Neuropharmacology

Conditional deletion of CB2 cannabinoid receptors from peripheral sensory neurons eliminates CB2-mediated antinociceptive efficacy in a mouse model of carrageenan-induced inflammatory pain

--Manuscript Draft--

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Abstract:	CB2 cannabinoid receptor activation suppresses pathological pain in animal models. CB2 agonists show promise as therapeutic agents because they lack unwanted side effects commonly associated with direct activation of CB1 receptors. However, the types of pain most responsive to CB2 agonists are incompletely understood and cell types which underlie CB2-mediated anti-allodynic efficacy remain largely unknown. Our laboratory previously reported that the CB2 receptor agonist LY2828360 attenuated the maintenance of neuropathic pain induced by toxic challenge with chemotherapeutic and anti-retroviral agents in mice. Whether these findings generalize to models of inflammatory pain is not known. Here we show that LY2828360 (10 mg/kg i.p.) reversed the maintenance of carrageenan-induced mechanical allodynia in female mice. Anti-allodynic efficacy was fully preserved in global CB1 knock out (KO) mice but absent in CB2 KO mice. The anti-allodynic efficacy of LY2828360 was absent in conditional KO mice lacking CB2 receptors in peripheral sensory neurons (AdvillinCRE/+; CB2f/f). Intraplantar administration of LY2828360 (30 µg i.pl.) reversed carrageenan-induced mechanical allodynia in CB2f/f but not AdvillinCRE/+; CB2f/f mice of both sexes. Thus, CB2 receptors in peripheral sensory neurons underlie the therapeutic effects of LY2828360 injection in the paw. Lastly, qRT-PCR analyses revealed that LY2828360 reduced carrageenan-induced increases in IL-1β and IL-10 mRNA in paw skin. Our results provide evidence that LY2828360 suppresses inflammatory nociception in mice through a neuronal CB2-dependent mechanism that requires peripheral sensory neuron CB2 receptors and suggest that the clinical applications of LY2828360 as an anti-hyperalgesic agent should be re-evaluated.
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Cover letter

THE LINDA AND JACK
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November 30, 2022

Dear Editor,

We herein submit for your consideration a new manuscript entitled “Conditional deletion of CB2 cannabinoid receptors from excitatory neurons and peripheral sensory neurons eliminates CB2-mediated antinociceptive efficacy in a mouse model of carrageenan-induced inflammatory pain” by Kelsey G. Guenther, Zhili Xu, Julian Romero, Cecilia J. Hillard, Ken Mackie, and Andrea G. Hohmann. CB_2 receptor agonists have shown promise as therapeutic agents because they suppress pathological pain and lack psychotropic effects. However, the types of pain responsive to CB_2 agonists and the cell types that underlie CB_2-mediate anti-allodynic effects remain poorly understood. We asked whether LY2828360, a G protein-biased CB2 agonist that failed in a clinical trial for osteoarthritis pain, would suppress carrageenan-induced inflammatory nociception. We also identified cell types and mechanism underlying these effects. Our studies tested the following hypotheses: 1) the CB_2 receptor agonist LY2828360 is a viable treatment for acute inflammatory pain; 2) excitatory neurons and peripheral sensory neurons expressing CB_2 receptors contribute to the anti-allodynic effects of CB_2 receptor agonists in an inflammatory model; 3) injection of LY2828360 locally at the site of tissue injury (in the paw) produces anti-allodynic effects; 4) activation of CB_2 receptors with LY2828360 suppresses mRNA expression levels of cytokines and chemokines involved in carrageenan’s inflammatory response. To test these hypotheses, we first examined the ability of the CB_2 receptor agonist LY2828360 to alleviate mechanical allodynia induced by intraplantar administration of carrageenan. LY2828360 was effective at reducing carrageenan-induced mechanical allodynia in WT mice and CB_1 receptor KO mice but was not effective in CB_2 receptor knockout (KO) mice, consistent with a CB_2 receptor mechanism of action. To investigate cell types underlying CB_2 agonist efficacy in this model, we generated mouse lines with conditional KO (cKO) of CB_2 receptors from microglia/macrophages ($CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mice), excitatory neurons ($NEX^{CRE/+}$; $CB_2^{f/f}$ mice) or primary sensory neurons ($Advillin^{CRE/+}$; $CB_2^{f/f}$ mice). LY2828360 was effective in reducing carrageenan-induced mechanical allodynia in $CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mice but was ineffective in $NEX^{CRE/+}$; $CB_2^{f/f}$ or $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice. Our studies suggest that activation of CB_2 receptors in excitatory neurons and peripheral sensory neurons but not microglia/macrophages are necessary for anti-allodynic effects of CB_2 receptor agonist LY2828360 in our carrageenan-induced inflammatory pain model. Next, we demonstrated that local administration of CB_2 receptor agonist LY2828360 into the site of injury (inflamed carrageenan paw), suppressed carrageenan-induced inflammatory nociception, consistent with a local site of action. Lastly, qRT-PCR was used to analyze the mRNA level of proinflammatory and anti-inflammatory cytokines and chemokines following carrageenan administration and treatment with a CB_2 agonist. LY2828360 treatment decreased carrageenan-induced increases in mRNA expression levels of IL-1β and IL-10 in the paw skin but the effects of LY2828360 were not observed in paw skin of $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice similarly treated with carrageenan.

Our results provide a compelling rationale for exploiting peripheral CB_2 mechanisms for suppressing inflammatory pain and has significant translational relevance. For these reasons, we believe our manuscript will be of great interest to the readers of *Neuropharmacology* and warrants rapid publication. Thank you in advance for considering our manuscript for publication. All authors have approved the manuscript enclosed. All experiments were approved by our Institutional Animal Care and Use Committee.

Sincerely,

Andrea G. Hohmann, Ph.D.
Linda and Jack Gill Chair of Neuroscience and Professor
Department of Psychological and Brain Sciences
Indiana University, Bloomington, IN 47405

Ms. Ref. No.: NEUROPHARM-D-22-00892
Title: Conditional deletion of CB2 cannabinoid receptors from excitatory neurons and peripheral sensory neurons eliminates CB2-mediated antinociceptive efficacy in a mouse model of carrageenan-induced inflammatory pain
Neuropharmacology

Dear Dr. Spigelman
Thank you for the helpful reviews of our manuscript. We have now responded to the critiques of both reviewers and hope that you will find our revised manuscript acceptable for publication in Neuropharmacology. Changes to the text are highlighted in yellow, as requested.
The referees' reports are enclosed (please see below). Both referees have made some important and constructive comments that need to be addressed.

Response to reviewers of NEUROPHARM-D-22-00892
Reviewer #1: *In this manuscript, the authors present a compelling case that the selective CB2 receptor agonist LY2828360 reduces inflammatory pain via a CB2 mechanism involving peripheral nociceptors and excitatory neurons. The authors used a combination of transgenic mouse models to selectively delete CB2 in different cell types, and route of administration to determine the different sites of activity. Despite previous work demonstrating that CB2 selective agonists block various types of inflammation and pain, this paper provides new evidence localizing the analgesic effects to peripheral nociceptors (as stimulated by the inflammatory events occurring in the carrageenan-injected paw). The paper is clearly written and well organized. My comments are relatively minor.*
Minor concerns:
Methods 2.1: Please clarify whether the wildtype mice were littermates of the knockouts.

We now clarify that WT and global KO mice used in each study were littermates. (page 5, line 125). Similarly, CB2f/f mice were generated from the same crosses that produced the conditional KO mouse lines used for each experiment (page 5, line 123).

Methods 2.2: What was the purity of the LY2828360?

We have specified the purity of LY2828360 in our methods section (page 5, line 130)

Methods 2.3: What type of metal was used for the mesh table? How far apart were the gaps between the wire that the mice stood on top of for the von frey testing?

We have added further description of the von Frey table in the Assessment of Mechanical Allodynia section of the methods (page 6, line 144)

Very minor:
2.4: Please define HBSS, which is presumably Hank's balanced salt solution.

We have now defined Hank's balanced salt solution (page 6, line 156).

2.5.1 (first sentence): Habituation was not a repeating cycle (i.e., it wasn't periodic), so "period" can be deleted.

We have made the requested modification. (page 7, line 179) by deleting the word "period."
4. Gabapentin is used as a positive control for allodynia, which makes good sense because it is used off-label for neuropathic pain. I may have missed it, but the Discussion mentions it only as an anticonvulsant, which is accurate but may not be clear to readers.

We have added clarification in the discussion that Gabapentin is used to treat neuropathic pain in addition to being an anticonvulsant (page 20, line 434).

Reviewer #2: *In review of the manuscript titled "Conditional deletion of CB2 cannabinoid receptors from excitatory neurons and peripheral sensory neurons eliminates CB2-mediated antinociceptive efficacy in a*

mouse model of carrageenan-induced inflammatory pain" the authors test whether a selective CB2 agonist acts in the periphery vs the central nervous system to produce antiallodynic effects using CB2 receptor KOs that are in either peripheral nerves, vs excitatory neurons, vs CNS glial cells. Studies identified that the LY2828360's activity in inhibiting carrageenan-induced allodynia was via peripheral excitatory neurons and not via central nervous system glial cells. In addition, the studies demonstrate effects in female animals similar to males. There are several concerns listed below that should be considered prior to publication.

1) Concerns with a lack of a dose response curve with only one dose of the three doses tested of the LY2828360 producing antiallodynic effects. Higher doses should have been tested.

Dose response of LY2828360 was evaluated in WT animals using 1, 3 and 10 mg/kg doses (i.p.) of LY2828360. We previously reported that LY2828360 reduced paclitaxel-induced mechanical and cold allodynia in $CB_2^{f/f}$ mice at doses as low as 0.3 and 1 mg/kg i.p., respectively (Lin et al. (2022) Pain 163: 834-851). We also reported that the 1 mg/kg dose of LY2828360 fully normalized mechanical and cold responsiveness to control levels in C57BlJ/6 mice (Lin et al. (2018) Molecular Pharm 93: 49-62). The present study test included a dose 10-fold higher than the dose which fully reversed mechanical and cold allodynia in a paclitaxel-induced neuropathy model (Lin et al. (2018) Molecular Pharm 93: 49-62) and an anti-retroviral toxic neuropathy model (Carey et al. (2023) Pharma Rese 187: 106560) in our previously published work. We specifically tested the maximally efficacious dose observed in our control mice in the three different conditional knockout mouse lines employed in our paper that were all treated identically with intraplantar carrageenan. Our goal was to ascertain the cell types containing CB_2 receptors that were likely to contribute to anti-allodynic effects of LY2828360 using doses that are both therapeutically relevant and CB_2-mediated. We now include a caveat in our discussion section to note that our study does not preclude the possibility that therapeutic efficacy could be unmasked in $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice using higher (i.e. suprathreshold) doses. (page 20, line 448).

2) There is a lack of evidence that any of the conditional CB2 KO animals used in these studies had a decrease in CB2 receptor protein. There should be some demonstration in the loss of the receptor in KO mice that is co-stained with the specific markers of the peripheral nerve, or glial cells, etc.

The reviewer makes an important point. However, it is problematic to use available CB_2 antibodies for this purpose (For further discussion see Lin et al. (2022) Pain 163: 834-851; Carey et al. (2023) Pharma Res 187: 106560). Indeed, generation and use of our $CB_2^{f/f}$ mouse and conditional KO mouse lines was, in fact, motivated by the fact that available CB2 receptor antibodies exhibit staining in CB_2 KO mice (i.e. global CB_2 KOs are functional, rather than complete, knockouts; because available antibodies are not directed at the epitope deleted in the KO, nonspecific staining is consequently observed in tissues lacking the target) making interpretation of cell types containing CB_2 receptors that mediate the anti-nociceptive effects of CB_2 agonists difficult. In our previous publication we showed that both CB_2 and GFP mRNA levels were reduced in dorsal root ganglia of $Advillin^{CRE/+}$; $CB_2^{f/f}$ relative to $CB_2^{f/f}$ mice (Carey et al. (2023) Pharm Res 187: 106560), thereby validating the use of this mouse line in the present studies. Importantly, in the present studies, anti-allodynic efficacy of LY2828360 (10 mg/kg i.p.) was absent in $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice and fully preserved in $CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mice. The $CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mouse line has been used by both the Hillard and Hohmann labs to study a role for CB_2 receptors residing on CX3CR1 positive microglia in other dependent measures in the context of treatment with drugs of abuse (e.g. Behlke et al. 2022 ICRS abstract). A fuller characterization of this mouse will be published in a separate report. Inclusion of these latter experiments in the present manuscript would be beyond the scope of the present report, which we believe should remain focused on our model of carrageenan-evoked inflammatory nociception. We provide this context in our discussion section and note that our results do not preclude a role for other cell types/classes of microglia in contributing to the anti-allodynic effects of LY2828360 (page 20, line 450).

3) Due to the outcomes that the LY2828360 is acting in the periphery, additional measurements of paw inflammation in the absence and presence of LY would be useful.

The global and cKO mouse lines (and littermate controls) used in the present study were bred in house and are not commercially available. Generating these mouse lines required considerable grant resources and took several years to complete. The present studies were initiated during the COVID pandemic in 2020 when our laboratory was advised that Laboratory Animal Resources at Indiana University may be unable to provide care for our transgenic mouse lines and euthanasia of our mouse colonies may be required. Under these conditions, my lab made the decision to test as many mouse lines as efficiently as possible in acute studies to ensure that our animals would not be euthanized without being used for data collection. Under these conditions, we elected to not measure paw edema in any study. Evaluation of paw edema would ideally be performed in animals not subjected to prior behavioral testing at the peak of anti-allodynic effect of LY2828360. Mice used in our in vivo studies were subjected to the same behavioral testing protocols for evaluation of carrageenan-induced inflammatory nociception and the timecourse of LY2828360-induced anti-allodynic efficacy; this protocol also avoided inducing possible handling stress or restraint (required to measure paw edema) that could otherwise alter endogenous analgesic tone and complicate interpretation of the effects of LY2828360 in different mouse lines. We now state that more work is necessary to examine the impact of LY2828360 on hindpaw edema (page 23, line 516).

4) The manuscript (fourth paragraph, first sentence in discussion) states IL-10 cytokine as an inflammatory cytokine yet it is very well documented to be an anti-inflammatory cytokine that tends to decrease the other inflammatory cytokines including TNFalpha and IL-1beta. The discussion states a decrease in mRNA for the cytokines that implies IL-10 as well although there are previous publications demonstrating the CB2 receptor activation increases IL-10 (Saroz, Yurii; et al., (2019). ACS Pharmacology & Translational Science. 2 (6): 414-428).

We have corrected this typographical error. The distinction between pro- and anti-inflammatory cytokines is now clarified in the discussion (page 22, line 482). We observed an increase in IL-10, IL-1beta and TNFalpha mRNAs following intraplantar carrageenan injection and a decrease in IL-1beta and IL-10 mRNAs in carrageenan-injected animals following LY2828360 administration ipsilateral paw skin. Our results may reflect the timepoint at which we collected the tissue for qPCR studies, as modulation of these cytokines is a dynamic process and we measured mRNA expression levels at a single timepoint. Future studies should collect tissue at different time points to gain a more complete understanding of the cytokines undergoing either upregulation or downregulation as a function of inflammatory nociception and therapeutic treatment. We did not measure IL-10 protein in our studies. mRNA for the anti-inflammatory cytokine IL-10 may decrease in response to increases in protein levels of IL-10. We now provide further context and caveats related to the findings from our qPCR studies (page 22, Line 492). Finally, we note that we are unaware of any publications that measured IL-10, IL-1beta and TNF-alpha mRNA levels in the paw skin of mice subjected to intraplantar injection of carrageenan and evaluated the impact of a CB2 agonist on these measures. The excellent reference identified by the Reviewer evaluated human primary peripheral blood mononuclear cells.

5) Studies are a bit mixed as far as sexes in which many studies are female only (no comparison to males) and figures are not clearly identifying male and female (e.g. Figure 4) in which the legend mentions both sexes but figures do not indicate which are males vs females.

We specifically identify use of female or male mice in all studies in both our Methods Section and our figure captions. As noted above, the conditional KO mouse lines (Advillin^{CRE}, CX3CR1^{CRE}) are not commercially available and mixed sex groups were included where appropriate due to breeding limitations. Note that we specifically used both male and female mice in separate studies to show that intraplantar injection of LY2828360 suppressed carrageenan-induced inflammatory nociception through a peripheral mechanism with similar efficacy in both sexes (Experiment 9). This observation provided a rationale for using mixed sexes in mRNA studies in Experiment 13.

6) Where sexes combined on all the mRNA studies (Figure 7 and 8)?

Sex was only combined in Figure 8 (experiment 13), based upon results of our behavioral studies showing similar anti-allodynic efficacy of LY2828360 in mice of both sexes and due to limitations in breeding. We have updated the figure captions to include this important information. Both male and female $CB_2^{f/f}$ and Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice similarly develop mechanical allodynia due to carrageenan (as seen in experiment 10 and 11). We also previously reported that LY2828360 suppresses anti-retroviral toxic neuropathy in male and female $CB_2^{f/f}$ mice and these effects were also absent in Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice of each sex (Carey et al. (2023) Pharma Res 187: 106560). We also clarify that our results were not powered to detect sex differences in studies in which mixed sexes were combined (page 22, line 490).

7) The number of animals in some experiments (e.g. n=3, 4 and 5) are very low with no indication of whether a power analysis was performed to determine useful statistics.

We agree with the reviewer that sample size was too small for the study using NEX CRE CB2f/f mouse line, therefore we have removed this data from the manuscript. This removes one experiment from methods/results and 2 figure panels from figure 4.

8) There is a lack of recognition of references for other studies demonstrating CB2 receptor activity in peripheral antinociceptive effects other than the authors own papers including (Aline Quartilho, et al., "Inhibition of Inflammatory Hyperalgesia by Activation of Peripheral CB2 Cannabinoid Receptors. Anesthesiology 2003; 99:955-960; N. Clayton et al., "CB1 and CB2 cannabinoid receptors are implicated in inflammatory pain" Pain (96) 3, 2002, 253-260; T.Philip et al., "Inhibition of pain responses by activation of CB2 cannabinoid receptors" Chemistry and Physics of Lipids, (121) 1-2, 2002, Pages 191-200,)

We primarily focused our introduction and discussion on papers from our laboratory because these represent the only published studies to use the CB2f/f mouse line and conditional KO mouse lines employed in the present report. We also discussed prior publications employing carrageenan-induced inflammatory nociception in rats. We have now expanded our discussion to include the additional publications employing rats in other acute and inflammatory pain models that are cited above. We include additional references showing efficacy of CB2 receptor agonists administered locally in the paw in the presence and absence of inflammation induced by capsaicin and complete Freund's adjuvant (page 21, line 465). The Clayton et al. paper evaluated a CB2 agonist via a systemic route of administration and is cited elsewhere in our manuscript (page 3, line 72; page 23, line 507).

9) The discussion lacks recognition of many, many other publications demonstrating CB2 activity in the CNS in models of pain and inflammation, and should at least be discussed based on the different findings here.

In the present studies, we specifically employed a CB2 agonist (LY2828360) that failed for efficacy in a Phase 2 clinical trial of knee pain due to osteoarthritis but was found to be safe in humans to increase translational relevance of our studies; we also previously reported that LY2828360 was potent and efficacious in suppressing neuropathic nociception in two different preclinical mouse models in our lab. Because LY2828360 is a G protein-biased CB2 agonist that does not recruit arrestins or internalize CB2 receptors, it remains unclear whether the effects of LY2828360 are mimicked by previous CB2 agonists used in the literature. Species differences (rat vs. mice vs. human), biased signaling of CB2 agonists and different CB2 expression levels induced by different states of inflammation and injury add complexity to the reviewers request. For example, we specifically reported that a ligand described in the literature as a specific CB2 agonist (e.g. GW405833) produced anti-allodynic effects that were preserved in CB2 KO mice, absent in CB1 KO mice and blocked by CB1 but not CB2 antagonists (Li et al. (2019) Molec Pharm 95: 155-168). We refer interested readers to review papers published by ourselves and others in our discussion section (page 19 line 420). We believe that the present manuscript should primarily remain focused on carrageenan-induced inflammatory nociception, effects of our G protein-biased CB2 agonist LY2828360 and evaluation of CB2f/f and conditional KO mouse lines employed here in preclinical pain models.

10) Would be nice to have the LY2828360 compound affinity and selectivity values stated in the introduction instead of words such as "little affinity" for CB1 receptors.

We have added Ki value of LY2828360 for CB2 receptor and EC50 information of LY2828360 for CB1 and CB2 receptor in the introduction (page 4, line 84).

11) Misspelling of carrageenan in results section-3.6 Experiment 5: title."carrageenancar"

We have corrected the spelling of carrageenan (page 14, line 298).

Highlights

- CB_2 receptor agonist LY2828360 reduced carrageenan-induced mechanical allodynia
- Anti-allodynic effects of LY2828360 are CB_2 receptor dependent
- Peripheral sensory neuron CB_2 receptors are required for efficacy of LY2828360
- LY2828360 can exert anti-allodynic effect locally at the site of injury
- LY2828360 suppresses cytokines involved in carrageenan's inflammatory response

Title page (with author information)

Conditional deletion of CB2 cannabinoid receptors from peripheral sensory neurons eliminates CB2-mediated antinociceptive efficacy in a mouse model of carrageenan-induced inflammatory pain

Kelsey G. Guenther[1,2], Zhili Xu[1,2], Julian Romero[3], Cecilia J. Hillard[4], Ken Mackie[1,2,5], and Andrea G. Hohmann[1,2,5]

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Number of Words in Abstract: 243

Number of Figures: 8

Number of Tables: 3

Number of Pages of Text: 40

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Abstract

CB_2 cannabinoid receptor activation suppresses pathological pain in animal models. CB_2 agonists show promise as therapeutic agents because they lack unwanted side effects commonly associated with direct activation of CB_1 receptors. However, the types of pain most responsive to CB_2 agonists are incompletely understood and cell types which underlie CB_2-mediated anti-allodynic efficacy remain largely unknown. Our laboratory previously reported that the CB_2 receptor agonist LY2828360 attenuated the maintenance of neuropathic pain induced by toxic challenge with chemotherapeutic and anti-retroviral agents in mice. Whether these findings generalize to models of inflammatory pain is not known. Here we show that LY2828360 (10 mg/kg i.p.) reversed the maintenance of carrageenan-induced mechanical allodynia in female mice. Anti-allodynic efficacy was fully preserved in global CB_1 knock out (KO) mice but absent in CB_2 KO mice. The anti-allodynic efficacy of LY2828360 was absent in conditional KO mice lacking CB_2 receptors in peripheral sensory neurons ($Advillin^{CRE/+}$; $CB_2^{f/f}$). Intraplantar administration of LY2828360 (30 μg i.pl.) reversed carrageenan-induced mechanical allodynia in $CB_2^{f/f}$ but not $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice of both sexes. Thus, CB_2 receptors in peripheral sensory neurons underlie the therapeutic effects of LY2828360 injection in the paw. Lastly, *qRT-PCR* analyses revealed that LY2828360 reduced carrageenan-induced increases in IL-1β and IL-10 mRNA in paw skin. Our results provide evidence that LY2828360 suppresses inflammatory nociception in mice through a neuronal CB_2-dependent mechanism that requires peripheral sensory neuron CB_2 receptors and suggest that the clinical applications of LY2828360 as an anti-hyperalgesic agent should be re-evaluated.

Conditional deletion of CB2 cannabinoid receptors from peripheral sensory neurons eliminates CB2-mediated antinociceptive efficacy in a mouse model of carrageenan-induced inflammatory pain

Kelsey G. Guenther[1,2], Zhili Xu[1,2], Julian Romero[3], Cecilia J. Hillard[4], Ken Mackie[1,2,5], and Andrea G. Hohmann[1,2,5]

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Abstract

CB_2 cannabinoid receptor activation suppresses pathological pain in animal models. CB_2 agonists show promise as therapeutic agents because they lack unwanted side effects commonly associated with direct activation of CB_1 receptors. However, the types of pain most responsive to CB_2 agonists are incompletely understood and cell types which underlie CB_2-mediated anti-allodynic efficacy remain largely unknown. Our laboratory previously reported that the CB_2 receptor agonist LY2828360 attenuated the maintenance of neuropathic pain induced by toxic challenge with chemotherapeutic and anti-retroviral agents in mice. Whether these findings generalize to models of inflammatory pain is not known. Here we show that LY2828360 (10 mg/kg i.p.) reversed the maintenance of carrageenan-induced mechanical allodynia in female mice. Anti-allodynic efficacy was fully preserved in global CB_1 knock out (KO) mice but absent in CB_2 KO mice. The anti-allodynic efficacy of LY2828360 was absent in conditional KO mice lacking CB_2 receptors in peripheral sensory neurons ($Advillin^{CRE/+}$; $CB_2^{f/f}$). Intraplantar administration of LY2828360 (30 μg i.pl.) reversed carrageenan-induced mechanical allodynia in $CB_2^{f/f}$ but not $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice of both sexes. Thus, CB_2 receptors in peripheral sensory neurons underlie the therapeutic effects of LY2828360 injection in the paw. Lastly, *qRT-PCR* analyses revealed that LY2828360 reduced carrageenan-induced increases in IL-1β and IL-10 mRNA in paw skin. Our results provide evidence that LY2828360 suppresses inflammatory nociception in mice through a neuronal CB_2-dependent mechanism that requires peripheral sensory neuron CB_2 receptors and suggest that the clinical applications of LY2828360 as an anti-hyperalgesic agent should be re-evaluated.

Keywords: CB_2 receptor, cannabinoid, inflammation, pain, dorsal root ganglion

1. Introduction

Engaging targets within the endocannabinoid system can supress pain behavior in preclinical and clinical studies (for review see Lötsch et al., 2018). However, activation of CB_1 cannabinoid receptors also produces unwanted side effects (e.g., sedation, altered mental state, memory impairment, and dependence (Howlett et al., 2002; Cooper and Haney, 2009)) that limit clinical utility. By contrast, activation of CB_2 cannabinoid receptors suppresses pathological pain in several rodent models without producing adverse CB_1-mediated cannabimimetic effects (see Guindon and Hohmann, 2008 for review). We previously reported that CB_2 activation reduces neuropathic nociception without producing tolerance or physical dependence (Deng et al., 2015). However, most early studies of CB_2 analgesic efficacy employed male rodents exclusively and whether CB_2 antinociceptive efficacy is comparable between sexes remains poorly understood. In male rats, CB_2 activation reduces mechanical and heat hypersensitivity in the carrageenan model of inflammatory nociception (Nackley, Makriyannis and Hohmann, 2003; Clayton et al., 2002; Elmes et al., 2005) and suppresses transmission of inflammation-evoked neuronal activity measured both immunohistochemically and electrophysiologically (Nackley, Makriyannis and Hohmann, 2003; Nackley, Suplita and Hohmann, 2003; Nackley et al., 2004). CB_2 agonism is unlikely to produce deleterious psychoactive effects, possibly due to relative paucity of CB_2 receptors in the central nervous system (CNS) and primary localization of these receptors to immune tissues and cells (Munro et al., 1993; Galiègue et al., 1995; Van Sickle et al., 2005). Thus, CB_2 agonists may be preferable to analgesic medications with high abuse potential such as opioids (Gutierrez et al., 2011; Xi et al., 2011; Iyer et al., 2020). Both efficacy of CB_2 activation in pain models as well as the lack of apparent adverse side effects makes CB_2 a favourable target for the development of pharmacotherapies for pain (for review see Soliman et al. 2021). However, a key challenge for clinical translation, given the failure of CB_2 agonists in several clinical pain trials (Cabañero, Martín-García and Maldonado, 2021), is determining which pain states are best treated by CB_2 agonists.

LY2828360 is a potent CB_2 receptor agonist that exhibits similar affinity for human and rat CB_2 receptors (Ki = 40.3 nM) and has little affinity for the human CB_1 receptor (EC50s = 20.1 and > 100,000 nM for CB_2 and CB_1 respectively; Hollinshead et al., 2013). We showed that LY2828360 displays strong Gi/o-protein bias, resulting in the inhibition of cAMP and activation of ERK1/2 signaling, without β-arrestin recruitment or CB_2 receptor internalization (Lin et al., 2018). LY2828360 attenuated neuropathic nociception produced by the chemotherapeutic agent paclitaxel (Lin et al., 2018; Lin et al., 2022) or the anti-retroviral agent zalcitabine (Carey et al., 2023) and inhibited the development of tolerance to the anti-allodynic effects of morphine in mice (Lin et al., 2018; Carey et al., 2023). Whether this profile of anti-allodynic efficacy generalizes to models of inflammatory pain is unknown. Understanding the range of potential therapeutic indications for this compound is important because LY2828360 failed in a Phase 2 clinical trial for osteoarthritis pain but was demonstrated to be safe for use in humans (Pereira et al., 2013).

The present studies examined the ability of LY2828360 to reduce carrageenan-induced inflammatory nociception and established the mechanism of action underlying these effects. LY2828360 was tested for its ability to reduce carrageenan-induced inflammatory nociception in CB_1 knockout (KO) and CB_2 KO mice to assess mediation by CB_2. To identify specific cell types involved in the anti-allodynic ability of LY2828360, we generated mouse lines with conditional KO (cKO) of CB_2 receptors from microglia or macrophages expressing the protein coding gene C-X3-C Motif Chemokine Receptor 1 ($CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mice) or peripheral sensory neurons ($Advillin^{CRE/+}$; $CB_2^{f/f}$ mice) and asked whether anti-allodynic efficacy of LY2828360 was altered following conditional deletion of CB_2 from each cell type. Finally, we asked whether local administration of LY2828360 reduces carrageenan-induced mechanical allodynia in wildtype (WT) mice and in mice lacking CB_2 in peripheral sensory neurons. Finally, the ability of LY2828360 to modulate mRNA expression levels of cytokines and chemokines involved in the inflammatory response to carrageenan was examined in lumbar spinal cord, paw skin and spleen of both WT and $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice.

2. Materials and Methods

2.1. Subjects

Female and male mice between 72 and 274 days of age at the start of the experiment were used, with all mice age matched for each experiment. Mice were housed in a temperature-controlled colony room on a 24-hr light/dark cycle (lights on at 7am; lights off at 7pm), such that behavioral testing occurred during the light phase of the cycle. Mice were maintained on ad libitum food and water. CB_2 knockout (KO) mice and wild type (WT) mice on a C57BL/6J background and CB_1 KO mice on a CD1 background were used. Mice with conditional KO of CB_2 receptors in microglia, excitatory neurons and peripheral sensory afferents were also used. All cKO mouse lines were on a C57BL/6J background. To conditionally delete CB_2 from the desired cells, female mice carrying two alleles of the floxed CB_2 gene ($CB_2^{f/f}$; López et al., 2018) were bred with male mice carrying two floxed CB_2 alleles and one allele of Cre-recombinase in either the CX3CR1 (Yona et al., 2013) or Advillin (Zhou et al., 2010) locus, as appropriate for the experiment. Mice used from this breeding strategy were $CX3CR1^{CRE/+}$; $CB_2^{f/f}$ (deletion of CB_2 from microglia), $Advillin^{CRE/+}$; $CB_2^{f/f}$ (deletion of CB_2 from peripheral sensory afferents) and $CB_2^{f/f}$ controls (no deletion of CB_2, $CB_2^{f/f}$ only). $CB_2^{f/f}$ mice were generated from the same crosses that produced the conditional KO mice lines used for each experiment and were tested concurrently. KO mice were bred at Indiana University. Wildtype (WT) littermates were included for each experiment as appropriate comparators. All experiments were approved by the Indiana University Animal Care and Use Committee.

2.2. Drugs

LY2828360 (8-(2-chlorophenyl)- 2-methyl-6-(4-methylpiperazin-1-yl)-9-(tetrahydro-2H-pyran-4-yl)-9H- purine) was synthesized by Sai Biotech (Mumbai, India; purity > 98%). LY2828360 was administered intraperitoneally (i.p.) at doses of 1, 3 and 10 mg/kg, and administered in a volume of 10 ml/kg in a vehicle (VEH) consisting of 10% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), and the remaining 90% consisted of emulphor (Alkamuls EL-620; Solvay), 95% ethanol (Sigma-Aldrich)

and 0.9% saline (Aqualite System; Hospira, Inc., Lake Forest, IL) at a 1:1:18 ratio. LY2828360 was prepared at a concentration of 3 µg/µl in the same vehicle used for i.p. administration and administered at a volume of 10 µl into the paw via direct intraplantar (i.pl.) injection (30 µg/paw). Gabapentin was administered i.p. at a dose of 50 mg/kg, prepared at a concentration of 10 mg/ml in solution with saline. To induce inflammation, λ-Carrageenan (Sigma-Aldrich) was mixed in saline (1% wt/vol solution) and delivered into the right hind paw by intraplantar injection at a volume of 20 µl.

2.3. Assessment of Mechanical Allodynia

Mechanical allodynia was assessed by measuring the paw withdrawal threshold (in grams) to punctate mechanical stimulation, assessed using an electronic von Frey anesthesiometer (IITC Life Sciences Inc., Woodland Hills, CA) as described in our previously published work (Lin et al. 2018; Lin et al., 2022; Carey et al., 2023). First, mice were placed on an elevated testing table with a stainless-steel wire mesh platform (with 0.6 x 0.6 cm gaps in the wire mesh) underneath clear Plexiglass chambers (10.5 x 9 x 7 cm) for 1 hour of habituation prior to testing. Following habituation, baseline measurements of paw withdrawal thresholds were taken using the von Frey anesthesiometer. Immediately following baseline measurements, carrageenan (20 µl) was injected into the right hind paw. Subsequent von Frey measurements were taken at various time points (see specific experiments in procedure section for details). To measure paw withdrawal threshold, a semi-flexible plastic filament with a 0.8 mm diameter was applied vertically to the plantar surface of the hind paws until withdrawal of the paw; a digital readout of the threshold for paw withdrawal was displayed on the anesthesiometer meter and recorded by the experimenter. Each paw was measured twice at every time point, including for baseline measurements (in a right-left-right-left order).

2.4. Tissue collection and quantitative real-time polymerase chain reaction (qRT-PCR)

Mice were perfused with Hank's Balanced Salt solution (HBSS) 30-min following injection of each pharmacological treatment, and then spleen, lumbar spinal cord, and paw skin (ipsilateral and contralateral paws separated) were flash frozen. Spleen and spinal cord RNA was extracted using TRizol

(Invitrogen)/RNeasy (Qiagen) RNA mini Kit according to the manufacturer's instructions, and paw skin RNA was extracted using a RLT buffer:2-mercapto ethanol mixture in a 100:1 ratio/RNeasy RNA mini Kit. RNA (3000 ng for spleen, 500 ng for spinal cord/paw skin) was used in a Luna Universal One-Step RT-qPCR Kit (New England Biolabs) with the following cycling conditions: 55°C for 10 min, 95°C for 1 min, and 40 cycles of 95°C for 10 sec and 60°C for 1 min. RNA levels in extracts were quantified by cycle threshold (Ct), where Ct levels are inversely proportional to the amount of target nucleic acid in the sample. Relative gene expression for CB_2, interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNFα) and monocyte chemoattractant protein 1 (MCP1) mRNAs were normalized to the housekeeping gene GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Sequences for qRT-PCR primers were: IL-1β sense 5'-CGT GGA CCT TCC AGG ATG AG-3'; IL-1β anti-sense 5'-CAT CTC GGA GCC TGT AGT GC-3'; IL-10 sense 5'-GGA CTT TAA GGG TTA CTT GGG TTG CC-3'; IL-10 anti-sense 5'-CAT TTT GAT CAT CAT GTA TGC TTC T-3'; TNFα sense 5'-CGT CGT AGC AAA CCA CCA AG-3'; TNFα anti-sense 5'-TAG CAA ATC GGC TGA CGG TG-3'; MCP1 sense 5'-GAA GGA ATG GGT CCA GAC AT-3'; MCP1 anti-sense 5'-ACG GGT CAA CTT CAC ATT CA-3'; CB_2 sense 5'-ACG GTG GCT TGG AGT TCA AC-3'; CB_2 anti-sense 5'- GCC GGG AGG ACA GGA TAA T-3'; GAPDH sense 5'-AGG TCG GTG TGA ACG GAT TTG-3'; GAPDH anti-sense 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'.

2.5. Experimental Procedure

2.5.1. General experimental procedures

Paw withdrawal thresholds were measured in duplicate in each paw prior to carrageenan administration (baseline), after allowing for 1 hour of habituation. Carrageenan was injected i.pl. immediately following completion of baseline measurements. Paw withdrawal thresholds were subsequently measured either 3- or 4-hours post-carrageenan to document the presence of inflammatory nociception prior to pharmacological treatments, based upon the protocol described below. Timing of pharmacological manipulations was optimized for specific experiments as described below.

2.5.2. Experiment 1: Dose response of LY2828360 effect in carrageenan-induced inflammatory pain

We evaluated the dose response of LY2828360 in suppressing the maintenance of carrageenan-induced inflammation in C57BL/6J female mice. Mice (n = 6 per group) were injected intraperitoneally (i.p.) with either 1, 3 or 10 mg/kg LY2828360 or VEH 30 mins prior to drug testing. Testing of drug effects began 4-hours after carrageenan administration.

2.5.3. Experiment 2: Are the anti-allodynic effects of LY2828360 preserved in CB_1 KO mice?

To rule out the possibility that LY2828360 is producing anti-allodynic effects through CB_1 receptors, we examined the ability of LY2828360 (10 mg/kg i.p.) to suppress inflammatory nociception in female CB_1 receptor KO mice (n = 7-8 per group). LY2828360 (10 mg/kg i.p.) or vehicle was injected 30-mins prior to assessment of pharmacological effects 4 and 6 hours after carrageenan injection.

2.5.4. Experiment 3: Is anti-allodynic efficacy of a reference agent preserved in CB_2 KO mice?

The ability of gabapentin to reduce carrageenan-induced mechanical allodynia in female WT (C57 strain) mice and CB_2 KO mice (n = 8 per group) was determined. Gabapentin (50 mg/kg i.p.) or vehicle was injected 30-mins prior to the 6-hour post-carrageenan measurement. Subsequent measurements occurred at 7 and 24-hours post-carrageenan.

2.5.5. Experiment 4: Role of CB_2 receptors in the anti-allodynic effect of LY2828360

To ascertain whether the anti-allodynic effects of LY2828360 were CB_2-mediated, female CB_2 KO mice were treated with either LY2828360 (10 mg/kg i.p.) or its vehicle (n = 6 per group) during the maintenance phase of carrageenan-induced inflammatory nociception. Experimental procedures were conducted identically as those in Experiment 2.

2.5.6. Experiment 5: Are the anti-allodynic effects of LY2828360 preserved in $CB_2^{f/f}$ mice receiving intraplantar injection of carrageenan?

The ability of LY2828360 to reduce carrageenan-induced mechanical allodynia relative to vehicle treatment was assessed in female $CB_2^{f/f}$ control mice (n = 10 per group). Experimental procedures were the same as in Experiment 3.

2.5.7. Experiment 6: Impact of cKO of CB_2 receptors in microglia and macrophages on the anti-allodynic effect of LY2828360

To ascertain whether LY2828360 is exerting its anti-allodynic effect by engaging CB_2 receptors on microglia or macrophages, we administered LY2828360 or its vehicle to female $CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mice (n = 4-7 per group). Experimental procedures were the same as in Experiment 3. LY2828360 (10 mg/kg i.p.) or vehicle was administered 30-mins prior to the 6-hour post-carrageenan measurement.

2.5.8. Experiment 7: Effect of carrageenan in mice with cKO of CB_2 from peripheral sensory neurons

To ensure that carrageenan-induced inflammatory nociception develops normally following conditional deletion of CB_2 receptors from peripheral sensory neurons, carrageenan-induced mechanical allodynia was measured in female $CB_2^{f/f}$ and $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice (n = 7 per group). Paw withdrawal thresholds were reassessed 2, 4, 6 and 24-hours post carrageenan.

2.5.9. Experiment 8: Impact of cKO of CB_2 from peripheral sensory neurons on anti-allodynic effect of LY2828360

To ascertain the possible contribution of CB_2 receptors in peripheral sensory neurons to the anti-allodynic effects of LY2828360, we asked whether antiallodynic effects of LY2828360 would be attenuated in female $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice (n = 7 per group). Experimental procedures were conducted the same as in Experiment 3.

2.5.10. Experiment 9 and 10: Local paw injection of LY2828360 into the paw of $CB_2^{f/f}$ and $Advillin^{CRE/+}$; $CB_2^{f/f}$ male and female mice

To ascertain whether LY2828360 suppresses carrageenan-induced inflammatory nociception through a peripheral mechanism, vehicle or LY2828360 (30 μg i.pl.) was injected locally into the paw ipsilateral or contralateral to the paw injected with carrageenan. Mechanical paw withdrawal thresholds were assessed before and after intraplantar injections of carrageenan, drug or vehicle in four separate groups of mice: *female* $CB_2^{f/f}$ mice (n = 5 per group), *female* $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice (n = 5 per group),

male $CB_2^{f/f}$ mice, (n = 5 per group) and *male* $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice (n = 4-5 per group). Mice were injected with either LY2828360 or VEH unilaterally into the plantar hindpaw surface either ipsi- or contra-lateral to the carrageenan-injected paw. Intraplantar injections were performed 30 min prior to a 6-hour post-carrageenan measurement. Additional measurements were taken at 7- and 24-hours post-carrageenan.

2.5.11. Experiment 11: Impact of intraplantar injection of LY2828360 in the absence of carrageenan

To ascertain whether LY2828360 was antinociceptive in the absence of carrageenan, LY2828360 (30 µg i.pl.) or vehicle was administered locally into the paw of naïve male C57 WT mice (n = 5 per group). Experimental procedures were conducted as in Experiment 7, with the exception that an equivalent volume of saline was injected into the paw in lieu of carrageenan.

2.5.12. Experiment 12: Impact of LY2828360 on mRNA expression levels of inflammatory-related markers following intraplantar injection of carrageenan

To ascertain whether LY2828360 reduces mRNA levels of inflammatory markers in the spleen, spinal cord, or paw skin, LY2828360 (10 mg/kg i.p.) or VEH was administered to female C57 WT mice (n = 8 per group) 3.5 hrs after intraplantar carrageenan injection. Tissue was also collected from a group of otherwise naïve animals (n=8) as a comparator.

2.5.13. Experiment 13: qRT-PCR analysis to assess the impact of intraplantar carrageenan in the spinal cord of mice lacking CB_2 in $Advillin^{CRE}$ expressing cells

To ascertain whether carrageenan increases inflammatory markers in the lumbar spinal cord of male and female $CB_2^{f/f}$ and $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice, equivalent volumes (20 µl) of carrageenan or saline were administered into a single hind paw (n = 5-6 per group, mixed sex). Then, 6 hrs post-injection mice were euthanized using live decapitation and their spinal cord was extracted and flash frozen.

2.5.14. Experiment 14: Impact of LY2828360 on mRNA expression levels of inflammatory markers following intraplantar injection of carrageenan in mice lacking CB_2 in Advillin^{CRE} expressing cells

To ascertain whether LY2828360 reduces inflammatory markers in the spleen, spinal cord, or paw skin of female $CB_2^{f/f}$ and Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice, LY2828360 (10 mg/kg i.p.) or VEH was administered (n = 5 per group) 3.5 hrs after intraplantar carrageenan injection.

3. Results

3.1. General Behavioral Results

In general, prior to pharmacological treatments, carrageenan lowered paw withdrawal thresholds in the *ipsilateral* (i.e. carrageenan-injected) paw relative to baseline (pre-injection) levels (Table 1), but no difference was observed between group or time points in the paw *contralateral* to carrageenan injection in any study (data not shown). Following pharmacological treatments, differences in *contralateral* paw withdrawal thresholds were only detected in a subset of studies (Table 2).

Table 1. Summary of statistical analyses evaluating development of carrageenan-induced hypersensitivity in the carrageenan-injected *ipsilateral* paw prior to pharmacological treatments (mechanical withdrawal thresholds).

Figure	Group	Time	Interaction
1A	$F_{3,19} = 0.06245$, $p = 0.9790$	-	-
2A	$F_{1,13} = 0.1325$, $p = 0.7218$	$F_{1,13} = 0.02251$, $p = 0.8830$	$F_{1,13} = 0.2915$, $p = 0.5984$
3A	$F_{1,14} = 2.444$, $p = 0.1403$	**$F_{1,14} = 42.59$, $p < 0.0001$******	$F_{1,14} = 0.8586$, $p = 0.3698$
3C	$F_{1,14} = 2.2588$, $p = 0.6189$	**$F_{1,14} = 30.25$, $p < 0.0001$******	$F_{1,14} = 2.352$, $p = 0.1474$
3E	$F_{1,10} = 3.428$, $p = 0.0938$	**$F_{1,10} = 7.06$, $p = 0.0240$***	$F_{1,10} = 0.004534$, $p = 0.9476$
4A	$F_{1,17} = 0.9811$, $p = 0.3358$	**$F_{1,17} = 11.42$, $p = 0.0036$****	$F_{1,17} = 0.01227$, $p = 0.9131$
4C	$F_{1,12} = 0.6703$, $p = 0.4289$	**$F_{1,12} = 28.54$, $p = 0.0002$*****	$F_{1,12} = 0.3348$, $p = 0.5736$
4E	$F_{1,12} = 0.0001301$, $p = 0.9911$	**$F_{1,12} = 29.78$, $p = 0.0001$*****	$F_{1,12} = 0.1282$, $p = 0.7265$
5A	$F_{1,11} = 0.3124$, $p = 0.5874$	**$F_{1,11} = 10.93$, $p = 0.0070$***	$F_{1,11} = 4.24$, $p = 0.0640$
5C	$F_{1,10} = 3.217$, $p = 0.1031$	$F_{1,10} = 4.543$, $p = 0.0589$	$F_{1,10} = 1.141$, $p = 0.3105$
5E	$F_{1,8} = 1.797$, $p = 0.2169$	**$F_{1,8} = 70.26$, $p < 0.0001$******	$F_{1,8} = 2.068$, $p = 0.1883$
5G	$F_{1,8} = 1.76$, $p = 0.2213$	$F_{1,8} = 3.733$, $p = 0.0894$	$F_{1,8} = 0.1367$, $p = 0.7212$
6A	$F_{1,11} = 0.02834$, $p = 0.8694$	**$F_{1,11} = 22.65$, $p = 0.0006$*****	$F_{1,11} = 3.876$, $p = 0.0747$
6C	$F_{1,10} = 3.439$, $p = 0.0934$	**$F_{1,10} = 10.49$, $p = 0.0089$****	$F_{1,10} = 0.001533$, $p = 0.9695$
6E	$F_{1,8} = 0.4482$, $p = 0.5220$	**$F_{1,8} = 26.76$, $p = 0.0009$*****	$F_{1,8} = 1.888$, $p = 0.2067$
6G	$F_{1,6} = 0.6613$, $p = 0.4472$	**$F_{1,6} = 10.22$, $p = 0.0187$***	$F_{1,6} = 0.07212$, $p = 0.7973$

Table 2. Summary of statistical analyses of mechanical withdrawal thresholds in the *contralateral* paw following pharmacological treatments

Figure	Group	Time	Interaction
1B	$F_{3,19}$ = 2.046, p = 0.1415	-	-
2B	$F_{1,13}$ = 0.1908, p = 0.6694	$F_{1,13}$ = 2.648, p = 0.1277	$F_{1,13}$ = 0.00134, p = 0.9714
3B	**$F_{1,14}$ = 5.834, p = 0.0300***	$F_{2,28}$ = 0.4645, p = 0.6332	$F_{2,28}$ = 0.6038, p = 0.5537
3D	$F_{1,14}$ = 1.958, p = 0.1835	**$F_{2,28}$ = 4.114, p = 0.0271***	$F_{2,28}$ = 0.04266, p = 0.9583
3F	$F_{1,10}$ = 0.8129, p = 0.3885	$F_{1,10}$ = 0.04466, p = 0.8369	$F_{1,10}$ = 0.06597, p = 0.8025
4B	$F_{1,17}$ = 0.3021, p = 0.5897	$F_{2,34}$ = 0.1654, p = 0.8482	$F_{2,34}$ = 0.551, p = 0.5815
4D	$F_{1,12}$ = 0.0002904, p = 0.9867	$F_{2,24}$ = 0.7491, p = 0.4835	$F_{2,24}$ = 0.07709, p = 0.9260
4F	$F_{1,12}$ = 1.903, p = 0.1929	$F_{2,24}$ = 0.5071, p = 0.6086	$F_{2,24}$ = 1.671, p = 0.2093
5B	$F_{1,12}$ = 1.845, p = 0.1993	$F_{2,24}$ = 0.127, p = 0.8813	$F_{2,24}$ = 0.7871, p = 0.4666
5D	**$F_{1,10}$ = 23.31, p = 0.0007*** **	$F_{2,20}$ = 1.737, p = 0.2016	$F_{2,20}$ = 1.921, p = 0.1726
5F	$F_{1,8}$ = 0.02062, p = 0.8894	$F_{2,16}$ = 2.692, p = 0.0982	**$F_{2,16}$ = 3.721, p = 0.0471***
5H	$F_{1,8}$ = 0.3147, p = 0.5902	$F_{2,16}$ = 0.2489, p = 0.7826	$F_{2,16}$ = 2.94, p = 0.0818
6B	$F_{1,12}$ = 0.7557, p = 0.4017	$F_{2,24}$ = 3.07, p = 0.0744	$F_{2,24}$ = 0.5455, p = 0.5900
6D	$F_{1,10}$ = 2.181, p = 0.1705	$F_{2,20}$ = 0.08355, p = 0.9202	$F_{2,20}$ = 1.112, p = 0.3529
6B	**$F_{1,8}$ = 6.008, p = 0.0399***	$F_{2,16}$ = 0.5274, p = 0.6001	$F_{2,16}$ = 0.2469, p = 0.7841
6D	$F_{1,6}$ = 0.1611, p = 0.7020	$F_{2,12}$ = 0.5367, p = 0.5981	$F_{2,12}$ = 2.368, p = 0.1358

3.2. Experiment 1: Dose response of LY2828360 effect in carrageenan-induced inflammatory pain

LY2828360 increased paw withdrawal thresholds ($F_{3,19}$ = 3.904, p = 0.0250) in the carrageenan-injected (*ipsilateral*) paw. Dunnett's multiple comparisons test revealed that 10 mg/kg i.p. LY2828360 increased paw withdrawal thresholds relative to VEH treatment (p = 0.0150), with no difference between VEH and either 1 mg/kg (p = 0.9253) or 3 mg/kg (p = 0.8904) LY2828360 doses detected (Fig. 1A).

3.3. Experiment 2: Are the anti-allodynic effects of LY2828360 preserved in CB_1 KO mice?

In CB_1 KO mice, LY2828360 (10 mg/kg i.p.) increased paw withdrawal thresholds in the carrageenan-injected (*ipsilateral*) paw across the observation interval (Group: $F_{1,13}$ = 34.82, p < 0.0001; Time: $F_{1,13}$ = 34.82, p = 0.2142; Interaction: $F_{1,13}$ = 0.6893, p = 0.4214); Sidak's multiple comparisons test revealed that LY2828360 increased paw withdrawal thresholds at the 4-hr (p = 0.0009) and 6-hr post carrageenan time-point (p = 0.0279) relative to VEH (Fig. 2A).

3.4. Experiment 3: Is anti-allodynic efficacy of a reference agent preserved in CB_2 KO mice?

In C57 WT control mice, gabapentin (50 mg/kg i.p.) increased paw withdrawal thresholds in the carrageenan-injected paw, paw withdrawal thresholds changed over time and the interaction was

significant (Group: $F_{1,14} = 20$, $p = 0.0005$; Time: $F_{2,28} = 7.929$, $p = 0.0019$; Interaction: $F_{2,28} = 7.184$, $p = 0.0030$); Sidak's multiple comparisons test revealed that gabapentin increased mechanical paw withdrawal thresholds compared to VEH at the 6-hr ($p < 0.0001$) and 7-hr post carrageenan timepoints ($p = 0.0010$) (Fig. 3A). In CB_2 KO mice, gabapentin (50 mg/kg i.p.) increased paw withdrawal thresholds in the carrageenan-injected paw in a time-dependent manner (Group: $F_{1,14} = 16.36$, $p = 0.0012$; Time: $F_{2,28} = 3.266$, $p = 0.0531$; Interaction: $F_{2,28} = 4.682$, $p = 0.0176$) compared to vehicle at both the 6- ($p = 0.0001$; Sidak's multiple comparison test) and 7-h post carrageenan timepoints ($p = 0.0021$) (Fig. 3C).

3.5. Experiment 4: Role of CB2 in the anti-allodynic effect of LY2828360

In CB_2 KO mice, LY2828360 (10 mg/kg i.p.) did not alter paw withdrawal thresholds in the carrageenan-injected paw relative to vehicle treatment (Group: $F_{1,10} = 0.05587$, $p = 0.8179$; Time: $F_{1,10} = 1.919$, $p = 0.1961$; Interaction: $F_{1,10} = 2.115$, $p = 0.1765$) (Fig. 3E).

3.6. Experiment 5: Are the anti-allodynic effects of LY2828360 preserved in $CB_2^{f/f}$ mice receiving intraplantar injection of carrageenan?

In $CB_2^{f/f}$ control mice, LY2828360 (10 mg/kg i.p.) increased paw withdrawal thresholds in the carrageenan-injected paw, paw withdrawal thresholds changed over time and the interaction approached significance (Group: $F_{1,17} = 7.832$, $p = 0.0123$; Time: $F_{2,34} = 10.93$, $p = 0.0002$; Interaction: $F_{2,34} = 3.145$, $p = 0.0558$); Sidak's multiple comparisons test revealed that LY2828360 increased mechanical paw withdrawal thresholds compared to VEH at the 6-hr timepoint ($p = 0.0029$) (Fig. 4A).

3.7. Experiment 6: Impact of cKO of CB_2 receptors in microglia and macrophages on the anti-allodynic effect of LY2828360

In $CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mice, paw withdrawal thresholds in the carrageenan-injected paw differed across time (Time: $F_{2,24} = 8.956$, $p = 0.0012$). LY2828360 (10 mg/kg i.p.) trended to alter paw withdrawal thresholds as a function of group and the interaction was significant (Group: $F_{1,12} = 4.562$, $p = 0.0540$; Interaction: $F_{2,24} = 4.729$, $p = 0.0186$);); Sidak's multiple comparisons test revealed that LY2828360

increased mechanical paw withdrawal thresholds compared to VEH at the 6-hr timepoint (p = 0.0046) indicating that anti-allodynic efficacy was preserved in this cKO mouse line (Fig. 4C).

3.8. Experiment 7: Effect of carrageenan in mice with cKO of CB_2 from peripheral sensory neuron

In the absence of pharmacological manipulations, carrageenan lowered paw withdrawal thresholds in the *ipsilateral* (carrageenan-injected) paw of Advillin$^{+/+}$; $CB_2^{f/f}$ and Advillin$^{CRE/+}$; $CB_2^{f/f}$ relative to baseline (Time: $F_{5,60}$ = 22.11, p < 0.0001), consistent with the development of mechanical allodynia. This effect occurred irrespective of genotype (group) and the interaction was not significant (Genotype: $F_{1,12}$ = 1.794, p = 0.2052); Interaction: $F_{5,60}$ = 0.5866, p = 0.7101). Paw withdrawal thresholds did not differ as a function of genotype, time, or interaction in the non-inflamed *contralateral* paw (Group: $F_{1,12}$ = 0.1758, p = 0.6825; Time: $F_{5,60}$ = 1.272, p = 0.2876; Interaction: $F_{5,60}$ = 1.361, p = 0.2519) (Data not shown).

3.9. Experiment 8: Impact of cKO of CB_2 receptors from peripheral sensory neurons on the anti-allodynic effect of LY2828360

In Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice, paw withdrawal threshold in the carrageenan-injected paw differed across time (Time: $F_{2,24}$ = 4.916, p = 0.0162), but LY2828360 (10 mg/kg i.p.) did not alter paw withdrawal thresholds as a function of group and the interaction was not significant (Group: $F_{1,12}$ = 0.2013, p = 0.6617; Interaction: $F_{2,24}$ = 0.4772, p = 0.6263) (Fig. 4E). Thus, anti-allodynic efficacy of LY2828360 was absent in cKO mice following deletion of CB_2 from peripheral sensory neurons.

3.10. Experiment 9: Local paw injection of LY2828360 into the ipsilateral paw of $CB_2^{f/f}$ and Advillin$^{CRE/+}$; $CB_2^{f/f}$ male and female mice

In *female* $CB_2^{f/f}$ mice receiving local injections in ipsilateral paw, LY2828360 (30 µg i.pl.) increased paw withdrawal thresholds in the carrageenan-injected paw, and paw withdrawal thresholds changed over time (Group: $F_{1,11}$ = 16.23, p = 0.0020; Time: $F_{2,22}$ = 12.83, p = 0.0002; Interaction: $F_{2,22}$ = 1.629, p = 0.2190); Sidak's multiple comparisons test revealed that LY2828360 increased mechanical paw withdrawal thresholds compared to VEH at the 6-hr (p = 0.0107) and 7-hr (p = 0.0051) timepoints (Fig. 5A). In *female* Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice receiving injections in ipsilateral paw, paw withdrawal

threshold in the carrageenan-injected paw differed across time (Time: $F_{2,20} = 17.93$, $p < 0.0001$), but LY2828360 (30 μg i.pl.) did not alter paw withdrawal thresholds as a function of group or interaction (Group: $F_{1,10} = 0.682$, $p = 0.4282$; Interaction: $F_{2,20} = 0.2997$, $p = 0.7443$) (Fig. 5C).

In *male* $CB_2^{f/f}$ mice receiving injections in ipsilateral paw, paw withdrawal thresholds in the carrageenan-injected paw differed across time and the interaction was significant (Time: $F_{2,16} = 29.16$, $p < 0.0001$; Interaction: $F_{2,16} = 6.338$, $p = 0.0094$), but LY2828360 (30 μg i.pl.) did not alter paw withdrawal thresholds as a function of group (Group: $F_{1,8} = 2.781$, $p = 0.1339$); Sidak's multiple comparisons test revealed that paw withdrawal thresholds were higher in the ipsilateral-LY2828360 paw injection groups relative to ipsilateral-VEH and at the 6-hr ($p = 0.0410$) timepoint (Fig. 5E). In *male* Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice receiving injections in ipsilateral paw, paw withdrawal threshold in the carrageenan-injected paw differed across time (Time: $F_{2,16} = 7.42$, $p = 0.0052$), but LY2828360 (30 μg i.pl.) did not alter paw withdrawal thresholds as a function of group or interaction (Group: $F_{1,8} = 0.0011$, $p = 0.9744$; Interaction: $F_{2,16} = 0.0845$, $p = 0.9194$) (Fig. 5G). Thus, local injection of LY2828360 in the carrageenan-injected paw failed to suppress mechanical allodynia in either *female* or *male* Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice.

3.11. Experiment 10: Local paw injection of LY2828360 in <u>contralateral</u> paw of $CB_2^{f/f}$ and Advillin$^{CRE/+}$; $CB_2^{f/f}$ male and female mice

In *female* $CB_2^{f/f}$ mice receiving injections in contralateral paw, paw withdrawal thresholds in the carrageenan-injected paw differed across time (Time: $F_{2,22} = 16.68$, $p < 0.0001$), but LY2828360 (30 μg i.pl.) did not alter paw withdrawal thresholds as a function of group and the interaction was not significant (Group: $F_{1,11} = 4.074$, $p = 0.0686$; Interaction: $F_{2,22} = 0.739$, $p = 0.4891$ (Fig. 6A). In *female* Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice receiving injections in contralateral paw, withdrawal thresholds in the carrageenan-injected paw did not differ as a function of group, time or interaction (Group: $F_{1,10} = 2.198$, $p = 0.1690$; Time: $F_{2,20} = 0.3946$, $p = 0.6791$; Interaction: $F_{2,20} = 0.05654$, $p = 0.9452$) (Fig. 6C).

In *male* $CB_2^{f/f}$ mice receiving injections in contralateral paw, withdrawal thresholds in the carrageenan-injected paw did not differ as a function of group, time or interaction (Group: $F_{1,8} = 0.09111$,

p = 0.7705; Time: $F_{2,16}$ = 0.3005, p = 0.7446; Interaction: $F_{2,16}$ = 0.01146, p = 0.9886) (Fig. 6E). In *male* Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice receiving injections in contralateral paw, LY2828360 (30 µg i.pl.) increased paw withdrawal thresholds in the carrageenan-injected paw, and paw withdrawal thresholds changed over time but the interaction was not significant (Group: $F_{1,6}$ = 14.01, p = 0.0096; Time: $F_{2,12}$ = 19.76, p = 0.0002; Interaction: $F_{2,12}$ = 0.2941, p = 0.7504) (Fig. 6G).

3.12. Experiment 11: Impact of intraplantar injection of LY2828360 in the absence of carrageenan

In the absence of carrageenan injection, paw withdrawal thresholds did not differ as a function of group, time, or interaction in the saline-injected paw of C57 WT mice before (Group: $F_{1,8}$ = 0.2198, p = 0.6517 ; Time: $F_{1,8}$ = 1.216, p = 0.2532 ; Interaction: $F_{1,8}$ = 1.526, p = 0.2518) or after (Group: $F_{1,8}$ = 0.07089, p = 0.7968 ; Time: $F_{2,16}$ = 1.431, p = 0.2681; Interaction: $F_{2,16}$ = 0.5819, p = 0.5702) pharmacological treatments. Paw withdrawal thresholds did not differ as a function of group, time, or interaction in the non-injected paw of C57 WT mice that received local injections of saline either before (Group: $F_{1,8}$ = 0.9938, p = 0.3480 ; Time: $F_{1,8}$ = 4.295, p = 0.0719 ; Interaction: $F_{1,8}$ = 0.2152, p = 0.6551) or after (Group: $F_{1,8}$ = 0.02304, p = 0.8831 ; Time: $F_{2,16}$ = 0.08789, p = 0.9163 ; Interaction: $F_{2,16}$ = 0.5543, p = 0.5851) pharmacological treatments (Data not shown).

3.13. Experiment 12: Impact of LY2828360 on mRNA expression levels of inflammatory-related markers following intraplantar injection of carrageenan

Carrageenan injection in the paw increased mRNA expression levels of cytokines IL-1β ($F_{2,21}$ = 28.59, p < 0.0001), IL-10 ($F_{2,21}$ = 46.16, p < 0.0001), TNFα ($F_{2,21}$ = 25.76, p < 0.0001) and the chemokine MCP1 ($F_{2,21}$ = 22.65, p < 0.0001) in the injected (*ipsilateral*) paw without changing levels of CB_2 mRNA ($F_{2,21}$ = 2.219, p = 0.1336) (Fig. 7A). Tukey's multiple comparison test revealed that carrageenan increased mRNA expression levels of IL-1β, IL-10, TNFα and MCP1 in the VEH (i.p.) injected group vs. the naïve (no carrageenan) group (p < 0.0001 for all comparisons). LY2828360 also decreased mRNA expression levels of IL-1β and IL-10 in the carrageenan-treated groups that received LY2828360 (i.p) relative to VEH (i.p.) injections (p < 0.04 for all comparisons). No changes were observed in mRNA

expression levels of any of these markers in paw skin derived from the non-injected *contralateral* paw ($p > 0.2$ for all comparisons; Fig. 7B). Carrageenan injection increased mRNA expression levels of IL-1β ($F_{2,21} = 8.944$, $p = 0.0015$) and MCP1 ($F_{2,21} = 4.435$, $p = 0.0247$) in the lumbar spinal cord without changing levels of IL-10 ($F_{2,21} = 2.752$, $p = 0.0868$), TNFα ($F_{2,21} = 0.2378$, $p = 0.7905$) or CB_2 ($F_{2,21} = 1.024$, $p = 0.3764$) mRNA (Fig. 7C). Tukey's multiple comparison test revealed that carrageenan increased mRNA expression of IL-1β in the VEH (i.p.) injected group relative to the naïve group ($p = 0.0173$). Carrageenan injection increased mRNA expression levels of IL-1β ($F_{2,19} = 18.74$, $p < 0.0001$) in the spleen without changing levels of IL-10 ($F_{2,20} = 2.567$, $p = 0.1018$), TNFα ($F_{2,20} = 0.7415$, $p = 0.4890$), MCP1 ($F_{2,20} = 1.116$, $p = 0.3472$) or CB_2 ($F_{2,20} = 0.2507$, $p = 0.7807$) mRNA (Fig. 7D). Tukey's multiple comparison test revealed that carrageenan increased mRNA expression of IL-1β in the VEH injected group vs naïve group ($p = 0.0006$).

3.14. Experiment 13: qRT-PCR analysis to assess the impact of intraplantar carrageenan in the lumbar spinal cord of mice lacking CB_2 in Advillin^{CRE} expressing cells

Table 3. Summary of statistical analyses of qRT-PCR data used to assess the impact of LY2828360 on inflammatory markers following intraplantar injection of carrageenan in mice lacking CB_2 in Advillin^{CRE} expressing cells (Experiment 15)

Marker	Genotype	Drug	Interaction
Ipsilateral paw			
IL-1β	$F_{1,16} = 0.07951$, $p = 0.7816$	$F_{1,16} = 0.02824$, $p = 0.8687$	$F_{1,16} = 0.6682$, $p = 0.4257$
IL-10	$F_{1,16} = 0.3808$, $p = 0.5458$	$F_{1,16} = 0.5689$, $p = 0.4616$	$F_{1,16} = 0.09311$, $p = 0.7642$
TNFa	$F_{1,16} = 1.317$, $p = 0.2680$	$F_{1,16} = 0.09746$, $p = 0.7589$	$F_{1,16} = 0.1154$, $p = 0.7385$
MCP1	$F_{1,16} = 0.0001717$, $p = 0.9897$	$F_{1,16} = 0.7937$, $p = 0.3862$	$F_{1,16} = 0.3792$, $p = 0.5467$
Contralateral paw			
IL-1β	$F_{1,15} = 1.197$, $p = 0.2911$	$F_{1,15} = 0.1709$, $p = 0.6852$	$F_{1,15} = 2.981$, $p = 0.1048$
IL-10	$F_{1,16} = 0.4288$, $p = 0.5219$	$F_{1,16} = 0.1886$, $p = 0.6699$	$F_{1,16} = 1.99$, $p = 0.1775$
TNFa	$F_{1,16} = 3.017$, $p = 0.1016$	$F_{1,16} = 0.3696$, $p = 0.5518$	$F_{1,16} = 0.05098$, $p = 0.8242$
MCP1	$F_{1,16} = 0.2327$, $p = 0.6361$	$F_{1,16} = 1.07$, $p = 0.3163$	$F_{1,16} = 0.1399$, $p = 0.7133$
Lumbar spinal cord			
IL-1β	$F_{1,15} = 0.1067$, $p = 0.7485$	$F_{1,15} = 0.538$, $p = 0.4746$	$F_{1,15} = 1.079$, $p = 0.3155$
IL-10	$F_{1,15} = 1.904$, $p = 0.1878$	$F_{1,15} = 0.3604$, $p = 0.5572$	$F_{1,15} = 0.4234$, $p = 0.5251$
TNFa	$F_{1,15} = 1.736$, $p = 0.2075$	$F_{1,15} = 0.3809$, $p = 0.5464$	$F_{1,15} = 2.095$, $p = 0.1684$
MCP1	$F_{1,16} = 0.003402$, $p = 0.9542$	$F_{1,16} = 0.7486$, $p = 0.3997$	$F_{1,16} = 0.7826$, $p = 0.3894$
Spleen			
IL-1β	$F_{1,15} = 1.535$, $p = 0.2343$	$F_{1,15} = 2.46$, $p = 0.1376$	$F_{1,15} = 1.695$, $p = 0.2126$
IL-10	$F_{1,15} = 0.88$, $p = 0.3631$	$F_{1,15} = 0.1186$, $p = 0.7353$	$F_{1,15} = 1.236$, $p = 0.2838$
TNFa	$F_{1,15} = 3.732$, $p = 0.0725$	$F_{1,15} = 0.3165$, $p = 0.5820$	$F_{1,15} = 0.6876$, $p = 0.4200$
MCP1	$F_{1,15} = 0.05494$, $p = 0.8178$	$F_{1,15} = 0.1425$, $p = 0.7111$	$F_{1,15} = 0.4086$, $p = 0.5323$

Intraplantar carrageenan increased MCP1 mRNA levels relative to intraplantar saline in the lumbar spinal cord of Advillin$^{CRE/+}$; CB$^{f/f}$ mice ($F_{1,20} = 5.541$, $p = 0.0289$), but no difference in genotype or

interaction was observed (Genotype: $F_{1,20}$ = 3.03, p = 0.0971; Interaction: $F_{1,20}$ = 2.259, p = 0.1485) (Fig. 8C). Sidak's multiple comparison test revealed that carrageenan increased MCP1 mRNA levels increased in the lumbar spinal cord of carrageenan-injected Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice compared to saline-injected Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice (p = 0.0197). Levels of IL-1β (Genotype: $F_{1,19}$ = 0.4429, p = 0.5137; Treatment: $F_{1,19}$ = 0.4526, p = 0.5092; Interaction: $F_{1,19}$ = 0.6662, p = 0.4245) and TNFα (Genotype: $F_{1,20}$ = 0.846, p = 0.3686; Treatment: $F_{1,20}$ = 4.237, p = 0.0528; Interaction: $F_{1,20}$ = 0.6048, p = 0.4459) mRNA expression levels did not differ in lumbar spinal cord as a function of carrageenan injection or genotype and the interaction was not significant (Fig. 8A-B).

3.15. Experiment 14: Impact of LY2828360 on mRNA expression levels of inflammatory markers following intraplantar injection of carrageenan in mice lacking CB_2 in Advillin^{CRE} expressing cells

mRNA expression levels of IL-1β, IL-10, TNFα and MCP1 did not differ as a function of genotype, drug treatment or interaction in carrageenan-injected $CB_2^{f/f}$ and Advillin$^{CRE/+}$; $CB2^{f/f}$ mice receiving LY2828360 (10 mg/kg i.p.) or vehicle in any tissue evaluated (i.e. ipsilateral paw skin, contralateral paw skin, lumbar spinal cord or spleen). However, mRNA expression levels of TNFα trended to increase in the spleen of Advillin$^{CRE/+}$; $CB2^{f/f}$ mice relative to $CB^{2f/f}$ mice similarly receiving intraplantar carrageenan injections (Genotype: $F_{1,15}$ = 3.732, p = 0.0725) (Table 3).

4. Discussion

Activation of the cannabinoid CB_2 receptor suppresses pathological pain in a variety of animal models (e.g. Guindon and Hohmann, 2008; Murineddu et al., 2013; Cabañero et al., 2021; Soliman et al. 2021). However, lack of knowledge about the types of pain most responsive to CB_2 agonists and cell types that underlie the therapeutic effects of CB_2 agonists, amongst other factors (e.g. biased signaling of CB_2 agonists, species differences in agonist efficacy), have hindered prospects for successful clinical translation. We previously reported that the slowly-signaling G protein-biased CB_2 receptor agonist LY2828360 effectively reduced paclitaxel-(Lin et al., 2018; Lin et al., 2022) and anti-retroviral- (Carey et

al. 2023) induced neuropathic nociception. However, it was not known whether LY2828360 attenuated inflammatory nociception and if its efficacy varied by sex. Here we show that the CB_2 receptor agonist LY2828360 effectively reduces mechanical allodynia in a carrageenan-induced inflammatory pain model at a dose of 10 mg/kg i.p., but not at lower doses, suggesting that it is less potent in reversing carrageenan-induced inflammatory nociception compared to paclitaxel- or 2,3-dideoxycytidine (ddC)-induced neuropathic nociception (Lin et al., 2018; Lin et al., 2022; Carey et al., 2023). This effect was CB_2 receptor dependent as anti-allodynic efficacy was absent in CB_2 receptor KO mice and preserved in CB_1 receptor KO mice. Thus, the antiallodynic effects of LY2828360 are mediated by CB_2 receptors and independent of CB_1 receptors. By contrast, the anticonvulsant drug gabapentin which is also prescribed as a treatment for neuropathic pain (Bennett and Simpson, 2004) was equally efficacious in suppressing carrageenan-induced allodynia in both wild type and CB_2 receptor KO mice. This observation suggests that, unlike LY2828360, the anti-allodynic effect of gabapentin in suppressing the maintenance of carrageenan-induced inflammatory nociception was independent of the CB_2 receptor.

To further investigate the cell types responsible for the anti-allodynic effect of LY2828360, we tested its efficacy in mice lacking CB_2 receptors in specific populations of cells after establishment of carrageenan-induced inflammatory nociception. LY2828360 (10 mg/kg i.p.) failed to suppress carrageenan-induced mechanical allodynia in mice with conditional deletion of the CB_2 receptor from peripheral sensory neurons ($Advillin^{CRE/+}$; $CB_2^{f/f}$ mice) at the dose tested but retained efficacy in mice with conditional deletion of CB_2 from microglia and macrophages expressing CX3CR1 ($CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mice) and control mice ($CB_2^{f/f}$). Thus, activation of CB_2 receptors in peripheral sensory neurons is necessary for the anti-allodynic effects of LY2828360 in a mouse model of carrageenan-induced inflammatory pain, whereas CB_2 receptors in CX3CR1-expressing microglia/macrophages are not required for anti-allodynic efficacy under our experimental conditions. Our results do not preclude the possibility that higher doses of LY2828360 or different CB_2 agonists might be effective in $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice or implicated in other pain models. Additionally, given our $CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mouse model

is specific to microglia or macrophages expressing the protein coding gene C-X3-C Motif Chemokine Receptor 1 our results do not preclude a role for other cell types/classes of microglia in contributing to the anti-allodynic effect in our studies. The retained efficacy of LY2828360 in CX3CR1$^{CRE/+}$; $CB_2^{f/f}$ mice is likely due to lack of involvement of CB_2 receptors in CX3CR1-expressing microglial/macrophages in the ability of this agonist to reduce carrageenan-induced inflammatory pain and not because of a failure of the CB_2 receptor conditional KO mouse model used; this mouse model has been used successfully to unmask behavioral phenotypes mediated by CB_2 agonists in other studies in our labs (Behlke et al., 2022 and unpublished data). Moreover, because $CB_2^{f/f}$ and Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice do not differ in their development of carrageenan-induced inflammatory pain, we conclude that the results seen in mice lacking CB_2 receptors in peripheral sensory neurons is specifically due to the loss of efficacy of LY2828360 in the cKO and not due to loss of CB_2 receptors in sensory neurons affecting the development of inflammatory nociception. We previously reported that otherwise naïve Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice show lower levels of mRNA for CB_2 and GFP in DRG, but not paw skin, lumbar spinal cord or spleen, indicating that the cKO is selective for CB_2 in peripheral sensory neurons (Carey et al., 2023).

Local administration of CB_2 receptor agonists in the paw reduces pain behavior in naïve rats in the plantar test as well as in diverse models of inflammatory pain (Malan et al., 2001, 2002; Quartilho et al., 2003; Gutierrez et al., 2007). In the present studies, peripheral mechanisms of anti-allodynic efficacy were also evaluated by using local administration of LY2828360 into the carrageenan-treated hind paw of $CB_2^{f/f}$ mice and Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice. LY2828360 injected into the carrageenan-inflamed paw reduced mechanical allodynia compared to VEH (i.pl.) treatment in $CB_2^{f/f}$ but not Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice. This observation provides further evidence that anti-allodynic efficacy of LY2828360 is dependent on CB_2 receptors arising from DRG sensory neurons and provides additional evidence that LY2828360 can exert its effect at the local site of injury. Conversely, when LY2828360 was injected into the paw opposite to the site of injury (contralateral paw) it did not alter responsiveness relative to VEH groups in either Advillin$^{CRE/+}$; $CB_2^{f/f}$ or Advillin$^{+/+}$; $CB_2^{f/f}$ mice. These observations suggest that LY2828360 was

unlikely to reach the systemic circulation when injected locally into the paw and confirm our results suggesting that intraplantar injection of LY2828360 in the carrageenan-injected paw is suppressing allodynia via a local site of action. Our data also suggest that an intraplantar LY2828360 (30 µg i.pl.) injection does not produce an antinociceptive effect (i.e. in our assessments of mechanical paw withdrawal thresholds) in the absence of carrageenan, an effect that we confirmed by injecting LY2828360 into the paw of WT mice in the absence of carrageenan-induced inflammation.

Carrageenan injected locally in the paw increased mRNA expression levels of pro-inflammatory cytokines IL-1β and TNFα, the anti-inflammatory cytokine IL-10 and the chemokine MCP1 selectively in the paw skin ipsilateral to carrageenan injection. Activation of the CB_2 receptor by LY2828360 suppressed mRNA expression levels of cytokines that are involved in carrageenan's response, specifically the pro-inflammatory cytokine IL-1β and the anti-inflammatory cytokine IL-10 in the carrageenan-injected paw skin compared to VEH treatment. Moreover, unlike in the WT mice, LY2828360 did not modulate these cytokines in either $CB_2^{f/f}$ or $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice. Differing results between these two studies may be because of different time points of tissue collection of these two experiments or a genotype difference between the WT and the $CB_2^{f/f}$ mice. Additionally, our results were not powered to detect sex differences in studies in which mixed sexes were combined.

Few studies examining the effect of carrageenan on IL-10 in the paw skin have been published and none have examined the impact of CB_2 receptor agonists on IL-10 modulation in this tissue. qRT-PCR studies have shown both a decrease or no change in mRNA levels of the anti-inflammatory cytokine IL-10 in the paw skin following carrageenan injection (Zhang et al., 2020; Gong et al., 2009) and increase in response to CB_2 receptor activation (eg. Robinson et al., 2015; Saroz et al., 2019). The carrageenan-induced increase in IL-10 mRNA observed in our study may be due to dose of carrageenan employed, species used or reflect the timepoint at which we collected tissue for our qRT-PCR studies. Modulation of these cytokines is a dynamic process a single timepoint is not representative of the entire process. Future

studies using qRT-PCR should use tissues collected at several timepoints to get a more comprehensive understanding of how these cytokines are modulated by carrageenan and CB_2 receptor activation in paw skin tissue.

Our results provide the first evidence that LY2828360 suppresses inflammatory nociception through a CB_2-dependent mechanism in mice. Our findings align with previous research from our lab and others suggesting that CB_2 receptor agonists can reduce inflammatory pain behaviours and neurochemical markers of inflammation-evoked neuronal activation in lumbar spinal cord (Nackley, Makriyannis and Hohmann, 2003; Clayton et al., 2002; Elmes et al., 2005), extending the results to both sexes. LY2828360 is of particular interest as a CB_2 receptor agonist because it has recently been shown to reduce neuropathic nociception through a CB_2-mediated mechanism without producing tolerance and can also prevent tolerance to morphine (Lin et al., 2018; Lin et al., 2022; Carey et al., 2023). LY2828360 exhibits functional selectivity as a G protein-biased agonist that does not cause internalization of the CB_2 receptor or arrestin recruitment (Lin et al., 2018). More work is necessary to determine if the biased nature of this agonist specifically engages therapeutically relevant pathways. It is possible that the choice of therapeutic indication (i.e. osteoarthritis) rather than the small molecule or receptor target accounts for why LY2828360 failed for efficacy in a phase 2 clinical trial for knee pain due to osteoarthritis (Pereira et al., 2013). Lastly, more work is necessary to examine the impact of LY2828360 on hindpaw edema. Our results contribute to an emerging body of literature (Lin et al., 2018; Lin et al., 2022; Carey et al., 2023) which collectively suggest that the clinical application of this small molecule CB_2 agonist should be re-evaluated in neuropathic or inflammatory pain conditions.

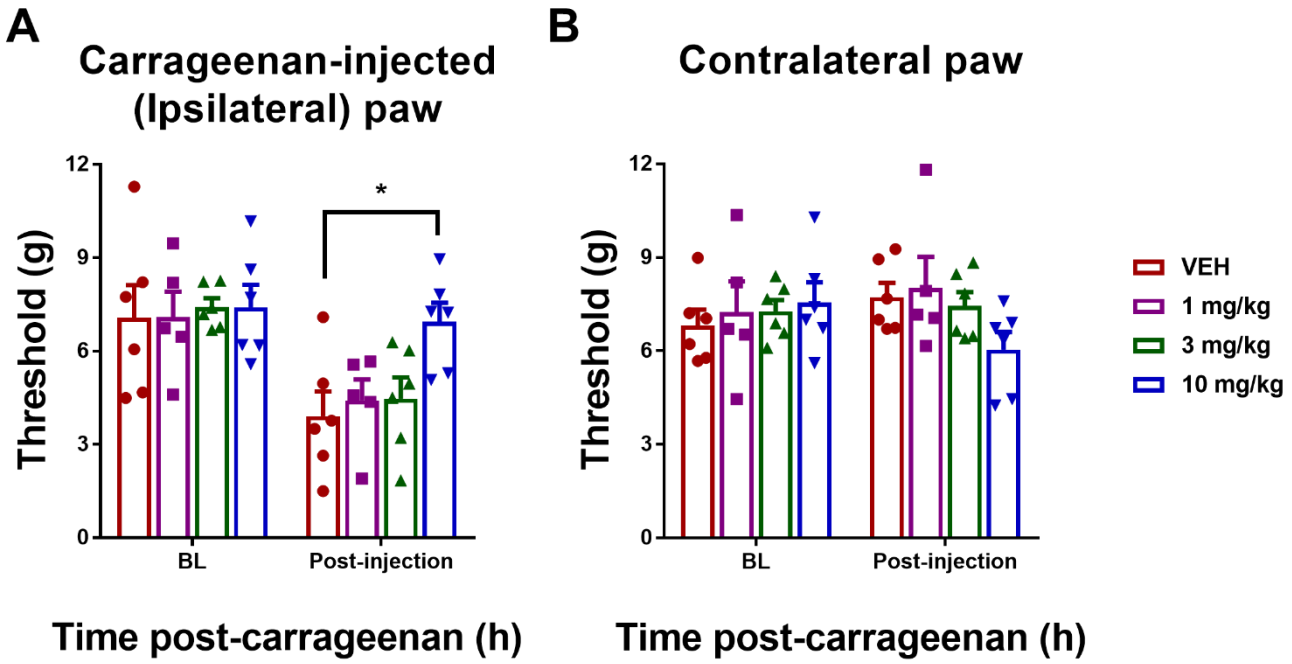
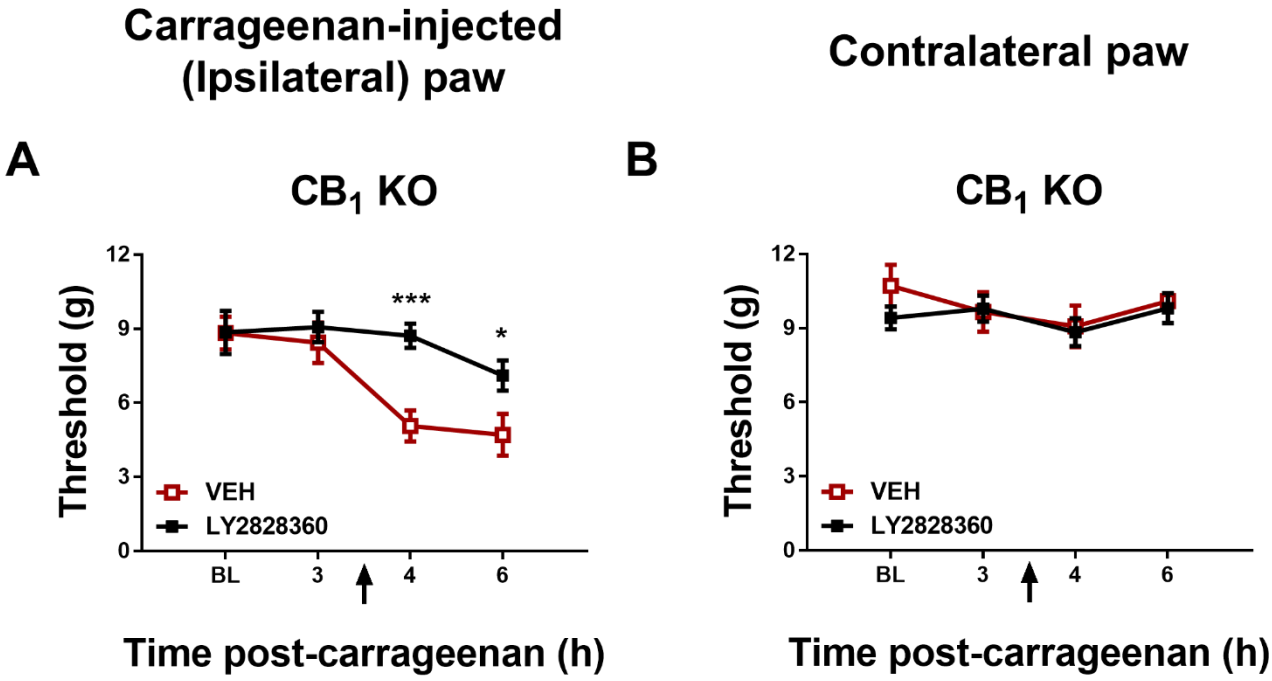


Fig. 1. Dose response of LY2828360 in suppressing carrageenan-induced mechanical allodynia in female C57 WT mice (n = 6 per group). LY2828360 reduced carrageenan-induced mechanical allodynia at a dose of 10 mg/kg i.p. but not at lower doses (1 or 3 mg/kg i.p.). LY2828360 (10 mg/kg) increased paw withdrawal thresholds in the ipsilateral paw (A) but not the contralateral paw (B) in a mouse model of carrageenan-induced inflammatory pain. Data show mean (± SEM) threshold for the *ipsilateral* and *contralateral* paw in Experiment 1. *$p < 0.05$ vs. VEH; ###$p < 0.001$ vs. baseline (BL).



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531 **Fig. 2.** LY2828360 (10 mg/kg i.p.) increased mechanical paw withdrawal thresholds in the ipsilateral (A)

532 but not contralateral (B) paw of female CB_1 KO mice in a mouse model of carrageenan-induced

533 inflammatory pain (n = 7-8 per group). Data show mean (± SEM) threshold for the *ipsilateral* and

534 *contralateral* paw in Experiment 2. *$p < 0.05$, ***$p < 0.001$ vs. VEH.

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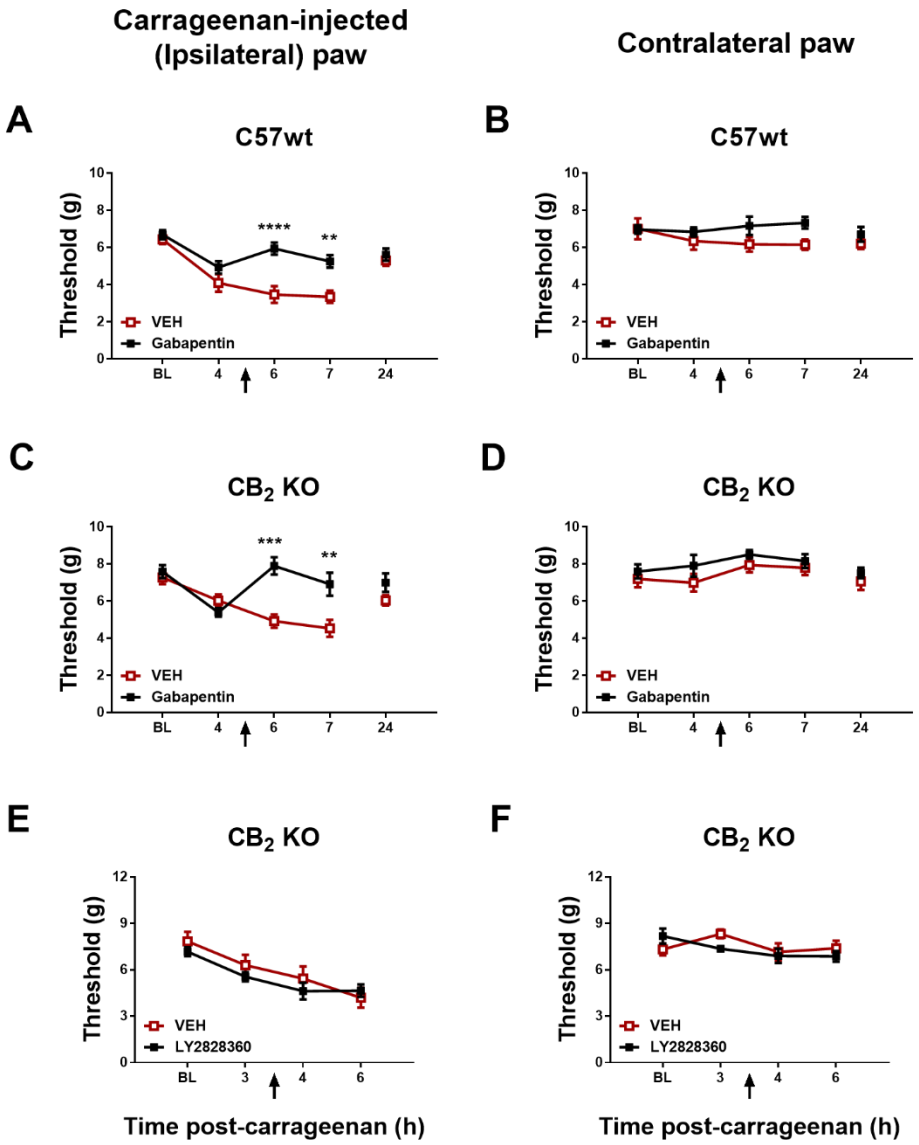

Fig. 3. Gabapentin (50 mg/kg i.p.) increased mechanical paw withdrawal thresholds in the paw ipsilateral (A) or contralateral (B) to carrageenan injection in female C57 WT and CB_2 KO mice (n = 8 per group). LY2828360 (10 mg/kg i.p.) did not alter paw withdrawal thresholds in the paw ipsilateral (A) or contralateral (B) to carrageenan injection in female CB_2 KO mice (n = 6 per group). Mean (± SEM) threshold for the *ipsilateral* and *contralateral* paw in Experiment 3 and 4. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. VEH.

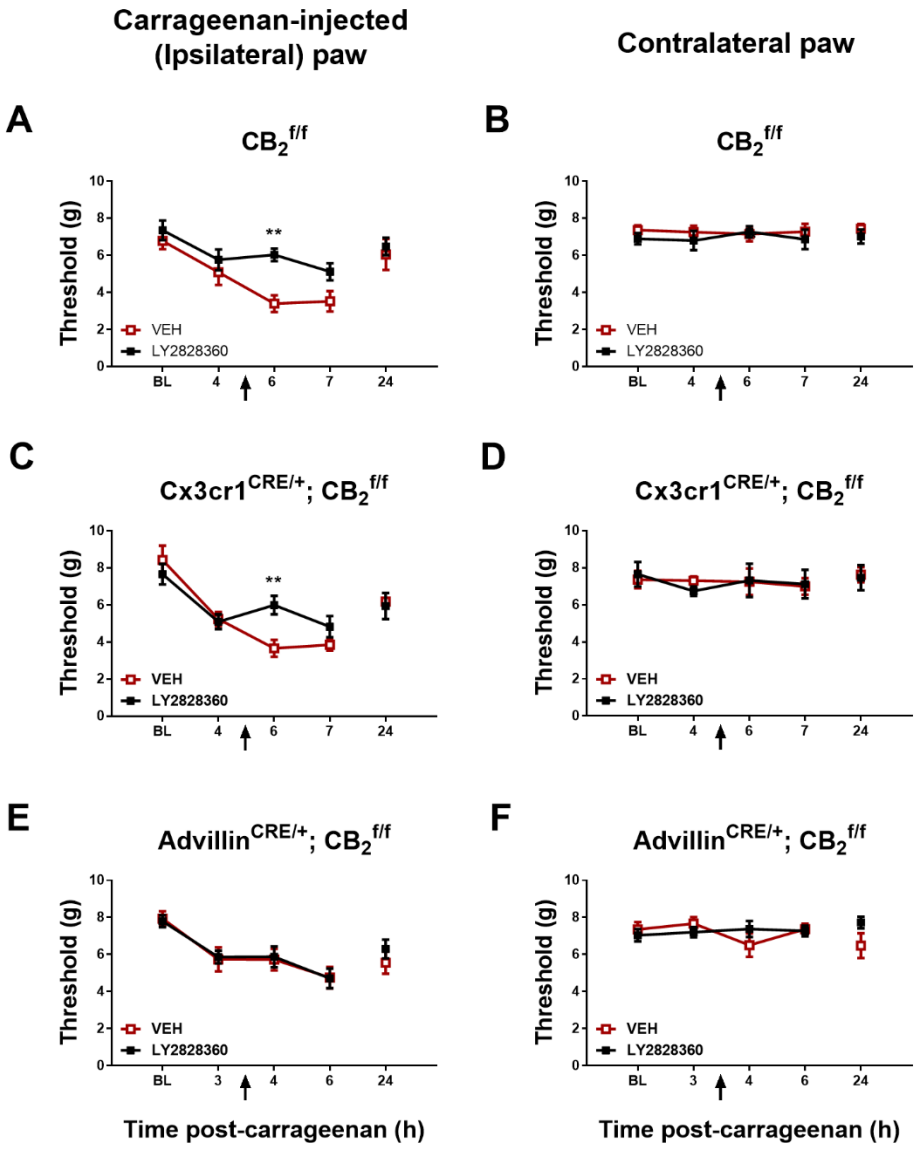

Fig. 4. LY2828360 (10 mg/kg i.p.) increased mechanical paw withdrawal thresholds in paw ipsilateral but not contralateral to carrageenan injection in female $CB_2^{f/f}$ and $CX3CR1^{CRE/+}$; $CB_2^{f/f}$ mice (n = 4-10 per group). LY2828360 (10 mg/kg i.p.) did not alter paw withdrawal thresholds in paw ipsilateral or contralateral to carrageenan injection in female $Advillin^{CRE/+}$; $CB_2^{f/f}$ mice (n = 7 per group). Mean (± SEM) threshold for the *ipsilateral* and *contralateral* paw in Experiment 5, 6, 7 and 9. $^{**}p < 0.01$ vs. VEH.

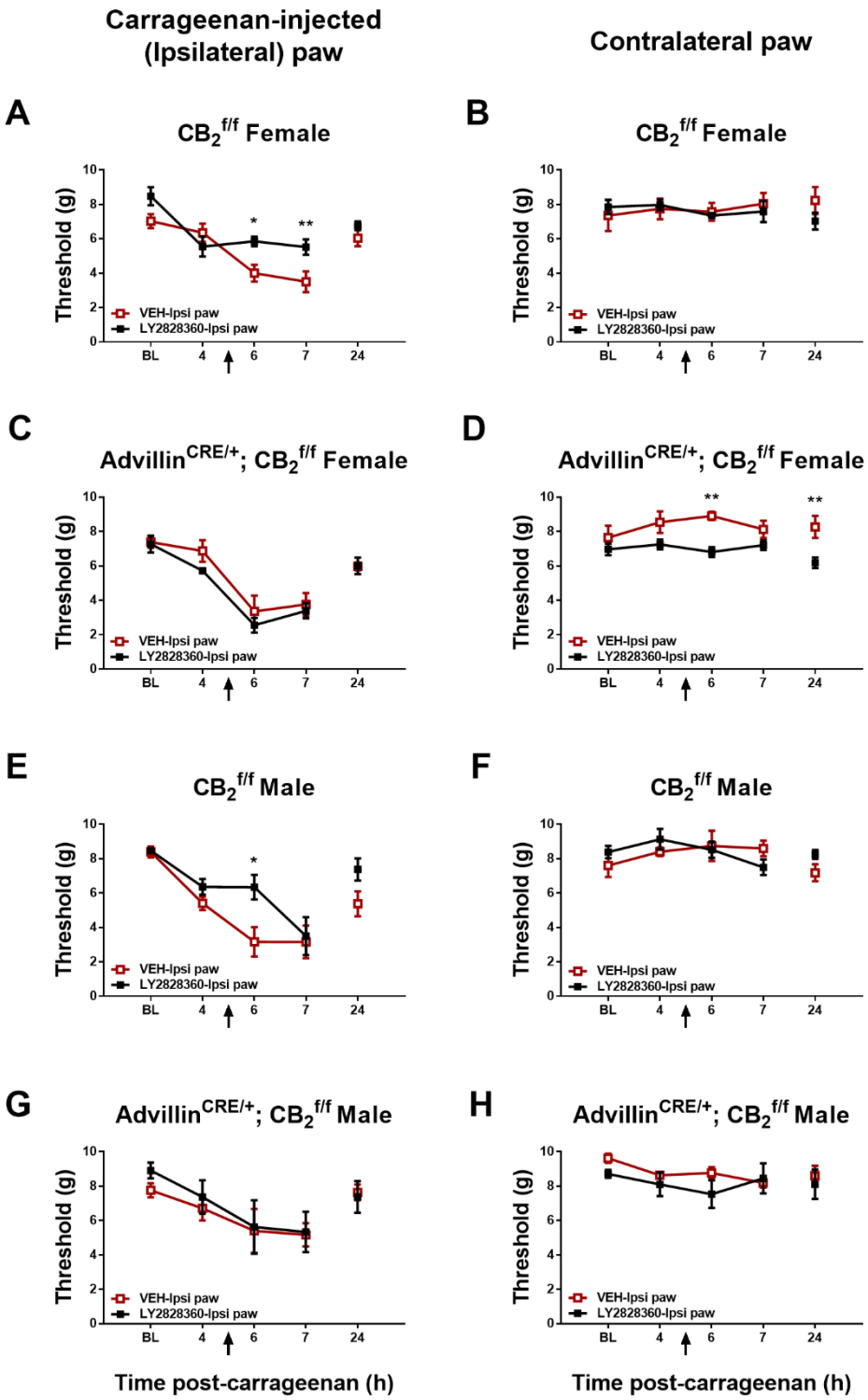

Fig. 5. Impact of *ipsilateral* intraplantar LY2828360 (30 µg i.pl.) injection on paw withdrawal thresholds in the paw ipsilateral or contralateral to carrageenan injection in male and female $CB_2^{f/f}$ mice and

555 Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice (n = 4-5 per group). Mean (± SEM) threshold for the *ipsilateral* and

556 *contralateral* paw in Experiment 9. *$p < 0.05$, **$p < 0.01$ vs. VEH-ipsi paw.

557

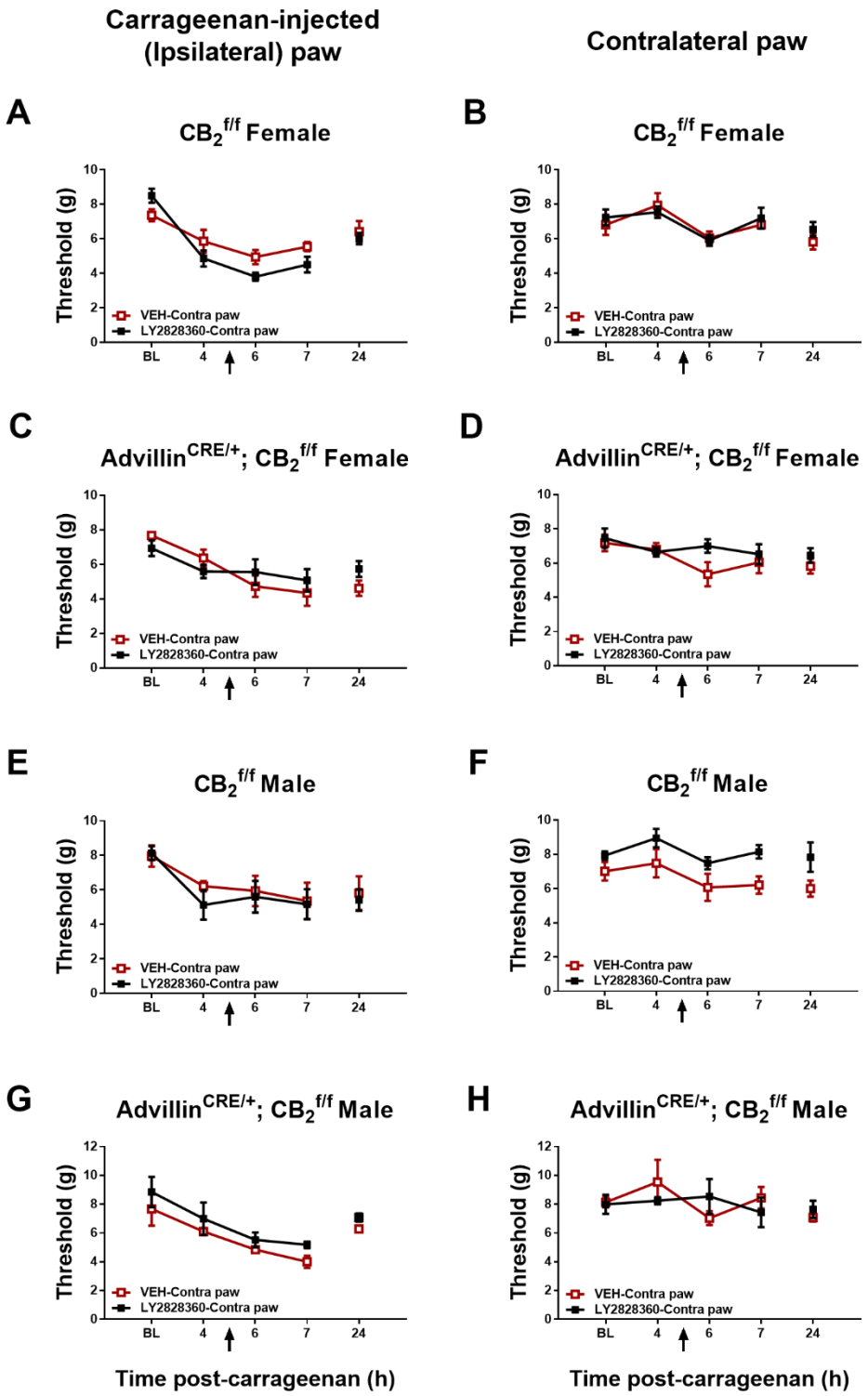

Fig. 6. Impact of *contralateral* intraplantar LY2828360 (30 µg i.pl.) injection on paw withdrawal thresholds in the paw ipsilateral or contralateral to carrageenan injection in female and male $CB_2^{f/f}$ and

562 Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice (n = 4-5 per group). Mean (± SEM) threshold for the *ipsilateral* and

563 *contralateral* paw in Experiment 10.

564

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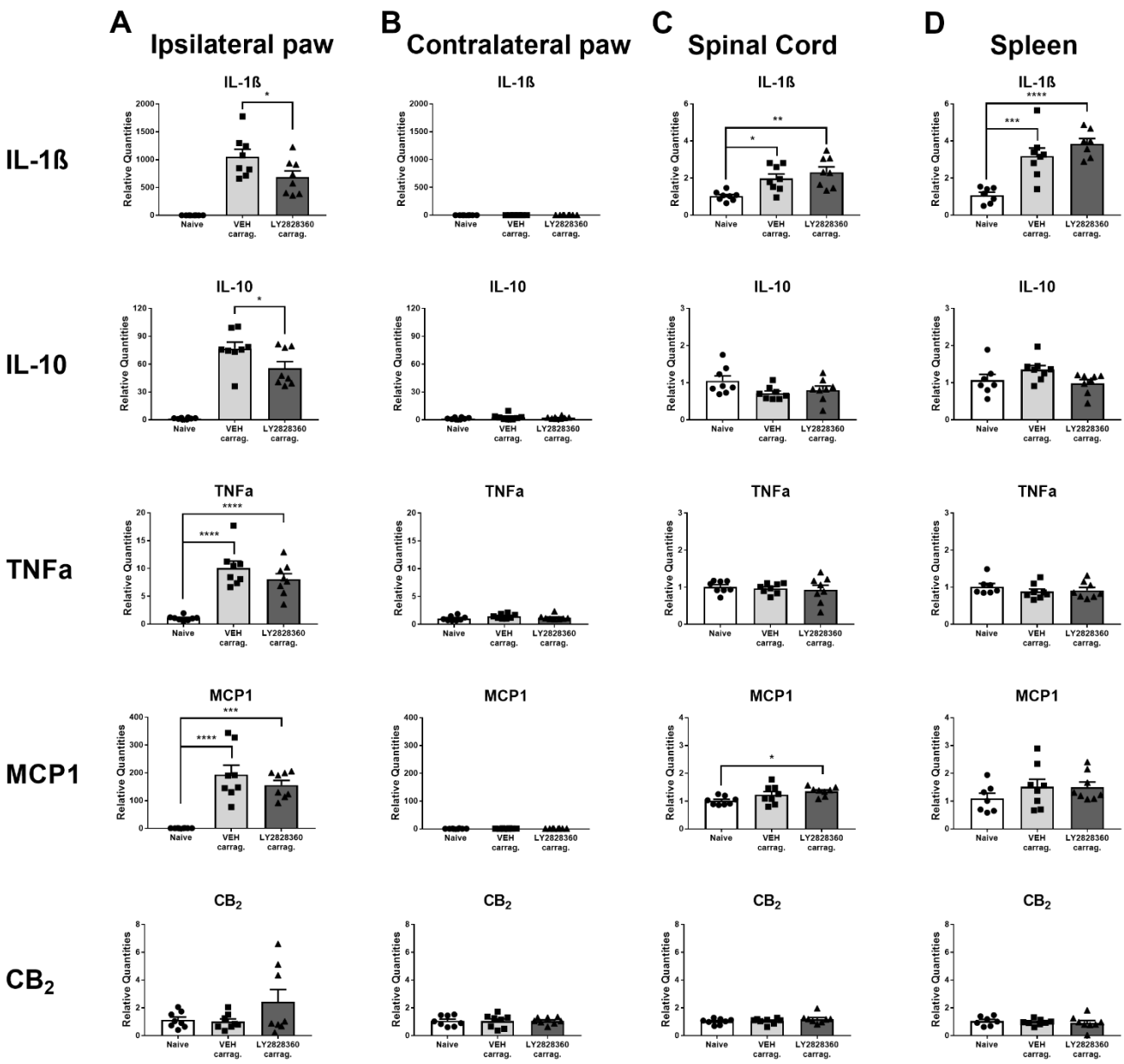

Fig. 7. Intraplantar carrageenan increases levels of IL-1β, IL-10, TNFα and MCP1 mRNAs in the ipsilateral paw. Intraplantar carrageenan increased IL-1β and MCP1 mRNAs in the lumbar spinal cord and increased IL-1β mRNA in the spleen of female WT mice. LY2828360 (10 mg/kg i.p.) reduced IL-1β and IL-10 mRNA expression compared to VEH in the ipsilateral paw but did not affect mRNA expression levels of any marker in the contralateral paw, spinal cord or the spleen compared to VEH (n = 8 per

574 group). Mean (± SEM) $2^{-\Delta\Delta CT}$ values for Experiment 12. Asterisk indicates a group difference; *$p < 0.05$,

575 ***$p < 0.001$, ****$p < 0.0001$.

576

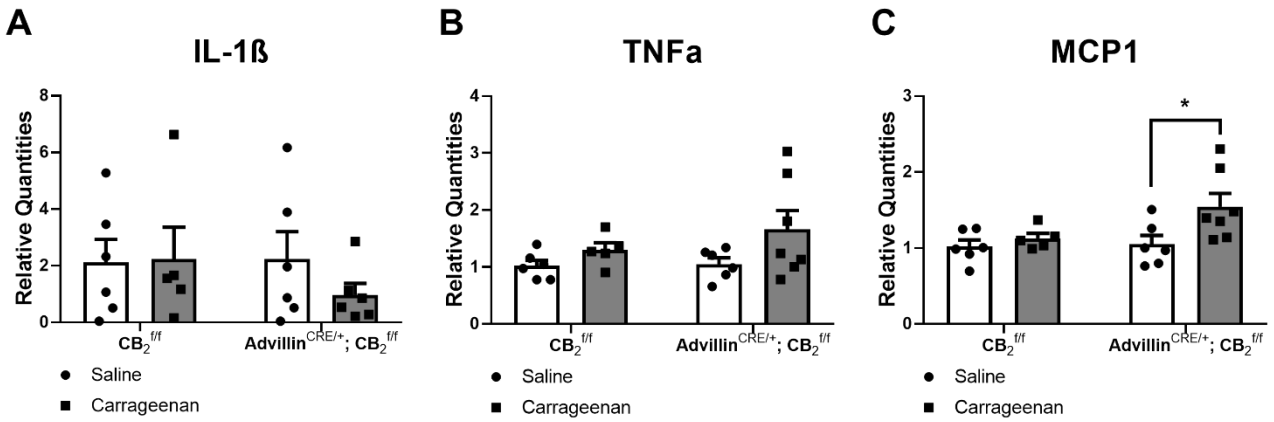

Fig. 8. Carrageenan increased MCP1 mRNA expression levels in the lumbar spinal cord of male and female Advillin$^{CRE/+}$; $CB_2^{f/f}$ mice. Mean (± SEM) $2^{-\Delta\Delta CT}$ values for Experiment 13 (n = 5-6 per group). Asterisk indicates a group difference; *$p < 0.05$, *$p < 0.05$ vs. saline.

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