AWARD NUMBER: **W81XWH-16-1-0533**

TITLE: Targeting the CRMP2-Ca2+ Channel Complex for Abortive Treatment of Migraine and Post-Traumatic Headache

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REPORT DATE: September 30, 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
SEPT 2018	Annual	1 SEP 2017 – 31 AUG 2018

4. TITLE AND SUBTITLE

Targeting the CRMP2-Ca2+ Channel Complex for Abortive Treatment of Migraine and Post-Traumatic Headache

5a. CONTRACT NUMBER
W81XWH-16-1-0533

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Migraine is one of the world's most common neurological disorders. Current acute migraine treatments have sub-optimal 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 due to 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 and post-traumatic headache. CRMP2 has been demonstrated to regulate N-type voltage gated Ca^{2+} channel (CaV2.2) activity and Ca^{2+}-dependent CGRP release in sensory neurons. The co-expression of CRMP2 with CaV2.2 and CGRP in trigeminal ganglia (TG) 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 anti-epileptic drug (*R*)-Lacosamide (Vimpat®), inhibited CRMP2 phosphorylation by cyclin dependent kinase 5 (Cdk5) in rat TG slices and decreased depolarization-evoked Ca^{2+} influx in TG cells in culture.We found that(*S*)-LCM significantly blocked capsaicin-evoked CGRP release from dural nerve terminals in the rat ex vivo cranial cup preparation. Additionally, cephalic and extracephalic cutaneous allodynia (CA) induced in rats by activation of dural nociceptors with a cocktail of inflammatory mediators (IM), was prevented and reversed by oral and intraperitoneal administrations demonstrating its high potential in mitigating migraine.

15. SUBJECT TERMS

S-Lacosamide, migraine, CRMP2, Cav2.2, allodynia, capsaicin-evoked CGRP release, cranial cup, inflammatory mediators, nitric oxide donor, sodium channels, calcium channels,

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	Unclassified	27	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION:**

Soldiers have a three-fold higher incidence of migraine than civilians. Post-traumatic headache (PTH) can occur following a traumatic brain injury (TBI) though the underlying mechanisms that are not understood. PTH often presents with a migraine phenotype and affects upwards of 97% of all soldiers with TBI. Current treatments are often inadequate. New treatments are urgently needed for migraine and PTH. CGRP has been linked to migraine pathophysiology. The release of CGRP from either the peripheral or central processes of neurons depends on activation of Ca^{2+} channels including the CaV2.2 (N-type) channel, which is critically modulated by collapsin response mediator protein 2 (CRMP2). We have recently found that (*S*)-lacosamide ((*S*)-LCM), an *enantiomer* of the clinically approved anti-epileptic drug (*R*)-LCM, or Vimpat®, has preferential activity on Ca^{2+} channels through the modulation of CRMP2 phosphorylation. The goal of this work is to determine if (*S*)-LCM is effective in preclinical models of migraine by inhibiting interactions of CRMP2 and N-type calcium channels providing a rationale for advancement to human trials for migraine and for PTH.

2. **KEYWORDS:**

(*S*)-Lacosamide, migraine, CRMP2, Cav2.2, allodynia, CGRP release, cranial cup, nitric oxide donor, voltage-gated sodium channels, voltage-gated calcium channels, excitability, constellation pharmacology

3. **ACCOMPLISHMENTS:**
   - **What were the major goals of the project?**

We hypothesized that (*S*)-LCM, through inhibition of Cdk5-mediated phosphorylation of CRMP2, inhibits CaV2.2 activity and consequently diminishes CGRP release. By limiting CGRP release, which is increased in the jugular blood of migraineurs during attack[32], (*S*)-LCM will be an effective abortive treatment for migraine and PTH. (*S*)-LCM will be tested *in vitro* for functional activity in blocking Ca^{2+} currents in cultured rat TG neurons identified to innervate the dura in *Aim 1*. The molecular signature of TGs as well as the selectivity-profile of (*S*)-LCM will also be determined in *Aim 1*. This will allow us to assess if (*S*)-LCM targets a particular class of neurons. The efficacy, and duration of action, of (*S*)-LCM in abolishing inflammatory mediator (IM)-induced cutaneous allodynia (CA) in rats will be determined in *Aim 2.1*. The effects of (*S*)-LCM on expression of nitric oxide (NO) donor- induced CA and CGRP levels from jugular blood will be tested in rats with sumatriptan-induced latent sensitization (injury-free medication overuse headache model) in *Aim 2.2*. *Aim 3.1* will assess cephalic pain, ongoing pain, and neurochemical changes in a repetitive mild traumatic brain injury (rmTBI) rodent model as well as efficacy of (*S*)-LCM on these output measures. Finally, we will evaluate neurobehavioral toxicity of (*S*)-LCM to determine its therapeutic index in *Aim 4*. All aims will be evaluated in both male and female rodents to detect potential gender differences.

The following aims has been proposed to be completed at the Khanna and Porreca Laboratories during the entire funding period:

- **Aim 1 (KHANNA lab): Efficacy and mechanism of (*S*)-LCM in blocking Ca^{2+} currents in cultured TG neurons innervating dura from rats.** In this Aim, we will confirm the mechanism of action of (S)-LCM by investigating ionic changes in TGs from vehicle or sumatriptan-treated rats and on TGs from mice subjected to sham injury or to repetitive mild TBI in order to explore changes that may be relevant to "migrainous" biology.
  - **Aim 1.1**. Assessing inhibition of Cdk5-mediated phosphorylation of CRMP2 in TGs that innervate the dura mater.

- **Aim 1.2**. Assess the inhibition of CRMP2-CaV2.2 association in these TG cells.
- **Aim 1.3**. Test the inhibition of Ca^{2+} currents by (S)-LCM in cultured identified dural TG neurons.
- **Aim 1.4**. Test the effects of (S)-LCM on excitability of TG neurons.
- **Aim 1.5**. Perform a phenotypic screening of TG neurons innervating the dura mater utilizing the "constellation pharmacology" paradigm

- **<u>Aim 2 (PORRECA Lab)</u>: Determine if (*S*)-LCM prevents periorbital and hindpaw cutaneous allodynia (CA) and blood CGRP elevation in rats.** In this aim, the *in vivo* efficacy of (*S*)-LCM will be evaluated in 2 cephalic pain models using 2 different routes at multiple times. CA and CGRP plasma levels will be used as output measures.
- Determine if (S)-LCM prevents periorbital and hindpaw cutaneous allodynia (CA) and blood CGRP elevation in rats. In this aim, the in vivo efficacy of (S)-LCM in inhibiting CA and CGRP plasma levels will be evaluated in 2 cephalic pain models using two different routes at multiple time points in male and female rats.
  - **Aim 2.1**. Does (S)-LCM abolish the development of CA induced by IM in rats?
  - **Aim 2.2**. Determine if (S)-LCM prevents nitric oxide (NO) donor-induced CA and blood CGRP elevation in rats with triptan-induced latent sensitization.
- **<u>Aim 3 (PORRECA Lab)</u>: Assessing cephalic pain and efficacy of (S)-LCM in a repetitive mild traumatic brain injury (rmTBI) rodent model.** The goal of this aim is to characterize the presence and neurochemical characteristics of PTH following rmTBI in mice as a model of PTH and to evaluate efficacy of (S)-LCM to reverse CA.
  - In **Aim 3.1** Assess the efficacy of (*S*)-LCM in abolishing TBI-induced allodynia in mice.
  - **Aim 3.2** will assess the possible presence of ongoing headache in mice with rmTBI. Establish CPP with iv ketorolac in TBI mice and then test the effect of (*S*)-LCM on blocking ketorolac-induced CPP.
  - **Aim 3.3** will determine the consequences of rmTBI on the function of nerves innervating the dura mater using the cranial cup preparation. Detect the release of CGRP by dural afferents in TBI mice and determine the effect of (*S*)-LCM.

- **<u>Aim 4 (KHANNA lab)</u>: Does (*S*)-LCM have significant adverse effects at therapeutic doses for treatment of cephalic pain and PTH?**
  - In **Aim 4**, we will assess the potential liabilities of (S)-LCM preclinically as a step in advancement to humans using a battery of widely accepted rodent models.

- **What was accomplished under these goals?**
  1) Major activities: During this funding period, we have performed experiments proposed for Aim 2.
  2) Objectives: Our objectives during this period were to determine if (*S*)-LCM prevents (pre-treatment) and alters (post-treatment) IM or NO donor-induced cutaneous allodynia in migraine animal models, and determine if (*S*)-LCM regulates plasma CGRP level in the animal models likewise.
  3) Significant results: We have demonstrated that pre-treatments of (S)-LCM (30 mg/kg, i.p. or p.o.) at 30 mins prior-IM blocked IM-induced cutaneous periorbital and hindpaw allodynia, while post-treatment at 30 mins or 2 hours post-IM did not. Experiments with the NO donor did not allow a conclusion of whether post-treatment of (*S*)-LCM would affect cutaneous periorbital and hindpaw allodynia in MOH models or increased plasma CGRP levels.

Methods for **Aim 1**: Methods for **Aim 1**:

Animals: Pathogen-free, adult male Sprague–Dawley rats (150–200 g; Harlan Laboratories) were housed in temperature (23±3 °C) and light (12-h light/12-h 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 three per cage but individually housed after the dural cannulation on a 12 h 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.

Western blotting: For examining the effect of (S)-LCM on CRMP2 phosphorylation state, TGs were dissected from adult rats and treated for 30 min at 37°C with 200 µM (S)-LCM diluted in Dulbecco's modified essential media (DMEM; Cat#11965, Life technologies). Next, tissues were lysed by sonication in RIPA buffer (50mM Tris-HCl, pH 7.4, 50mM NaCl, 2mM MgCl2, 1% [vol/vol] NP40, 0.5% [mass/vol] sodium deoxycholate, and 0.1% [mass/vol] sodium dodecyl sulfate and protease (Cat#B14002; Biotool) and phosphatase inhibitors (Cat#B15002, Biotool), and BitNuclease (Cat#B16002, Biotool)). Protein concentrations were determined using the BCA protein assay (Cat#PI23225, Thermo Fisher Scientific). Approximately 5µg 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), 5% non-fat dry milk. Primary antibodies used for probing were CRMP2 (Cat#C2993, Sigma, St Louis, MO), CRMP2 pSer522 (Cat#CP2191, ECM Biosciences), and Actin (Cat#A2066, Sigma, St Louis, MO) and were diluted in TBST with 5% bovine serum albumin. Immunoblots were revealed by enhanced luminescence (Cat#WBKLS0500, Millipore) 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).

Primary trigeminal ganglia (TG) neuronal cultures. Sensory TG neurons from SD rats were excised aseptically and placed in Hank buffered salt solution (HBSS, 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 min incubation (37°C) in a DMEM solution containing neutral protease (3.125 $mg.ml^{-1}$, Cat#LS02104, Worthington) and collagenase Type I (5 $mg.ml^{-1}$, Cat#LS004194, Worthington). The dissociated cells were resuspended in complete TG medium (i.e., DMEM containing penicillin (100 U/mL), streptomycin (100 µg/mL), 30 $ng.ml^{-1}$ nerve growth factor, and 10% fetal bovine serum (Hyclone)). 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) as a drop of 20 µl on the center of each coverslip, then placed in a 37°C, 5 % CO_2 incubator for 45–60 min 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

Calcium imaging: DRG neurons were loaded at 37°C with 3µM Fura-2AM (Cat#F-1221, Life technologies, stock solution prepared at 1mM in DMSO, 0.02% pluronic acid, Cat#P-3000MP, Life technologies) for 30 minutes (K_d= 25µM, λ_{ex} 340, 380 nm/λ_{em} 512 nm) to follow changes in intracellular calcium ($[Ca^{2+}]_i$) in Tyrode's solution (at ~310 mOsm) containing 119 mM NaCl, 2.5mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 25mM HEPES, pH 7.4 and 30mM glucose. The solution was supplemented with 500nM tetrodotoxin (TTX, voltage-gated Na^+ channel inhibitor) and 1 µM nifedipine (L-type voltage-gated Ca^{2+} channel inhibitor). Incubation with either (*R*)-LCM (200 µM) or (*S*)-LCM (200 µM, 20 µM, 2 µM, 200 nM, 20 nM, or 2 nM) was done during the loading of the cells with Fura-2AM and the drugs were also added to the excitatory solution. All calcium-imaging experiments were done at room temperature (~23°C), except those involving bath applications of innocuous (17°C) or cold (4°C) temperature stimuli. To isolate the contributions of particular channel subtypes, we used the following subunit-selective blockers (all purchased from Alomone Labs, Jerusalem): Nifedipine (10 μM, L-type); ω-agatoxin GIVA (200 nM, P/Q-type) [4]; ω-conotoxin-GVIA (500 nM, N-type) [2]; SNX-482 (200 nM, R-type) [5]; and 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2, 1 μM, T-type) [1]. Baseline was acquired for 1 minute followed by stimulation (15 sec) with an excitatory

solution (at ~310 mOsm) comprised of 32mM NaCl, 90mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 25mM HEPES, pH 7.4 and 30mM glucose. Fluorescence imaging was performed with an inverted microscope, Nikon Eclipse T*i*-U (Nikon Instruments Inc.), using objective Nikon Super Fluor MTB FLUOR 10x 0.50 and a Photometrics cooled CCD camera CoolSNAP ES^2 (Roper Scientific) controlled by NIS Elements software (version 4.20, Nikon instruments). The excitation light was delivered by a Lambda-LS system (Sutter Instruments). The excitation filters (340±5 nm 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.

Whole-cell voltage-clamp electrophysiology: Recordings were obtained from acutely dissociated DRG neurons as described. To isolate calcium currents, Na^+ and K^+ currents were blocked with 500 nM tetrodotoxin (TTX; Alomone Laboratories) and 30 mM tetraethylammonium chloride (TEA-Cl; Sigma). Extracellular recording solution (at ~310 mOsm) consisted of the following (in mM): 110 *N*-methyl-D-glucamine (NMDG), 10 $BaCl_2$, 30 TEA-Cl, 10 HEPES, 10 glucose, pH at 7.4, 0.001 TTX, 0.01 nifedipine. The intracellular recording solution (at ~310 mOsm) consisted of the following (in mM): contained 150 CsCl2, 10 HEPES, 5 Mg-ATP, 5 BAPTA, pH at 7.4. Fire-polished recording pipettes, 2 to 5 MV resistances were used for all recordings. Whole-cell recordings were obtained with a HEKA EPC-10 USB (HEKA Instruments Inc.); data were acquired with a Patchmaster (HEKA) and analyzed with a Fitmaster (HEKA). Capacitive artifacts were fully compensated, and series resistance was compensated by ~70%. Recordings made from cells with greater than a 5 mV shift in series resistance compensation error were excluded from analysis. All experiments were performed at room temperature (~23°C).

Constellation pharmacology: DRG neurons were loaded at 37°C with 3μM Fura-2AM for 30 minutes in Tyrode's solution. After a 1 minute baseline measurement Ca^{2+} influx was stimulated by the addition of the following receptor agonists: 400 nM menthol, 50 μM histamine, 10 μM adenosine triphosphate (ATP), 200 μM allyl isothiocyanate (AITC), 1 mM acetylcholine (Ach), 100 nM capsaicin diluted in Tyrode's solution (without TTX or Nifedipine). At the end of the constellation pharmacology protocol, cell viability was assessed by depolarization-induced Ca^{2+} influx using and an excitatory KCl solution comprised of 32mM NaCl, 90mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 25mM HEPES, pH 7.4, 30mM glucose. After the 1-minute baseline measurement, each trigger was applied for 15-seconds in the order indicated above in 6-minute intervals. Following each trigger, bath solution was continuously perfused over the cells to wash off excess of the trigger. This process was automated using the software WinTask x64 (Version 5.1, WinTask) that controlled the perfusion of the standard bath solution and triggers through Valvelink 8.2 software (Automate Scientific). For the (*S*)-LCM condition, 10 μM (*S*)-LCM was added to the Tyrode's solution during the loading with Fura-2AM, as well as to each of the solutions containing a trigger. Fluorescence imaging was performed under the same conditions noted above for calcium imaging. A cell was defined as a 'responder' if its fluorescence ratio of 340nm/380nm was greater than 10% of the baseline value calculated using the average fluorescence in the 30 seconds preceding application of the trigger.

Results for Aim 1

Aim 1.1: Treatment of trigeminal ganglia with 200 µM of (S)-LCM inhibited CRMP2 phosphorylation (Figure 1).

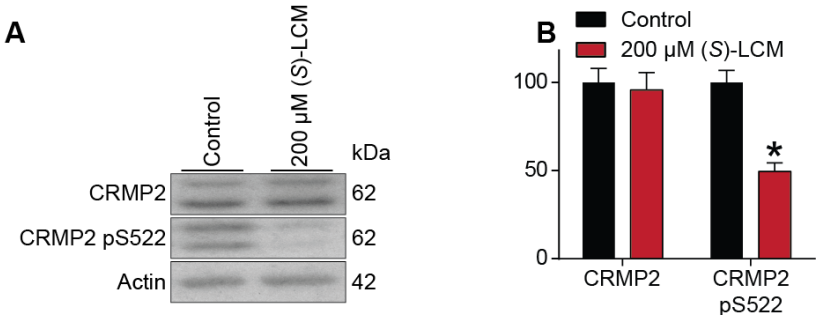


Figure 1: (*S*)-LCM inhibits CRMP2 phosphorylation in adult rat trigeminal ganglion (TG) neurons. (**A**) Representative Western blots of lysates prepared from TGs incubated for 30 min with vehicle (water) or (*S*)-LCM (200 µM), probed with the indicated CRMP2 and CRMP2 pS522 antibodies. The positions 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 TG per condition). Cdk5 phosphorylated-CRMP2 (pS522) (normalized to CRMP2 levels and actin) was significantly decreased by (*S*)-LCM treatment. Asterisks indicate statistical significance compared with control (*p<0.05, Mann-whitney test).

Aim 1.3: Treatment of trigeminal ganglia with 200 µM of (S)-LCM inhibited depolarization-evoked Ca^{2+} influx in trigeminal sensory neurons (Figure 2).

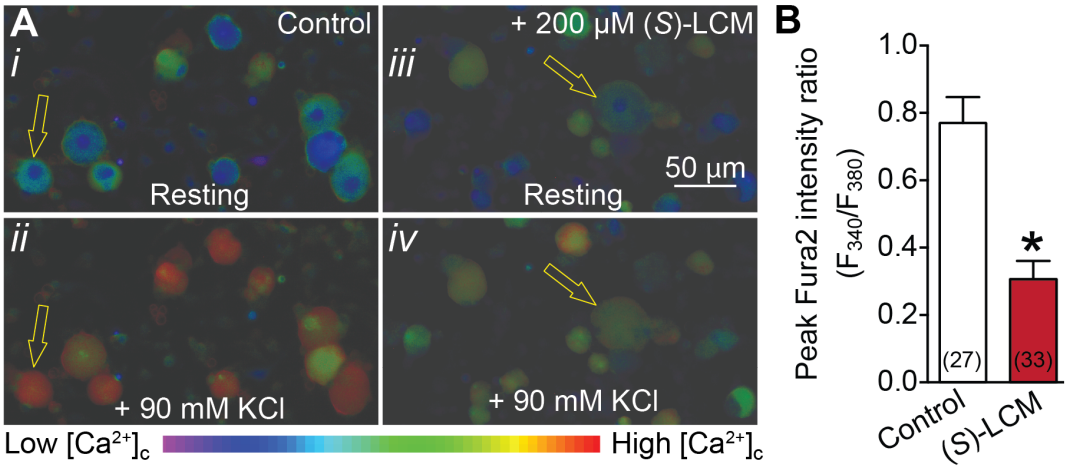


Figure 2: (*S*)-LCM inhibits Ca^{2+} influx in adult rat trigeminal ganglion (TG) neurons. (**A**) Pseudocolored fluorescent images of a field of TG neurons visualized for Fura-2AM, before (resting, *i* and *iii*) and after (90 mM KCl) stimulation for control (*ii*)- and 200 µM of (*S*)-LCM (*iv*)-treated trigeminal neurons. Following a 1-min baseline measurement, neurons were stimulated with 90mM KCl for 15 s and the response measured for 3 additional minutes. Scale bar is 50 µm. Fluorescent scale shows the relative intracellular calcium concentration $[Ca^{2+}]_c$ in each neuron. (**B**) Bar graph showing the normalized peak fluorescence response (adjusted for background) of TGs incubated for 30 min with vehicle (water; control) or 200 µM of (*S*)-LCM. Values represent the average ± SEM, n=27-33 cells per condition. Asterisks indicate statistical significance compared with control cells (p<0.05, 1-way analysis of variance with Dunnett's post hoc analysis).

Aim 1.4: Treatment of trigeminal ganglia with 200 µM of (S)-LCM inhibited depolarization-evoked Ca^{2+} influx in trigeminal sensory neurons (Figure 3)

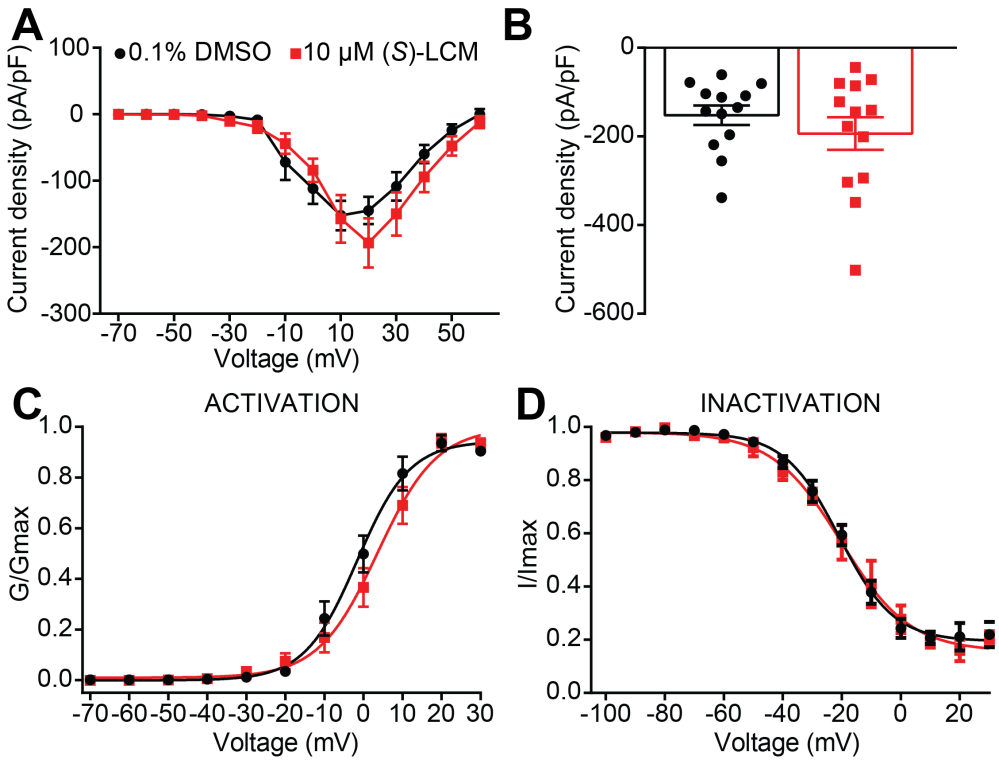


Figure 3: Low concentration of (*S*)-LCM has no effect on Ca^{2+} currents in adult rat trigeminal ganglion (TG) neurons. (**A**) Mean IV curve of current density normalized to the cell capacitance (pA/pF) for each voltage step tested in adult TG neurons treated with 10 µM (S)-LCM or vehicle. (**B**) Bar graph with scatter plot of peak Ca^{2+} current density at 10mV in adult TG neurons treated with 10 µM (S)-LCM or vehicle. (**C**) Activation and (**D**) inactivation properties of the recorded Ca^{2+} currents for the indicated conditions in adult TG neurons. In all conditions no difference was found between vehicle and 10 µM (S)-LCM treated cells (Mann-Whitney test). Asterisks indicate statistical significance compared with control cells ($p<0.05$, 1-way analysis of variance with Dunnett's post hoc analysis).

Aim 1.5: Functional fingerprinting of TG neuron populations treated with 10 µM of (S)-LCM. (Figure 4-5)

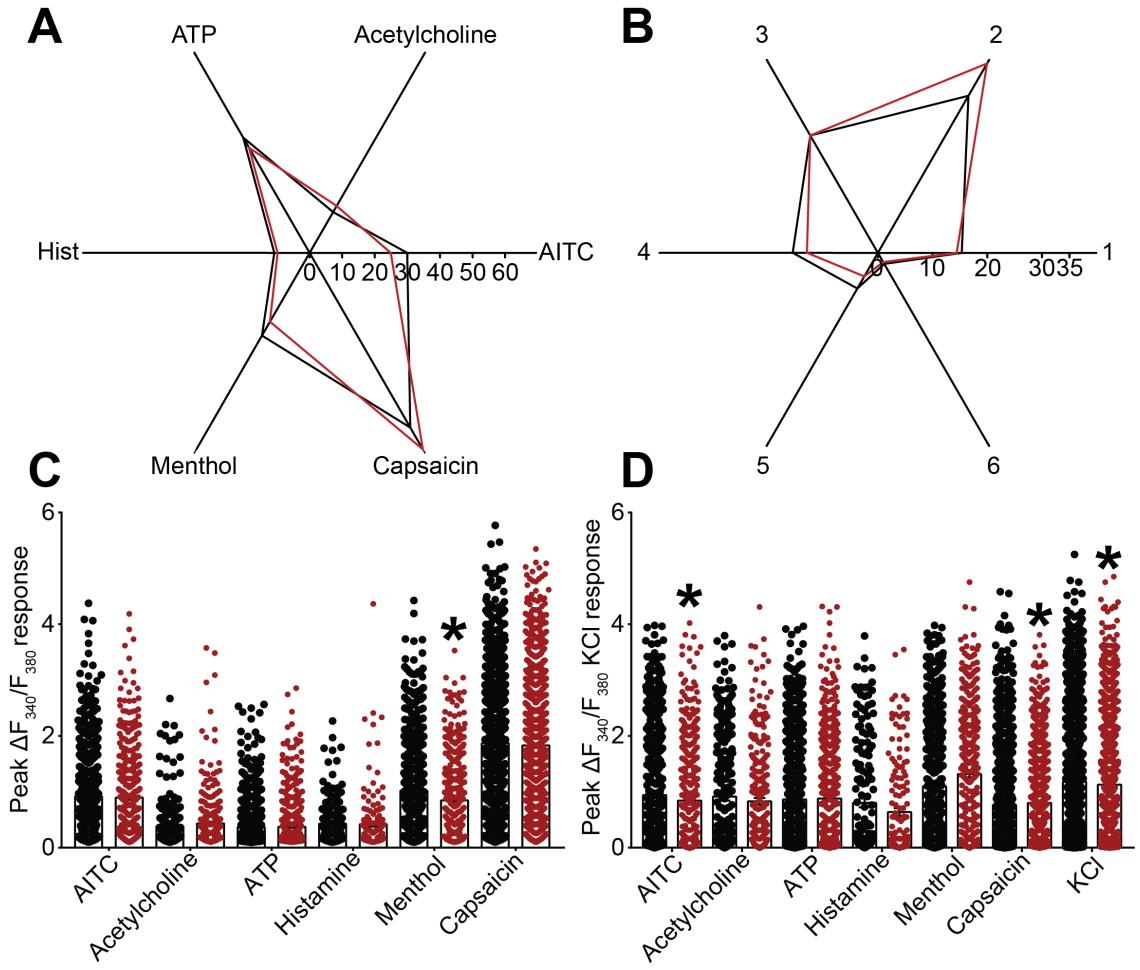


Figure 4: Constellation pharmacology reveals TG neuron sub-population targeting by (S)-LCM. (**A**) spider plot of the percentage of cells responding to the indicated receptor agonist used in this study. No significant change in functional overall TG neuron populations were found in cells treated with 10 µM of (S)-LCM (red lines) compared to cells treated with vehicle (black lines). (**B**) Spider plot of the percentage of TG neurons responding to the indicated number of receptor agonists. The number 1 indicated that the TG neuron responded to only depolarization. No significant change in functional overall TG neuron responsivity was found in cells treated with 10 µM of (S)-LCM (red lines) compared to cells treated with vehicle (black lines). (**C**) Bar graph with scatter plot of peak Ca^{2+} influx in TG neurons responding to the indicated trigger and treated with either vehicle or 10 µM of (S)-LCM. (S)-LCM treatment decreased the Ca^{2+} influx evoked by menthol. (**D**) Bar graph with scatter plot of the peak Ca^{2+} influx in response to depolarization with 90 mM KCl in neurons treated with either 10 µM of (S)-LCM or vehicle. Peak responses were classified based on the functional profile of each neurons. Treatment with 10 µM (S)-LCM inhibited depolarization evoked Ca^{2+} influx in neurons responding to capsaicin and AITC. Overall, (S)-LCM treatment decreased the depolarization evoked Ca^{2+} influx. Asterisks indicate statistical significance compared with control cells ($p<0.05$, 1-way analysis of variance with Dunnett's post hoc analysis). N=1586 cells in the vehicle group and n= 1589 cells on the 10 µM (S)-LCM group.

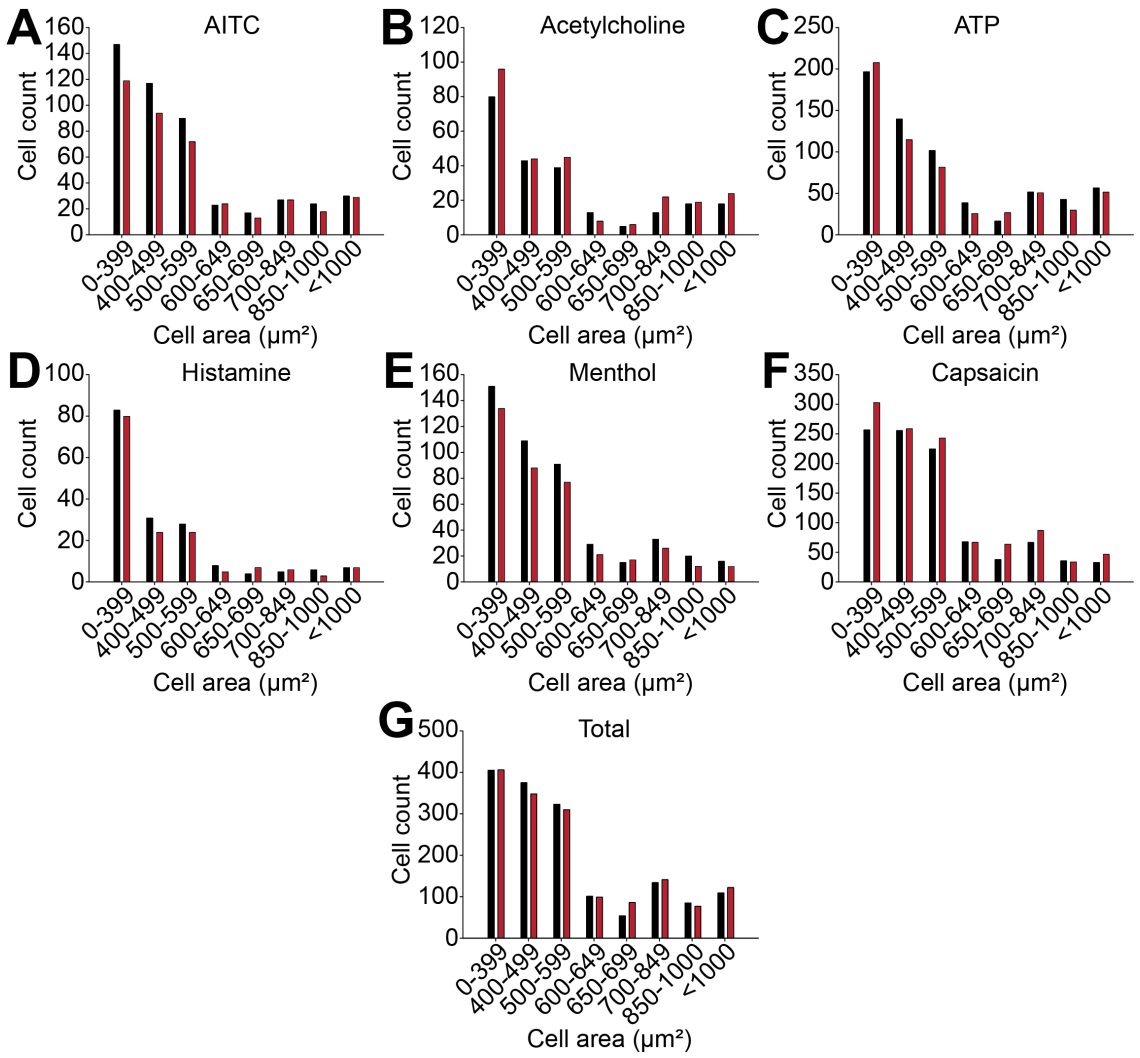


Figure 5. Size distribution of neurons responsive to specific constellation pharmacology triggers. Data for select neuronal classes is shown. For all panels, the x-axis represents the cell area (classified according to the indicated intervals) and the y-axis denotes the number of cells corresponding to the indicated trigger combination for each condition (0.002% DMSO in black or 10 µM (*S*)-LCM in red). Bar graphs of sizes of neurons representing (**A**) AITC, (**B**) Acetylcholine, (**C**) ATP, (**D**) Histamine, (**E**) Menthol or (**F**) Capsaicin responding cells. No significant change of the size of neurons responding to the indicated triggers, was found in 10 µM (S)-LCM treated compared to vehicle treated neurons. Size analysis of the entire population (**G**) is a control for the consistency of the TG neuron cultures used in the constellation pharmacology experiment and shows no difference between 10 µM (S)-LCM and vehicle treated TG neurons.

Methods and results for **Aim 2.1**: Male, Sprague Dawley rats (Harlan) weighing 200–300 g were implanted with a guide cannula sitting on the dural surface without penetrating dura. After 7 days' recovery, the animals were tested for mechanical withdrawal thresholds using von Frey (VF) filaments applied to the face (periorbital allodynia) and hindpaw: baselines. After the baseline measurement, the animals were received injections of a cocktail of 10 µL inflammatory mediator (IM, pH 5.0) onto the dura. Following the dural injections, periorbital and hindpaw withdrawal thresholds were measured beginning 1 hour after the injection up to 4 or 5 hours. The investigator blinded to the treatments.

Dural IM produced robust cephalic and extracephalic pain, and oral administration of (S)-LCM at 30 mg/kg dose blocked IM-induced cutaneous periorbital and hindpaw allodynia when given at 30 mins prior to IM. (Figure 6).

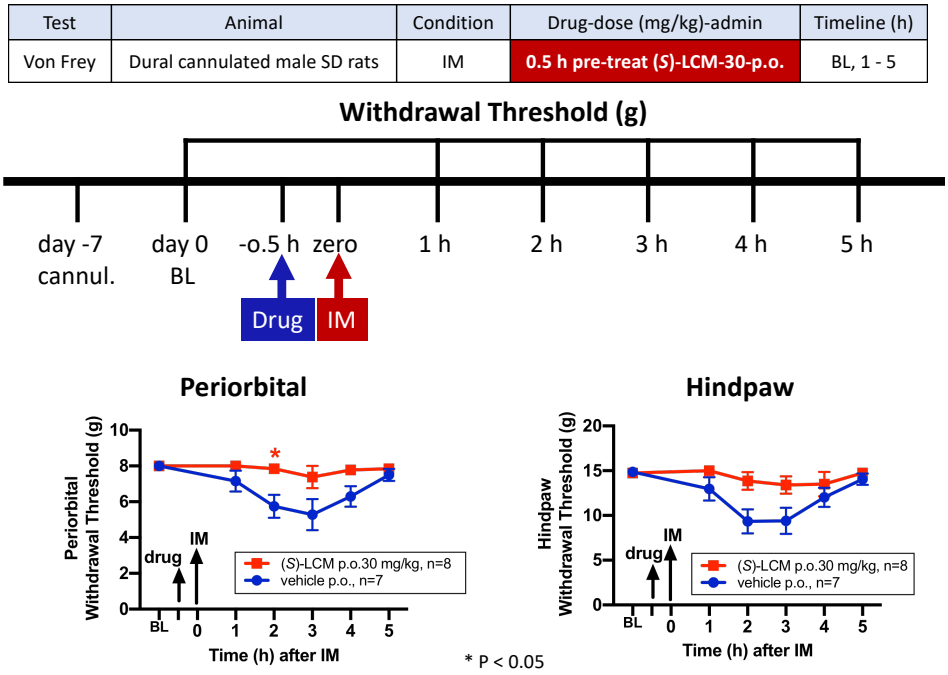


Figure 6. Blockade of IM-induced cephalic and extracephalic allodynia by oral (*S*)-LCM. *Experimental conditions & procedure*: SD rats were implanted with a guide cannular on the dural surface and after 7 days were baselined for periorbital and hindpaw tactile threshold (8 g for periorbital and 15 g for hindpaw measurements). Rats were administered (*S*)-LCM (30 mg/kg, p.o.) or vehicle (10% DMSO in water). An injection of IM onto the dural surface was done at 30 mins post-drug. The periorbital and hindpaw tactile thresholds were measured hourly for 5 hours following IM injection. *Experimental results*: (*S*)-LCM (30 mg/kg, p.o.) retained high periorbital and hindpaw withdrawal thresholds after IM injection, suggesting strong cephalic and extracephalic anti-allodynic effects of (*S*)-LCM. Group sizes are 7 and 8 shown on the graphs. Multiple t tests were used with significance level at $p < 0.05$.

Intraperitoneal administration of (S)-LCM at 30 mg/kg dose also blocked IM-induced cutaneous periorbital and hindpaw allodynia when given at 30 mins prior to IM. (Figure 7).

Test	Animal	Condition	Drug-dose (mg/kg)-admin	Timeline (h)
Von Frey	Dural cannulated male SD rats	IM	**0.5 h pre-treat (*S*)-LCM-30-i.p.**	BL, 1 - 5

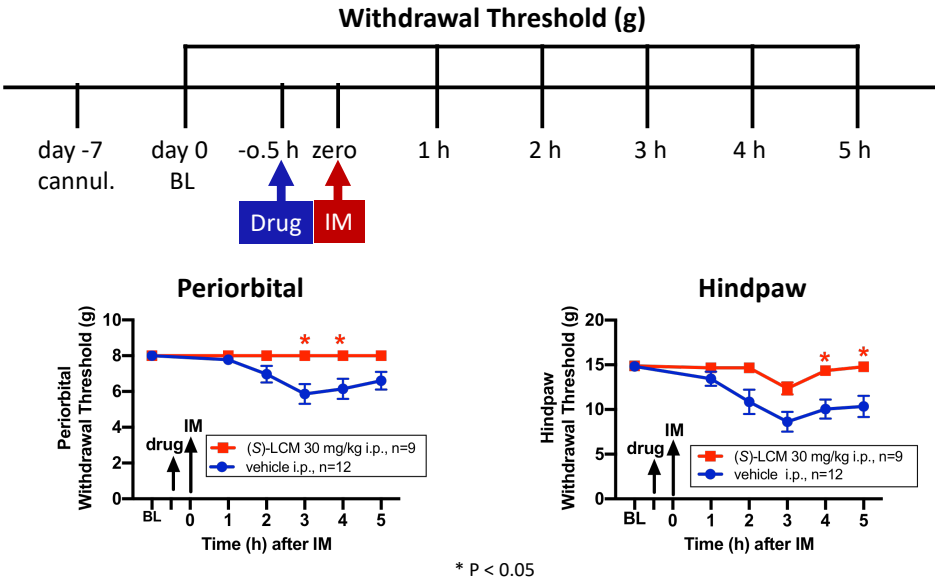


Figure 7. Blockade of IM-induced cephalic and extracephalic allodynia by intraperitoneal (*S*)-LCM. *Experimental conditions & procedure*: SD rats were implanted with a guide cannular on the dural surface and after 7 days were baselined for periorbital and hindpaw tactile threshold (8 g for periorbital and 15 g for hindpaw measurements). Rats were administered (*S*)-LCM (30 mg/kg, i.p.) or vehicle (10% DMSO in water). An injection of IM onto the dural surface was done at 30 mins post-drug. The periorbital and hindpaw tactile thresholds were measured hourly for 5 hours following IM injection. *Experimental results:* prior treatment of (*S*)-LCM (30 mg/kg, i.p.) retained high periorbital and hindpaw withdrawal thresholds after IM injection, suggesting strong cephalic and extracephalic anti-allodynic effects of (*S*)-LCM. Group sizes are 9 and 12 shown on the graphs. Multiple t tests were used with significance level at $p < 0.05$.

Oral administration of (S)-LCM (30 mg/kg) at 2 hours after IM did not alter IM-induced cutaneous hindpaw allodynia but reversed periorbital allodynia (Figure 8).

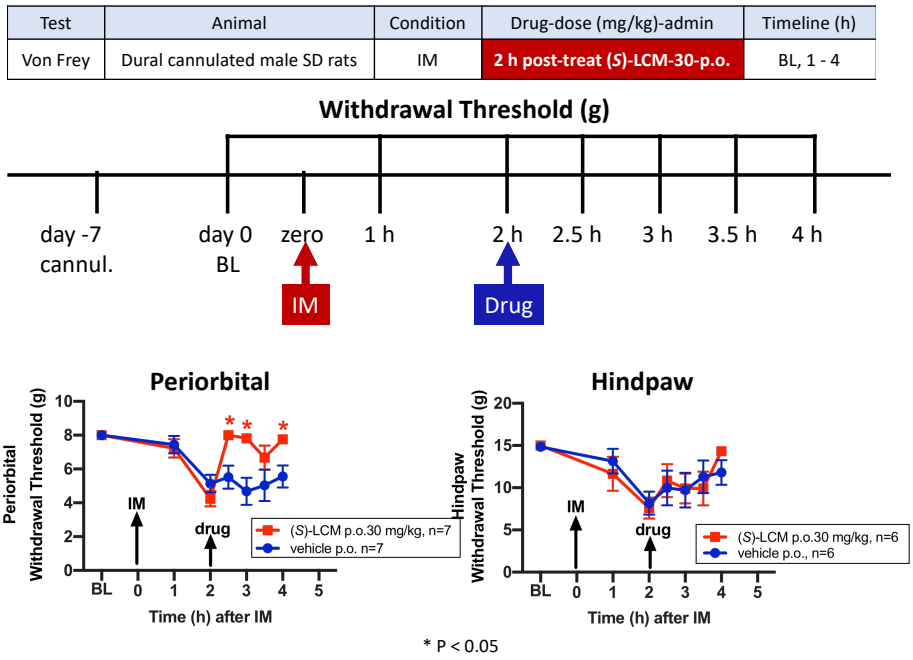


Figure 8. Reversal of IM-induced allodynia by oral (*S*)-LCM. *Experimental conditions & procedure*: SD rats were implanted with a guide cannular on the dural surface, after 7 days were baselined for periorbital and hindpaw tactile threshold (8 g for periorbital and 15 g for hindpaw measurements), and received an injection of IM onto the dural surface. The periorbital and hindpaw tactile thresholds were measured hourly for 2 hours, and (*S*)-LCM (30 mg/kg, p.o.) or vehicle (10% DMSO in water) were administered at 2 hours post-IM. The periorbital and hindpaw tactile thresholds were measured every 30 mins up to 4 hours (2 hours post (*S*)-LCM). *Experimental results*: (*S*)-LCM (30 mg/kg, p.o.) did not alter hindpaw allodynia (right), but reversed periorbital allodynia (left) induced by IM. This suggested the effective blockade of cephalic but not extracephalic allodynia by 30 mg/kg (*S*)-LCM. Group sizes are 6 and 7 shown on the graphs. Multiple t tests were used with significance level at $p < 0.05$.

Intraperitoneal administration of (S)-LCM (30 mg/kg) at 30 mins after IM did not alter IM-induced allodynia (Figure 9).

Test	Animal	Condition	Drug-dose (mg/kg)-admin	Timeline (h)
Von Frey	Dural cannulated male SD rats	IM	**0.5 h post-treat (*S*)-LCM-30-i.p.**	BL, 1 - 5

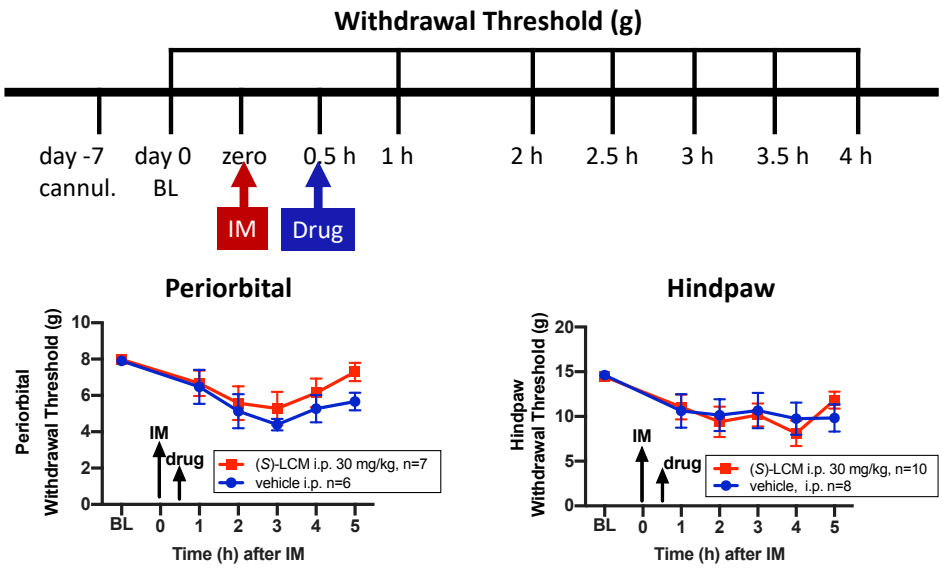


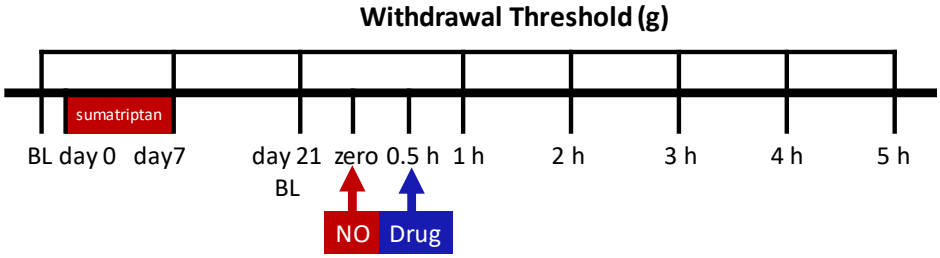
Figure 9. Effect of post-treatment with intraperitoneal (*S*)-LCM on IM-induced cephalic and extracephalic allodynia. *Experimental conditions & procedure*: SD rats were implanted with a guide cannular on the dural surface, after 7 days were baselined for periorbital and hindpaw tactile threshold (8 g for periorbital and 15 g for hindpaw measurements), and received an injection of IM onto the dural surface. (*S*)-LCM (30 mg/kg, i.p.) or vehicle (10% DMSO in water, i.p.) were administered at 30 mins post-IM. The periorbital and hindpaw tactile thresholds were measured hourly up to 5 hours. *Experimental results*: There was no significant alteration of cephalic and extracephalic allodynia observed by post-treated (*S*)-LCM at 30 mg/kg dose (i.p.). Group sizes are 6 to 10 shown on the graphs.

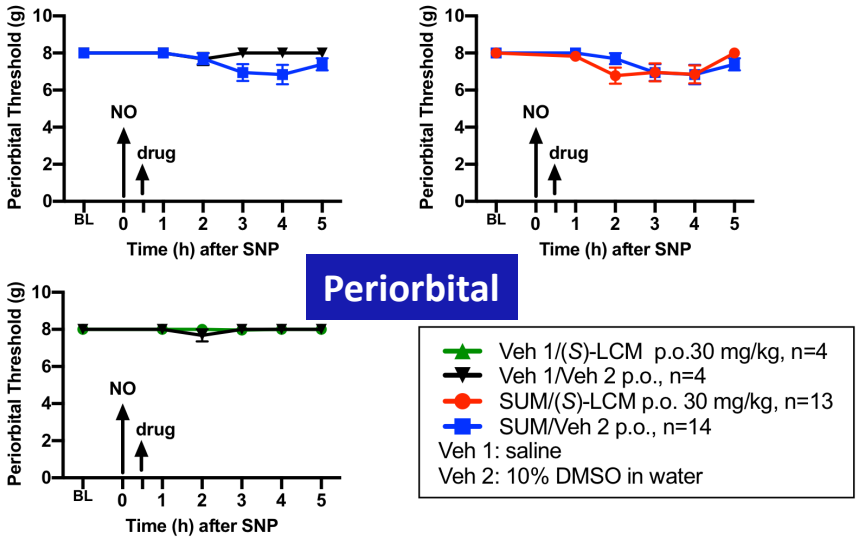
Methods and results for **Aim 2.2**: Female, Sprague Dawley rats were received s.c. infusions of sumatriptan (SUM, 0.6 mg/kg/day) or saline for 7 days by implanted minipumps, followed by a 14 day drug-free period. On day 21, rats were baselined for periorbital and hindpaw tactile thresholds and were injected sodium nitroprusside (SNP, 3 mg/kg, i.p.), a NO donor. At 30 mins post-NO, (*S*)-LCM (30 mg/kg) or 10% DMSO in water was injected orally, and periorbital and hindpaw withdrawal thresholds were measured beginning 1 hour after the NO injection up to 5 hours. The investigator was blinded to the treatments.

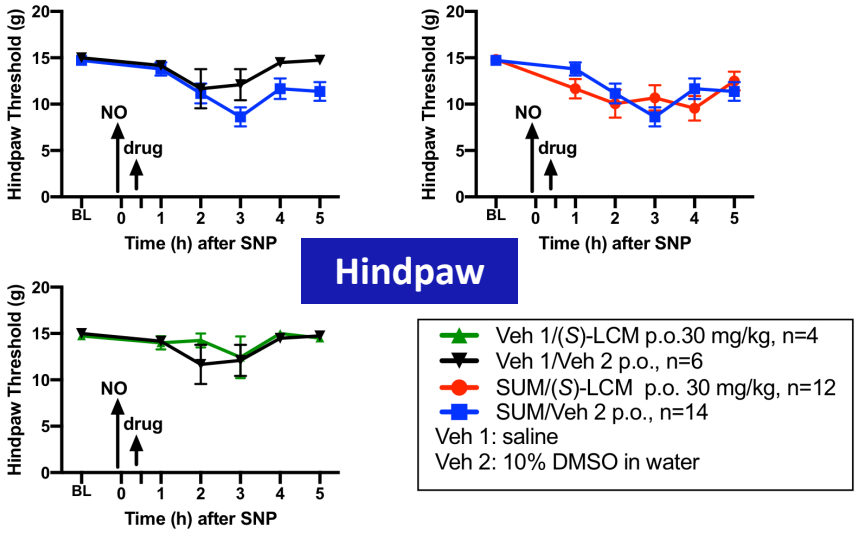
On day 21, following injection of SNP (3 mg/kg, i.p.) and blood was collected from jugular vein under isofluorane anesthesia at 0.5 or 1.5 h post-SNP, and was centrifuged to extract plasma. CGRP levels in the plasma was measured using a Cayman CGRP ELISA kit.

Following return of facial and hindpaw thresholds to baseline after the drug free period, the SNP NO-donor was administered and periorbital and hindpaw thresholds were evaluated. Unexpectedly, significant allodynia was not observed preventing a conclusion as to whether post-treatent with (S)-LCM at 30 mg/kg dose (p.o.) altered sensory thresholds. (Figure 10)

Test	Animal	Condition	Drug-dose (mg/kg)-admin	Timeline (h)
Von Frey	**Female SD rats**	**Sumatriptan-0.6(mg/kg/day) 7 days-s.c. SNP-3(mg/kg)-i.p.**	**0.5 h post-treat (*S*)-LCM-30-p.o.**	BL, 1 - 5







(see previous page) Figure 10. Effect of post-treated oral (*S*)-LCM on NO donor-induced cephalic and extracephalic allodynia in female rats with triptan-induced latent sensitization. *Experimental conditions & procedure*: Female SD rats were baselined for periorbital and hindpaw tactile threshold (8 g for periorbital and 15 g for hindpaw measurements), and received s.c. infusions of sumatriptan (0.6 mg/kg/day) or saline using a minipump for 7 days. On day 7, rats were baselined second time, followed by a 14 day drug-free period. On day 21, rats were received a NO-donor, sodium nitroprusside (SNP, 3 mg/kg, i.p.), followed by oral (*S*)-LCM (30 mg/kg) or vehicle (10% DMSO in water). The periorbital and hindpaw tactile thresholds were measured hourly for 5 hours post-NO. *Experimental results:* In this experiment, there was no significant alteration of cephalic and extracephalic allodynia observed following SNP and for this reason, the possible effect of post-treatment with (*S*)-LCM at 30 mg/kg dose (p.o.) could not be evaluated. Group sizes were 4 to 14 shown on the graphs.

NO-donor challenge significantly increased plasma CGRP levels in long-term sumatriptan exposed rats. (Figure 11)

Test	Animal	Condition	Drug-dose(mg/kg)-admin	Timeline (h)
ELISA	Female SD rats	Sumatriptan 3(mg/kg/day) 7 day s.c. - SNP 3 (mg/kg) i.p.	none	1.5

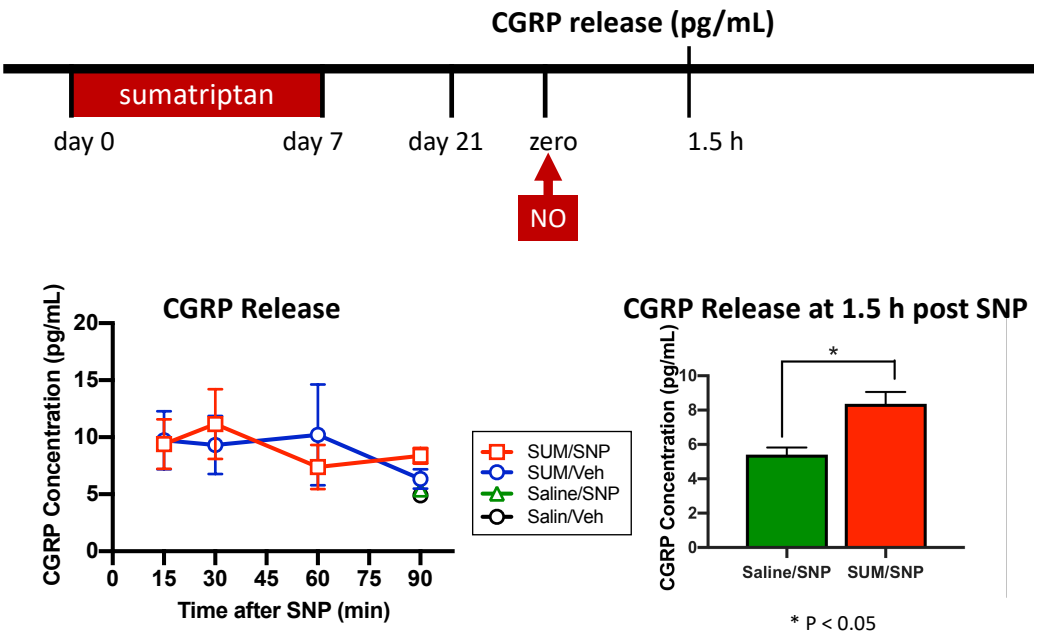


Figure 11. Effect of a NO donor on CGRP release in rats with triptan-induced latent sensitization. *Experimental conditions & procedure*: Female SD rats were received s.c. infusions of sumatriptan (0.6 mg/kg/day) or saline using a minipump for 7 days. On day 21 rats were received sodium nitroprusside (SNP, 3 mg/kg, i.p) or vehicle (10% DMSO in water). Blood was collected from jugular vein under isoflurane anesthesia at 15, 30, 60, 90 mins post-SNP, and was centrifuged to extract plasma. CGRP levels in the plasma was measured using a Cayman CGRP ELISA kit. Group sizes were 4 each. (left) Experimental procedure was repeated for CGRP release at 1.5 h post SNP using 5 rats (right) *Experimental results*: Time course collection did not show effect on CGRP release, but there is a significant effect of sumatriptan on NO donor-induced CGRP release observed in two groups*. Multiple t tests were done with significance level at $p < 0.05$.

There is no significant effect of (S)-LCM on plasma CGRP release observed. (Figure 12)

Test	Animal	Condition	Drug-dose(mg/kg)-admin	Timeline (h)
ELISA	Female SD rats	Sumatriptan 3(mg/kg/day) 7 day s.c. - SNP 3 (mg/kg) i.p.	0.5 h post-treat (*S*)-LCM-30-p.o.	1.5

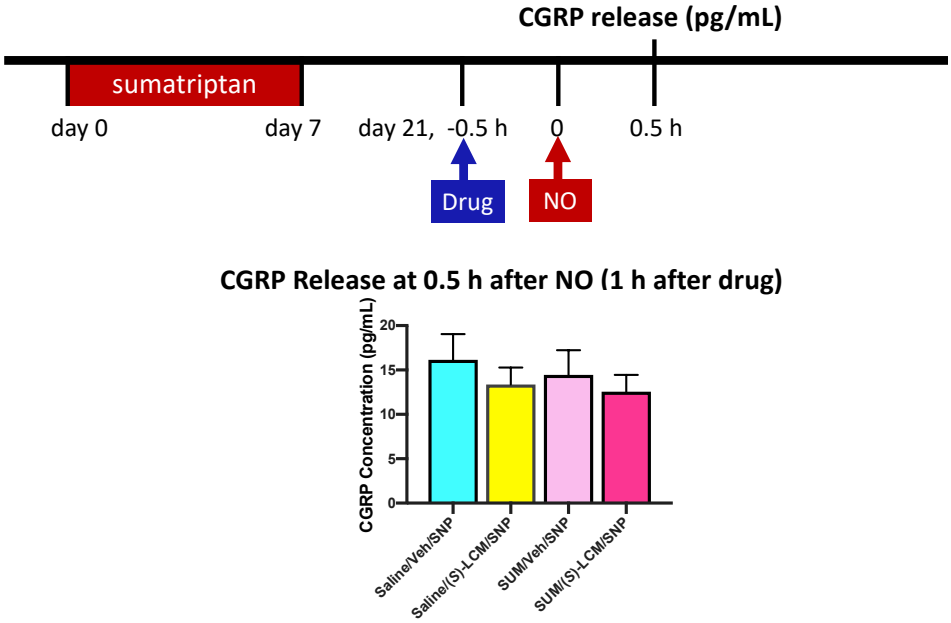


Figure 12. Effect of (*S*)-LCM on NO-donor induced CGRP release in rats with triptan-induced latent sensitization. *Experimental conditions & procedure*: Female SD rats were received s.c. infusions of sumatriptan (0.6 mg/kg/day) or saline using a minipump for 7 days. On day 21 rats were received (*S*)-LCM (30 mg/kg, p.o.) or vehicle (10% DMSO in water), followed by injection of SNP (3 mg/kg, i.p) at 30 mins post-drug. Blood was collected from jugular vein under isoflurane anesthesia at 30 mins post-SNP, and was centrifuged to extract plasma. CGRP levels in the plasma was measured using a Cayman CGRP ELISA kit. Group sizes were 6 each collect. *Experimental results*: CGRP blood levels in this experiment were higher than in the previous study. There was no significant effect on CGRP release in plasma observed in 4 different groups even with slight reductions between (*S*)-LCM groups and non-treated groups. As shown in the previous studies, drug treatment time might be a critical point for the CGRP release.

Methods and results for **Aim 3.1**: We have completed Aim 3.1 in year 1 using mild TBI mouse model and showed that minor deficits in motor coordination and locomotor hyperactivity recovered over time, but when the previously injured mice received bright light stress, the cephalic and extracephalic allodynia were precipitated again. (refer to the Year 1 Progress Report)

Methods and results for **Aim 4**: We have performed the rotarod tests in year 1 and confirmed that intrathecal administration of (*S*)-LCM at high dose (3 μg/5 μL) did not cause motor deficits or sedation. (refer to the Year 1 Progress Report)

Summary: This funding period has allowed us to meet many of our primary goals. We have not experienced technical problems, and our experiments are progressing in a timely fashion.

- **What opportunities for training and professional development has the project provided?**

  Nothing to report.

- **How were the results disseminated to communities of interest?**

  Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - The Khanna lab will continue on our goals for Aim
  - The Porrreca lab will continue on our goals for Aims 3.2, 3.3, and 4 in the next funding period.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
   - **What was the impact on the development of the principal discipline(s) of the project?**
     - Our previous work in year 1 showed the remarkable efficacy of (*S*)-LCM in mitigating cephalic and extracephalic allodynia in IM model and MOH latent sensitization model. We also characterized mild traumatic brain injury (mTBI)-induced cephalic and extracephalic allodynia as well as latent sensitization to bright light stress. One application of the mild TBI caused long-lasting sensitization of the animals to bright light stress even after the initial cephalic pain resolved suggesting relevance to mechanisms of post-traumatic headache.
     - We demonstrated that prior treatments of (*S*)-LCM via i.p. or p.o. were most effective in blocking IM-induced cephalic and extracephalic allodynia compared to post-treatment suggesting higher potential as a prophylactic therapy.
     - We demonstrated that high dose (S)-LCM can inhibit CRMP2 phosphorylation and calcium influx in TG neurons. The constellation pharmacology experiment shows the effect of (S)-LCM on select TG neuron populations.
   - **What was the impact on other disciplines?**

     Nothing to Report.

   - **What was the impact on technology transfer?**

     Nothing to Report.

- **What was the impact on society beyond science and technology?**

  Nothing to Report.

5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**
   - **Actual or anticipated problems or delays and actions or plans to resolve them**
   - **Changes that had a significant impact on expenditures**
   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
   - **Significant changes in use or care of human subjects**
   - **Significant changes in use or care of vertebrate animals.**
   - **Significant changes in use of biohazards and/or select agents**

   Nothing to Report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*
   - **Publications, conference papers, and presentations**
     - **Journal publications.**
     - **Books or other non-periodical, one-time publications.**
     - **Other publications, conference papers, and presentations.**

       Nothing to report.

   - **Website(s) or other Internet site(s)**

     Nothing to report.

   - **Technologies or techniques**

     Nothing to report.

   - **Inventions, patent applications, and/or licenses**

     Nothing to report.

   - **Other Products**

     Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Name:	**Frank Porreca**
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2

Contribution to Project:	Dr. Porreca advised the whole team and provided oversight for the entire project.
Funding Support:	NA
Name:	**Yeon Sun Lee**
Project Role:	Senior Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Lee designed the study, coordinated the work flow, analyzed the data and wrote the report.
Funding Support:	NA
Name:	**Nathan Eyde**
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	8
Contribution to Project:	Mr. Eyde executed the experiments, and collected the behavioral data.
Funding Support:	NA
Name:	**Chaoling Qu**
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	8
Contribution to Project:	Ms. Qu executed the experiments, and collected the behavioral data.
Funding Support:	NA
Name:	**Rajesh Khanna**
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-9066-2969
Nearest person month worked:	2

Contribution to Project:	Dr. Khanna advised the whole team and provided oversight for the entire project.
Funding Support:	NA
Name:	**Aubin Moutal**
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Dr. Moutal executed the constellation pharmacology experiments
Funding Support:	NA
Name:	**Wennan Li**
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Li executed the electrophysiology in both DRG and TG neurons.
Funding Support:	NA
Name:	**Song Cai**
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Li executed the electrophysiology in both DRG and TG neurons.
Funding Support:	NA

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - **Organization Name:**
  - **Location of Organization:**
  - **Partner's contribution to the project***)*
    - **Financial support;**

- **In-kind support**;
- **Facilities**;
- **Collaboration**;
- **Personnel exchanges**
- **Other.**

Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:**

Two separate reports for Drs. Khanna and Porreca have been submitted to https://ers.amedd.army.mil for each award.

.

   - **QUAD CHARTS:**

NA

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[7] Pradhan AA, Smith ML, McGuire B, Tarash I, Evans CJ, Charles A. Characterization of a novel model of chronic migraine. Pain 2014;155(2):269-274.

APPENDICES:

None.