

Inhibition of spinal astrocytic GPR177 attenuates neuropathic pain by modulating C3/C3aR signaling in male rats

Corresponding Author: Dr Yong-Xing Yao

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 current manuscript by Zhang et al. investigates the role of GPR177 in the spinal dorsal horn and its involvement in the pathogenesis of neuropathic pain. The authors demonstrate that GPR177 is primarily expressed in C3-expressing astrocytes within the spinal dorsal horn. Furthermore, the study shows that silencing GPR177 expression using siRNA suppresses the upregulation of C3 observed in the pain model and results in significant alleviation of pain behavior. Collectively, these findings suggest that the astrocytic GPR177/C3/C3aR axis plays a critical role in the development of neuropathic pain. However, there are several concerns regarding the study that need to be addressed.

Major issues;

1. The authors demonstrated via double immunofluorescence staining that GPR177 is primarily expressed in astrocytes in naïve rats. However, it is possible that the cellular localization of GPR177 changes in the spinal dorsal horn following peripheral nerve injury due to excessive sensory input, thereby contributing to the pathogenesis of chronic pain. Therefore, the authors should examine the cellular localization of GPR177 not only in naïve rats but also in neuropathic pain model rats during both the induction and maintenance phases of pain. Furthermore, the authors should quantify these co-localization results and compare the expression rates across the different conditions.
2. While I understand that GPR177 expression is upregulated, the authors should also analyze the expression levels of its endogenous ligand(s) during the induction and maintenance phases of the pain pathology. Additionally, they should investigate whether intrathecal administration of the endogenous ligand drives the activation of the C3/C3aR signaling axis.
3. Sex differences are well-documented in the pathogenesis of pain. Therefore, the authors should determine whether the GPR177/C3/C3aR axis is a male-specific mechanism or a common pathway shared by both sexes. It is essential to conduct these experiments using female rats to validate the generalizability of the findings.
4. While the authors show that GPR177 upregulation correlates with C3 expression, the molecular mechanism linking GPR177 (a Wnt transporter) to C3 production remains unclear. The Discussion should address whether this regulation is mediated via canonical/non-canonical Wnt signaling pathways or other mechanisms.
5. Regarding the quantification of Western blot data, with the exception of Fig. 5, the values for the Sham group appear to be normalized to exactly 1 without variance. The authors should analyze and present the experimental error for the control group as well, similar to the analysis presented in Fig. 5, to allow for an accurate statistical evaluation.
6. Even when GPR177 expression is silenced using siRNA, the authors should verify whether the downstream C3a-C3aR pathway is effectively suppressed. This should be demonstrated by analyzing the expression levels of C3a and C3b using ELISA.
7. Regarding the intrathecal Compstatin experiment, it appears that the protein expression levels of GPR177, GFAP, and C3 did not increase following CCI treatment in this specific dataset. The authors must explain this discrepancy and why the upregulation observed in previous figures was not reproduced here.
8. In the Discussion, the authors state, "Our findings align with previous studies showing increased GPR160 expression in the ipsilateral spinal dorsal horn of CCI rats." However, the current study focuses on GPR177, not GPR160, and the

relevance of this comparison is unclear. The authors need to provide context regarding GPR160 and clearly explain the relationship or relevance between previous findings on GPR160 and their current results on GPR177.

9. In the Discussion, the authors state: "Since both GPR177 and TRPs are members of the G protein-coupled receptor superfamily, we speculated that GPR177 might be similarly modulated... akin to TRPV family members." TRP channels are generally classified as ion channels that respond to external stimuli, not as members of the G protein-coupled receptor superfamily. The authors must correct this factual error and revise their argument to accurately reflect the biological classification of these proteins.

Minor issues;

1. To ensure reproducibility, the authors should add catalog numbers and RRIDs for all materials, such as antibodies, used in this study.

Reviewer #2

(Remarks to the Author)

Inhibition of spinal astrocytic GPR177 attenuates neuropathic pain by modulating C3/C3aR signaling in male rats

Hui Zhang, Ang Li, Meng-Jing Yang, Yu-Fan Liu, Yan Yang and Yong-Xing Yao

The authors investigated a potential mechanism by which GPR177 may contribute to neuropathic pain behavior in male rats with traumatic nerve injury. Here they found GPR177 was largely expressed in spinal cord astrocytes and when its expression was reduced by siRNA, neuropathic pain was attenuated. Moreover, the loss of GPR177 correlated to reductions in GFAP expression, indicating reduced astrocyte activity. Their findings also show a link between astrocyte GPR177 and astrocyte expression of C3 and C3/C3aR expression. This study provides a novel link between GPR177 in astrocytes and astrocyte C3/C3aR in traumatic nerve injury-induced neuropathic pain.

These novel findings are interesting and may provide insight into the astrocyte activity in chronic pain states as well as strategies to target this pathway to alleviate chronic pain.

The data is clear and experiments are well-controlled. The conclusions for the most part are supported by the data. However, there is one major concern that dampens enthusiasm for the study and a few minor issues that can be addressed.

The authors assert that GPR177 in astrocytes engages C3/C3aR signaling to contribute to pain. However, the only data supporting this is C3aR expression data. There are no functional studies to show that C3aR was engaged. Their data with compstatin show greater reversal of neuropathic pain behaviors than their GPR177 siRNA. This could come down to drug efficacy vs. siRNA efficacy, but it could also point to the other function of C3 in the CNS - aberrant synaptic pruning, which is also linked to neuropathic pain. This was neither ruled out by the use of C3aR antagonists, genetic reduction of C3aR in their model or discussed as potential alternative explanation. Therefore, the conclusions that this is C3aR-mediated needs more support.

Minor:

1. The authors should provide justification for only studying male. They recognize it as a limitation, but do not state why they believe males were the best starting point.

2. More information on the Von Frey test is needed. Was the threshold the first filament to yield a positive response or was it based on the algorithms such as the Dixon method? If it was the first positive response, did this have to occur 100% of the time between the 5 trials or 50%?

3. siRNA sequences should be included to allow the reader to determine the appropriateness of the sequence and what it covers.

4. Antibody and ELISA catalog numbers should be included to allow the reader to interpret the data and to repeat if they so wish.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all my concerns and the manuscript is much improved.

Reviewer #2

(Remarks to the Author)

The author's have satisfactorily addressed all issues raised. The additional data, discussion, and RRIDs strengthen the manuscript.

One minor suggestion would be to define abbreviations (e.g., MT, TL, etc.) at the first instance in the results since the methods follow the results and discussion.

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Reply to Reviewer and Editor's Comments:

We thank the reviewers and editor for their valuable comments. The reviewers' insights have significantly improved the manuscript. We revised the manuscript accordingly. The revisions and changes are indicated in blue in a separate document. In addition, point-by-point responses to the comments are provided below.

Reviewer #1:

Major issues;

1. The authors demonstrated via double immunofluorescence staining that GPR177 is primarily expressed in astrocytes in naïve rats. However, it is possible that the cellular localization of GPR177 changes in the spinal dorsal horn following peripheral nerve injury due to excessive sensory input, thereby contributing to the pathogenesis of chronic pain. Therefore, the authors should examine the cellular localization of GPR177 not only in naïve rats but also in neuropathic pain model rats during both the induction and maintenance phases of pain. Furthermore, the authors should quantify these co-localization results and compare the expression rates across the different conditions.

Response: Thank you for your valuable suggestion. We fully agree that examining the cellular localization of GPR177 in both naïve and neuropathic pain model rats will strengthen our study. Therefore, during revision, we performed additional immunofluorescence staining for GPR177 with GFAP, NeuN, and Iba-1 under CCI condition. Quantitative analysis indicated that in naive state, more than 96% of GFAP+ cells co-localized with GPR177, whereas less than 3% of NeuN+ cells and 2% of Iba1+ cells co-localized with GPR177. The expression profile was similar in CCI model rats. These results were presented as bar graphs in the revised figures (Fig. 1E and S1–2) and discussed in the corresponding section.

We believe these additions will address your concern and provide a clearer understanding of GPR177's cell-type-specific role in neuropathic pain. Thank you again for your helpful feedback.

2. While I understand that GPR177 expression is upregulated, the authors should also analyze the expression levels of its endogenous ligand(s) during the induction and maintenance phases of the pain pathology. Additionally, they should investigate whether intrathecal administration of the endogenous ligand drives the activation of the C3/C3aR signaling axis.

Response: Thank you for your valuable comments. We fully understand your interest in the expression and functional role of the endogenous ligand(s) of GPR177 in pain pathology.

We would like to respectfully point out that GPR177 is currently classified as an orphan receptor—its endogenous ligand(s) have not yet been identified. As stated in the manuscript, no known endogenous ligand has been reported for GPR177 in the literature or in our preliminary studies. Therefore, it is not feasible to analyze the expression levels of its endogenous ligand(s) during the induction and maintenance phases of pain, nor to intrathecally administer such a ligand to investigate C3/C3aR signaling activation.

Given the lack of known endogenous ligands, our study focused on the functional significance of upregulated GPR177 expression and its downstream signaling cascade. We agree that once the endogenous ligand(s) of GPR177 are discovered, the experiments suggested by the reviewer would be highly informative and should be pursued.

We hope this clarifies the current limitation. We have added a brief statement in the Discussion section to acknowledge this point. Thank you again for your constructive feedback.

3. Sex differences are well-documented in the pathogenesis of pain. Therefore, the authors should determine whether the GPR177/C3/C3aR axis is a male-specific mechanism or a common pathway shared by both sexes. It is essential to conduct these experiments using female rats to validate the generalizability of the findings.

Response: Thank you for your insightful comment regarding the potential sex specificity of the GPR177/C3/C3aR axis. We fully agree that sex differences are an important consideration in pain research.

In response to your suggestion, we have performed additional experiments using female rats during the revision period. The results are now presented in the Supplementary Materials (Fig. S3). These data allow us to preliminarily assess whether the observed mechanism is male-specific or shared across sexes.

Furthermore, we have revised the “Limitations” subsection to transparently acknowledge the initial exclusion of female animals, summarize the new findings, and discuss their implications for future studies. We believe these additions strengthen the manuscript and provide a meaningful foundation for further investigation into sex-related differences in this signaling pathway.

We sincerely appreciate your constructive feedback, which has helped improve the rigor and generalizability of our study.

4. While the authors show that GPR177 upregulation correlates with C3 expression, the molecular mechanism linking GPR177 (a Wnt transporter) to C3 production remains unclear. The Discussion should address whether this regulation is mediated via canonical/non-canonical Wnt signaling pathways or other mechanisms.

Response: Thank you for your insightful comment regarding the molecular mechanism linking GPR177 (a Wnt transporter) to C3 production. We fully agree that clarifying this relationship is important for understanding the signaling axis. We acknowledge that we overlooked discussing the regulatory relationship between GPR177 and canonical/non-canonical Wnt signaling pathways, as well as other mechanisms in the original manuscript. In the revised Discussion, we have added a dedicated paragraph addressing the potential mechanisms, which are highlighted in blue font.

We sincerely appreciate your constructive feedback, which has significantly improved the depth and clarity of our manuscript.

5. Regarding the quantification of Western blot data, with the exception of Fig. 5, the values for the Sham group appear to be normalized to exactly 1 without variance. The authors should analyze and present the experimental error for the control group as well, similar to the analysis presented in Fig. 5, to allow for an accurate statistical evaluation.

Response: Thank you for your careful observation. We have re-analyzed the Western blot data for all figures and now present the Sham group values as mean $\pm$ SEM. The revised quantification bars include individual data points and error bars for the control group. These corrections have been made in the updated figures and legends. We appreciate your valuable feedback.

6. Even when GPR177 expression is silenced using siRNA, the authors should verify whether the downstream C3a-C3aR pathway is effectively suppressed. This should be demonstrated by analyzing the expression levels of C3a and C3b using ELISA.

Response: Thank you for your constructive suggestion. We fully agree that verifying the suppression of the downstream C3a-C3aR pathway upon GPR177 silencing is critical to confirm the specificity of the axis.

In response, we have performed ELISA assays to analyze C3a and C3b levels in the spinal cord tissue after GPR177 monoclonal antibody treatment (Chen G, et al., J Clin Invest, 2015). The results are presented in the revised manuscript (Fig. 5J–K). We observed that GPR177 neutralization significantly reduced C3a and C3b expression compared to the control group, indicating effective suppression of the downstream complement cascade.

These data further support the functional link between GPR177 and the C3/C3aR signaling axis. We have incorporated these findings into the Results section and updated the Discussion accordingly.

We appreciate your insightful feedback, which has strengthened the mechanistic evidence of our study.

7. Regarding the intrathecal Compstatin experiment, it appears that the protein expression levels of GPR177, GFAP, and C3 did not increase following CCI treatment in this specific dataset. The authors must explain this discrepancy and why the upregulation observed in previous figures was not reproduced here.

Response: Thank you for your careful observation. We sincerely appreciate the opportunity to clarify this point.

We would like to explain that there is an important difference in the control groups between the two experimental settings. In the earlier experiments (Fig. 2), the control group was a sham group that received sham surgery, and CCI induced upregulation. In contrast, in the intrathecal compstatin experiment, the control group received intrathecal DMSO (the vehicle for compstatin) in CCI model rats (Fig. 6F–H). Compared to CCI, CCI+DMSO did not alter the protein expression.

We hope this explanation resolves your concern. We are grateful for your thorough review, which has helped us to improve our manuscript.

8. In the Discussion, the authors state, “Our findings align with previous studies showing increased GPR160 expression in the ipsilateral spinal dorsal horn of CCI rats.” However, the current study focuses on GPR177, not GPR160, and the relevance of this comparison is unclear. The authors need to provide context regarding GPR160 and clearly explain the relationship or relevance between previous findings on GPR160 and their current results on GPR177.

Response: Thank you for your careful observation and valuable suggestion. We fully understand your concern regarding the mention of GPR160 in the Discussion.

We would like to clarify that the reference to GPR160 was intended to provide broader context for the emerging role of orphan GPRs in pain pathology. Both GPR160 and GPR177 are orphan receptors whose expression is significantly upregulated in the spinal cord under neuropathic pain conditions (e.g., CCI). The previous study on GPR160 demonstrated that its upregulation contributes to pain hypersensitivity, which parallels our current findings on GPR177. However, we agree that the direct juxtaposition without clear rationale may cause confusion.

To address this, we have revised the corresponding sentence in the Discussion to explicitly state that we mention GPR160 as an example of another orphan GPR implicated in pain, not to imply a direct mechanistic link or comparison. The revised text now reads: “Our findings align with previous studies showing increased expression of orphan GPRs, such as GPR160, in the ipsilateral spinal dorsal horn of CCI rats. This suggests that upregulation of orphan receptors may represent a common pathological feature in

neuropathic pain, though the specific downstream pathways of each receptor remain distinct.”

We believe this revision provides the necessary context and clarifies the relevance of the comparison. Thank you again for helping us improve the clarity of our manuscript.

9. In the Discussion, the authors state: “Since both GPR177 and TRPs are members of the G protein-coupled receptor superfamily, we speculated that GPR177 might be similarly modulated... akin to TRPV family members.” TRP channels are generally classified as ion channels that respond to external stimuli, not as members of the G protein-coupled receptor superfamily. The authors must correct this factual error and revise their argument to accurately reflect the biological classification of these proteins.

Response: Thank you for pointing out this factual error. We sincerely apologize for the inaccuracy. TRP channels are cation channels, not members of the G protein-coupled receptor superfamily. We have corrected this statement in the revised Discussion by removing the erroneous classification and revising the argument. The corrected text now reads:

“Although GPR177 is an orphan GPR and TRP channels are structurally unrelated ion channels, both have been implicated in pain signaling. We speculated that GPR177 might be similarly modulated in response to cold, heat, and mechanical stimuli, akin to TRP family members.”

We appreciate your careful review, which has helped improve the scientific accuracy of our manuscript.

Minor issues;

1. To ensure reproducibility, the authors should add catalog numbers and RRIDs for all materials, such as antibodies, used in this study.

Response: Thank you for your constructive suggestion regarding the reproducibility of our study. We fully agree that providing catalog numbers and RRIDs for all key materials, including antibodies, is essential for transparency and reproducibility.

In the revised manuscript, we have now included the catalog numbers and Research

Resource Identifiers (RRIDs) for all antibodies and major reagents used in this study. These details have been added to the Methods section and are also listed in Supplementary Table S1 for quick reference.

We appreciate your careful review, which has helped improve the rigor and reproducibility of our work.

Reviewer #2:

The authors assert that GPR177 in astrocytes engages C3/C3aR signaling to contribute to pain. However, the only data supporting this is C3aR expression data. There are no functional studies to show that C3aR was engaged. Their data with compstatin show greater reversal of neuropathic pain behaviors than their GPR177 siRNA. This could come down to drug efficacy vs. siRNA efficacy, but it could also point to the other function of C3 in the CNS - aberrant synaptic pruning, which is also linked to neuropathic pain. This was neither ruled out by the use of C3aR antagonists, genetic reduction of C3aR in their model or discussed as potential alternative explanation. Therefore, the conclusions that this is C3aR-mediated needs more support.

Response: Thank you for your critical and insightful comment. We fully agree that C3aR expression data alone are insufficient to conclude that C3aR signaling mediates the observed pain behaviors. To address this, we have performed additional experiments during the revision.

Specifically, we administered C3aR antagonist SB290157 (intrathecal, 10 µg/day for 2 days) to CCI rats. Our data show that C3aR blockade significantly reversed mechanical allodynia and thermal hyperalgesia, with an effect size comparable to that of Compstatin (Fig. S4). Furthermore, we investigated the effect of silencing spinal GPR177 on the expression level of a synaptic pruning markers CR3 and found no significant change following GPR177 knockdown, suggesting that the C3-C3aR axis, rather than aberrant synaptic pruning, may be the primary mechanism in our model (Fig. S5).

These results directly demonstrate that C3aR engagement is functionally necessary for the GPR177-driven pain phenotype. We have revised the Results and Discussion

sections accordingly, and we now also discuss the possibility of alternative C3 functions, noting that our current data support a C3aR-dependent mechanism while acknowledging that other pathways remain to be fully ruled out.

We sincerely appreciate your guidance, which has substantially strengthened our mechanistic conclusions.

Minor:

1. The authors should provide justification for only studying male. They recognize it as a limitation, but do not state why they believe males were the best starting point.

Response: Thank you for your thoughtful comment. We fully agree that sex is a fundamental biological variable and that including both sexes is essential for the generalizability and translational relevance of preclinical research.

Our initial decision to use only male rats was made to minimize potential confounding effects from the estrous cycle in females, which can introduce variability in pain-related behaviors and molecular endpoints, as noted in previous studies (Higginbotham et al., Neuron, 2025). Establishing a stable baseline in males allowed us to define the core signaling pathway before extending to more complex sex comparisons.

In response to your suggestion, we have now performed preliminary experiments in female rats and included these data in the supplementary materials. Additionally, we have expanded the Limitations section in the revised manuscript to transparently address the initial exclusion of females, summarize these new findings, and outline the need for future dedicated studies on sex-specific mechanisms.

We thank you again for this important input, which has improved the rigor and transparency of our study.

2. More information on the Von Frey test is needed. Was the threshold the first filament to yield a positive response or was it based on the algorithms such as the Dixon method? If it was the first positive response, did this have to occur 100% of the time between the 5 trials or 50%?

Response: Thank you for your careful question regarding the von Frey test methodology.

We have clarified this in the revised Materials and methods section. A series of calibrated von Frey filaments (4.0–26.0 g) are applied perpendicularly to the plantar surface of the hind paw. Testing begins with the 4.0 g filament. A positive response (rapid withdrawal, licking, or shaking) was defined as the mechanical threshold. This assessment was repeated three times at 5-minute intervals per session, and the average of the three calculated thresholds was taken as the final value for each animal.

We appreciate your thorough review, which has improved the methodological transparency of our manuscript.

3. siRNA sequences should be included to allow the reader to determine the appropriateness of the sequence and what it covers.

Response: Thank you for your valuable suggestion. We agree that providing the siRNA sequences is essential for transparency and reproducibility. In the revised manuscript, we have now included the full siRNA sequences (sense and antisense strands) in the Materials and methods section under “SiRNA and drug administration.”

4. Antibody and ELISA catalog numbers should be included to allow the reader to interpret the data and to repeat if they so wish.

Response: Thank you for your valuable suggestion. In the revised manuscript, we have now included the catalog numbers for all antibodies and ELISA kits used in this study. These details have been added to the Materials and methods section. Furthermore, Research Resource Identifiers (RRIDs) are also summarized in Supplementary Table S1 for quick reference.

Reply to Editor and Reviewer's Comments:

We thank the editor and reviewers for their insightful comments, which have significantly improved the manuscript. We have revised the manuscript accordingly. The revisions and changes are indicated in blue in a separate document. In addition, point-by-point responses to the comments are provided below.

Editor's Comments:

Please edit your revised manuscript according to requests listed in the online table.

Response: We thank the editor for providing the detailed revision requests. We have carefully reviewed all the points and have revised the manuscript accordingly. All changes have been incorporated to address the requests listed in the table. We look forward to hearing from you and would be happy to make any further changes, if required.

Reviewer #2:

One minor suggestion would be to define abbreviations (e.g., MT, TL, etc.) at the first instance in the results since the methods follow the results and discussion.

Response: Thank you for your thoughtful suggestion. We have revised the manuscript to define all abbreviations, including MT and TL, at their first mention in the Results section. We appreciate your feedback, which has improved the manuscript's clarity.