nature portfolio

Peer Review File

Sensory plasticity of dorsal horn silent neurons as a critical mechanism for neuropathic pain

Corresponding Author: Dr Cedric Peirs

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)
This manuscript demonstrates an impressive integration of an ex vivo skin-to-spinal cord preparation with large-scale two-photon Ca^{2+} imaging to explore sensory processing within dorsal horn circuits under both normal and neuropathic conditions. The authors show that mechanical allodynia after peripheral nerve injury primarily results from the recruitment of a previously silent population of excitatory neurons, rather than the conversion of nociceptive-specific neurons into WDR-like responders. This represents an important conceptual advance supported by robust experimental evidence. Overall, the study is technically rigorous, well executed, and highly relevant to the pain research field.

Comments
• The authors demonstrate that, in the disinhibition model, the percentage of Aβ and Aδ neurons changed, whereas in the cuff model, only Aβ fibers changed. Please discuss what could be the reason that the cuff model of chronic pain does not fully recapitulate the disinhibition model.
• Similarly, the neurons of deep laminae are not altered in the cuff model as compared to the disinhibition model. Please explain.
• How do the authors reconcile the differences between changes in the neurons responding to noxious stimuli in Lamina I-IIi in the spinal disinhibition and chronic pain model?
• The authors should clearly state that the model does not simulate spiking or synaptic dynamics, but instead uses experimentally constrained modality codes to infer likely transitions (e.g., Silent→WDR).
• The underlying assumptions—such as permitting only 0→1 transitions, disallowing 1→0 losses, and interpreting neuropathy purely as partial disinhibition—should be explicitly described and briefly justified.
• A short clarification in the Discussion that distinguishes what is constrained (population-level distributions) versus what is inferred (cell-wise transitions) would improve transparency.
• The concept of “silent neurons” is central to the manuscript. It would help to explicitly define early in the Results that “silent” refers to neurons lacking GCaMP-detectable suprathreshold responses, while acknowledging that subthreshold synaptic inputs may still exist and referring the reader to the Discussion part.
• How mechanical stimulus forces were calibrated across experiments.
• Please show the stability of neuronal responses over repeated trials (even a short quantitative statement)
• The modeling treats each lamina independently, yet the text occasionally implies inference about cross-laminar circuitry. A brief clarification stating that cross-laminar interactions are inferred from the imaging data and not explicitly modeled would prevent misinterpretation.

Minor Comments:
o 50 – No full stop
o 175 – Need a reference that shows that the tibial branch innervates most of the glabrous pad.
o Line 479 – less extent and not extend
o Line 480 – it should be written as innocuous mechanical hyperactivity, as noxious mechanical pinch input did not cause any alterations in the lamina I-IIi neurons.
o 611 – What are the references for other studies?
o 620 – Peaks instead of pics
o 647, 649 – Delete ‘that’, as it is written in 645 ‘given that’.
o Please insert figure numbers in the discussion for easy understanding by the readers.

o 682 – It should be vesicular glutamatergic transporter.
o 687 – they, not their
o 701 – mechanical is written twice

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
This manuscript presents an innovative study using an ex vivo somatosensory preparation combined with two-photon calcium imaging to monitor dorsal horn neuronal activity across laminae. The work tackles central and long-standing questions in the pain field, and specifically, how different sensory modalities are encoded within distinct laminar circuits, and how inhibition physiologically gates sensory information to prevent innocuous inputs from accessing nociceptive pathways. The integration of physiological skin stimulation with large-scale functional imaging is a major strength, as is the computational modeling that helps interpret circuit-level reorganization. Overall, this is a technically sound and conceptually relevant contribution that advances our understanding of dorsal horn plasticity in neuropathic pain and of the functional role of spinal inhibition in gating sensory information.

Major Concerns
1. Concnerning the Interpretation of “silent neurons”, the authors conclude that ~50% of dorsal horn neurons are “silent” under control conditions and that the absence of a GCaMP6f signal indicates absence of action potential firing. This interpretation requires substantial revision. GCaMP6f sensitivity varies widely across neuronal populations and experimental contexts. While the sensor can detect single action potentials under optimal conditions (as suggested by the study quoted by the authors - Chen et al., 2013), this is not universally true. For example, Huang et al. (eLife, 2021) showed that fewer than 10% of isolated single spikes were detectable with GCaMP6f in cortical neurons under two-photon excitation, illustrating that failure to observe a calcium transient cannot be taken as evidence that no spike occurred.
This limitation is likely more pronounced in the spinal cord, where deeper laminae contain substantial myelin, leading to significant optical scattering and reduced signal-to-noise relative to cortex. Many dorsal horn excitatory neurons exhibit small somatic Ca^{2+} transients and sparse single-spike firing. Additionally, heterogeneity in Ca^{2+} channel density and buffering may further reduces the detectability of small events.
Given these well-recognized constraints, neurons classified here as “silent” may indeed receive synaptic input and fire action potentials that fall below the detection threshold. Thus, the current terminology overstates what the method can infer. Revising the terminology and acknowledging these methodological limitations will substantially improve the rigor and accuracy of the manuscript.

2. Following the previous point, I have also some concern on Aβ-driven activation in deep dorsal horn neurons. The authors report that only ~5% of excitatory dorsal horn neurons respond to Aβ stimulation in control conditions, primarily in laminae I–IIo. While some superficial activation of Aβ inputs is now recognized, the near absence of deep dorsal horn responses contradicts extensive prior electrophysiological and anatomical evidence.
Decades of work demonstrate that Aβ low-threshold mechanoreceptors robustly activate deep dorsal horn neurons, particularly wide dynamic range (WDR) neurons in laminae III–V. Importantly, the authors themselves cite Lavertu et al. (Brain, 2014), who showed that approximately half of lamina V spinothalamic projection neurons exhibit low mechanical thresholds and broad WDR-like tuning. These projection neurons are identified (excitatory) projection neurons and known to integrate Aβ input.
Thus, the conclusion that Aβ-driven responses in deep dorsal horn excitatory neurons are rare or that low-threshold stimuli preferentially target deep inhibitory neurons is not well supported by data.
Indeed, following concern #1, an alternative explanation is that the calcium imaging approach systematically under-detects activity in deep excitatory neurons due to strong light scattering in myelinated tissue, weaker somatic Ca^{2+} transients in deep WDR neurons, and sparse or low-probability firing in response to Aβ stimuli.
I suggest the authors discuss these limitations more explicitly and acknowledge that their method likely underestimates the extent of Aβ engagement in deeper laminae. Complementary approaches, e.g., c-Fos mapping using equivalent stimulation, could help validate whether the deep excitatory population is truly unresponsive under these conditions.

3. The authors use bicuculline and strychnine to block GABA_A and glycine receptor–mediated inhibition. However, GABA_B receptor–mediated inhibition remains intact and is known to contribute to spinal nociceptive processing, especially following injury (see Malcangio, Neuropharmacology 2018). This should be acknowledged or integrated as it may influence the interpretation of disinhibition experiments and their comparison to neuropathic pain states.

4. A key strength of the study is the comparison of how distinct sensory modalities are transformed by disinhibition. However, the manuscript does not fully develop this point. The authors state (p.16) that disinhibition increases neuronal activity "regardless of modality," suggesting uniform inhibitory control across sensory channels. Yet the data (Fig. 3A–C and Supplementary Fig. 7) suggest quantitatively stronger effects for mechanical stimuli relative to thermal modalities. This aligns with prior work (e.g., Ferrini et al., Nat Commun 2020), with the authors data in nerve-injured animals and with clinical phenotypes in neuropathic pain, where mechanical allodynia is typically the most prominent modality change. Additionally, in Supplementary Fig. 4 cold-responsive neurons appear to constitute a relatively distinct population that is less responsive to mechanical stimuli under control conditions but becomes mechanosensitive after disinhibition. I recommend a more explicit discussion (or additional analysis) of these modality-specific differences, particularly under baseline conditions, as they represent an important and underexplored aspect of the dataset.

5. The manuscript draws parallels between changes observed after pharmacological disinhibition and those seen after nerve injury, implying a mechanistic link. However, this connection remains inferential in the current form. The authors could strengthen this conclusion by testing whether enhancing inhibition reverses the recruitment of "silent" neurons after nerve injury. Even partial evidence in this direction would substantially reinforce the hypothesized causal role of disinhibition.

Minor Points
Line 82: Inhibitory neurons do not exert a "protective" role; "gating" or "filtering" would be more accurate.
Line 205: The statement "69% of all responding neurons were mechanosensitive" is misleading. Figure 1E–F indicates that many of these neurons also respond to additional modalities. Please revise for clarity.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
This manuscript carried out an ex vivo somatosensory preparation combined with 2-photon Ca2+ imaging and investigated excitatory neuronal activity in whole dorsal horn (DH) under normal conditions, after spinal disinhibition or nerve injury. The authors showed that most DH excitatory neurons were converted into highly polymodal neurons after pharmacologically induced spinal disinhibition or peripheral chronic constriction injury. More interesting, they found a number of previously silent neurons that responded to a wide dynamic range of sensory modalities in both superficial and deep DH laminae after spinal disinhibition or nerve injury. This manuscript proposed that these new identified silent neurons following nerve injury are likely the main drivers of neuropathic pain. The manuscript is well written and the experimental design logical.
There are several comments below that could help improve the manuscript.
1. It remains unclear whether the phenomenon observed is broadly applicable across neuropathic pain conditions. Chronic contraction injury model (Cuff) of neuropathic pain, although used, does not capture nerve injury induced neuropathic pain in patients. This model is artificial and includes a
combination of compression and inflammation. It would be better to have clinically relevant models of neuropathic pain (e.g., SNI) to confirm the observed phenomenon.
2. Given that Ca2+ imaging was conducted 3 weeks after nerve injury, the observed phenomenon is likely associated with the maintenance of neuropathic pain. Is the same phenomenon observed during the induction of neuropathic pain?
3. As the authors pointed out in the Discussion, there are some limitations using an ex vivo preparation. Although the validations were provided under physiological conditions, it is still unclear whether descending inhibitory pathways from supraspinal regions following nerve injury influence the conclusion drawn. This point should be discussed.
4. Due to technical limitations, caution should be taken in concluding that neuropathic pain does not primarily result from the transformation of excitatory nociceptive neurons into wide dynamic range neurons.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed our concerns. In our opinion, the revised manuscript substantially improves the clarity and readability of the work.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I really appreciate the substantial efforts made by the authors, particularly the additional experiments and the revised discussion, which address several of the concerns raised and more clearly acknowledge the technical limitations of the approach.
Overall, these additions significantly strengthen the manuscript.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
All comments raised have been addressed. This reviewer is satisfied with all responses.

(Remarks on code availability)

General Response

We would like to thank the reviewers for their positive comments and appreciation of the work as well as their constructive suggestions, which have now led to a significantly improved version of the manuscript. This version includes several important controls and consideration of calcium imaging limitations. These and other points that were raised by the reviewers are now addressed in detail below.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript demonstrates an impressive integration of an ex vivo skin-to-spinal cord preparation with large-scale two-photon Ca^{2+} imaging to explore sensory processing within dorsal horn circuits under both normal and neuropathic conditions. The authors show that mechanical allodynia after peripheral nerve injury primarily results from the recruitment of a previously silent population of excitatory neurons, rather than the conversion of nociceptive-specific neurons into WDR-like responders. This represents an important conceptual advance supported by robust experimental evidence. Overall, the study is technically rigorous, well executed, and highly relevant to the pain research field.

Comments

•***Comment 1.1:*** *The authors demonstrate that, in the disinhibition model, the percentage of Aβ and Aδ neurons changed, whereas in the cuff model, only Aβ fibers changed. Please discuss what could be the reason that the cuff model of chronic pain does not fully recapitulate the disinhibition model.*

Response: The primary reason for these differences is likely that pharmacological disinhibition (bicuculline + strychnine) produces a complete blockade of inhibitory receptors (GABA-A and Glycine receptors) across all laminae. In contrast, nerve injury likely induces a partial loss of inhibitory controls that often affects distinct DH populations. In fact, we previously found that the dorsal horn contains several microcircuits that can even lead to similar symptoms (Peirs et al. 2021, 10.1016/j.neuron.2020.10.027), potentially due to altered activity of different populations of inhibitory neurons.

We have now included new experiments assessing the effect of GABA to DH neurons after nerve injury, which are now included in Fig. S13. We found that GABA was unable to reduce the activity of neurons receiving Aβ-fiber inputs, at doses that were effective in sham animals. In contrast, there was no difference in the effect of GABA on neurons receiving exclusively Aδ- and/or C-fiber inputs in sham vs neuropathic animals, consistent with the idea that only

inhibition onto Aβ-fiber inputs is affected after nerve injury. Similarly, the partial nature of disinhibition in the cuff model appears to be insufficient to recruit new nociceptive neurons in the superficial dorsal horn, in contrast to what we observed after pharmacological disinhibition. This is consistent with our behavioural data, where cuff animals did not show significant differences in noxious sensitivity compared to sham controls, 3 weeks after the injury.
Why such specificity in spinal disinhibition after nerve injury is an important question, and is most likely related to the intrinsic properties of populations of inhibitory neurons. It seems that neurons expressing PV are particularly sensitive to chronic pain conditions, probably related to their unique ability to sustain bursts of action potentials of incredible high frequency, which is altered after nerve injury (Qiu et al. 2024, 10.1073/pnas.2403777121). Interestingly, these PV neurons have been implicated in chronic pain not only in the spinal cord but also in other areas such as the anterior cingulate cortex (Kang et al. 2015, 10.1186%2Fs13041-015-0170-6).

We have now included these new data to the manuscript and substantially expanded our Discussion (lines 675-710) to better explain the differences observed using the cuff model compared to pharmacological disinhibition.

• *Comment 1.2: Similarly, the neurons of deep laminae are not altered in the cuff model as compared to the disinhibition model. Please explain.*

Response: This is an interesting remark, and several hypotheses may explain why nerve injury seems to alter preferentially neurons of the superficial laminae. Several studies in rodents and human have showed that nerve injury is associated with an increase in spontaneous activity in sensory neurons, especially nociceptors long after the onset of the neuropathy (Devor 2009, 10.1007/s00221-009-1724-6). This activity may actually account for spontaneous burning pain described by neuropathic patients. We previously found that such ectopic activity in nociceptors sensitizes neurons only in superficial laminae, due to the intense release of reactive oxygen species in this area (Peirs et al., 2016, 10.1097/ALN.0000000000001000). Consistently, another study reported a decrease in PV expression only in superficial laminae after peripheral injury (Zacharova et al., 2009, 10.33549/physiolres.931513), strengthening the idea that superficial DH neurons are more susceptible to be disinhibited under pathological conditions. Another hypothesis could relate on the type of DH disinhibition that is induced by the nerve injury. It is indeed well known that glycinergic inhibitory neurons are enriched in the deep laminae, while GABAergic inhibitory neurons are more numerous in the superficial laminae (Zeilhofer et al., 2009, 10.1007/978-1-4419-0226-9_3). However, this is currently little evidence that GABAergic, rather than Glycinergic, transmission is reduced after nerve injury (Zeilhofer 2005, 10.1007/s00018-005-5107-2), especially given that most, if not all, glycinergic neurons co-express GABA (Peirs et al. 2020, 10.1007%2Fs00702-020-02159-1).

We have now expanded our Discussion to address this important point (lines 697-710).

• *Comment 1.3: How do the authors reconcile the differences between changes in the neurons responding to noxious stimuli in Lamina I-IIi in the spinal disinhibition and chronic pain model?*

Response: As mentioned above, this could be owed to the partial nature of disinhibition induced by the cuff model, which appears to be insufficient to recruit new nociceptive neurons in the superficial dorsal horn, unlike what we saw after complete pharmacological disinhibition. Interestingly this is consistent with our behavioural data, as cuff animals did not show significant differences in noxious sensitivity compared to sham controls, 3 weeks after the injury. This is now included in the Discussion (lines 676-686).

• *Comment 1.4: The authors should clearly state that the model does not simulate spiking or synaptic dynamics, but instead uses experimentally constrained modality codes to infer likely transitions (e.g., Silent→WDR).*

Response: We have revised the Manuscript accordingly and stated this clearly in the Materials and Methods section (lines 1200-1205) and stated that "In addition, the model did not simulate spiking or synaptic dynamics but was rather focused on experimentally measured population-level. Thus, the experimental population-level distributions of DH sensory profiles were constrained, while the specific cell-wise transitions required to shift the network from control to neuropathic states were algorithmically inferred."

• *Comment 1.5: The underlying assumptions—such as permitting only 0→1 transitions, disallowing 1→0 losses, and interpreting neuropathy purely as partial disinhibition—should be explicitly described and briefly justified.*
Response: We now describe and justify this constraint in the Materials and Methods section (lines 1224-1228) and acknowledged this limitation in the conclusion: "Although our computational model was restrained to focus on spinal disinhibition under neuropathic conditions, other mechanisms of central sensitization that have been well described could be implanted in the algorithm in future studies."

• *Comment 1.6: A short clarification in the Discussion that distinguishes what is constrained (population-level distributions) versus what is inferred (cell-wise transitions) would improve transparency.*

Response: We have updated the Manuscript accordingly and mentioned this clearly in the Materials and Methods section (lines 1202-1205): "In addition, the model did not simulate spiking or synaptic dynamics but was rather focused on experimentally measured population-level. Thus, the experimental population-level distributions of DH sensory profiles were constrained, while the specific cell-wise transitions required to shift the network from control to neuropathic states were algorithmically inferred."

• ***Comment 1.7:*** *The concept of "silent neurons" is central to the manuscript. It would help to explicitly define early in the Results that "silent" refers to neurons lacking GCaMP-detectable suprathreshold responses, while acknowledging that subthreshold synaptic inputs may still exist and referring the reader to the Discussion part.*

Response: We defined "silent" neurons in the results section as requested and referred the reader to the discussion part: "Unexpectedly, we found that among all responding neurons after disinhibition, 50.3% ± 4.9 of them had no previous detectable Ca2+ transients in response to suprathreshold electric stimulation of the dorsal root in control conditions. We thus considered these neurons as "previously silent", though they may also receive subthreshold afferent inputs undetectable by Ca2+ imaging (see Discussion)". (Lines 270-275)

We also addressed the limit of detecting subthreshold synaptic inputs by performed additional experiments. We combined current clamp whole-cell patch clamp recording of dorsal horn neurons together with two-photon Ca2+ imaging, which are now including in supplementary figure 12. We found that all DH neurons could reliably evoke calcium transients at rheobase, but that transients were hardly detected following injections of smaller currents. This limit is now clearly addressed in the Discussion section under technical consideration (lines 574-636).

• ***Comment 1.8:*** *How mechanical stimulus forces were calibrated across experiments.*

Response: We have updated the material and Methods section as following: "Innocuous static stimulation was delivered using a round, blunt rubber stimulator (area = 12.6 mm^2). The stimulator was placed on the skin surface without applying additional pressure, delivering a force of ~0.2 g/mm^2 while taking care to avoid inducing motion artifacts during acquisition. Stimuli were delivered at ~0.5 Hz following a tone, using the pattern shown in Fig. S1d to consistently cover most of the plantar surface."

• ***Comment 1.9:*** *Please show the stability of neuronal responses over repeated trials (even a short quantitative statement)*

Response: We have included a new analysis of DH responses following repeated identical stimulation trains in Table 5, and added the following sentence to the discussion: "We also assessed whether the same population of neurons would be activated following two identical stimulations and found no significant difference in the percentage of responding neurons or in the amplitude of the calcium transients, indicating that the same neurons could be reliably activated between trains of stimuli (Fig. S13)." (Lines: 596-600).

• ***Comment 1.10:*** *The modeling treats each lamina independently, yet the text occasionally implies inference about cross-laminar circuitry. A brief clarification stating that cross-laminar interactions are inferred from the imaging data and not explicitly modeled would prevent misinterpretation.*

Response: We have added the following clarification to the Methods: "Cross-laminar interactions were inferred and not incorporated into the model, which treated each lamina independently." (Lines 1199–1205).

Minor Comments:
Comment 1.11: *50 – No full stop*
Response: We have added full stop line 51.

Comment 1.12: *175 – Need a reference that shows that the tibial branch innervates most of the glabrous pad.*
Response: We have modified to: "which innervates the glabrous skin (Corder et al. 2010)." Line 169.

Comment 1.13: *Line 479 – less extent and not extend*
Response: We have corrected "less extend" to "less extent" line 474.

Comment 1.14: *Line 480 – it should be written as innocuous mechanical hyperactivity, as noxious mechanical pinch input did not cause any alterations in the lamina I-IIi neurons.*
Response: We have corrected this line 474.

Comment 1.15: *611 – What are the references for other studies?*

Response: We modified the manuscript as following: “To compensate for the low speed of laser scanning compared to fast calcium transients, we recorded during 10 seconds with repeated stimulations at 2Hz, a frequency previously shown to reliably evoke action potentials in DH post-synaptic neurons without synaptic failure, even for neurons receiving C-fiber inputs (Dickie et al. 2017).” Line 628.

Comment 1.16: *620 – Peaks instead of pics*
Response: We have corrected to “peaks” line 629.

Comment 1.17: *647, 649 – Delete ‘that’, as it is written in 645 ‘given that’.*
Response: We have deleted the redundant "that" lines 659 and 663.

Comment 1.18: *Please insert figure numbers in the discussion for easy understanding by the readers.*
Response: Figure numbers were added throughout.

Comment 1.19: *682 – It should be vesicular glutamatergic transporter.*
Response: We have corrected this line 728.

Comment 1.20: *687 – they, not their*
Response: we have corrected "their" to "they" line 733.

Comment 1.21: *701 – mechanical is written twice*
Response: We have removed the duplicated word line 745.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank Reviewer#2 for her/his contribution in reviewing the Manuscript.

Reviewer #3 (Remarks to the Author):

This manuscript presents an innovative study using an ex vivo somatosensory preparation combined with two-photon calcium imaging to monitor dorsal horn neuronal activity across laminae. The work tackles central and long-standing questions in the pain field, and specifically, how different sensory modalities are encoded within distinct laminar circuits, and how inhibition physiologically gates sensory information to prevent innocuous inputs from accessing nociceptive pathways. The integration of physiological skin stimulation with large-scale functional imaging is a major strength, as is the computational modeling that helps interpret circuit-level reorganization. Overall, this is a technically sound and conceptually relevant contribution that advances our understanding of dorsal horn plasticity in neuropathic pain and of the functional role of spinal inhibition in gating sensory information.

Major Concerns

Comment 3.1. Concnerning the Interpretation of "silent neurons", the authors conclude that ~50% of dorsal horn neurons are "silent" under control conditions and that the absence of a GCaMP6f signal indicates absence of action potential firing. This interpretation requires substantial revision. GCaMP6f sensitivity varies widely across neuronal populations and experimental contexts. While the sensor can detect single action potentials under optimal conditions (as suggested by the study quoted by the authors - Chen et al., 2013), this is not universally true. For example, Huang et al. (eLife, 2021) showed that fewer than 10% of isolated single spikes were detectable with GCaMP6f in cortical neurons under two-photon excitation, illustrating that failure to observe a calcium transient cannot be taken as evidence that no spike occurred.

This limitation is likely more pronounced in the spinal cord, where deeper laminae contain substantial myelin, leading to significant optical scattering and reduced signal-to-noise relative to cortex. Many dorsal horn excitatory neurons exhibit small somatic Ca^{2+} transients and sparse single-spike firing. Additionally, heterogeneity in Ca^{2+} channel density and buffering may further reduces the detectability of small events.

Given these well-recognized constraints, neurons classified here as "silent" may indeed receive synaptic input and fire action potentials that fall below the detection threshold. Thus, the current terminology overstates what the method can infer. Revising the terminology and acknowledging these methodological limitations will substantially improve the rigor and accuracy of the manuscript.

Response: We thank the reviewer for this important concern and fully agree that GCaMP6f has well-recognized sensitivity limitations in certain tissues. We acknowledge in the paper that the GCaMP6f sensor, while sensitive enough to detect activity following a single action potential under optimal conditions (Chen et al., 2013, 10.1038/nature12354), likely missed sub-

threshold variations of membrane potential that do not elicit sufficient Ca^{2+} increase to produce a detectable transient in the Results (lines 287-291) and the Discussion (lines 581-597).

Nevertheless, we have performed additional experiments with 2-photon Ca^{2+} imaging of individual DH neurons in spinal cord slices with dorsal root stimulations, which are now included in Fig.S11. We found that a reliable GCamp6f signal was detected following a single dorsal root stimulation at 25µA or 500µA (Fig.S11a). In addition, delivering a train of 20 stimulations at 1Hz reliably followed the train of stimulations in both conditions (Fig.S11b). Finally delivering a train of 20 stimulations at 2Hz similar to the protocol applied in our paradigm triggered a long lasting Ca^{2+} transient with individual peaks corresponding to the train of stimulations. Similarly, we performed additional patch clamp experiments coupled with 2-photon Ca^{2+} imaging and found that Ca^{2+} signals faithfully followed the action potentials firing induced by injecting step currents through current clamp in superficial as well as deep laminae (Fig.S12a-b). These data indicate that the GCaMP6f sensor can be a faithful proxy for detecting single AP in excitatory DH neurons and can detect a single dorsal root stimulation. We do acknowledge however in the Results (lines 287-291) and the Discussion (lines 581-597) that subthreshold changes in ion currents below rheobase were likely undetectable through Ca^{2+} imaging.

Taken together, we have therefore: (i) revised the definition of "silent neurons" thoroughly to make clear that these are neurons with no GCaMP6f-detectable suprathreshold response (lines 270-275); and (ii) we acknowledge that despite the high sensitivity of the GCaMP6f sensor, certain sparse or low-frequency spiking may go undetected, and that optical scattering in deeper myelinated laminae may further reduces signal-to-noise. These caveats are now stated early in the methods, Results and expanded in the Discussion section (lines 633-634).

Comment 3.2. Following the previous point, I have also some concern on Aβ-driven activation in deep dorsal horn neurons. The authors report that only ~5% of excitatory dorsal horn neurons respond to Aβ stimulation in control conditions, primarily in laminae I–IIo. While some superficial activation of Aβ inputs is now recognized, the near absence of deep dorsal horn responses contradicts extensive prior electrophysiological and anatomical evidence. Decades of work demonstrate that Aβ low-threshold mechanoreceptors robustly activate deep dorsal horn neurons, particularly wide dynamic range (WDR) neurons in laminae III–V. Importantly, the authors themselves cite Lavertu et al. (Brain, 2014), who showed that approximately half of lamina V spinothalamic projection neurons exhibit low mechanical thresholds and broad WDR-like tuning. These projection neurons are identified (excitatory) projection neurons and known to integrate Aβ input.

Thus, the conclusion that Aβ-driven responses in deep dorsal horn excitatory neurons are rare or that low-threshold stimuli preferentially target deep inhibitory neurons is not well supported by data.

Indeed, following concern #1, an alternative explanation is that the calcium imaging approach systematically under-detects activity in deep excitatory neurons due to strong light scattering in myelinated tissue, weaker somatic Ca^{2+} transients in deep WDR neurons, and sparse or low-probability firing in response to Aβ stimuli.
I suggest the authors discuss these limitations more explicitly and acknowledge that their method likely underestimates the extent of Aβ engagement in deeper laminae.
Complementary approaches, e.g., c-Fos mapping using equivalent stimulation, could help validate whether the deep excitatory population is truly unresponsive under these conditions.

Response: We appreciate this critical and insightful observation regarding our findings concerning the percentage and the distribution of Aβ responsive neurons in the DH, which may appear conflicting with electrophysiological studies demonstrating robust WDR activation by Aβ inputs in laminae III–V.
While we do acknowledge that optical scattering due to dense myelination in deeper DH laminae, or weak somatic transients evoked by Aβ fibers in large WDR neurons, may lower our capacity to detect Aβ-responsive neurons in the deep DH Laminae (now added to the discussion), the important difference in the proportion of neurons responding to Aβ stimulations is likely due to the sample bias related to in vivo recording, compared to all excitatory neurons selectively targeted in our study. In fact, previous unbiased functional mapping studies using p-ERK or c-Fos activation following innocuous mechanical stimuli align closely with our observations. These studies consistently show a relatively low number of responsive neurons in deep laminae (LIII–V) compared to the superficial DH (LI–IIo) under control conditions (Miraucourt et al., 2007, 10.1371/journal.pone.0001116; Peirs et al., 2016, 10.1097/ALN.0000000000001000). Notably, even after pharmacological disinhibition, this spatial distribution pattern is conserved; with a significantly higher density of activated neurons remaining in the superficial laminae compared to the deep DH. Additionally, a recent transcriptomic analysis of spinal projection neurons identified at least 3 populations that express GABA and not VGLUT2, which are particularly enriched in the deep dorsal horn (Lin et al., 2026, 10.1016/j.celrep.2025.116717). While the existence of this previously unappreciated proportion of inhibitory projection neurons may have important outcomes in our understanding of the spinal cord circuitry, we did not include this last observation in the discussion, as these results are yet to be confirmed at the level of the lumbar spinal cord.

We have updated our Discussion accordingly to render more transparency, and suggest further investigations to clarify this important point (lines 649-655).

Comment 3.3. *The authors use bicuculline and strychnine to block GABA_A and glycine receptor–mediated inhibition. However, GABA_B receptor–mediated inhibition remains intact and is known to contribute to spinal nociceptive processing, especially following injury (see Malcangio, Neuropharmacology 2018). This should be acknowledged or integrated as it may*

influence the interpretation of disinhibition experiments and their comparison to neuropathic pain states.

Response: We have highlighted this information in the Discussion (line 582).

Comment 3.4. *A key strength of the study is the comparison of how distinct sensory modalities are transformed by disinhibition. However, the manuscript does not fully develop this point. The authors state (p.16) that disinhibition increases neuronal activity "regardless of modality," suggesting uniform inhibitory control across sensory channels. Yet the data (Fig. 3A–C and Supplementary Fig. 7) suggest quantitatively stronger effects for mechanical stimuli relative to thermal modalities. This aligns with prior work (e.g., Ferrini et al., Nat Commun 2020), with the authors data in nerve-injured animals and with clinical phenotypes in neuropathic pain, where mechanical allodynia is typically the most prominent modality change. Additionally, in Supplementary Fig. 4 cold-responsive neurons appear to constitute a relatively distinct population that is less responsive to mechanical stimuli under control conditions but becomes mechanosensitive after disinhibition. I recommend a more explicit discussion (or additional analysis) of these modality-specific differences, particularly under baseline conditions, as they represent an important and underexplored aspect of the dataset.*

Response: We really appreciate this valuable comment. Indeed, one of the most interesting aspects of the study is determining which sensory modalities are "wired" onto individual neurons, and which of these synaptic inputs are gated under control conditions. This information is indeed essential for estimating the extent to which modality-specific neurons may converge under pathological conditions. Importantly, these findings also formed the basis of the computational model we applied, which allowed us to conclude how neuropathic circuits may have evolved from the control conditions.

To address this point, and to rightfully report that mechanical but also thermal modalities were gained, we have revised and updated the manuscript accordingly: i) we added a new analysis identifying which modalities were present under control conditions and were gained following disinhibition (Fig.S4d); ii) we revised the description of the change in DH neuron modalities to "Such increase in neuronal activity was observed across all tested modalities, although with different extents"; and iii) we added an explicit description in the results of the cold modality, noting that it was the least modality gain following disinhibition (lines 348–353).

Comment 3.5. *The manuscript draws parallels between changes observed after pharmacological disinhibition and those seen after nerve injury, implying a mechanistic link. However, this connection remains inferential in the current form. The authors could strengthen this conclusion by testing whether enhancing inhibition reverses the recruitment of "silent" neurons after nerve injury. Even partial evidence in this direction would substantially reinforce the hypothesized causal role of disinhibition.*

Response: We fully agree that this is an important experiment and thank the reviewer for suggesting it. To address this question, we have performed additional experiments to address this point (Fig.S13) by testing the effect of enhancing the inhibition with an increasing concentration of GABA ranging from 0.1 to 1 µM. We found that in slices from sham mice, the application of GABA significantly reduced the max peak amplitude and the percentage of responsive neurons to different stimulation intensities in a dose dependent manner. Interestingly, the same treatment applied to slices from cuff mice induced a reduction in DH activity in a fiber type manner. The max peak response and the percentage of responders where drastically reduced by increasing the concentration of GABA following 100 µA and 500µA peripheral stimulations, similar to what we saw in sham mice. In contrast, responses to Aβ stimulation with 25µA remained largely unaffected by the increasing concentration of GABA, highlighting a possible disruption in the inhibitory control of these afferent inputs following nerve injury. We have included these results in the manuscript and discussed them accordingly (lines 682-686).

Minor Points

Comment 3.6. *Line 82: Inhibitory neurons do not exert a "protective" role; "gating" or "filtering" would be more accurate.*

Response: We have corrected to "under the control of inhibitory interneurons".

Comment 3.7. *Line 205: The statement "69% of all responding neurons were mechanosensitive" is misleading. Figure 1E–F indicates that many of these neurons also respond to additional modalities. Please revise for clarity.*

Response: We have revised to: "Under these conditions, 69% of all responding neurons were mechanosensitive, either uni- or poly- modal, consistent with a recent study that analyzed the sensory modality of superficial DH neurons (Warwick et al. 2022)". (Lines 197-200).

Reviewer #4 (Remarks to the Author):

This manuscript carried out an ex vivo somatosensory preparation combined with 2-photon Ca2+ imaging and investigated excitatory neuronal activity in whole dorsal horn (DH) under normal conditions, after spinal disinhibition or nerve injury. The authors showed that most DH excitatory neurons were converted into highly polymodal neurons after pharmacologically induced spinal disinhibition or peripheral chronic constriction injury. More interesting, they found a number of previously silent neurons that responded to a wide dynamic range of

sensory modalities in both superficial and deep DH laminae after spinal disinhibition or nerve injury. This manuscript proposed that these new identified silent neurons following nerve injury are likely the main drivers of neuropathic pain. The manuscript is well written and the experimental design logical.
There are several comments below that could help improve the manuscript.

Comment 4.1. *It remains unclear whether the phenomenon observed is broadly applicable across neuropathic pain conditions. Chronic contraction injury model (Cuff) of neuropathic pain, although used, does not capture nerve injury induced neuropathic pain in patients. This model is artificial and includes a combination of compression and inflammation. It would be better to have clinically relevant models of neuropathic pain (e.g., SNI) to confirm the observed phenomenon.*

Response: We thank the reviewer for bringing up this complex issue about animal models. We do acknowledge that all preclinical neuropathic pain models carry several limitations. The cuff model, however, reproduces key features of clinical compressive neuropathy, the most prevalent form of neuropathic pain in patients; including altered dorsal root ganglion excitability, axonal degeneration patterns consistent with human nerve entrapment, and most importantly symptoms, comorbidities and pharmacological therapeutic sensitivity of neuropathic chronic pain (Mosconi & Kruger, 1996, 10.1016/0304-3959(95)00077-1; Yalcin et al., 2014, 10.3791/51608; Zhu & Henry, 2012, 10.1186/1471-2202-13-15). While the SNI model also produces a robust phenotype (Decosterd et al., 2002, 10.1016/S0304-3959(02)00275-0), it involves transection of two nerve branches causing drastic denervation, spinal cell death, and dorsal horn reorganization, which in our hands produced widespread cell death at L4–L5 segments (unpublished data). This impacted our capacity to perform reliable functional Ca^{2+} imaging with our ex vivo preparation which largely implies on a reliable connectivity between the skin and the DH. Nevertheless, our core finding, the recruitment of previously silent excitatory neurons, was independently reproduced in the pharmacological disinhibition model, and the convergence of results across both paradigms strongly supports the generalizability of our conclusions. Nonetheless, we have included a statement in the discussion acknowledging the limitation associated with the neuropathic model and that future studies should test the reproducibility of our results in other neuropathic models and other chronic pain models (lines: 822-825).

Comment 4.2. *Given that Ca2+ imaging was conducted 3 weeks after nerve injury, the observed phenomenon is likely associated with the maintenance of neuropathic pain. Is the same phenomenon observed during the induction of neuropathic pain?*

Response: Our Ca^{2+} imaging was performed indeed 3 weeks post-surgery, a well-validated time point at which animals show stable ipsilateral static and dynamic mechanical allodynia (present from 1 week post-surgery and lasting at least 3 weeks). Whether silent neuron recruitment occurs progressively during the induction phase (days 3–14) is not addressed by the present data, as it would relates more to inflammatory mediators released in the first week or two following the injury, than to persistent neuropathic pain. We have added this explicitly as future directions: " It will also be important to determine the temporal dynamics of the silent neuron recruitment as the disease progresses after the injury, and if this can be prevented in vivo. Additionally, future studies should investigate if the recruitment of silent neurons as a main driver of neuropathic pain is a common feature of the pathology using other neuropathic pain models, and if it also relates to other chronic pain conditions." (Lines: 819-822).

Comment 4.3. *As the authors pointed out in the Discussion, there are some limitations using an ex vivo preparation. Although the validations were provided under physiological conditions, it is still unclear whether descending inhibitory pathways from supraspinal regions following nerve injury influence the conclusion drawn. This point should be discussed.*

Response: We agree that this is an important limitation. Descending noradrenergic and serotonergic pathways tonically modulate spinal nociceptive transmission and have been shown to be dysregulated in neuropathic pain. Their absence in the ex vivo preparation could indeed lead to overestimate the proportion of silent neurons that become active after disinhibition, which we now clearly acknowledge in the manuscript: *"the influence from descending noradrenergic and serotonergic pathways from supraspinal areas, or other key factors such as $GABA_B$ contribution, circulating hormones or immune cells were absent in this preparation."* We have also expanded this discussion in the Limitations section, compared our results to data obtained in vivo, and highlighted that validation of our conclusions in vivo should be a priority, if/when technically possible (lines 580-585 and 819-822).

Comment 4.4. *Due to technical limitations, caution should be taken in concluding that neuropathic pain does not primarily result from the transformation of excitatory nociceptive neurons into wide dynamic range neurons.*

Response: We agree and have tempered this conclusion. We acknowledge that Ca^{2+} imaging cannot detect changes in firing frequency of existing WDR neurons, and that such changes could contribute to neuropathic hypersensitivity without appearing as phenotypic conversion. We also highlight that the Silent→WDR mechanism is mostly supported by the computational model, with its own technical limitation. We now acknowledge this limit in the manuscript, and have tempered this statement in the abstract and highlights. We also discuss the fact that changes in firing frequency of existing NS neurons (which are difficult to detect with slow-

scanning Ca^{2+} imaging) could also contribute to noxious hypersensitivity without appearing as a change in the proportion of responding neurons (please see Technical considerations, lines 574-636).

END OF REVISIONS