed in temperature (23 ± 3°C) and light (12-hour light/12-hour dark cycle; lights on 07:00-19:00) controlled rooms with standard rodent chow and water available ad libitum. The Institutional Animal Care and Use Committee of the College of Medicine at the University of Arizona approved all experiments. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain. Animals were randomly assigned to treatment or control groups for the behavioral experiments. Animals were initially housed 3 per cage, but individually housed after the dural cannulation on a 12-hour light-dark cycle with food and water ad libitum. All behavioral experiments were performed by experimenters who were blinded to the experimental groups and treatments.

### 2.2. Immunohistofluorescence and epifluorescence imaging

Trigeminal ganglia were dissected from adult rats and then fixed using 4% paraformaldehyde for 4 hours at room temperature (RT). Trigeminal ganglia were next transferred into a 30% sucrose solution and left at 4°C until the sinking of the tissues could be observed (~3 days). Tissues were cut in 10 μm thickness using the Bright OTF 5000 Microtome Cryostat (Hacker Instruments and Industries, Inc, Winnsboro, SC), and fixed onto gelatin coated glass slides and kept at −20°C until use. Before antibody staining, slides were dried at room temperature for 30 minutes and incubated with phosphate buffered saline (PBS) containing 200 mM $NH_4Cl$ for 30 minutes. Next, the slides were incubated with PBS containing 3% sodium deoxycholate for 30 minutes at RT; these 2 PBS incubations were performed to reduce the background fluorescence of the tissue. Trigeminal ganglion (TG) slices were permeabilized and saturated using PBS containing 3% bovine serum albumin (BSA) and 0.3% triton X-100 solution for 30 minutes at RT, and then antibodies were added overnight. The antibodies used were CaV2.2 (Cat#TA308673; Origene, Rockville, MD); CGRP (Cat#C8198; Sigma, St Louis, MO); CRMP2 (Cat#C2993; Sigma or Cat#11096; Immuno-Biological Laboratories, Minneapolis, MN); CRMP2 pSer522 (Cat#CP2191; ECM Biosciences, Versailles, KY); and βIII-tubulin (Cat#G712A; Promega, Madison, WI). The slices were then washed 3× in PBS, and incubated with PBS containing 3% BSA and 0.3% triton X-100 containing secondary antibodies (Alexa 488 goat anti-rabbit or Alexa 594 goat anti-mouse secondary antibodies [Life Technologies]) for at least 3 hours at RT. After 3 washes (PBS, 10 minutes, RT), either 4',6-diamidino-2-phenylindole was used to stain the nuclei of cells or neurotrace (Cat#N21479; Thermo Fisher Scientific) was used to stain neuronal soma. Slides were mounted and stored at 4°C until analysis. Immunofluorescent micrographs were acquired on an Olympus BX51 microscope with a Hamamatsu C8484 digital camera using a 4× UplanFL N, 0.13 numerical aperture or a 20× UplanSApo 0.75 numerical aperture objective. The freeware image analysis program Image J (http://rsb.info.nih.gov/ij/) was used to generate merged images.

### 2.3. Western blotting

For examining the effect of (*S*)-LCM on CRMP2 phosphorylation state, TGs were dissected from adult rats and treated for 30 minutes at 37°C with 200 μM (*S*)-LCM diluted in Dulbecco's modified essential media (DMEM; Cat#11965; Life technologies, Carlsbad, CA). Then, tissues were lysed by sonication in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 1% [vol/vol] NP40, 0.5% [mass/vol] sodium deoxycholate, and 0.1% [mass/vol] sodium dodecyl sulfate and protease [Cat#B14002; Biotool, Houston, TX] and phosphatase inhibitors [Cat#B15002; Biotool], and BitNuclease [Cat#B16002; Biotool]). Protein concentrations were determined using the bicinchoninic acid protein assay (Cat#PI23225; Thermo Fisher Scientific, Waltham, MA). Approximately 5 μg of total proteins were loaded on an SDS-PAGE and then transferred to polyvinylidene difluoride membranes and blocked at room temperature for 1 hour in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% tween 20) and 5% nonfat dry milk. Primary antibodies used for probing were CRMP2 (Cat#C2993; Sigma), CRMP2 pSer522 (Cat#CP2191; ECM Biosciences, Versailles, KY), and Actin (Cat#A2066, Sigma) and were diluted in TBST with 5% BSA. Immunoblots were revealed by enhanced luminescence (Cat#WBKLS0500; Millipore, Billerica, MA) before exposure to a photographic film. Films were scanned, digitized, and quantified using Un-Scan-It gel version 6.1 scanning software (Silk Scientific Inc, Orem, UT).

### 2.4. Primary trigeminal ganglia neuronal cultures

Sensory TG neurons from Sprague–Dawley rats were excised aseptically and placed in Hank buffered salt solution (Life technologies) containing penicillin (100 U/mL) and streptomycin (100 μg/mL, Cat#15140; Life technologies). The ganglia were further dissected to remove all non-neuronal structures before enzymatic dissociation by a 45-minute incubation (37°C) in a DMEM solution containing neutral protease (3.125 mg/mL$^{-1}$, Cat#LS02104; Worthington, Lakewood, NJ) and collagenase type I (5 mg/mL$^{-1}$, Cat#LS004194; Worthington). The dissociated cells were resuspended in complete TG

medium (ie, DMEM containing penicillin [100 U/mL], streptomycin [100 μg/mL], 30 ng/mL$^{-1}$ nerve growth factor, and 10% fetal bovine serum [Hyclone, Logan, UT]). For $Ca^{2+}$ imaging (see below), the cells were seeded on poly-D-lysine (Cat#P6407; Sigma) coated glass coverslips (Cat#72196-15; Electron Microscopy Sciences, Hatfield, PA) as a drop of 20 μL on the center of each coverslip, then placed in a 37°C, 5% $CO_2$ incubator for 45 to 60 minutes to allow cells to attach. Then, the cultures were flooded by gently adding complete TG medium on the edge of each well to avoid detaching any weakly adherent cell. All cells were used within 24 hours after seeding.

### 2.5. Calcium imaging

Trigeminal ganglion neurons were loaded at 37°C with 3 μM Fura-2AM (Cat#F-1221; Life technologies, stock solution prepared at 1 mM in dimethyl sulfoxide, 0.02% pluronic acid, Cat#P-3000 MP; Life technologies) for 30 minutes ($K_d$ = 25 μM, $\lambda_{ex}$ 340, 380 nm/$\lambda_{emi}$ 512 nm) to follow changes in intracellular calcium ($[Ca^{2+}]_c$) in Tyrode solution (at ~310 mOsm) containing 119 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, pH 7.4 and 30 mM glucose. The solution was supplemented with 500 nM tetrodotoxin (voltage-gated $Na^+$ channel inhibitor) and 1 μM nifedipine (L-type voltage-gated $Ca^{2+}$ channel inhibitor). Incubation with 200 μM (*S*)-LCM was done during the loading of the cells with Fura-2AM and was also added to the excitatory solution. All calcium imaging experiments were done at room temperature (~23°C). Baseline was acquired for 1 minute followed by stimulation (15 seconds) with an excitatory solution (at ~310 mOsm) comprised of 32 mM NaCl, 90 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, pH 7.4 and 30 mM glucose. Fluorescence imaging was performed with an inverted microscope, Nikon Eclipse T*i*-U (Nikon Instruments Inc, Melville, NY), using objective Nikon Nikon S Plan Fluor ELWD 20 × 0.45 and a Photometrics cooled CCD camera CoolSNAP $ES^2$ (Roper Scientific, Planegg, Germany) controlled by NIS Elements software (version 4.20, Nikon Instruments Inc). The excitation light was delivered by a Lambda-LS system (Sutter Instruments, Novato, CA). The excitation filters (340 ± 5 and 380 ± 7 nm) were controlled by a Lambda 10-2 optical filter change (Sutter Instruments). Fluorescence was recorded through a 505-nm dichroic mirror at 535 ± 25 nm. To minimize photobleaching and phototoxicity, the images were taken every 10 seconds during the time-course of the experiment, using the minimal exposure time that provided acceptable image quality. The changes in $[Ca^{2+}]_c$ were monitored by following the ratio of $F_{340}/F_{380}$, calculated after subtracting the background from both channels.

### 2.6. Cranial cup and calcitonin gene related peptide release assay

The ex vivo cranial cup prep was prepared according to methods described previously.[22,24] Rats were killed by $CO_2$ overdose and decapitated. The skull with attached dura was dissected out as a whole cup. The 2 hemispheres and the TG were removed without injuring the meninges underneath. The entire skull with attached dura was used as the vehicle to hold solutions. The head cavities were then mounted in a humid chamber at 37°C. The skull cavities were filled with 700 μL oxygenated synthetic interstitial fluid (SIF; 37°C) comprised of 108 mM NaCl, 3.48 mM KCl, 3.5 mM $MgSO_4$, 26 mM $NaHCO_3$, 11.7 mM $NaH_2PO_4$, 1.5 mM $CaCl_2$, 9.6 mM Na gluconate, 5.5 mM glucose, and 7.6 mM sucrose, pH 7.4. After 5 minutes, the solution was discarded and SIF was renewed for 2 more washes. After a total of 3 washes, the collection protocol was started. Two baselines (#1 and #2), 1 pretreatment (#3), 1 cotreatment (#4), and 2 post-treatment fractions (#5 and #6) (5 minutes, 700 μL each) were collected for measurement of CGRP release. Samples were immediately stored in a −20°C freezer. (*S*)-lacosamide (200 μM) or vehicle (0.9% saline) was added to the pretreatment and cotreatment fractions (#3 and 4). Capsaicin (1 μM; Sigma) was added to the SIF as a cotreatment with (*S*)-LCM or vehicle at the fourth fraction to evoke CGRP release. The concentration of CGRP released into the SIF was measured by enzyme-linked immunospecific assay (Cayman Chemical, Ann Arbor, MI).

### 2.7. Dural cannulation in vivo

The procedure was performed according to our previous reports.[26,27] Briefly, naive rats were anesthetized by ketamine/xylazine (80/12 mg/kg, i.p.; Western Medical Supply, Arcadia, CA/Sigma) and fixed on a stereotaxic frame. A burr hole (about 1 mm diameter) was created at 1 mm rostral and lateral to bregma. A single guide cannula (Plastics One, Roanoke, VA) was implanted above the dura mater and glued firmly to form a tight seal around the skull. Two additional burr holes were drilled to implant 2 stainless steel screws to either side of the skull to secure the guide cannula with dental cement and acrylics. Care was taken not to disrupt the dura mater at any time. Stainless steel dummy cannulas were inserted to keep the guide cannula free of debris. Gentamycin (8 mg/kg, s.c.) was given to counteract infection. After surgery, rats were housed individually and allowed to recover for minimum 6 days. The appropriate placement of the guide cannula was also verified post hoc to ensure lack of dural penetration. Data from the animals with defective cannulas were discarded (<10%).

### 2.8. Dural application of inflammatory mediators

Inflammatory mediator (IM) solution was prepared from histamine, serotonin, bradykinin, and prostaglandin E $E_2$ (all purchased from Thermo Fisher Scientific) 1 mM each and dissolved in 10 mM HEPES, at pH 5.0. Inflammatory mediator was slowly injected onto the dural surface through the dwelling cannula consistent with our previous report.[27] Care was taken to preserve the integrity of dura. The volume was 10 μL per rat. Successful delivery of IM was indicated by the slow advance of the solutions to the epidural space without any leakage from surrounding areas of the guide cannula or the edge of the cement.

### 2.9. Evaluation of periorbital and hind paw tactile allodynia

Behavioral thresholds to innocuous tactile stimuli were determined by applying calibrated von Frey filaments perpendicularly to the periorbital region at the center of the forehead or the plantar surface of the hind paw, until the head moved away or a withdrawal response was elicited.[27] The withdrawal thresholds of the periorbital region or hind paw were measured in response to probing of the plantar surface, with a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL), in logarithmically spaced increments ranging from 0.41 to 15 g (cutoff was set at 8 or 15 g for periorbital or hind paw region, respectively). Each filament was applied perpendicularly to the center of the forehead or plantar surface of both left and right hind paws of rats held in suspended wire-mesh cages. Withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength ("up and down" method), analyzed using a Dixon nonparametric test, and expressed as the mean withdrawal threshold.[14,18,42,59] It should be noted that pain is a human experience and that the enhanced responsiveness to a normally ineffective strength of

von Frey filaments in rodents are described as "allodynia" within the limitations of animal models.

### 2.10. Data analysis

Statistical significance of differences between means was determined by either parametric or nonparametric analysis of variance followed by post hoc comparisons (Dunnett or Tukey tests) using GraphPad Software. Differences were considered to be significant if $P \leq 0.05$.

## 3. Results

### 3.1. CRMP2 is co-expressed with CaV2.2 and CGRP in trigeminal ganglia

N-type voltage-gated $Ca^{2+}$ channel activity has been shown to trigger CGRP release.[3,34] Because CaV2.2 activity is dependent on CRMP2 expression,[9,15] we determined if CRMP2 and CaV2.2 were expressed within the same regions of TGs. Costaining for CRMP2 and CaV2.2 was performed together with Neurotrace that marks neuronal soma. We detected CaV2.2 in neuronal soma within the ophthalmic (V1) and maxillary (V2) nerve branch regions of TG, but not in the neuronal projections (**Fig. 1A**); retrolabeling studies demonstrate that the V1 region is the target of dural afferents and thus likely involved in headache-related pain pathways.[39] All CaV2.2-positive neurons also expressed CRMP2 (**Fig. 1A**). The proximity between these proteins suggests the possibility of functional coupling that could underlie nociceptive signaling.

Because dissociation of the CRMP2/CaV2.2 interaction results in a loss of CaV2.2 activity and decreased CGRP release,[8] we determined if CGRP expression correlated with CRMP2 in TGs. Calcitonin gene related peptide expression was observed in the soma of some neurons within the V1 and V2 regions of the TGs (**Fig. 1B**); these neurons also expressed CRMP2 (**Fig. 1B**). Collectively, the immunohistochemistry observations support the idea that CaV2.2, CRMP2, and CGRP exist in bi- and tri-partite complexes, lending support to the hypothesis that they are functionally positioned to coordinate headache-related nociceptive signals.

### 3.2. Expression of Cdk5-phosphorylated CRMP2 is restricted to neuronal projections in trigeminal ganglia

As CRMP2 expression has not been previously investigated in TGs, we first characterized CRMP2 expression in this structure. Trigeminal ganglion slices were costained with antibodies against CRMP2 and βIII-tubulin, a neuronal marker. We observed neuronal expression of CRMP2, together with βIII-tubulin, in the V1 and V2 nerve branch regions of TG (**Fig. 2A**). Collapsin response mediator protein 2 was localized to both soma and axonal projections of TG neurons (**Fig. 2A**, *inset*), with high colocalization between the 2 proteins (**Fig. 2A**). Next, we investigated the expression of Cdk5-phosphorylated CRMP2 (ie, CRMP2-pS522) in TG. Similar to CRMP2, we noted neuronal expression for CRMP2-pS522 in the V1 and V2 regions of TG (**Fig. 2B**). However, almost no colocalization was observed between CRMP2-pS522 and tubulin (**Fig. 2B**, *inset*), consistent with findings that phosphorylated CRMP2 has decreased association with βIII-tubulin.[16] Cdk5-phosphorylated CRMP2 was absent from TG soma but present in the axonal projections (**Fig. 2B**, *inset*). Because CRMP2 phosphorylation by Cdk5 promotes biochemical association[10] and activity with CaV2.2,[10,50,51] these results support the idea that CRMP2-pS522 drives CaV2.2 channel into axonal projections.

### 3.3. (S)-lacosamide inhibits CRMP2 phosphorylation and depolarization-evoked $Ca^{2+}$ influx in trigeminal sensory neurons

Trigeminal ganglia were dissected and incubated with 200 μM (*S*)-LCM for 30 minutes at 37°C. Collapsin response mediator protein 2 levels were unchanged between lysates of TGs incubated with (*S*)-LCM compared with control TGs treated with water (**Fig. 3A, B**). In contrast, TGs treated with 200 μM (*S*)-LCM

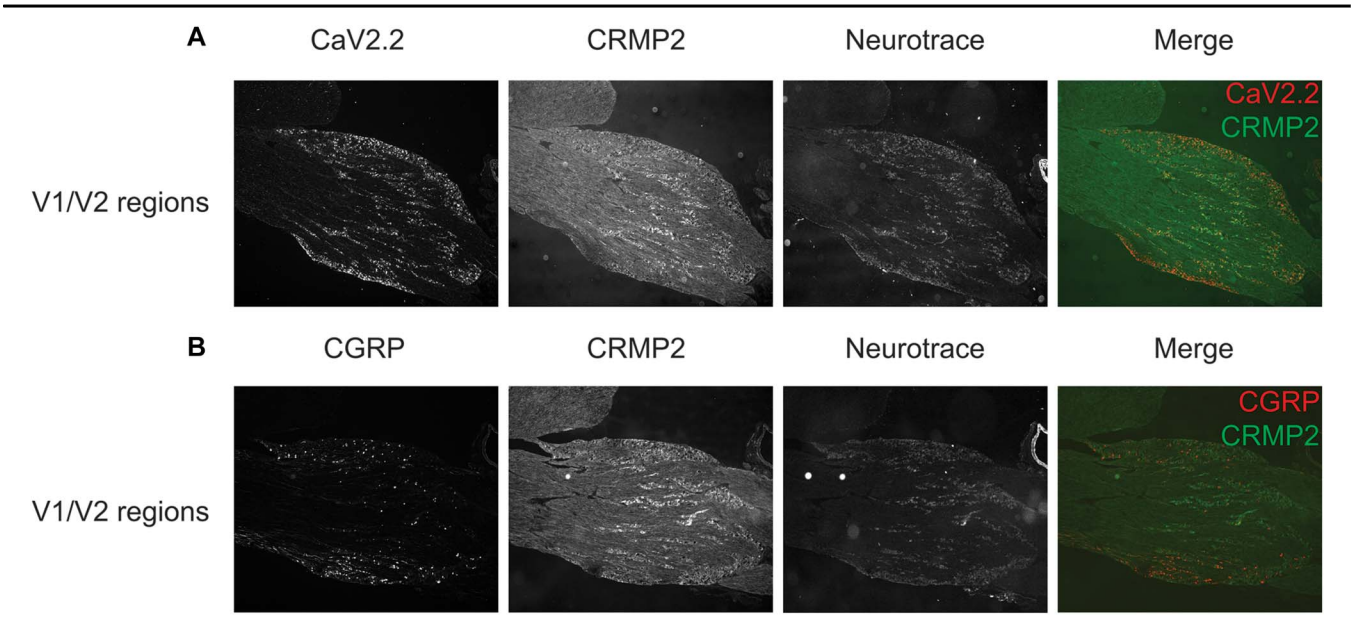


Figure 1. CaV2.2 and CGRP are co-expressed with CRMP2 in adult rat trigeminal ganglion (TG) neurons. (A) Double-immunofluorescent staining for CaV2.2 and CRMP2 in the TG. Neurotrace was used to mark neuronal somas. Merged images show the staining pattern surrounding the ophthalmic (V1) and maxillary (V2) nerve branch regions of the TG. CaV2.2 colocalizes with CGRP in the cytoplasmic regions of the TG neurons. (B) Micrographs of a 10-μm section of an adult TG double-immunostained with CGRP and CRMP2. CGRP is present in a few cells in the V1/V2 regions (inset) where it colocalizes with CRMP2 (merged panel). CaV2.2, N-type voltage-gated $Ca^{2+}$ channel; CGRP, calcitonin gene related peptide; CRMP2, collapsin response mediator protein 2.

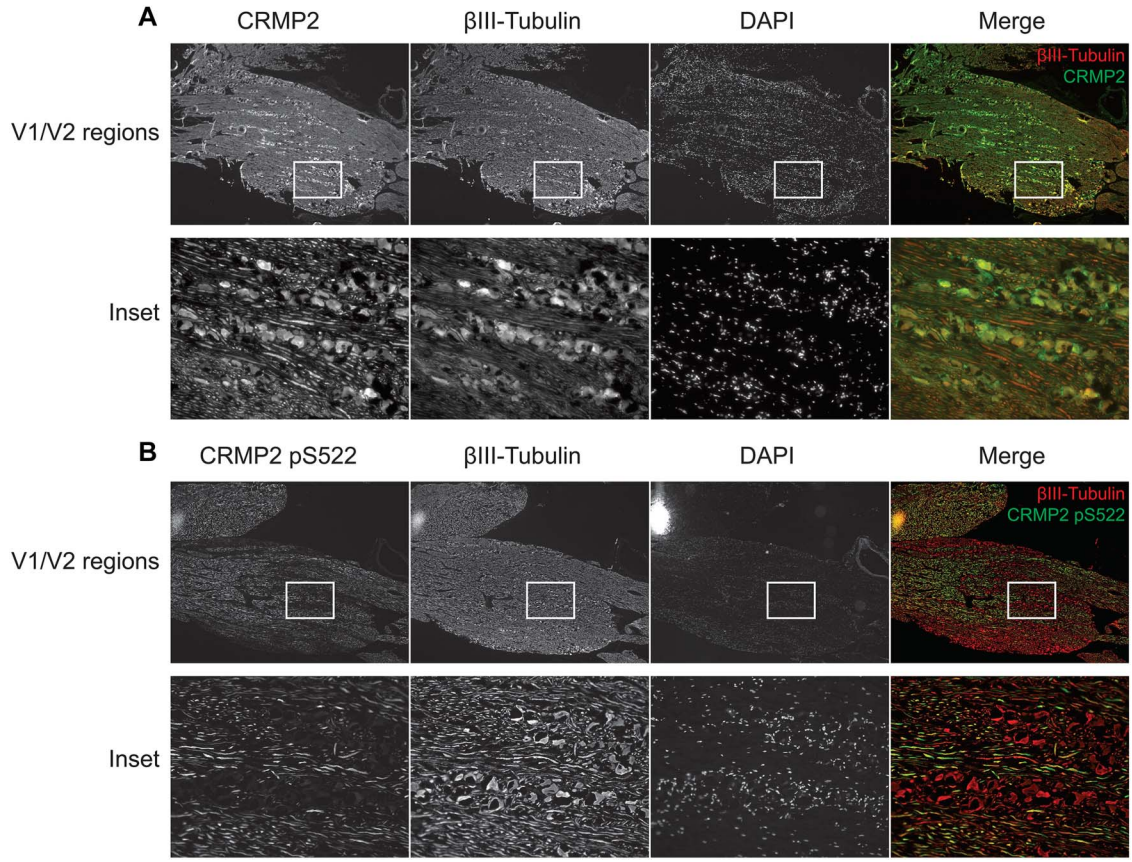


Figure 2. CRMP2 and phosphorylated CRMP2 expression in adult rat trigeminal ganglion (TG) neurons. (A) Micrographs of a 10-μm section of an adult TG double-immunostained with CRMP2 and βIII-tubulin (neuronal marker). A merged image (CRMP2/βIII-tubulin/DAPI [nuclear stain]) shows that many CRMP2-positive cells also express βIII-tubulin immunoreactivity. Higher magnification (inset) shows the staining pattern surrounding the ophthalmic (V1) and maxillary (V2) nerve branch regions of TG. CRMP2 colocalizes with βIII-tubulin in both somas and neuronal processes of TG neurons. (B) Micrographs of a 10-μm section of an adult TG double-immunostained with CRMP2 pS522 and βIII-tubulin. Phosphorylated CRMP2 staining is absent from the soma of TG neurons and instead appears predominantly in nerve fibers, where it colocalizes with βIII-tubulin (merge and inset). CaV2.2, N-type voltage-gated $Ca^{2+}$ channel; CGRP, calcitonin gene related peptide; CRMP2, collapsin response mediator protein 2; DAPI, 4',6-diamidino-2-phenylindole.

treatment had significantly reduced (by ~48%) levels of Cdk5-phosphorylated CRMP2 (pS522) compared with control TGs (**Fig. 3A, B**). Thus, (*S*)-LCM can be used to limit CRMP2 phosphorylation in TG sensory neurons.

Because CRMP2 phosphorylation by Cdk5 is important for CaV2.2 activity in cortical[10,51] and DRG neurons,[50] we tested if limiting CRMP2 phosphorylation levels using (*S*)-LCM could also inhibit $Ca^{2+}$ influx in TG neurons. Trigeminal ganglion primary neuron cultures were prepared and cells were loaded with Fura2-AM ratiometric dye before $Ca^{2+}$ imaging and stimulation with high 90 mM KCl (to recruit mostly CaV2 channels[72]). Neurons were stimulated for 15 seconds, which produced a transient increase of intracellular $Ca^{2+}$ concentration evidenced by an increase of the Fura2 fluorescence ratio ($F_{340}/F_{380}$) (**Fig. 3C**). Depolarization of TG neurons, incubated for 30 minutes with 200 μM (*S*)-LCM at 37°C, yielded a $K^+$-evoked $Ca^{2+}$ influx that was ~40% lower than in control TGs (**Fig. 3D**). Thus, (*S*)-LCM inhibits the activity of voltage-gated $Ca^{2+}$ channels by blocking CRMP2 phosphorylation by Cdk5, reinforcing the role of CRMP2 in nociceptive signaling.

### *3.4. Inhibition of capsaicin-evoked CGRP release from dural afferents by (S)-lacosamide in the cranial cup preparation*

Basal CGRP release was ~5.75 ± 0.68 pg/mL (**Fig. 4A**, fractions 1 and 2). Capsaicin (1 μM) evoked a robust release of CGRP from the dural afferents (**Fig. 4A**, fraction 3). Pretreatment and cotreatment of (*S*)-LCM (200 μM) significantly attenuated CGRP release by ~24% ($P < 0.05$ vs vehicle control; **Fig. 4A**, fraction 3); this level of inhibition of capsaicin-induced CGRP release is similar to that with inhibitors in DRGs in culture.[58] Thus, these findings lend support to the hypothesis that (*S*)-LCM inhibits CRMP2 phosphorylation and $Ca^{2+}$ influx, which converges to inhibit dural CGRP release.

### *3.5. Inhibition of inflammatory mediator–induced cutaneous allodynia by (S)-lacosamide*

Dural injection of an IM cocktail resulted in the development of periorbital and hind paw allodynia within an hour, with a peak effect at 2 to 3 hours after injection of the IM cocktail (**Fig. 5A**) and returning to baseline after 5 hours (**Fig. 5A, C**). Oral administration of (*S*)-LCM (30 mg/kg) significantly inhibited the development of cutaneous and hind paw allodynia induced by IM in rats when given 30 minutes after dural IM (10 μL) applied through a previously implanted cannula. The effect of (*S*)-lacosamide was significant 2 hours after IM administration and lasted for 2 to 3 hours (**Fig. 5A, C**). The area over the curve, indicative of the overall effect of (*S*)-LCM administration, for periorbital but not hind paw allodynia, was significantly decreased by (*S*)-LCM (**Fig. 5B, D**). Concurrent with results demonstrating inhibition of dural CGRP release, the behavioral results show that (*S*)-LCM prevents the development of cephalic pain.

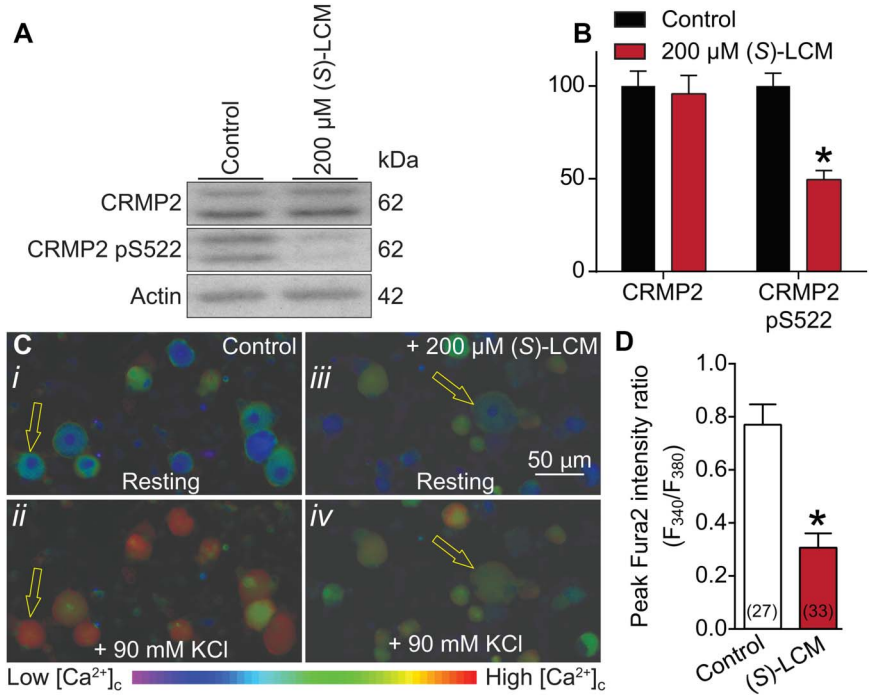


Figure 3. (*S*)-LCM inhibits CRMP2 phosphorylation and $Ca^{2+}$ influx in adult rat trigeminal ganglion (TG) neurons. (A) Representative Western blots of lysates prepared from TGs incubated for 30 minutes with vehicle (water) or (*S*)-LCM (200 μM), probed with the indicated CRMP2 and Cdk5 phosphorylated-CRMP2 (CRMP2-pS522) antibodies. The positions of molecular weight markers (kilodaltons, kDa) are indicated on the right. Actin is used as a loading control. (B) Summary of the mean relative levels of CRMP2 (normalized to actin) in arbitrary units (a.u.). (*S*)-LCM (200 μM) treatment did not significantly affect CRMP2 expression (n = 3 TGs per condition). Cdk5 phosphorylated-CRMP2 (pS522) (normalized to CRMP2 levels and actin) was significantly decreased by (*S*)-LCM treatment. (C) Pseudocolored fluorescent images of a field of TG neurons visualized for Fura-2AM, before (resting, *i* and *iii*) and after (90 mM KCl) stimulation for control (*ii*)- and 200 μM of (*S*)-LCM (*iv*)-treated trigeminal neurons. Following a 1-minute baseline measurement, neurons were stimulated with 90 mM KCl for 15 seconds and the response was measured for 3 additional minutes. Scale bar is 50 μm. Fluorescent scale shows the relative intracellular calcium concentration $[Ca^{2+}]_c$ in each neuron. (D) Bar graph showing the normalized peak fluorescence response (adjusted for background) of TGs incubated for 30 minutes with vehicle (water; control) or 200 μM of (*S*)-LCM. Values represent the average ± SEM, n = 27 to 33 cells per condition. Asterisks indicate statistical significance compared with control cells ($P < 0.05$, 1-way analysis of variance with Dunnett post hoc analysis). CaV2.2, N-type voltage-gated $Ca^{2+}$ channel; CGRP, calcitonin gene related peptide; CRMP2, collapsin response mediator protein 2; (*S*)-LCM, (*S*)-lacosamide.

## 4. Discussion

Migraine remains a significant unmet clinical need. Although the mechanisms that initiate migraine remain uncertain, activation of the trigeminovascular system is considered to be essential for migraine pain.[6,52] Trigeminovascular activation may provoke release of multiple excitatory neurotransmitters from dural afferent terminals including CGRP.[32,33,46] Activation and sensitization of thinly myelinated and unmyelinated nociceptive afferent fibers that innervate the dura can elicit pain.[6,52] Additionally, sensitization of the second-order neurons of the trigeminal *n. caudalis*[6,52] can occur resulting in enhanced nociceptive inputs to higher brain centers including the thalamus, hypothalamus, and cortical sites, collectively manifesting as migraine pain.[6,11,52,53] A majority of migraineurs experience CA during their migraine, suggesting sensitization of pain pathways.[13,19] Because perivascular stimulation of the dura results in referred cephalic pain,[32,73] animal models have used artificially applied inflammatory stimuli to the dura to activate and sensitize afferent fibers,[12,17,25] using CA as an outcome measure.

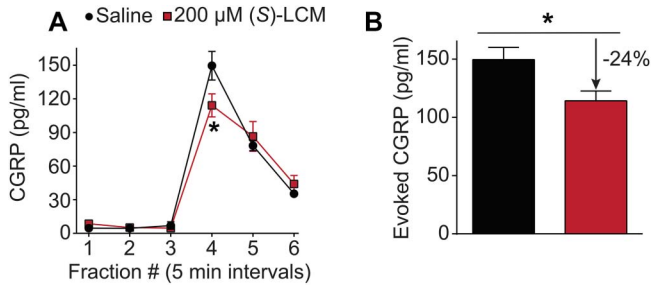


Figure 4. (*S*)-LCM inhibits CGRP release from dural afferents. (A) Cranial cup preparation from naive adult rats was used to assess capsaicin (1 μM)-induced CGRP release from dural nerve terminals. Capsaicin increased CGRP release from the dura, which was significantly attenuated by pre- and co-incubation of (*S*)-LCM (200 μM) with capsaicin (5 minutes/fraction). (*S*)-LCM alone did not have any effect on CGRP release (*$P < 0.05$ vs control; 2-way ANOVA post hoc Sidak test). (B) Bar graph shows that the peak evoked CGRP release for fraction #4 was decreased by 24% by (*S*)-LCM (200 μM) treatment compared with control (*$P < 0.05$ vs control, Student *t* test). ANOVA, analysis of variance; CGRP, calcitonin gene related peptide; (*S*)-LCM, (*S*)-lacosamide.

Calcitonin gene related peptide has been firmly established as a cardinal mediator of migraine. Blood levels of CGRP, but not of Substance P (SP), are elevated during migraine attacks.[35] The intravenous infusion of CGRP provokes migraine in migraineurs, but not in normal subjects.[55] Precipitating migraine in susceptible individuals by administration of nitroglycerin causes elevations in CGRP in the jugular venous blood.[29,40,69] It is likely that CGRP does not directly activate trigeminal dural afferents but potentiates the release of nociceptive agents into the perivascular space.[31,45] Clinical investigations have now demonstrated that small molecule CGRP receptor antagonists (eg, AMG334[67]) are also efficacious against migraine[37,54]; however, development of this therapeutic class remains uncertain because of safety issues.

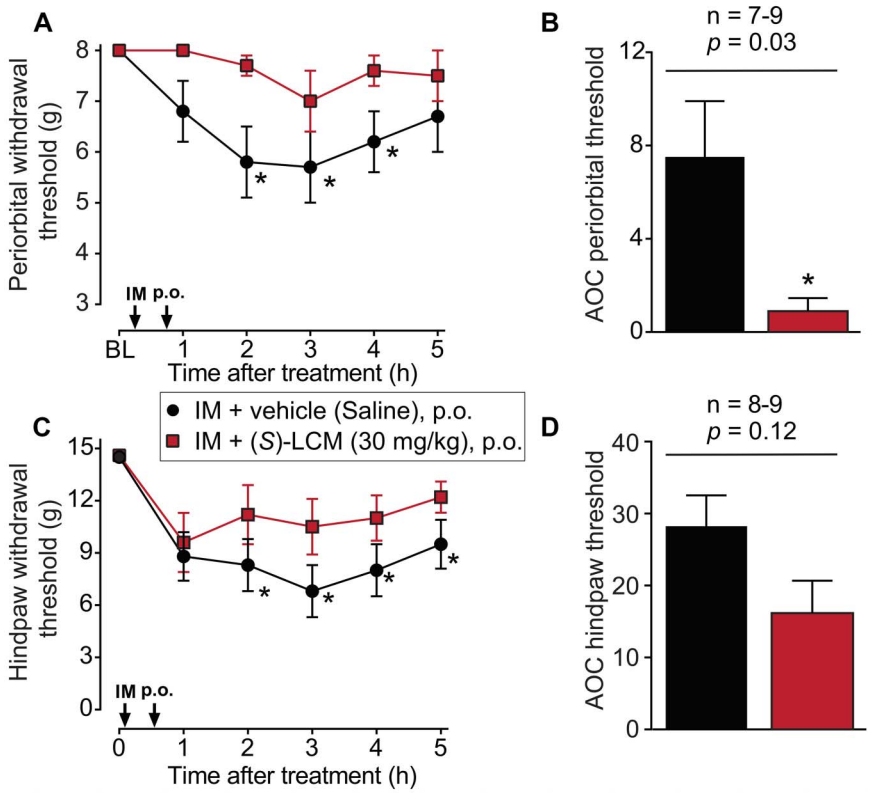


Figure 5. (*S*)-LCM decreases cutaneous allodynia (CA) elicited by dural application of inflammatory mediators (IMs). Synthetic interstitial fluid (SIF) or IMs were applied to the dura mater through a previously implanted cannula. Tactile threshold was assessed with von Frey filaments before and hourly after IM application for 5 hours. Rats exposed to dural IM developed periorbital (A) and hind paw allodynia (C) within 2 hours, which lasted for an additional 3 hours. (*S*)-LCM (30 mg/kg, p.o.) significantly inhibited CA (A) as well as hindpaw allodynia (C) when given 30 minutes after dural IM (*$P < 0.05$ vs control); 2-way ANOVA post hoc Sidak test. AOC using the trapezoid method for facial allodynia (B) and hind paw allodynia (D) showed a reduction of allodynia after (*S*)-LCM (30 mg/kg, p.o., given 30 minutes post IM-injection) treatment. AOC values were calculated between 1 and 5 hours. ANOVA, analysis of variance; AOC, Area over the time-response curve; (*S*)-LCM, (*S*)-lacosamide.

The precise site of action of small molecule CGRP antagonists remains to be confirmed, but studies using positron emission tomography imaging in humans showed poor penetration of telcagepant to the brain at therapeutically effective doses.[38] Calcitonin gene related peptide antibodies (eg, ALD403,[20] LY2951742,[21] and TEV48125)[7] are in development for migraine prophylaxis.[65] Notably, these antibodies are unlikely to gain significant access to the central nervous system suggesting the relevance of peripheral sites in the therapeutic effect. Collectively, these observations indicate that modulation of trigeminal dural afferents or their postsynaptic pathways, or both, can provide effective therapy for migraine.

Our results identify CRMP2 expression in both neuronal somas and projections, whereas phosphorylated CRMP2 was observed only in neuronal projections. Concomitant expression of CaV2.2, CRMP2, and CGRP was detected in a subset of neurons from the ophthalmic branch of TGs that receive input from the dura mater.[39] These results suggest a coordinated mechanism involving interactions between CRMP2 and CaV2.2 to facilitate the release of pro-nociceptive CGRP that consequently leads to cephalic pain. In this model, phosphorylated CRMP2 may play a role in localizing CaV2.2 to presynaptic terminals where the channel can participate in nociceptive transmission.

In a series of studies, we identified the axonal CRMP2 as a novel regulator of CaV2.2 activity; direct binding between CaV2.2 and CRMP2 leads to CRMP2-mediated increase in $Ca^{2+}$ current density and increased transmitter release in sensory neurons.[8] Because CRMP2 expression is upregulated in the zygomaticotemporal branch of the trigeminal nerve of migraineurs,[36] and CaV2.2 mediates a significant fraction of evoked release of CGRP,[74] it follows that targeting CRMP2 in cephalic pain is a rational strategy. To do so, we identified a cell-penetrating CRMP2-derived peptide (tat-CBD3) that disrupted the CaV2.2/CRMP2 interaction, preventing release of CGRP in the spinal cord. This peptide did not alter baseline sensory thresholds but was antihyperalgesic in models of persistent pain,[8] supporting CaV2.2-CRMP2 as a novel node for development of pain therapeutics. Importantly, this peptide did not show the side effects of ziconotide, suggesting that indirect modulation of CaV2.2 activity may be advantageous for increased safety. Notably, tat-CBD3 applied onto the rat dura mater before intranasal capsaicin, dose-dependently inhibited induced blood flow changes, confirming efficacy of CRMP2/CaV2.2 perturbation in dural neurogenic vasodilatation.[8]

To rapidly translate this concept to clinical use, we searched for molecules interacting with CRMP2 which were amenable to a rapid development path for abortive treatment of migraine. An additional criterion in our search was preferential targeting of CRMP2 to calcium, rather than, voltage-gated $Na^+$ channels. This search led to the discovery of (*S*)-LCM, an enantiomer of the clinically approved antiepileptic drug Vimpat. Vimpat is used clinically for adjunctive control of epileptic seizures.[5] Vimpat modulates slow inactivation of $Na^+$ channels[28,63] with an $IC_{50}$ of

80 μM.[71] Importantly, this activity profile of (*R*)-lacosamide ((*R*)-LCM) predominates over modulation of $Ca^{2+}$ function. Critically, we and others have demonstrated that at doses active in vivo for inhibition of seizures, (*R*)-LCM *does not* modulate $Ca^{2+}$ activity,[70] suggesting that modulation of CGRP release and efficacy in migraine treatment is unlikely. In contrast to (*R*)-LCM, we discovered that (*S*)-LCM has preferential activity on $Ca^{2+}$ channels through the modulation of CRMP2 phosphorylation.[51] Here, (*S*)-LCM, by reducing Cdk5-mediated CRMP2 phosphorylation, reduced CaV2.2 activity by ~48% in TG sensory neurons. That the observed decrease in TG neurons is less than that previously observed in DRG sensory neurons[50] could be attributed to a lesser contribution of CaV2.2 to the overall $Ca^{2+}$ influx in TG neurons. We previously reported that (*S*)-LCM specifically inhibits CaV2.2 in DRGs.[50] It is possible that other (ie, P/Q- or L-type[74]) voltage-gated calcium channels may contribute to the $Ca^{2+}$ influx in TGs. Nevertheless, CGRP release from TG neurons could be inhibited with ω-conotoxin, the CaV2.2 blocker, but not by blockers of other voltage-gated calcium channels.[2] Additionally, (*S*)-LCM significantly decreased capsaicin-evoked CGRP release from dural afferents to a degree similar to that observed in DRG cells, which is consonant with our previous finding of inhibition of capsaicin-triggered $Ca^{2+}$ influx by (*S*)-LCM,[50] thus validating the feasibility of indirect targeting CaV2.2, via CRMP2, as a novel means to curb CGRP release from the dura, a possible site of action relevant to migraine.

Pharmacokinetic data on (*R*)-LCM reported oral bioavailability together with brain penetration with a brain to plasma partition coefficient of 0.55.[43] (*R*)-lacosamide reaches a peak blood concentration after ~40 minutes with a half-life of ~3 hours.[43] Oral administration of (*S*)-LCM prevented the development of CA, which peaked at 2 hours after the IM injection. Cutaneous allodynia is observed in a great majority of patients during migraine and is likely the result of sensitization of central pain transmission pathways that can be engaged experimentally with IM application on the dura of rodents.[27] Concomitant expression of CaV2.2, CRMP2, and CGRP in the trigeminal system provides a likely mechanistic basis for the antinociceptive efficacy of (*S*)-LCM. Our studies did not determine the site of action of oral (*S*)-LCM which could reflect mechanisms within the brain, in the periphery or both.

The development of novel therapeutics for acute migraine therapy remains a high unmet clinical need and the small molecule (*S*)-LCM may serve this purpose. We have demonstrated that CRMP2 and its phosphorylation is a therapeutic target for headache and migraine pain (**Fig. 6**). We also characterized a novel axis of nociceptive signaling from the dura to the TGs consisting of CaV2.2 regulation by Cdk5 phosphorylated CRMP2 which leads to CGRP release in dural afferents. This pathway is relevant in ascending pain signaling from DRGs[50] or TGs to the brain. Additionally, the brain penetrance of (*S*)-LCM suggests possible additional actions at postsynaptic sites including the trigeminal nucleus caudalis and in higher order pain processing sites. Notably, (*S*)-LCM does not impair motor performance or elicit other observable side-effects in rodents.[50] If this molecule exhibits appropriate drug-like qualities, like its R-isomer, and if the safety profile can be confirmed, we expect that (*S*)-LCM may be suitable for clinical evaluation as an acute treatment for migraine.

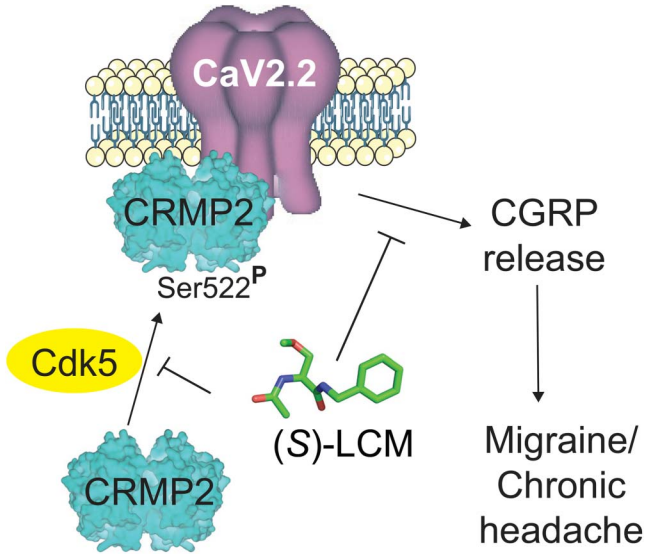


Figure 6. Signaling between CRMP2 and CaV2.2 controls CGRP release and cephalic pain. CRMP2 phosphorylation by Cdk5 is blocked by (*S*)-LCM leading to a reduction in calcium and consequently limiting CGRP release. By limiting CGRP release, which is increased in the jugular blood of migraineurs during an attack, we envision that (*S*)-LCM will be an effective abortive treatment for migraine and chronic headache. CaV2.2, N-type voltage-gated $Ca^{2+}$ channel; Cdk5, cyclin-dependent kinase 5; CGRP, calcitonin gene related peptide; CRMP2, collapsin response mediator protein 2; (*S*)-LCM, (*S*)-lacosamide.

## Conflict of interest statement

The authors have no conflicts of interest to declare.

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