

Targeting C1q Prevents Microglia-Mediated Synaptic Removal in Neuropathic Pain

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript from Yousefpour et al. describes a very interesting study on the role of microglia in engulfing and phagocytosing synaptic elements, leading to synapse pruning and remodelling of spinal excitatory and inhibitory connectivity. This study addresses very important questions in the field and employs several elegant methods. Microscopy/morphological analyses are performed at a very high level of expertise. Overall, this study has the potential to make an important impact on the field.

However, the study fails to convince at present owing to apparent internal contradictions and lack of mechanistic insights, which are necessary for significant advance in our understanding of neuropathic pain. These are discussed in detail below.

1. There are a number of concerns regarding the analysis of excitatory neurons:

(i) Substance P is expressed in a subset of excitatory neurons in the spinal cord, as described by the authors. Since it is only a small subset, the inferences derived from study about alterations in excitatory neurons are quite limited. Unfortunately, the text refers to 'excitatory neurons' and 'excitatory elements', although the experimental parameter studied is only derived from Tac1 neurons. This needs to be clearly acknowledged and discussed as a limitation and inferences should not be drawn for all excitatory neurons, but clearly labelled as Substance P-containing excitatory interneurons throughout the text.

(ii) Along these lines, please explain why mouse lines expressing Cre in global/other excitatory neurons were not employed in the study.

(iii) Perhaps this caveat is partly overcome in the analyses shown in Fig. 2, which pertain to Vglut2 expression in lysosomes – is this indicative of all excitatory populations in the spinal dorsal horn? Please provide references.

(iv) Furthermore, is Vglut2 expressed in peripheral sensory neurons (given that they are indeed glutamatergic) and would an inclusion of Vglut2 in microglial lysosomes thus lack distinction between spinal and peripheral neuronal components? The authors address this aspect in experiments shown in Fig. 2, but not in the experiments shown in Fig. 1, which include all Vglut2-positive terminals – this is confusing. For Fig. 2, the authors state that Vglut2 is only expressed in IB4-positive peripheral sensory neurons – it would be good to demonstrate this in DRG sections since it is so critical to the inferences drawn for the data shown in Fig. 2.

(v) The engulfment of Vglut2 is not significantly more than that of Vgat (Fig. 1i, j), suggesting that excitatory terminals are not engulfed to a lower extent than inhibitory, thus making the caveat given by the unequal comparison of Gad2-dtTomato with Tac1-dtTomato even more prominent.

Taken together, these data do not convincingly support the conclusion that inhibitory neurons are affected to a larger extent than excitatory neurons.

2. Is Gad2-Cre labelling all spinal GABAergic neurons? Please clarify whether Gad1 is also expressed in the spinal cord.

3. Was contralateral inclusion of dtTomato in microglial lysosomes also studied?

4. Spinal microglial contributions to nociceptive processing in neuropathic pain are reported in several previous studies to be strongly influenced by sex differences, including studies from the authors. Please clarify whether sex differences were observed regarding the role of microglia in synapse engulfment in neuropathic mice. The authors state that in the discussion that inhibiting C1q had the same effect in male and female mice. In addition, however, it would be critical to test whether the effects on microglial synapse pruning (Fig. 1 and Fig. 2) show sexual dimorphism.

5. Phagocytotic activity of microglia peaked at day 7 post-injury as per dtTomato inclusion (Fig. 1e, f), but was significantly only starting day 14 as per Vgat and Vglut2 inclusion (Fig. 1i, j). Can this difference be accounted for?

6. The authors state that NeuN is a marker for neuronal cell bodies. As per literature, it is densely localized in and marks neuronal nuclei.

7. The authors observed that depletion of spinal microglia inhibits hypersensitivity longer than the period of treatment with the drug. Surprisingly, based alone on this observation, they come to the following conclusion: 'Importantly, the effect of transient depletion of microglia during the peak of engulfment persisted longer than the treatment period, suggesting that microglia-mediated synapse engulfment has a long-lasting effect on pain behavior.' This would imply that synapse engulfment is the only function of microglia, which goes against a huge body of evidence showing that microglia-derived mediators leads to sensitization of neurons, activation of astrocytes and several such functions which lead to hypersensitivity. This error must be corrected. The depletion of microglia is equally indicative of all of the functions attributed to microglia and does not provide any evidence for a specific role for synapse engulfment in neuropathic pain.

8. In Fig. 2, the authors report data from structured illumination microscopy to directly study synapses positive for pre- and post-synaptic proteins. While the data appear to be sound, they suggest that entire synapses are engulfed and degraded by microglia, i.e., not just the pre-synaptic component. This is at odds with findings in Fig. 1 that post-synaptic components were not found in microglial lysosomes. It would be helpful to explain what is actually meant with synapse engulfment, and if the study makes a case for presynaptic engulfment, the mechanisms of this level of selectivity is reached need to be clarified and discussed. The topic of microglial pruning of synapses has been well studied in the brain, so perhaps these aspects are known, but the manuscript does not give a clear picture to the reader, with seemingly contradictory data in Fig. 1 and Fig. 2 leading to a lot of confusion.

9. The authors put in appreciable efforts towards clarifying the contributions of the C3 pathway vs the fractalkine pathway in synapse pruning and neuropathic pain using knockout (KO) mice in this study, and the data on the C3 pathway (using C3 KO mice) and C1q neutralization provide important information. However, the study does not provide further mechanistic insights. Because the involvement of the complement system in neuropathic pain has been demonstrated previously, including beneficial effects of targeting this pathway in neuropathic mice (e.g., Griffin et al. J. Neurosci 2007, Twining et al. 2005) and the role of microglia in synapse remodelling in pain states has been discussed in the field (e.g., Warwick et al. 2021), it would be important to reveal mechanisms, e.g.:

(i) Here, C3 KO mice and C1q neutralization led to lack of synapse pruning and reduction of neuropathic pain, suggesting that both are required and overall, the text implies that both are in series. However, the C1q pathway is believed to distinct from the C3 pathway and both converge on the C3 convertase, which then leads to C5a. Reduction of neuropathic pain by inhibition of C5a has been reported (Griffin et al. 2007). C3 can also activate C3aR independently of the C1q pathway. Thus, it is perplexing as to why both C3 KO and C1q neutralization leads to the same and complete phenotype in this study although both run in parallel and converge much further downstream. One would have expected partial reduction with either if both are important.

(ii) The study also stops short of explaining why there is a preferential loss of inhibitory synapses. C1q and C3 were found in all microglia, but C1q binds only to inhibitory synapses? Importantly, where are the effectors/receptors that mediate these functions localized and are these restricted to inhibitory synapses? Please report on the location of C3aR and C5aR.

(iii) Finally, C3 signaling is also known to prune postsynaptic (dendritic) spine components. How is selective phagocytosis of the presynaptic terminals achieved via the C3 pathway in the spinal cord?

(iv) While at least part of the results suggest that microglia engulf both excitatory and inhibitory synapses (Fig. 1), but the latter half of the study addressing the role of the pathways only points to inhibitory synapses. In the discussion, the authors discuss that while both types of synapses are pruned, only excitatory synapses are added on via other mechanisms. This leads to the question as to how excitatory synapses are being pruned.

Other questions/suggestions:

- Since the complement cascade might not be broadly known to the readership, a schematic depicting parallel pathways and/or inter-relationship between key players mentioned in the manuscript (C1q, C3, C3aR, C5a, C5aR, fractalkine, CXCR3) would be helpful.

- Please clarify the mode of administration of PLX3397 in the results text.

- The data indicate that although the number of Vglut2+ and Vgat+ inclusions was reduced in C3 KO mice, the volume of CD68+ lysosomal compartments increased to the same level in these mice as with wild type mice following nerve injury. What could be leading to the latter?

- The title of the study is weakened by previous studies showing that the downstream targets of C1q can be exploited for pain relief in neuropathic pain. From the reviewer's point of view, the most noteworthy finding of this study is not that targeting C1q reduces neuropathic pain, rather that microglial mechanisms lead to the known dysbalance between excitatory and inhibitory synapses via the complement pathway, leading to neuropathic pain.

Reviewer #2

(Remarks to the Author)

Overview: In the manuscript entitled 'Complement protein C1q is a therapeutic target for neuropathic pain', Yousefpour et al describe a novel role of microglia, through the complement pathway, in inhibitory synaptic reductions associated with neuropathic pain phenotypes following nerve injury. Although microglia have been ascribed a functional role in similar injury models, the underlying mechanisms driving inhibitory synapse loss often observed, and whether microglia are involved, have not been described. The authors show, through multiple approaches, that microglia significantly engulf synaptic material derived from both inhibitory and excitatory beginning 7 days post-injury. Pharmacological depletion of microglia leads to improved outcomes in a behavioral assay assessing neuropathic pain and prevents inhibitory synapse loss. The authors also demonstrate that the complement pathway proteins C3 and C1q are involved in microglial-mediated engulfment, and that pharmacological inhibition of C1q activation prevents inhibitory synapse loss and improves neuropathic pain outcomes. Overall, this is a robust manuscript detailing a novel and exciting role of microglia and complement in neuropathic injury, with rigorous experimental design, pharmacological and genetic approaches, and structural and behavioral readouts. Given the recent discovery of the role of microglia and C1q in developmental inhibitory synaptic pruning (Favuzzi et al, Cell 2021), the findings presented by Yousefpour et al further demonstrate the potential importance of these mechanisms as therapeutic targets in disease. While I do not have any major concerns, I believe the authors have over-interpreted several results that should be clarified and revised in both the text and figure legends, and addressed within the discussion. Specifically, the authors never demonstrate a functional role of microglial engulfment through CR3 as the main mechanism for the phenotypes they observe, although this is stated as the mechanism in the last sentence of the introduction. Further, the observed phenotypes between microglia depletion and pharmacological C1q inhibition vary considerably. It is possible that there are alternative interpretations of these data, and this needs to be considered. Below I have outlined specific concerns to be addressed:

Overall Strengths:

- Exciting and novel findings demonstrating a role of microglia, through the complement cascade, in inhibitory synaptic pruning and neuropathic pain
- Rigorous experimental design with good representation of males/females
- Genetic and pharmacological approaches to assess mechanisms
- o Especially important given the lack of current data on the role of microglial pruning during normal spinal cord development
- Clearly written and easy to follow

Overall weaknesses:

- No major concerns

• Minor concerns:

o General concerns to be addressed:

- Clearly indicate time points of analysis within figure legends.
- Clarify the timing of injury model (ie: acute versus chronic injury phases) and frame observations in this context. How could these data be interpreted across different stages of injury and/or recovery?
- For example, using their injury model, Yousefpour et al demonstrate in Figure 1 that significant increases in microglial engulfment of neuronal elements and presynaptic terminals within the ipsilateral spinal cord following SNI (Figs 1e, f and I, j, respectfully) can be detected beginning 7 days post injury. They continue to detect significant engulfment at 21 days post injury, the longest time point they report. Is spontaneous recovery observed in this injury model? Is there a time point following injury that microglial engulfment returns to baseline? How could persistent microglial engulfment/activation be affecting this model and the interpretations of these data? The authors should address these findings more clearly in the text and highlight potential caveats.
- The phenotypes of C1q inhibition and microglia depletion observed are different. While both show prevention of inhibitory synapse loss, these do not phenocopy one another, suggesting that there may be additional mechanisms of C1q and/or microglia in injury and recovery processes independent of one another. These issues should be addressed in the text and figure legend titles.

o Figure-specific concerns:

- Figure 2:
 - Would be helpful to have engulfment data of microglia that repopulate the dorsal horn following completion of PLX treatment.
 - ANOVA analysis of PLX over time: Fig 2d
- o Still see reduction in pain threshold in PLX-treated cohorts over time. Pharmacological depletion of microglia does not fully prevent neuropathic pain phenotype (based on behavioral analysis Fig 2D). There is improvement, but animals don't seem to be fully protected. Additionally, phenotype is similar while animals are on treatment (with few microglia at day 7) and at day 21 when microglia have repopulated, indicating that loss of microglia early after injury does not result in improved recovery over time.

- Fig 2F: See significant reduction in inhibitory synapses in vehicle, completely prevented in microglia-depleted cohorts, however animals still have pain phenotype. Indicates that, while microglia may be playing a direct role in the significant reduction of inhibitory synapses observed, this is not the only contributing factor to phenotype. Please clarify in text.
- Figure 3:
 - Given the abundance of microglial sequencing data publicly available, authors should compare their data sets to the disease-associated microglia signature (DAM; Keren-Shaul et al Cell 2017 and others) as several of these pathways highlighted have also been associated with this gene signature.
 - Figure legend/title needs to be revised. The data do not necessarily show that these 'pruning pathways are upregulated'. Gene expression of some factors is increased, and IHC analysis of proteins shows an increase in several factors as well, but this could be just correlative.
 - Do these changes in microglia persist over time? As referenced above, significant engulfment of synaptic elements persists at least until 21 days post injury. Do the microglia retain these changes? IHC and RNAScope analysis should be performed at these other time points to address this question and to better understand the role these changes may play in the injury over time.
- Figure 4:
 - The authors show no data on CR3 even though that is the title of the figure. Figure shows data from C3 KO animals, which could have complement-dependent activities independent of microglia engulfment. To definitively state this, experiments would need to be performed in CR3 KO mice. This should be clarified in the title and text.
 - For all synaptic analyses, it would be helpful to see (perhaps in supplemental figures) whether individual presynaptic or postsynaptic values are changing (independent of co-localized structural synapses). In several data sets (ie; Fig 4e, g; Fig6 l, k) there appears to be considerably more presynaptic labeling in experimental conditions that may not be reflected in the quantified data. It may be that changes in microglial targeting have unique effects on presynaptic injury and recovery.
- Figure 5:
 - In Fig5A, it would be helpful to see co-localization with Iba1 IHC or another microglial-specific marker to confirm expression is only in microglia.
 - Unclear how analysis was performed- were all cells counted? Was C1q mRNA detected in all cells? Please clarify.
 - Would be helpful to see time course of RNAScope, C1q IHC analysis across relevant time points during injury and recovery.
- Figure 6:
 - Fig 6b: phenotype appears much worse in this cohort than in Fig 2. Does variability in severity of injury influence data? Especially given the discrepancy in phenotypes between C1q inhibition and microglia ablation.
 - No difference in C1q-labeled vglut2 with C1q inhibition, however the authors observe a significant reduction in vglut2 engulfment.
 - Looks to be dramatically different changes in presynaptic staining with C1q inhibition. Would be helpful to show quantification of presynaptic labeling independent of 'synaptic' quantification co-localization with postsynaptic label as mentioned above.
 - The authors need to clarify potential differential roles of microglia throughout the injury process. Pharmacological C1q inhibition is performed across the duration of injury model, however the authors see no improvement until last time point assessed (21 days post injury). These data indicate that C1q may have a specific role in impeding the recovery process rather than preventing injury during acute phase of this model.
 - Similar to Fig 2F, the authors demonstrate that pharmacological inhibition of C1q prevents inhibitory synapse loss, but not neuropathic pain behavior. The authors should highlight this finding and provide alternative explanations in the discussion

Reviewer #3

(Remarks to the Author)

Summary:

Using extensive and detailed imaging, the authors evaluate how microglial pruning may be involved in spinal cord circuit plasticity after peripheral injury. They first establish that microglia prune both inhibitory and excitatory synapses from 7 to 21 days after SNI and further show an associated decrease in inhibitory synapse density. Using the CSF1R inhibitor, PLX3397, they demonstrate that microglia are necessary for these alterations and then home in on the microglia receptors CR3 and CX3CR1 as potential triggers based on existing RNAseq data. Using C3 KO and CX3CR1 KO mice, they next show that CR3 is the primary pathway involved in this phenomenon by further localizing C1q to synaptic elements tagged for pruning. Finally, they use the C1q blocking antibody, ANX M1.21 to reproduce the effect of C3 KO on synapse density, suggesting that this may be a therapeutic strategy. Overall, this is an interesting study that nicely outlines a contribution of microglial pruning to preferential loss of inhibitory synapses after nerve injury. My concerns are mainly related to the need for more detail with respect to selection of mouse strains, confirmation of behavioural outcomes and the need for additional experiments to support their conclusions.

Major comments:

- In one case the authors use a receptor knockout (Cx3CR1), in the other they use a ligand knockout (C3 KO). It seems that these could have very different outcomes simply on that basis. For example, can fractalkine bind to other receptors? Are there other ligands for C3R? Did the authors perform similar experiments in the CR3 KO mouse (as in Schafer et al, Neuron 2012)?

- What was the rationale for sparing the sural in some of the experiments and then sparing the tibia for others? This is very unusual given the regional distribution of these nerves with respect to their dorsal horn central projections which could confound results. Moreover, the authors do not even specify which experiment was performed with which exact SNI model. More information is needed, and ideally, all experiments should be performed in the same model.
- With the PLX3397 depletion, at what time point was synapse density quantified (Fig. 2e-h)? Also, what is the effect of depletion alone (without SNI) on synapse density? This is important because the authors demonstrate a 13% increase in excitatory synapses, do these mice exhibit hyperalgesia without injury?
- Authors quantify number of CR3+ microglia (Fig 3e2) but % expression of Cx3CR1 per microglial cell (Fig 3h2). Why was the metric changed rather than kept identical for the two different pruning pathway receptors?
- The C3 KO strain demonstrated almost identical increases in both excitatory and inhibitory synapses. A behavioural outcome (at a minimum allodynia) is therefore crucial to understand the ultimate functional consequence of these changes which may offset the SNI-induced loss of inhibition, or alternately, may overshoot with the increased excitatory synapses.
- Pre-emptive analgesia is not particularly clinically-relevant. Have the authors performed ANX dosing in a treatment (i.e., post-injury) paradigm? How was the dose of the ANX-M1.21 anti-C1q blocking antibody selected? Was a dose-response performed? The anti-allodynic effect shown in Figure 6b is subtle (increased from 0.10 to 0.25g), suggesting that either the contribution of synapse pruning to this outcome is minor, or that the dose needs to be optimized.
- The investigators used the C3 KO mouse (Jax #003641) which is on a mixed background (B6:129). Given what is known about strain differences in pain outcomes, with strain suggested to be a major confound in mice pain research (Lariviere WR, Chesler EJ and Mogil JS. JPET 2001) why did the authors use this mouse instead of the C3 KO mouse which is C57Bl/6J congenic (Jax #029661)?
- The authors do not demonstrate any functional correlate of the loss of inhibitory synapses. Does spontaneous excitatory postsynaptic current (sEPSC) frequency change? This would be important to understand because the effect on behaviour is rather minimal.
- The drug being studied in the final experiments (ANX-M1.21) was supplied by Annexon and author YAZ is employed by Annexon. This should be disclosed in the COI section.

Minor comments:

- Figure 3 IHC images are very small and hard to discern. In particular, the Cx3CR1 staining looks diffuse and not cell-specific, can larger or high-resolution images be provided?
- Is the "n" listed per group in the figure legends the number of mice, the number of sections or something else? Please specify for each figure/experiment.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have performed an extensive and careful revision. The responses to reviewers are convincing and highly credible. The additional experiments and data added during the revision have strengthened the manuscript. This is a very interesting and insightful study that will be interesting to a broad readership.

Reviewer #2

(Remarks to the Author)

This is a review of the revised manuscript "Targeting C1q Protects Spinal Synaptic Circuitry in Neuropathic Pain". The authors have improved upon the original article and have satisfactorily addressed many of the original concerns outlined in the prior review. Overall, I continue to find this work to be of interest and support its publication in Nature Communications.

Reviewer #3

(Remarks to the Author)

In this revised manuscript, the authors make a stronger case for microglial involvement in inhibitory synapse pruning, specifically for the C3/complement pathway. My remaining comments are mainly editorial as the manuscript could focus more clearly on the positive findings and summarize key points which get a bit lost in the many experiments.

1. The interpretation of the PLX studies is not altogether clear to me. For example, if there is a 13% increase in ipsilateral DH excitatory synapse density after depletion, how does that result in overall lower hypersensitivity?

2. The major finding is related to C3/complement-mediated synapse engulfment but as written (lines 221-244) the focus is on the negative CX3CR1 KO data. It would help clarify the findings if instead the focus was on the C3 KO experiments.
3. Given the developmental effects of the C3 KO, the preferred experiment would be in an inducible C3 cKO mouse line. Agree that the C1q blocking experiments are the next best option.
4. As mentioned previously, pre-emptive analgesia is not particularly clinically-relevant. Have the authors performed ANX dosing in a treatment (i.e., post-injury) paradigm? No additional experiments are requested, however, please do comment on this in the Discussion.
5. The lack of sex difference in the PLX experiments is supported by other recent studies (Donovan et al. Cell Reports 2024; Zhou et al. Cell Reports 2019; Kohno et al. Science 2022). These studies also performed versions of microglia depletion with possible alternate mechanisms proposed which may be worth adding to the Discussion given that reversal of allodynia was delayed and incomplete in the current study. In particular, the fact that the repopulated microglia may be involved in resolution (Kohno et al; Donovan et al).

Minor comments:

Figure 2d (and others) - y axis label needs to be corrected (withdrawal, not withdrawal)

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ANSWER TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

We would like to thank the reviewer for their thoughtful comments and constructive directions that led to complementary experiments and analyses towards improving our manuscript. Below are the answers to the reviewer's comments:

Comment:

There are a number of concerns regarding the analysis of excitatory neurons:

(i) Substance P is expressed in a subset of excitatory neurons in the spinal cord, as described by the authors. Since it is only a small subset, the inferences derived from study about alterations in excitatory neurons are quite limited. Unfortunately, the text refers to 'excitatory neurons' and 'excitatory elements', although the experimental parameter studied is only derived from Tac1 neurons. This needs to be clearly acknowledged and discussed as a limitation and inferences should not be drawn for all excitatory neurons, but clearly labelled as Substance P-containing excitatory interneurons throughout the text.

Response:

We agree with the reviewer that substance P-(SP) expressing neurons do not represent the entire population of excitatory interneurons in the dorsal horn and, if we implied otherwise, it is corrected now. In new experiments, we quantified engulfment of excitatory neuronal elements in Lmx1b-tdTomato mice in which all spinal cord dorsal horn excitatory neurons express fluorescent protein (see Szabo et al., J Neurosci 35, 5233-5246, 2015; Ding et al., Development 131, 3693-3703, 2004); Cheng et al., Nat Neurosci 7, 510-517, 2004); Lai et al., Development 143, 3434-3448, 2016). We selected day 7 post-SNI for the re-assessment of engulfment of excitatory neuronal elements in this mouse line because we observed the peak of engulfment at this time-point (Supplementary Figs. 1a1-7). Additionally, we compared engulfment of Tac1⁺ and Lmx1b⁺ excitatory neurons and found a comparable increase in engulfment of both Tac1⁺ and Lmx1b⁺ excitatory inputs in Lamina II of the spinal cord (Supplementary Fig. 1a6).

Please note that, for the remainder of this study, we relied on VGLUT2 as a marker for excitatory synapses, which labels all the excitatory synaptic terminals formed by spinal cord interneurons and some terminals of primary afferent nociceptors. Quantifications of SP⁺ excitatory and Lmx1b⁺ elements were performed to complement the quantification of VGLUT2 engulfment.

Altogether, the new data using genetically driven fluorescent protein expressed in all excitatory neurons represent overall engulfment of excitatory neural elements.

Comment:

Along these lines, please explain why mouse lines expressing Cre in global/other excitatory neurons were not employed in the study.

Response:

Our goals in this paper were: 1) to find whether SNI induces microglia-mediated synapse engulfment, and 2) to find the underlying mechanism for this process. When we initiated this study, we only had access to Tac1-tdTomato mice. Since the temporal pattern of Tac1⁺ input engulfment was comparable to VGLUT2⁺ input engulfment, we concluded that we had collected sufficient evidence in support of microglia-mediated pruning of excitatory inputs in the SNI model of neuropathic pain. However, based on the valid comment of the reviewer, we eventually obtained Lmx1b-tdTomato mice that exclusively express a red fluorescent protein in all spinal cord excitatory interneurons (including the Tac1⁺ and then quantified the overall amount of excitatory synapse engulfment in these mice (Supplementary Figs. 1a1-7). Our quantifications of engulfment of Lmx1b⁺ and Tac1⁺ neuronal elements were comparable in LII of the spinal cord at 7 days post-SNI (Supplementary Fig. 1a6).

Comment:

Perhaps this caveat is partly overcome in the analyses shown in Fig. 2, which pertain to Vglut2 expression in lysosomes – is this indicative of all excitatory populations in the spinal dorsal horn? Please provide references.

Furthermore, is Vglut2 expressed in peripheral sensory neurons (given that they are indeed glutamatergic) and would an inclusion of Vglut2 in microglial lysosomes thus lack distinction between spinal and peripheral neuronal components? The authors address this aspect in experiments shown in Fig. 2, but not in the experiments shown in Fig. 1, which include all Vglut2-positive terminals – this is confusing. For Fig. 2, the authors state that Vglut2 is only expressed in IB4-positive peripheral sensory neurons – it would be good to demonstrate this in DRG sections since it is so critical to the inferences drawn for the data shown in Fig. 2.

Response:

In the spinal cord, VGLUT2 is highly expressed in the terminals of all excitatory interneurons, and to a lesser extent, within the terminals of some primary afferent nociceptors, but at low level. The relevant references were now added to the text.

The figure on the right shows that depletion of microglia does not prevent loss of IB4⁺ primary afferent terminals. To ensure that SNI-mediated loss of VGLUT2⁺ primary afferent terminals does not affect quantifications of excitatory synapses of spinal neurons, we only quantified VGLUT2⁺IB4⁻ terminals

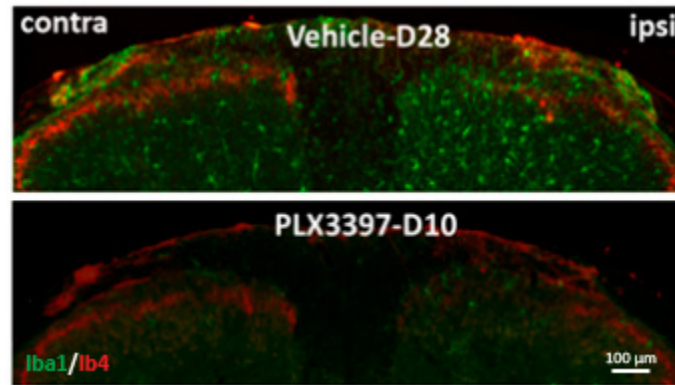


Figure related to comment: Representative images of spinal dorsal horn of SN mice labeled with Iba1 (green) and Ib4 (red)

(Supplementary Figs. 2a1-2 and 2b1-2). The result should not be changed by the extremely low number of peptidergic primary afferents, as the expression levels of VGLUT2 in that population (as for the IB4⁺) is very low and peptidergic afferents occur in very low numbers in inner lamina II in mouse than the IB4⁺.

Comment

Indeed, as mentioned above, in inner lamina II of the dorsal horn, where our quantification was done, VGLUT2 is expressed by spinal cord excitatory neurons, and IB4⁺ primary afferents, which also express MrgD⁺ (Zylka et al., Neuron 45, 17-25, 2005). SP primary afferents in mouse terminate in lamina I and outer lamina II, not in inner lamina II where we did the quantification. Another important factor to keep in mind is that the expression of VGLUT2 in terminals from primary afferents is very low, making it extremely difficult to detect by immunohistochemistry, contrary to what happens in the DRG. Therefore, most VGLUT2 immunoreactivity is indeed from interneurons. This is mentioned in multiple studies, and is discussed for mouse by Scherrer et al. (Proc. Natl. Acad. Sci. U. S. A 107, 22296-22301, 2010). We made sure that this is now clearly and correctly stated in the revised manuscript. The statement that VGLUT2 is only expressed in primary afferents that bind IB4 was wrong and is now eliminated from current manuscript.

Even keeping the above in mind, we went a step ahead in that for synapse quantification we used IB4 binding to exclude potentially VGLUT2 synapses with weak immunoreactivity which might be from the periphery in inner lamina II. However, this approach was not reliable for engulfment analysis since we were not sure if the absence of IB4 signal in microglial lysosomes was due to the absence of engulfment of IB4⁺ terminals or compromised reactivity of the fluorescent protein conjugated to IB4 within microglia.

To overcome this limitation, using MrgD-tdTom mice which express a fluorescent protein primarily in IB4⁺ excitatory primary afferents, we assessed engulfment of primary afferent

inputs and found no evidence for the engulfment of MrgD⁺ inputs at 7 days post-SNI (Fig. 1b1-3). Additionally, the Lmx1b-Tdtomato genetic mouse line was used to address the reviewer's comment, as they only express TdTomato in spinal excitatory neurons and not in primary afferents. Engulfment quantification in this line showed significant engulfment of excitatory neuronal elements (Supplementary Figs. 1a1-7).

All taken together, our results from MrgD-tdTom and Lmx1b-tdTomato mouse lines indicate that VGLUT2 engulfment observed in the SNI model is almost exclusively from spinal cord neurons and not from primary afferents.

Comment:

The engulfment of Vglut2 is not significantly more than that of Vgat (Fig. 1i, j), suggesting that excitatory terminals are not engulfed to a lower extent than inhibitory, thus making the caveat given by the unequal comparison of Gad2-dtTomato with Tac1-dtTomato even more prominent.

Response:

We did not directly compare changes in VGAT and VGLUT2 engulfment in the original version of the study. Based on this comment, we added these comparisons in the revised version (Supplementary Figs. 1c1 and 1c2). At day 7 post-SNI, engulfment of VGAT is greater than VGLUT2 and this is consistent with the comparisons of Gad2-tdTom with Tac1-tdTom and Lmx1b-tdTom at the same timepoint (Supplementary Fig. 1a7). However, at later timepoints we did not observe a significant difference between excitatory and inhibitory microglia-mediated synapse removal.

Comment:

Taken together, these data do not convincingly support the conclusion that inhibitory neurons are affected to a larger extent than excitatory neurons.

Response:

We respectfully disagree with the reviewer. Indeed, our results show that in the SNI model of neuropathic pain, microglia mediate loss of inhibitory synapses, but not excitatory ones. This statement is based on synapse quantifications in Figs. 2i and 2j, 3c2 and 3d2, and 6f2 and 6g2 that show that: 1) SNI results in inhibitory synapse loss, but not excitatory, and 2) depletion of microglia or C1q antibodies prevent inhibitory synapse loss and increases excitatory synapses. We elaborated on how inhibitory and excitatory synapses are differentially affected by synapse pruning, and how that could possibly affect spinal cord

sensory processing in the discussion section (lines 400-415).

Comment:

Is Gad2-Cre labelling all spinal GABAergic neurons? Please clarify whether Gad1 is also expressed in the spinal cord.

Response:

Gad2-Cre mice labels inhibitory neurons in lamina II of the dorsal horn which express Gad65 (Zeilhofer et al., 2012). Since the evidence for engulfment of inhibitory neuronal elements in Gad2-Cre mice was consistent with VGAT engulfment, we did not investigate engulfment in other mouse lines. Gad1 encodes for Gad67, and is also expressed in the spinal cord. Immunocytochemical studies of the spinal cord have shown that virtually all GABAergic neurons co-localize Gad65 and Gad 67, with levels of Gad65 being higher in the dorsal horn as shown in a study of Andrew Todd's group (Mackie et al. Neuroscience 119, 461-472, 2003). Therefore, using Gad2-Cre mice was the correct approach to label most if not all GABAergic neurons in lamina II.

Comment:

Was contralateral inclusion of dtTomato in microglial lysosomes also studied?

Response:

Yes, in all studies comparisons were performed between ipsilateral and contralateral sides. In sham animals, levels of microglial CD68⁺ (lysosomal marker) were considerably lower compared to contralateral side. Therefore, sham would not be a proper control for assessing ipsilateral engulfment.

Comment:

Spinal microglial contributions to nociceptive processing in neuropathic pain are reported in several previous studies to be strongly influenced by sex differences, including studies from the authors. Please clarify whether sex differences were observed regarding the role of microglia in synapse engulfment in neuropathic mice. The authors state that in the discussion that inhibiting C1q had the same effect in male and female mice. In addition, however, it would be critical to test whether the effects on microglial synapse pruning (Fig. 1 and Fig. 2) show sexual dimorphism.

Response:

This study was designed to assess sex-differences with sufficient power. The only sex difference that we observed was in the microglia depletion experiment (Supplementary Fig. 2d), in which we found sex-differences in the behavior effect at day 7 post-SNI during the treatment period. However, we did not observe sex-differences in subsequent off-treatment time points. Additionally, we did not observe significant sex-differences in other readouts (i.e., engulfment and synapse quantifications in all conditions, and anti-C1q treatment behavioral study). Although we consistently found more variability in treated female animals than in males, we found no significant difference between males and females. We made efforts to find the underlying cause of the observed differences in data variability between males and females by tracing the estrus cycle in females, but these efforts were largely inconclusive. In all graphs we showed all data points and labeled males and females with different colors to facilitate the understanding of the results.

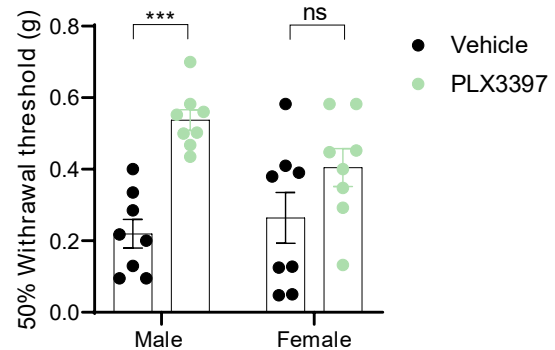


Figure related to this comment: Pain behavior in neuropathic mice treated either with PLX3397 or vehicle ($n = 8$ mice per group) at day 7 post-SNI during the treatment period.

Our conclusion based on the data is that there are no sex differences associated with the specific microglia-mediated pathway described in this study.

Comment:

Phagocytotic activity of microglia peaked at day 7 post-injury as per dtTomato inclusion (Fig. 1e, f), but was significantly only starting day 14 as per Vgat and Vglut2 inclusion (Fig. 1i, j). Can this difference be accounted for?

Response

In line with the tdTomato engulfment results, ipsilateral engulfment of VGAT and VGLUT2 was significant at days 7, 14, and 21 post-SNI, compared to contralateral (VGAT: D₇: $t_{88} = 8.5$, $P < 0.0001$; D₁₄: $t_{88} = 4.2$, $P = 0.0002$; D₂₁: $t_{88} = 4.1$, $P = 0.0003$; VGLUT2: D₇: $t_{88} = 4.7$, $P < 0.0001$; D₁₄: $t_{88} = 4.6$, $P < 0.0001$; D₂₁: $t_{88} = 2.9$, $P = 0.016$). We reviewed the Results section to make sure that the result is clearly stated.

Comment. The authors state that NeuN is a marker for neuronal cell bodies. As per literature, it is densely localized in and marks neuronal nuclei.

Response:

NeuN labels neuronal nuclei as correctly pointed out by the reviewer, although it also labels the cytoplasm with less intensity. Therefore, it is often used as a replacement for a Nissl

staining (see e.g. Ribeiro-da-Silva, A. in *The Rat Nervous System* - ed G. Paxinos 97-114, Elsevier Academic Press, 2015; Todd, A.J. *Nat Rev Neurosci* 11, 823-836, 2010). This information was added to the manuscript. Therefore, our statement was correct. This quantification was important to confirm that microglia were not removing neurons but were modifying neuronal connectivity.

Comment:

The authors observed that depletion of spinal microglia inhibits hypersensitivity longer than the period of treatment with the drug. Surprisingly, based alone on this observation, they come to the following conclusion: 'Importantly, the effect of transient depletion of microglia during the peak of engulfment persisted longer than the treatment period, suggesting that microglia-mediated synapse engulfment has a long-lasting effect on pain behavior.' This would imply that synapse engulfment is the only function of microglia, which goes against a huge body of evidence showing that microglia-derived mediators leads to sensitization of neurons, activation of astrocytes and several such functions which lead to hypersensitivity. This error must be corrected. The depletion of microglia is equally indicative of all of the functions attributed to microglia and does not provide any evidence for a specific role for synapse engulfment in neuropathic pain.

Response:

We fully agree with the reviewer that microglia contribute to pain behavior through many different pathways. We modified this statement according to the reviewer's comment to the following: "Although the analgesic effect of transient depletion of microglia was greater during the depletion period, it was still detectable when microglia repopulated the spinal cord. Microglia gradually retrieved their synapse engulfment capacity within the next 10 days after the treatment was ended" (lines 139-142).

Comment:

In Fig. 2, the authors report data from structured illumination microscopy to directly study synapses positive for pre- and post-synaptic proteins. While the data appear to be sound, they suggest that entire synapses are engulfed and degraded by microglia, i.e., not just the pre-synaptic component. This is at odds with findings in Fig. 1 that post-synaptic components were not found in microglial lysosomes. It would be helpful to explain what is actually meant with synapse engulfment, and if the study makes a case for presynaptic engulfment, the mechanisms of this level of selectivity is reached need to be clarified and discussed. The topic of microglial pruning of synapses has been well studied in the brain, so

perhaps these aspects are known, but the manuscript does not give a clear picture to the reader, with seemingly contradictory data in Fig. 1 and Fig. 2 leading to a lot of confusion.

Response:

In our synapse quantification approach, we collected data for the densities of presynaptic elements, postsynaptic elements, and the pairs between the two which represent the synapse number. We added Supplementary Figs. 2c-d to show the quantification data for pre-synaptic and post-synaptic synaptic components in addition to synaptic pairs, which was reported in Fig. 2i and 2j. The data suggested that changes in the postsynaptic density were consistent with changes in the presynaptic density for both synapse types. We performed synapse engulfment analysis for postsynaptic densities (Supplementary Figs. 1d2-3) but did not find any evidence for postsynaptic engulfment. Therefore, based on our data, we speculate that the reduction in postsynaptic densities was an indirect response to the loss of presynaptic inputs. Possibly, diminishing the presynaptic release machinery and the eventual loss of the presynaptic element precede the reorganization and disassembly of the postsynaptic scaffolding. This interpretation is in agreement with the literature (see e.g. Weinhard et al., Nat Commun 9, 1228, 2018).

As the reviewer mentioned, there are reports that microglia engulf postsynaptic (Paolicelli et al., Science 333, 1456-1458, 2011), and presynaptic elements (Schafer et al., Neuron 74, 691-705, 2012). To our knowledge, details about how pre-synaptic and post-synaptic components of the synapse are affected by synapse pruning processes are pathway and context dependent. Specifically, very little is known about these details in the context of inhibitory synapse removal. To our knowledge, inhibitory synapses are either displaced (Chen et al., Nat Commun 5, 4486, 2014). or removed presynaptically (Favuzzi et al., Cell 184, 4048-4063 e4032, 2021).

Although we described the absence of any evidence for microglia-mediated post-synaptic engulfment based on our findings in Supplementary Figs. 1d2-3, we are careful in the manuscript not to rule out the possibility of post-synaptic engulfment.

Comments:

The authors put in appreciable efforts towards clarifying the contributions of the C3 pathway vs the fractalkine pathway in synapse pruning and neuropathic pain using knockout (KO) mice in this study, and the data on the C3 pathway (using C3 KO mice) and C1q neutralization provide important information. However, the study does not provide further mechanistic insights. Because the involvement of the complement system in neuropathic pain has been demonstrated previously, including beneficial effects of targeting this pathway in neuropathic mice (e.g., Griffin et al. J. Neurosci 2007, Twining et al. 2005)

and the role of microglia in synapse remodelling in pain states has been discussed in the field (e.g., Warwick et al. 2021), it would be important to reveal mechanisms, e.g.: Here, C3 KO mice and C1q neutralization led to lack of synapse pruning and reduction of neuropathic pain, suggesting that both are required and overall, the text implies that both are in series. However, the C1q pathway is believed to be distinct from the C3 pathway and both converge on the C3 convertase, which then leads to C5a. Reduction of neuropathic pain by inhibition of C5a has been reported (Griffin et al. 2007). C3 can also activate C3aR independently of the C1q pathway. Thus, it is perplexing as to why both C3 KO and C1q neutralization leads to the same and complete phenotype in this study although both run in parallel and converge much further downstream. One would have expected partial reduction with either if both are important.

Response:

Based on our current understanding of the involvement of the classical complement pathway in synapse pruning, activation of C1 complex (C1qrs) results in C2, and C4 cleavage, which produces C3 convertase which then cleaves C3 resulting in generation of several C3 cleavage products (i.e., C3a, C3b, and C3bi) that are detected by microglia. It is not reported that the complement pathway proceeds further than this step in the context of synapse pruning. Therefore, we expect that full inhibition or genetic ablations of C1q, C4, and C3 produce a similar effect on synapse pruning. This is consistent with our observation and previous studies on this mechanism (see Figs. 4a2-d2, 4e4, 4f4, and 6f2-g2 and Schafer et al., Neuron 74, 691-705, 2012).

Based on the reviewer's suggestion, we explored the involvement of C5a, a common downstream product of the classical and alternative complement pathways, in synapse pruning. In a new experiment, we blocked C5a receptor signaling for 21 days using C5aR antagonist, PMX53 (3 mg/kg/day) and assessed microglia-mediated synapse pruning for inhibitory and excitatory synapses at multiple time-points. We found that antagonizing C5aR had no effect on microglia-mediated synapse removal (See figure below).

Relative to the effect of C5a and C3a on pain behavior, the mechanism through which C3aR and C5aR pathways contribute to pain is thought to be via reducing inflammation. Consistent with the previous findings, blocking C5aR has an instant and acute effect on pain behavior, which differs with the delayed behavioral effect we observed with anti-C1q neutralizing antibodies (Fig. 6b). Additionally, blocking C1q or genetic KO of C3 does not fully prevent C5a and C3a production and their effect on inflammatory pathways. In the absence of the classical complement pathway activation, which is the case with anti-C1q treatment, C5a and C3a can be generated via alternative and lectin pathways. In the absence of C3, the case of C3 KO, C5 can be generated through thrombin (Krisinger et al., Blood 120,

1717-1725, 2012). Therefore, C5a and C3a can still contribute to pain behavior in anti C1q-treated and C3 KO mice.

In our view, the acute behavioural effect of C5a and C3a inhibition is more consistent with the pro-inflammatory role of the complement system while the delayed behavioural effect of C1q inhibition can be better explained by the role of C1q in synapse pruning.

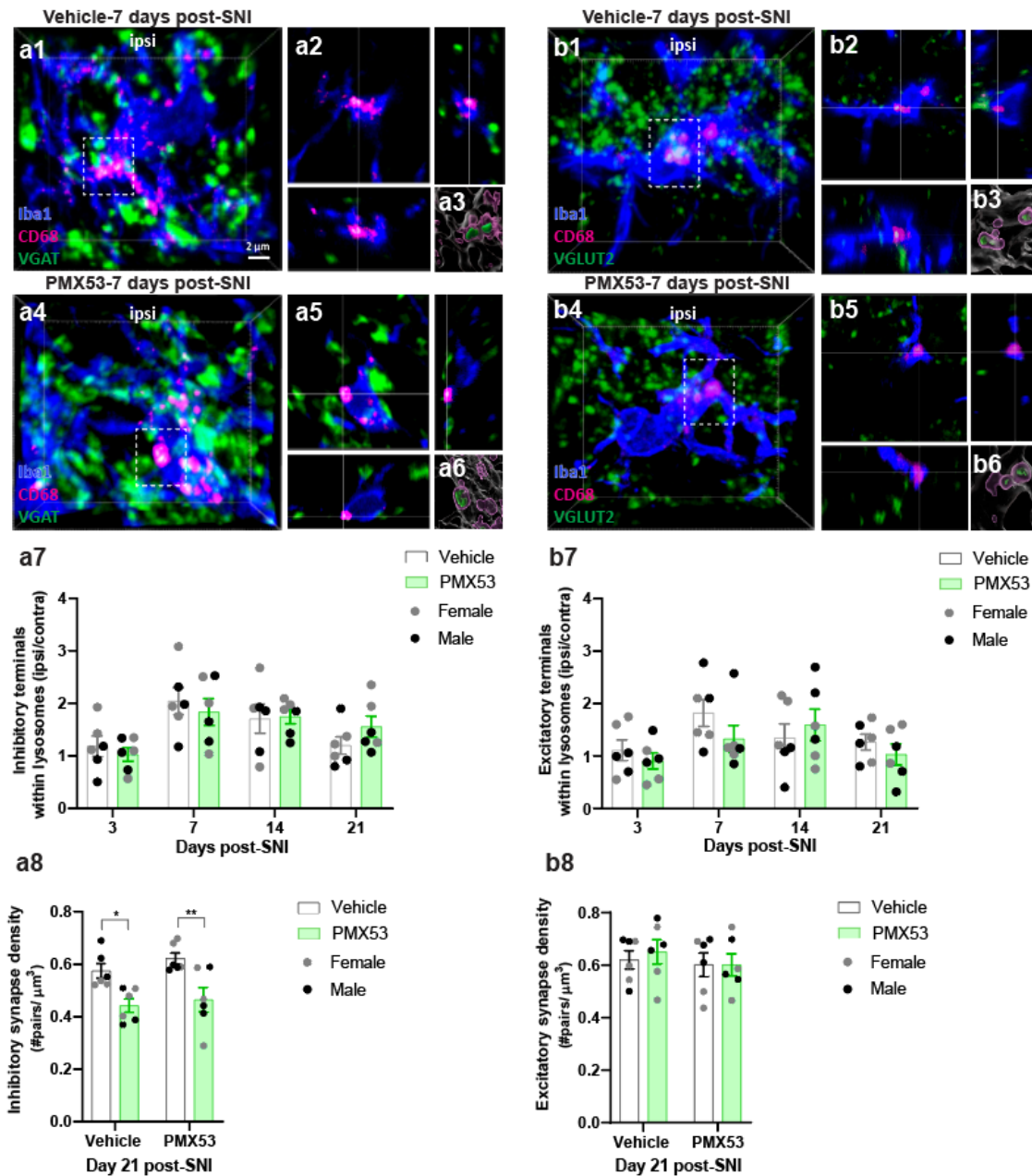


Figure related to above comment: Chronic C5a inhibition does not prevent spinal synapse engulfment and synapse loss: Three-dimensional fluorescent images of microglia (blue) sampled from ipsilateral dorsal horn labeled for lysosomal marker CD68 (magenta), marker of presynaptic inhibitory terminals (**a1 and a4**: VGAT; green) and marker of presynaptic excitatory terminals (**b1 and b4**: VGLUT2; green). **a2, a5, b2, and b5** are single plane enlarged images selected from confocal stacks illustrating the presence of synaptic signal inside lysosomes. **a3, a6, b3, and b6** are Representative 3D surface-rendered images illustrate the engulfment of presynaptic elements. **a7 and b7** Quantifications of temporal changes of engulfment of inhibitory (**a7**) and excitatory (**b7**) presynaptic terminals by microglia **a8 and b8** Quantifications of inhibitory and excitatory synapse density at day 21 post-SNI (n = 6 mice per group; 3 males and female mice) Means are plotted with individual data points $\pm$ SEM. * $p < 0.05$, and ** $p < 0.01$, analyzed with t-test.

Comment:

The study also stops short of explaining why there is a preferential loss of inhibitory synapses. C1q and C3 were found in all microglia, but C1q binds only to inhibitory synapses? Importantly, where are the effectors/receptors that mediate these functions localized and are these restricted to inhibitory synapses? Please report on the location of C3aR and C5aR.

Response:

In Figs. 5c1-3 and 6c1-2 we quantified C1q deposition on inhibitory and excitatory synapses and, in both cases, we observed an increase of C1q deposition on both synapse types.

Exploring molecules that attract C1q to the synapse site is an active area of research and so far in the context of excitatory synapses, phosphatidyl serin (PS) was shown to attract C1q (Scott-Hewitt et al., EMBO J 39, e105380, 2020). Whether the same target molecule is expressed by inhibitory synapses, and how its exposure is differentially regulated by inhibitory and excitatory synapses is an important question that remains to be answered. Separately, recent data on inhibitory synapse pruning suggest the involvement of a unique class of microglia that is sensitive to changes in GABA, as well as C1q (Favuzzi et al., Cell 184, 4048-4063 e4032, 2021).

We believe that answering the question of how C1q binds to a subset of inhibitory and excitatory synapses in the PNI model is an important study, which warrants an independent study and is beyond the scope of this study.

Microglia are shown to express C3aR and C5aR (Davoust et al., Glia 26, 201-211, 1999; Monk et al., Br J Pharmacol 152, 429-448, 2007). We also added new data that specifically showed that spinal microglia expressed these receptors and that their levels did not change after SNI (Supplementary Figs. 4d1-4)

Comment:

Finally, C3 signaling is also known to prune postsynaptic (dendritic) spine components. How is selective phagocytosis of the presynaptic terminals achieved via the C3 pathway in the spinal cord?

Response

Our findings suggest that in the SNI model of neuropathic pain, spinal C1q is selectively upregulated by peripheral nerve injury and is localized to a fraction of inhibitory and excitatory synapses. We have evidence suggesting that presynaptic sites are targeted by C1q and then are engulfed by microglia. Although, in our new analysis we show that anti-

C1q treatment also affects pre-synaptic elements, possibly as a consequence of pre-synaptic removal (Supplementary Figs. 6d, 6e). Upon C1q binding to the synapse, complement cascade is activated and C3 cleavage products are deposited, which attract microglia to the targeted synapse. Our data on synapse pruning and engulfment in C3 KO and microglia depletion experiment are consistent with this mechanism.

Comment

While at least part of the results suggest that microglia engulf both excitatory and inhibitory synapses (Fig. 1), but the latter half of the study addressing the role of the pathways only points to inhibitory synapses. In the discussion, the authors discuss that while both types of synapses are pruned, only excitatory synapses are added on via other mechanisms. This leads to the question as to how excitatory synapses are being pruned.

Response

As the reviewer correctly noted, we show engulfment of both inhibitory and excitatory synapses (Figs. 1, 4 and 6). But we only show that this engulfment activity results in inhibitory synapses loss, not excitatory (Figs. 2, 4 and 6). Notably, our microglia, and C1q depletion experiments, as well as our C3 and C4 KO studies show that inhibition of the complement-mediated synapse pruning cascade results in normalization of inhibitory synapses and increases excitatory synapse count (Figs 2, 4 and 6). All together, these findings suggest that excitatory synapse pruning happens in parallel to inhibitory synapse pruning. However, the excitatory synapse counts are unaffected presumably because excitatory synapses being regenerated resulting in the rewiring of the spinal circuitry. In the discussion, we proposed plausible mechanisms to explain why synapse pruning results in these two different outcomes (Discussion section: Microglia rewire dorsal horn circuitry in neuropathic pain). Additionally, we modified the text to ensure that our emphasis on the effect of microglia-mediated synapse pruning on loss of inhibitory synapses was not misleading.

Other questions/suggestions from reviewer:

Comment

- Since the complement cascade might not be broadly known to the readership, a schematic depicting parallel pathways and/or inter-relationship between key players mentioned in the manuscript (C1q, C3, C3aR, C5a, C5aR, fraktalkine, CXCR3) would be helpful.

Response

This is a great suggestion. We added a schematic depicting these parallel pathways to the paper (Supplementary Fig. 4d1).

Comment:

Please clarify the mode of administration of PLX3397 in the results text.

Response

The mode of administration was i.p. injection of PLX3397 and is now added to the result section (line 140).

Comment:

The data indicate that although the number of Vglut2+ and Vgat+ inclusions was reduced in C3 KO mice, the volume of CD68+ lysosomal compartments increased to the same level in these mice as with wild type mice following nerve injury. What could be leading to the latter?

Response

For assessing engulfment, we quantified the amount of synaptic proteins within the lysosomal compartments of microglia. In Supplementary Figs. 4b1-3, we assessed the volume of microglial lysosomes to confirm that the increase in microglial lysosomes in KO mice was comparable in the WT. These data showed that the reduction in synapse engulfment in C3 KO mice was not due to any possible reduction in CD68 expression by microglia and a deficit in lysosomal production was not biasing our quantification.

Although the increase in CD68 expression suggests an increase in microglial phagocytic activity, it does not necessarily mean elevated synapse engulfment via the complement pathway. Additionally, to our knowledge there is no evidence that C3 expression regulates or affects CD68 levels or lysosomal size.

Comment

The title of the study is weakened by previous studies showing that the downstream targets of C1q can be exploited for pain relief in neuropathic pain. From the reviewer's point of view, the most noteworthy finding of this study is not that targeting C1q reduces neuropathic pain, rather that microglial mechanisms lead to the known dysbalance between excitatory and inhibitory synapses via the complement pathway, leading to neuropathic pain.

Response:

We reconsidered the title of the manuscript, and we are very grateful for the good title suggestion.

The new title of the manuscript is: **Targeting C1q Protects Spinal Synaptic Circuitry in Neuropathic Pain**

Reviewer #2 (Remarks to the Author):

Overall response to comments: We thank the reviewer for encouraging, detailed, and constructive comments that helped clarifying the findings and led to some interesting new observations. Below are the responses to the reviewer's comments.

The reviewer had no major concerns. The following minor concerns were mentioned:

Comment

Clearly indicate time points of analysis within figure legends.

Response

In the new version, all the timepoints of the analyses are indicated in the figure legends.

Comment:

Clarify the timing of injury model (ie: acute versus chronic injury phases) and frame observations in this context. How could these data be interpreted across different stages of injury and/or recovery?

Response

Pain behavior does not recover within the timeframes that were studied here. Based on the pain behavior characterization of this model, at 7 days post-SNI, animals reach the maximum hypersensitivity that is maintained for months (>6 months) (Decosterd and Woolf, Pain 87, 149-158, 2000). Most studies using the SNI model were done within the time frame of a week to a month after the SNI induction. This is because many changes that take place in the first month after SNI are thought to be important for the long-term maintenance of neuropathic pain while other changes are transient with short-term impact on pain phenotype. Our hypothesis is that the early targeting of structural modifications in spinal synaptic circuitry is important to interfere with the establishment of long-term neuropathic pain. In this study, we identified that spinal synapse pruning is noticeable at 7 days after injury and remained significant for the following two weeks. This resulted in inhibitory synapse loss at 3 weeks post SNI. Based on this comment, in the new version of the manuscript, we asked the question if the observed synapse pruning was maintained for a

longer time and if it had any long-lasting effect on the spinal circuitry. We characterized the expression of complement-mediated synapse pruning components, engulfment, and synapse density at 5 months after SNI when chronic neuropathic pain was fully established (Figs. 3f-j and Supplementary Fig. 3). Five months after SNI, we found no significant microglia-mediated engulfment of inhibitory (Supplementary Figs. 3e1-2) and excitatory (Supplementary Figs. 3f1-2) terminals, which was consistent with downregulation of major synapse pruning-associated complement proteins (Figure 3f and Supplementary Figs. S3a-c). Notably, at this chronic time-point, a significant decrease in inhibitory synapses and an increase in excitatory synapses were observed in the ipsilateral LII as compared to the contralateral side suggesting the lasting effect of synapse engulfment on dorsal horn connectivity (Supplementary Figs. 3e3-4 and 3f3-4). These results suggest that although spinal synapse pruning is an early and transient event, it has a long-lasting impact on spinal circuitry. We thank the reviewer for putting us on the path of this new discovery.

Comment:

For example, using their injury model, Yousefpour et al demonstrate in Figure 1 that significant increases in microglial engulfment of neuronal elements and presynaptic terminals within the ipsilateral spinal cord following SNI (Figs 1e, f and I, j, respectfully) can be detected beginning 7 days post injury. They continue to detect significant engulfment at 21 days post injury, the longest time point they report. Is spontaneous recovery observed in this injury model? Is there a time point following injury that microglial engulfment returns to baseline? How could persistent microglial engulfment/activation be affecting this model and the interpretations of these data? The authors should address these findings more clearly in the text and highlight potential caveats.

Response:

As mentioned above, the SNI model of neuropathic is known to produce long-lasting pain behavior that is maintained for months (>6 months). However, in females, but not in males, reduced allodynia was reported after 14 months post-SNI (Muralidharan et al., J Clin Invest 132, e151817, 2022). Unfortunately, it was not feasible for us to prolong the study until these late time-points to examine synapse pruning during the recovery period in this model.

To address the comment regarding the more chronic effect of microglial engulfment, we assessed the following changes and added them to the manuscripts after 5 months post-SNI:

1. Changes in protein expressions in complement and Cx3CR1-mediated synapse pruning pathways (Figs. 3f and 3J and Supplementary Figs. 3b-d)

2. Changes over time in expression of complement and CX3CR1 related synapse pruning genes were studied for both pathways (Supplementary Fig. 3a)
3. Engulfment at 5 months post-SNI (Supplementary Figs. 3e1-2 and 3f1-2)
4. Inhibitory and excitatory synapse counts at 5 months post-SNI (Supplementary Figs. 3e3-4 and 3f3-4)
5. C1qa mRNA expression per microglia at 4 months post-SNI (Fig. 5a3)

Comment:

- The phenotypes of C1q inhibition and microglia depletion observed are different. While both show prevention of inhibitory synapse loss, these do not phenocopy one another, suggesting that there may be additional mechanisms of C1q and/or microglia in injury and recovery processes independent of one another. These issues should be addressed in the text and figure legend titles.

Response:

We agree with the reviewer that C1q inhibition and microglial depletion do not result in the same behavioral outcome. However, this is not unexpected because microglia contribute to pain behavior through many different pathways, and we propose that C1q-mediated synapse pruning is one of the many pathways by which microglia contributes to the pain pathology. However, the contribution of spinal C1q to neuropathic pain seems to be more specific and related to fewer pathways such as synapse pruning. We re-worded the text and figure legend titles to properly highlight the differences between the two behavioral effects.

Figure-specific concerns:

Comment:

Figure 2: Would be helpful to have engulfment data of microglia that repopulate the dorsal horn following completion of PLX treatment.

Response:

These data were generated and added to Fig. 2 (Fig. 2e-f)

Comment:

Concerns ANOVA analysis of PLX over time: Fig 2d

Response:

The requested analysis is added to the results section and below is the ANOVA table for the PLX treatment behavioral study with the requested analysis highlighted:

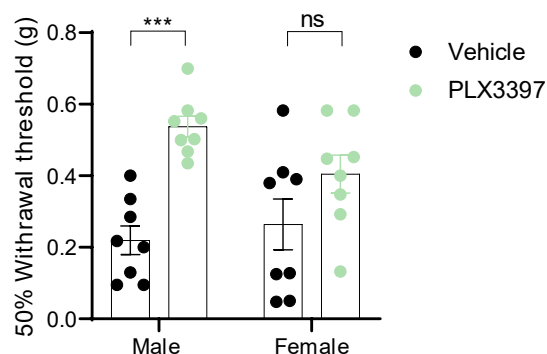
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.2327	3	0.07756	F (3, 120) = 3.463	P=0.0185
Treatment	1.736	3	0.5787	F (3, 120) = 25.83	P<0.0001
Time	0.5791	1	0.5791	F (1, 120) = 25.85	P<0.0001

Comment:

Still see reduction in pain threshold in PLX-treated cohorts over time. Pharmacological depletion of microglia does not fully prevent neuropathic pain phenotype (based on behavioral analysis Fig 2D). There is improvement, but animals don't seem to be fully protected. Additionally, phenotype is similar while animals are on treatment (with few microglia at day 7) and at day 21 when microglia have repopulated, indicating that loss of microglia early after injury does not result in improved recovery over time.

Response:

There is a sex-difference in the drug effect on pain behavior at day 7, which is consistent with the previous reports in the literature that depletion of microglia fully abolishes pain behavior in males and is not as effective in females (please see the graph below).



Pain behavior in neuropathic mice treated either with PLX3397 or vehicle (n = 8 mice per group) at day 7 post-SNI during the treatment period.

Since the sex difference observed during the treatment period at day 7 did not exist at later off-treatment time points (i.e., days 14 and 21 post-SNI), which were more relevant time-points to the mechanism being investigated, we did not separate the data by sex. However, we color-coded the data to demonstrate the behavioral differences between males and females.

At days 14 and 21 post-SNI, although we did not observe full recovery either, we still saw a partial drug effect compared to control suggesting that early depletion of microglia affected pain behavior at later time-points. This aspect of the results was consistent with the delayed partial effect of anti-C1q treatments on pain behavior that was later demonstrated.

Additionally, as synapse pruning is not the only function of microglia, the repopulated microglia would still contribute to pain through other mechanisms, and this may explain why full pain recovery is not achieved in these later timepoints.

Finally, we do agree with the reviewer that the magnitude of the behavior effect in figure 2d does not indicate recovery, and we are careful not to suggest otherwise in the text.

Comment:

•Fig 2F: See significant reduction in inhibitory synapses in vehicles, completely prevented in microglia-depleted cohorts, however animals still have pain phenotype. Indicates that, while microglia may be playing a direct role in the significant reduction of inhibitory synapses observed, this is not the only contributing factor to phenotype. Please clarify in text.

Response:

We agree with the reviewer. This observation is consistent with the lack of full recovery with anti-C1q treatment that also completely prevented synapse loss but partially affected pain behavior (Fig. 6b). We dedicated the last section of the discussion to highlight the partial and delayed effect of anti-C1q treatment on pain behavior.

This point is also clarified in the text (lines 173-175).

Comment:

Figure 3: Given the abundance of microglial sequencing data publicly available, authors should compare their datasets to the disease-associated microglia signature (DAM; Keren-Shaul et al Cell 2017 and others) as several of these pathways highlighted have also been associated with this gene signature.

Response:

This is a great suggestion. We analyzed microglial gene signatures for the two key pathways that we investigated using Tansley et al. (Nat Commun 13, 843, 2022) data set and reported the data in Supplementary Fig. 3a. Tansley et. al performed a more comprehensive comparison between SNI-induced microglia signature and other studies, and also reported the induction of DAMs in the SNI model of neuropathic pain.

Comment:

• Figure 3 legend/title needs to be revised. The data do not necessarily show that these 'pruning pathways are upregulated'. Gene expression of some factors is increased, and IHC analysis of proteins shows an increase in several factors as well, but this could be just correlative.

Response:

This is a valid point. Our analyses indicated that key components of the synaptic pruning pathways pruning pathways are upregulated. We have modified the title according to the reviewer's comment.

Comment:

Figure 3: Do these changes in microglia persist over time? As referenced above, significant engulfment of synaptic elements persists at least until 21 days post injury. Do the microglia retain these changes? IHC and RNAScope analysis should be performed at these other time points to address this question and to better understand the role these changes may play in the injury over time.

Response:

In the new version, we added quantifications for engulfment (Supplementary Figs. 3e1-2 and 3f1-2), synaptic changes (Figures S3e3-4, and Sf3-4), synapse pruning-related protein changes (Figures 3f-j, and S3b-d), and RNAScope analysis for C1qa expression (figure 5a3) at 21 days and 5 months post-SNI.

Comment:

Figure 4: The authors show no data on CR3 even though that is the title of the figure. Figure shows data from C3 KO animals, which could have complement-dependent activities independent of microglia engulfment. To definitively state this, experiments would need to be performed in CR3 KO mice. This should be clarified in the title and text.

Response:

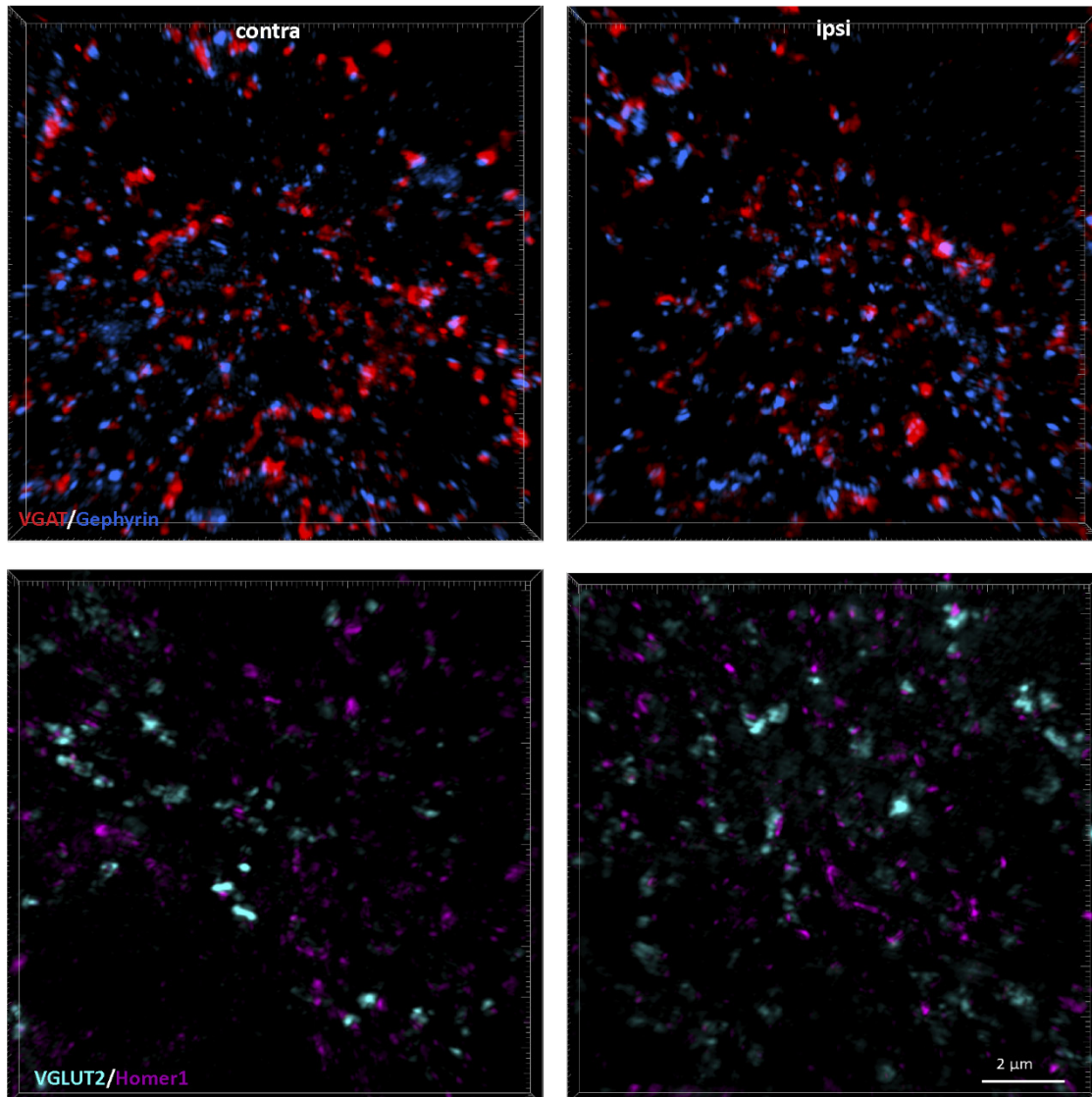
Unfortunately, we did not have access to CR3 KO mice. In the revised version of the paper, we replaced CR3 pathway with complement-mediated pathway.

Comment:

Figure 4: For all synaptic analyses, it would be helpful to see (perhaps in supplemental figures) whether individual presynaptic or postsynaptic values are changing (independent of co-localized structural synapses). In several data sets (i.e. Fig 4e, 4g; Fig6l, 6k) there appears to be considerably more presynaptic labeling in experimental conditions that may not be reflected in the quantified data. It may be that changes in microglial targeting have unique effects on presynaptic injury and recovery.

Response:

Thank you for this suggestion. We included the values for pre and post synaptic marker



quantifications in figures S2e-d and S6d-e. In fact, we found a higher density of labeling for post-synaptic markers compared to pre-synaptic markers for both synapse types. Possibly, because the labeling associated with presynaptic markers are relatively bigger than post-synaptic markers, it appears that there is more presynaptic labeling. We provide a larger magnification of the WT conditions in Figs. 4c1 and 4d1 for the reviewers only (see above) to help compare the labeling for pre- and post-synaptic densities. Also, in the new version of the manuscript we made the synapse quantification images for Figs. 2 and 6 as large as possible.

Comment regarding Figure 5:

- In Fig5A, it would be helpful to see co-localization with Iba1 IHC or another microglial-specific marker to confirm expression is only in microglia.

Response:

We performed Iba1 immunohistochemistry on the TMEM119-tdTomato mice and quantified the percentage of Iba1⁺ microglia that was not TMEM119⁺ in LI and LII (Supplementary Figs. 4a1-2). The quantification shows that about 90% of Iba1⁺ cells in LII are microglia.

Comment: (still concerning Figure 5)

Unclear how analysis was performed- were all cells counted? Was C1q mRNA detected in all cells? Please clarify.

Response:

C1qa mRNA was not detected in all cells. C1qa was hardly detectable in the contralateral cells of LII. In the ipsilateral cells, only a subset of DAPI⁺ nuclei had C1qa mRNA signal. As now is better clarified in the methods, in the figure 5a3 graph, we quantified the number of C1qa mRNA-associated puncta in those DAPI⁺ nuclei that are also C1qa⁺. The details of this quantification approach are added in the new version of the text.

Comment: (still concerning Figure 5)

- Would be helpful to see time course of RNAscope, C1q IHC analysis across relevant time points during injury and recovery.

Response:

In the current version, RNAscope analyses for C1q is added at day 7, day 21, and 5 months after injury (Fig. 5a3).

Comment: (concerning Figure 6)

Fig 6b: phenotype appears much worse in this cohort than in Fig 2. Does variability in severity of injury influence data? Especially given the discrepancy in phenotypes between C1q inhibition and microglia ablation.

Response:

Pain behavior is a function of multitude of factors including the experimenter, and the environmental factors. Pain behavior experiments, and PNI surgeries for microglia depletion and C1q inhibition experiments were performed by two different experimenters at two different times. The C1q inhibition experiment was replicated 2 times, and the results were consistent in both trials. We are happy to share the data for the first trial with the reviewer in case it is necessary.

Comment: (still concerning Figure 6)

- No difference in C1q-labeled vglut2 with C1q inhibition, however the authors observe a significant reduction in vglut2 engulfment.

Response:

Lack of statistical differences in C1q-labeled condition is because in the previous version, a 2-way ANOVA following Bonferroni post-hoc tests was performed to compare changes in C1q-labeled VGAT and VGLUT2 in vehicle and drug treated conditions. However, this test is not appropriate since VGAT and VGLUT2 changes are independent from each other. If we compare VGAT and VGLUT2 changes independent from each other with a t test, we find a significant reduction in both C1q-labeled VGAT and VGLUT2 synapses after treatment. In the new version, we chose the latter analysis (Fig. 6c2).

Comment: (still concerning Figure 6)

- Looks to be dramatically different changes in presynaptic staining with C1q inhibition. Would be helpful to show quantification of presynaptic labeling independent of 'synaptic' quantification co-localization with postsynaptic label as mentioned above.

Response:

Qualifications for pre-synaptic elements for anti-C1q treatment study are added in Supplementary Figs. 6d and 6e). Additionally, larger versions of synapse images were provided in Fig. 6.

Comment: (still concerning Figure 6)

- The authors need to clarify potential differential roles of microglia throughout the injury process. Pharmacological C1q inhibition is performed across the duration of injury model, however the authors see no improvement until last time point assessed (21 days post injury). These data indicate that C1q may have a specific role in impeding the recovery process rather than preventing injury during acute phase of this model.

Response:

The reviewer raises an important point regarding the delayed effect of C1q inhibition on pain behavior, which we also addressed in the last part of the discussion section. Since this model does not naturally recover, we think that C1q is more involved in the maintenance of chronic neuropathic pain.

Comment: (still concerning Figure 6)

- Similar to Fig 2F, the authors demonstrate that pharmacological inhibition of C1q prevents inhibitory synapse loss, but not neuropathic pain behavior. The authors should highlight this finding and provide alternative explanations in the discussion

Response:

It is likely that the aberrant increase of excitatory synapses in the anti-C1q-treated group contributes to the incomplete reversal of pain behavior. This is our main theory to explain the incomplete behavior effect despite the complete prevention of synapse loss. This point is integrated in the discussion (lines 421-425).

Reviewer #3 (Remarks to the Author):

We thank the reviewer for the insightful comments that helped us improving the manuscript.

Major comments from the reviewer:

Comment:

In one case the authors use a receptor knockout (Cx3CR1), in the other they use a ligand knockout (C3 KO). It seems that these could have very different outcomes simply on that basis. For example, can fractalkine bind to other receptors? Are there other ligands for C3R? Did the authors perform similar experiments in the CR3 KO mouse (as in Schafer et al, Neuron 2012)?

Response:

We did not perform CR3 KO studies. Our rationale for choosing the C3 KOs (new data from C4KOs are now added to the manuscript (Figs. 4e4 and 4f4)) and Cx3CR1 KOs was to assess the involvement of the complement and the Cx3CR1 synaptic pruning pathways with minimum interference with other pathways. Besides C3 cleavage products, C3R is reported to interact with leukocyte receptors, extracellular matrix, and other secreted molecules (Lamers et al., Front Immunol 12, 662164, 2021). Specifically, C3R role in leukocyte extravasation and interaction with extracellular matrix could affect leukocyte infiltration, as well as the mobility of microglia following PNI, which could affect the phagocytic function of microglia independently from C3R interactions with C3 products. Unlike C3R, C3 is expressed by glial cells and has a more well-defined role and relatively limited functions. Our rationale for not using Cx3CL1 was that this protein is reported to be expressed in neurons and we did not want to directly interfere with neuron-specific proteins.

Additionally, both C3 and CX3CR1 KOs were previously used as tools to interfere with C3R and CX3CR1 pathways (Schafer et al., Neuron 74, 691-705, 2012). However, in agreement with the reviewer, since we did not directly show the involvement of knocking out or

inhibiting C3R in the study, we removed the claim that suggests that we have directly modulated the C3R pathway. Instead, we refer to the pathway that was studied as complement-mediated synapse pruning.

Comment:

- What was the rationale for sparing the sural in some of the experiments and then sparing the tibia for others? This is very unusual given the regional distribution of these nerves with respect to their dorsal horn central projections which could confound results. Moreover, the authors do not even specify which experiment was performed with which exact SNI model. More information is needed, and ideally, all experiments should be performed in the same model.

Response:

The only experiment in which the tibial nerve was spared is the PLX3397 experiment (Fig. 2). The experiment in Fig. 2 was also replicated with sural nerve damage and comparable data were obtained (data are not shown in the paper but can be provided upon request). We were interested in finding whether there was an association between synapse changes and the territory of nerve damage, but we found no difference between the two variations of the SNI with respect to synapse pruning. The reason why we picked the tibial SNI model data over the sural SNI to incorporate in text was that in the tibial SNI model experiment both male and female mice were included and therefore was more informative. In the new version, we specified which PNI model was used for each experiment.

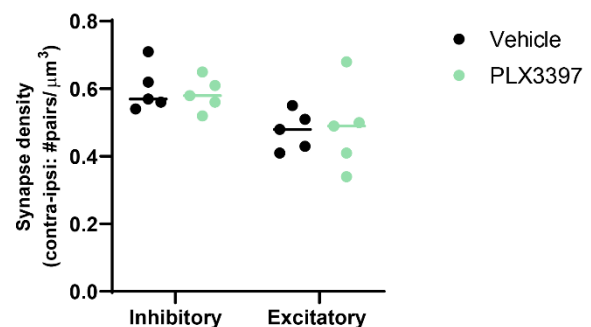
Comment:

With the PLX3397 depletion, at what time point was synapse density quantified (Fig. 2e-h)? Also, what is the effect of depletion alone (without SNI) on synapse density? This is important because the authors demonstrate a 13% increase in excitatory synapses, do these mice exhibit hyperalgesia without injury?

Response:

The synapse density was quantified at 21 days after PNI.

Depletion of microglia in sham animals does not result in changes in synapse numbers or hyperalgesia. The figure on the right shows synapse density in sham mice after 21 days following the same PLX3397 treatment protocol outlined in Fig. 2a.



It appears that, in the absence of microglia, PNI is necessary to induce the increase in excitatory synapses. It is likely that this increase contributes to the incomplete recovery of pain behavior when the pathway responsible for synapse pruning pathway is blocked.

Comment:

- Authors quantify number of CR3+ microglia (Fig 3e2) but % expression of Cx3CR1 per microglial cell (Fig 3h2). Why was the metric changed rather than kept identical for the two different pruning pathway receptors?

Response:

We have improved the CX3CR1 staining shown in Fig. 3h1 and provided quantification of the number of CX3CR1⁺ microglia in Figs. 3h2.

Comment:

The C3 KO strain demonstrated almost identical increases in both excitatory and inhibitory synapses. A behavioural outcome (at a minimum allodynia) is therefore crucial to understand the ultimate functional consequence of these changes which may offset the SNI-induced loss of inhibition, or alternately, may overshoot with the increased excitatory synapses.

Response:

We assessed pain behavior in both C3 and C4 KO mice and observed normal development of allodynia in both KO mice. One possibility, as suggested by the reviewer, could be due to the excessive production of excitatory synapses in these KO mice. We also considered the possibility of the effect of developmental processes on spinal circuits. This is because at the baseline level, we found a greater density of excitatory and inhibitory synapses in these KO mice compared to wild-type mice although the baseline mechanical thresholds appeared to be normal. Consistent with this hypothesis, we added new data that showed reduced engulfment of spinal synapses during postnatal development in C3 and C4 KO mice (Figs. 4e3 and 4f3).

Due to these potential developmental complications in C3 and C4 KO mice, we sought to use C1q neutralizing antibodies in wild-type mice to assess the effect of interfering with the complement synapse pruning pathway on synapse density and pain behavior.

Comment:

- Pre-emptive analgesia is not particularly clinically-relevant. Have the authors performed ANX dosing in a treatment (i.e., post-injury) paradigm? How was the dose of the ANX-M1.21 anti-C1q blocking antibody selected? Was a dose-response performed? The anti-allodynic effect shown in Figure 6b is subtle (increased from 0.10 to 0.25g), suggesting that either the

contribution of synapse pruning to this outcome is minor, or that the dose needs to be optimized.

Response:

Anti-C1q antibodies were administered at 100 mg/kg. At this dose, classical complement pathway is fully inhibited and full target engagement in the CNS is achieved. We have included the PK/PD data that show full inhibition of the complement cascade in Figure S6a. We have also used anti-drug (anti-antibody) antibodies and confirmed the presence of the drug in the spinal cord (Supplementary Figs. 6b1-7). This evidence suggests that it is unlikely that the magnitude of the behavior effect is due to insufficient dose since at this dose full complement blockage is achieved.

As stated in the discussion and highlighted by the reviewer in the previous comments, with targeting complement-mediated synapse pruning pathway, we affect both inhibitory and excitatory synapses and perhaps, to achieve a greater behavioral effect, only inhibitory synapse pruning should be targeted. However, the molecular mediators that differentiate inhibitory and excitatory synapse pruning are not known and identifying them is beyond the scope of this study. Our primary goal in this study was to demonstrate the role of classical complement pathway in spinal structural plasticity and in neuropathic pain. Further studies are required to address the effect of targeting complement system as a therapeutic approach and dissect mechanisms that are unique to inhibitory synapse loss.

Comment:

- The investigators used the C3 KO mouse (Jax #003641) which is on a mixed background (B6:129). Given what is known about strain differences in pain outcomes, with strain suggested to be a major confound in mice pain research (Lariviere WR, Chesler EJ and Mogil JS. JPET 2001) why did the authors use this mouse instead of the C3 KO mouse which is C57Bl/6J congenic (Jax #029661)?

Response:

The reviewer is raising a valid point, and we thank the reviewer for highlighting the strain differences. We have investigated the development of pain behavior in both C3 and C4 KOs. C4 KO was from C57Bl/6J background, and we have observed the normal development of allodynia in both mouse strains.

Comment:

- The authors do not demonstrate any functional correlate of the loss of inhibitory synapses. Does spontaneous excitatory postsynaptic current (sEPSC) frequency change? This would be important to understand because the effect on behaviour is rather minimal.

Response:

We do not possess in-house resources for electrophysiology. Unfortunately, our plans to generate physiology data with our external collaborator were interrupted by covid-19 pandemic and despite our efforts, it was not feasible to perform functional work in a reasonable time for this body of work.

Comment:

- The drug being studied in the final experiments (ANX-M1.21) was supplied by Annexon and author YAZ is employed by Annexon. This should be disclosed in the COI section.

Response:

Thanks for raising this point. We have listed the co-authors that were or are employed by Annexon Inc. in the COI section.

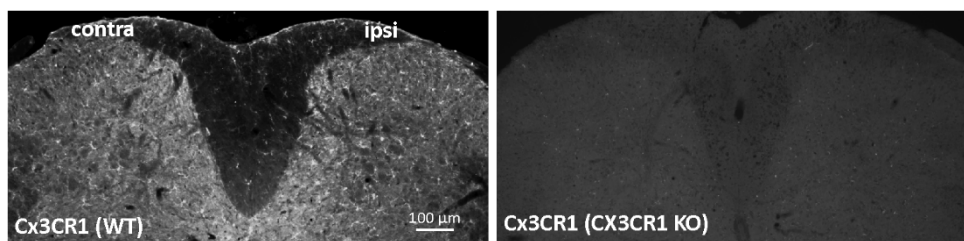
Minor comments:

Comment:

Figure 3 IHC images are very small and hard to discern. In particular, the Cx3CR1 staining looks diffuse and not cell-specific, can larger or high-resolution images be provided?

Response:

We have replaced the CX3CR1 image and below we show a larger view of the staining in the WT and CX3CR1 KO for the reviewer.



Comment:

Is the "n" listed per group in the figure legends the number of mice, the number of sections or something else? Please specify for each figure/experiment.

Response:

N is the number of animals in all the experiments. This is stated clearly in the revised version.

ANSWER TO COMMENTS

We are extremely pleased that the manuscript was found publishable after addressing some minor comments.

In preparing this revised version, we addressed the comments of reviewer 3 (below) as well as the Extended Comments from the Editor (in attached file).

We hope that this revised version meets editorial demands and is now ready for publication.

ANSWER TO REVIEWER #3 COMMENTS

We would like to thank this reviewer for the careful reassessment of our manuscript. Below are the answers to the reviewer's comments:

Comment:

Reviewer #3 (Remarks to the Author):

1. The interpretation of the PLX studies is not altogether clear to me. For example, if there is a 13% increase in ipsilateral DH excitatory synapse density after depletion, how does that result in overall lower hypersensitivity?

Thank you for this comment. We do not claim that synapse loss of both types directly results in reduced hypersensitivity in the PLX experiment. To clarify, we have revised the concluding section to explain the following points: (a) the key finding of the transient microglial depletion study is that both excitatory and inhibitory synapse removal are prevented, which ultimately resulted in the selective loss of dorsal horn inhibitory synapses, and (b) the relationship between microglial inhibition and reduced pain hypersensitivity cannot be conclusively attributed to synapse pruning at this stage of the study. We have also highlighted that our subsequent experiments will focus on establishing these connections more definitively.

2. The major finding is related to C3/complement-mediated synapse engulfment but as written (lines 221-244) the focus is on the negative CX3CR1 KO data. It would help clarify the findings if instead the focus was on the C3 KO experiments.

We appreciate the reviewer's feedback and have revised the text to emphasize the positive findings associated with the C3/complement pathway.

3. Given the developmental effects of the C3 KO, the preferred experiment would be in an inducible C3 cKO mouse line. Agree that the C1q blocking experiments are the next best option.

Thank you for recognizing the limitation regarding the KO mice. At the time of our study, the inducible C3 KO mouse model was not yet available.

4. As mentioned previously, pre-emptive analgesia is not particularly clinically-relevant. Have the authors performed ANX dosing in a treatment (i.e., post-injury) paradigm? No additional experiments are requested, however, please do comment on this in the Discussion.

That is an excellent question. ANX dosing was specifically designed to help dissect the underlying mechanism, therefore the treatment was administered prophylactically. We did not evaluate the post-injury effects of complement inhibition; however, based on the temporal characterization of synapse pruning (Figure 1), and complement expression levels (Figure 3), we anticipate that anti-C1q treatment would result in the most significant analgesic benefit—possibly even greater than the prophylactic effect—around the second week following peripheral nerve injury in this model, when synapse pruning and complement expression reach their peak.

We have now commented on this point in the discussion. Thank you for bringing up this interesting discussion point.

5. The lack of sex difference in the PLX experiments is supported by other recent studies (Donovan et al. Cell Reports 2024; Zhou et al. Cell Reports 2019; Kohno et al. Science 2022). These studies also performed versions of microglia depletion with possible alternate mechanisms proposed which may be worth adding to the Discussion given that reversal of allodynia was delayed and incomplete in the current study. In particular, the fact that the repopulated microglia may be involved in resolution (Kohno et al; Donovan et al)

We have incorporated the studies you mentioned to further elaborate on the discussion of sex differences. We appreciate you bringing these recent studies to our attention.

Minor comments:

Figure 2d (and others) - y axis label needs to be corrected (withdrawal, not withdrawal)

The typo is corrected