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TAF4A4 relieves injury-induced mechanical hypersensitivity through LDL receptors and modulation of spinal A-type K⁺ current

Graphical abstract

Highlights

- TAF4A4 reverses inflammatory, postoperative, and nerve injury-induced mechanical pain
- TAF4A4-mediated antihypersensitive effect requires functional LDL receptor family members
- SNI alters spinal Iₐ in opposite direction in excitatory and inhibitory interneurons
- TAF4A4 reverses SNI-induced alteration of Iₐ in both interneuron subtypes

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In brief

Yoo et al. demonstrate that intrathecal or systemic administration of human recombinant TAF4A4 reverses inflammatory, postoperative, and nerve injury-induced mechanical pain. The TAF4A4 antihypersensitive effect requires functional RAP-sensitive LDL receptors to restore normal mechanical sensitivity by rescuing the injury-induced alterations of Iₐ in two distinct subsets of spinal cord dorsal horn interneurons.
TAFA4 relieves injury-induced mechanical hypersensitivity through LDL receptors and modulation of spinal A-type K⁺ current

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SUMMARY

Pain, whether acute or persistent, is a serious medical problem worldwide. However, its management remains unsatisfactory, and new analgesic molecules are required. We show here that TAFA4 reverses inflammatory, postoperative, and spared nerve injury (SNI)-induced mechanical hypersensitivity in male and female mice. TAFA4 requires functional low-density lipoprotein receptor-related proteins (LRPs) because their inhibition by RAP (receptor-associated protein) dose-dependently abolishes its antihypersensitive actions. SNI selectively decreases A-type K⁺ current (Iₐ) in spinal lamina II outer excitatory interneurons (L-IIo ExINs) and induces a concomitant increase in Iₐ and decrease in hyperpolarization-activated current (Iₖh) in lamina II inner inhibitory interneurons (L-IIi InhINs). Remarkably, SNI-induced ion current alterations in both IN subtypes were rescued by TAFA4 in an LRP-dependent manner. We provide insights into the mechanism by which TAFA4 reverses injury-induced mechanical hypersensitivity by restoring normal spinal neuron activity and highlight the considerable potential of TAFA4 as a treatment for injury-induced mechanical pain.

INTRODUCTION

Pain is commonly classified as acute or chronic. Acute pain is short lived and essential for maintenance of our physical integrity, whereas chronic pain persists beyond the normal time of healing and adversely affects our well-being. Chronic inflammatory, neuropathic, or postoperative pain gives rise to long-lasting sensory abnormalities, such as hyperalgesia (extreme pain evoked by noxious stimuli) and mechanical allodynia (pain evoked by innocuous mechanical stimuli). These categories of pain differ in terms of etiology and clinical features, but they have several mechanisms in common, including alterations of neuro-immune interactions and neuron sensitization peripherally and centrally (Costigan et al., 2009). There is growing evidence to suggest that loss of inhibition may be a key mechanism underlying chronic pain (Bourane et al., 2015a, 2015b; Boyle et al., 2019; Coull et al., 2005; Duan et al., 2014; Peirs et al., 2015; Petitjean et al., 2015, 2019; Zeilhofer et al., 2012; Zhang et al., 2018). However, despite our extensive knowledge of the mechanisms and circuits underlying chronic pain in rodents, translation of these findings into effective treatments for chronic pain in humans remains unsatisfactory (Colloca et al., 2017). Indeed, non-steroidal anti-inflammatory drugs (NSAIDs) have limited efficacy against chronic pain, and opioids have multiple adverse effects, including potentially lethal respiratory depression, nausea, constipation, hyperalgesia, tolerance, and physical and psychological dependence (Benyamin et al., 2008). Thus, efforts to identify new targets with analgesic potential for treatment of chronic pain should be encouraged.

In the last few years, we have discovered striking features of the secreted protein TAFA4, suggesting that it might be an ideal drug for treatment of chronic pain. TAFA4 belongs to a family of five highly conserved secreted neurokines with a striking degree of amino acid identity among orthologs from divergent species...
TAF4A reverses injury-induced mechanical hypersensitivity in various pain models

In two previous studies (Delfini et al., 2013; Kambrun et al., 2018), we showed that intrathecal (IT) injection of TAF4A reversed carrageenan- and complete Freund’s adjuvant (CFA)-induced mechanical hypersensitivity in male mice. We further explored the potential of TAF4A as an analgesic molecule by extending our investigation to other preclinical pain models in male and female mice using IT and systemic subcutaneous (s.c.) injections. We used SNI as a model of neuropathic pain, paw incision as a model of postoperative pain, and intraplantar paw injection of carrageenan as a model of inflammatory pain. In the SNI model, recombinant TAF4A (170 pmol injected IT or 300 µg/kg injected s.c.) provided significant reversal of mechanical hypersensitivity, as shown by a significant increase in mechanical thresholds for up to 4 h after injection (Figures 1A, 1B, S1A, and S1B). The optimal concentration of TAF4A for s.c. administration was determined after injection of three different doses: 12, 60, and 300 µg/kg (Figure S1C). S.c. injection of 300 µg/kg TAF4A provided significant reversal of mechanical hypersensitivity in male and female mice in the SNI model. Furthermore, injection of 300 µg/kg of TAF4A to naive of SNI mice affected neither the locomotor activity nor the anxiety levels of the mice (Figure S1D). We therefore used this concentration for the paw incision and carrageenan-induced inflammation models. In both paradigms, on days 1 and 2 after injury, male and female mice displayed mechanical hypersensitivity, and IT injection of 170 pmol or s.c. administration of 300 µg/kg TAF4A provided significant reversal of mechanical hypersensitivity for up to 4 h after administration (Figures 1C–1F). In all three pain models, IT or s.c. administration of TAF4A had no effect on the mechanical thresholds of the contralateral paws (Figures 1A–1F). Consistent with these findings, IT injection of morphine had a significant analgesic effect in response to infrared heat stimulation, but IT administration of recombinant TAF4A or BSA had no effect on the heat threshold of the mouse’s tail (Figure S1E). Finally, using the SNI model, we compared the effect of TAF4A with those of SNC-80 (an agonist of the delta opioid receptor), DAMGO (an agonist of the µ opioid receptor), and bacofoxen (an agonist of the GABA3 receptor). We found that IT injection of TAF4A induced a reversal of SNI-induced mechanical hypersensitivity that was of a similar magnitude to that induced by SNC80, DAMGO, or bacofoxen but lasted significantly longer (Figure S1F). Overall, these results demonstrate that TAF4A does not exert general antinociceptive actions in non-injured animals but strongly reverses mechanical hypersensitivity caused by nerve injury, surgery, and inflammatory agents in male and female mice.
Figure 1. TAFA4 reverses the mechanical hypersensitivity induced by SNI, paw incision, and carrageenan

(A) A significant increase in the threshold for paw withdrawal in response to static mechanical stimulation (von Frey assay) was observed on the ipsilateral but not contralateral (Contra) side of SNI mice receiving IT injections of TAFA4 (n = 13) relative to the control group, which was treated with BSA (n = 14). TAFA4 had no effect on the mechanical thresholds of the Contra paw.

(B) TAFA4 also provided significant reversal of mechanical hypersensitivity when administered s.c. to mice undergoing SNI (n = 16). BSA-treated mice remained hypersensitive (n = 14). TAFA4 had no effect on the mechanical thresholds of the Contra paw.

(C and D) IT and s.c. administration of TAFA4 induced a significant increase in the threshold of paw withdrawal in response to static mechanical stimulation following paw incision on the ipsilateral but not Contra side (n = 14 for IT and n = 16 for s.c. administration) relative to the control group treated with BSA (n = 15 for both modes of administration).

(E and F) Carrageenan-induced mechanical hypersensitivity was reversed by IT or s.c. administration of TAFA4 (n = 14 for IT and n = 16 for SC administration) but not by administration of BSA (n = 14 for IT and s.c. administration).

Results are expressed as mean ± SEM. ***p < 0.001 for comparisons of TAFA4-treated and BSA-treated mice (multiple t tests). See also Figure S1.
Therefore, TAFA4 does not transduce its biological effect through FPR1 or through any of the chemokine and chemokine-like receptors tested.

A study by Khalaj et al. (2020) showed that TAFA1–TAFA4 and neurexins interact with each other by establishing intermolecular disulfide bonds during transport through the secretory pathway. This study demonstrates that TAFA proteins are key regulators of neurexin post-translational modifications but also suggest that TAFA4 proteins cannot be considered as traditional secreted ligands that bind neurexins. Therefore, we sought to identify the TAFA4 receptor. To do so, we performed a yeast two-hybrid (Y2H) assay with the full-length TAFA4 as bait, to screen an adult mouse brain expression library. We tested 94 million interactions in total, and 32 putative protein-protein interactions were identified. Eight of the identified clones encoded the E3 ubiquitin-protein ligase DZIP3, five encoded glutaredoxin-3, and 19 clones encoded parts of the 650-kDa LRP1 (9 clones) and LRP1b (10 clones) (Figure S2A).

We focused on the interaction of TAFA4 with LRP1 (Figure 3A) rather than the other putative partners for two reasons: (1) TAFA4 is a secreted protein and would therefore be able to bind to the large extracellular domain of LRP1 and LRP1b, and (2) literature searches showed that LRP1 played a greater physiological role than LRP1b. Indeed, mice lacking LRP1b are viable and fertile and have no damaging phenotypes (Marchang et al., 2004), whereas loss of LRP1 is embryonic lethal (Herz et al., 1992), and its conditional inactivation in Schwann cells, microglia, or neurons leads to impaired pain sensation as well as neurodegeneration (Brifault et al., 2019; Liu et al., 2010; Orita et al., 2013).

A glutathione S-transferase (GST) pull-down assay was performed to validate the physical interaction between TAFA4 and LRP1. The TAFA4/GST fusion protein was produced in a bacterial system, and secreted forms of domains II, III, and IV of LRP1 fused to the green fluorescent protein (GFP) were expressed in human embryonic kidney 293T cells. TAFA4 interacted strongly with domain IV, slightly with domain III, and not at all with domain II of LRP1 (Figure 3B). RAP, which is known to interact with and block ligand binding to a wide range of LDL receptors, such as LRPL, LRPLb, LRPL2, the very-low-density lipoprotein (VLDL) receptor, and apoE receptor 2 (Lee et al., 2007) was used as a control (Figure 3B). The TAFA4/LRP1 interaction was mediated neither by FPR1 nor chemokine receptors (Figure 2C). Therefore, TAFA4 does not transduce its biological effect through FPR1 or through any of the chemokine and chemokine-like receptors tested.

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interaction was further confirmed in fluorescence microscopy-based binding and saturation assays on RAW-264.7 cells, which are known to express high levels of LRP1 (Figures 3C and 3D). Finally, for functional confirmation that TAFA4 mediates its biological effects through LRP1, we performed behavioral pharmacology experiments in the SNI and paw incision models. In both paradigms, six cohorts of mice were subjected to injury, and injury-induced mechanical hypersensitivity was confirmed at appropriate time points. The mice in each cohort received an IT injection of 170 pmol recombinant TAFA4 alone, 160 pmol RAP alone, or 170 pmol TAFA4 in combination with decreasing amounts of RAP (160, 52, or 16 pmol) and BSA. TAFA4 alone provided significant reversal of injury-induced pain hypersensitivity, as shown by the significant increase in mechanical threshold. IT administration of RAP alone had no effect, whereas its co-administration with TAFA4 abolished TAFA4-mediated reversal of mechanical hypersensitivity in a dose-dependent manner (Figure 3E). Given that TAFA4 interacts with at least LRP1 and LRP1b, these data demonstrate that the antihypersensitive effect of TAFA4 requires functional RAP-sensitive LRPs.

TAFA4 reverses the SNI-mediated alterations to spinal I_A and I_h

We showed in a previous study that ablation of the TAFA4 gene causes prolonged injury-induced mechanical hypersensitivity (Delfini et al., 2013). This phenotype was attributed to selective dysregulation of I_h in unidentified L-II interneuron subtypes. In dorsal horn interneurons, I_h, mainly carried by Kv4 channels, is essential for modulation of neuronal excitability and nociceptive behaviors (Hu et al., 2006). This ion current also plays a crucial role in sensitization of L-II outer (L-IIo) excitatory interneurons (ExINs), as shown in a mouse model of capsaicin-induced paw inflammation (Zhang et al., 2018). We therefore investigated whether TAFA4 could reverse I_h alterations reflecting dorsal horn IN sensitization in a preclinical model of neuropathic pain. We chose to use the SNI model because accurate mapping of the spinal segment from which INs are selected for patch-clamp recording is possible in this model. Indeed, SNI induces massive microglial activation that can be visualized as fluorescently labeled IB4 in living tissue within the spinal segments in which axotomized afferents terminate (Boscia et al., 2013).

We used this approach and lumbar spinal cord slices from WT mice that were naive or subjected to SNI to screen a large number of INs by blind voltage-clamp recordings in L-IIo and L-II inner (L-III; Figure 4A). The classic voltage-step protocol (Figures
4B and 5B) used to evoke $I_A$ also triggers $I_h$ and T-type Ca$^{2+}$ currents ($I_T$), but $I_T$ was undetectable here because of its small amplitude. We focused on the Kv4 channels that generate tetraethylammonium chloride (TEA)-resistant $I_A$ by using an extracellular solution containing a high TEA-Cl concentration, as described previously (Strube et al., 2015). We found that, in WT mice, SNI strongly decreased $I_A$ amplitude in L-IIo with no detectable change in the $I_A$ amplitude in L-IIi (Figures 4C and 4D). $I_h$ was extremely small in most L-II neurons (except for a small subpopulation of L-II neurons with a small $I_A$; Figure 4B) and did not appear to be affected by SNI in either of the lamina (Figure 4D).

We then tried to identify the IN subtypes in which the SNI-mediated alteration of $I_A$ occurred, using gad1GFP mice (Tammakı et al., 2003). In these mice, 95% of inhibitory INs are labeled with GFP, making it possible to discriminate between inhibitory INs (InhINs; GFP$^+$) and excitatory INs (ExINs; GFP$^-$) (Figure 5A). We also chose to study these mice for the following two reasons: (1) changes to intrinsic properties might be expected in InhINs because SNI induces alterations to firing patterns and rheobase in the parvalbumin (PV$^+$) InhINs located in L-III and ventral L-IIi and characterized by a large $I_h$ (Boyle et al., 2019; Hughes et al., 2012), and (2) we hypothesized that the intrinsic electrophysiological properties of L-II-InhINs might be targeted by TAF4A, which is known to enhance GABAergic tone in L-II by increasing the frequency of spontaneous and miniature inhibitory postsynaptic currents (IPSCs) and decreasing the frequency of spontaneous and miniature excitatory postsynaptic currents (EPSCs) with no change in amplitude (Kambrun et al., 2018).
### Article

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**A**

![Dorsal Horn of Spinal Cord (DHSC)](legend on next page)

**B**

LIIo-ExIN  |  LIIi-InhIN
---|---
Naive  |  Naive
SNI  |  SNI

**C**

**E**

**F**

**I**

<table>
<thead>
<tr>
<th>Location</th>
<th>Neuron type</th>
<th>Mice model &amp; pharmacology (n)</th>
<th>IA (+70 mV; pA)</th>
<th>IA density (+70 mV; pA/pF)</th>
<th>Ih (-120 mV; pA)</th>
<th>Ih density (-120 mV; pA/pF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LII outer</strong></td>
<td><strong>ExIN</strong></td>
<td>Naive (46)</td>
<td>6367 ±378</td>
<td>294 ±21</td>
<td>-41.2±3.3</td>
<td>-1.9±0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SNI (26)</td>
<td>4366 ±430</td>
<td>192 ±18</td>
<td>-41.5±4.4</td>
<td>-1.9±0.2</td>
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<tr>
<td></td>
<td></td>
<td>SNI; TAF4A (11)</td>
<td>6079 ±966</td>
<td>333 ±44</td>
<td>-59.9±8.0</td>
<td>-3.6±0.6</td>
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<tr>
<td></td>
<td></td>
<td>SNI; TAF4A+RAP (13)</td>
<td>3976 ±751</td>
<td>216 ±34</td>
<td>-78.5±7.1</td>
<td>-4.7±0.6</td>
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<td></td>
<td><strong>InhIN</strong></td>
<td>Naive (25)</td>
<td>2589 ±227</td>
<td>95.4±8.7</td>
<td>-62±10.1</td>
<td>-2.2±0.3</td>
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<td>SNI (14)</td>
<td>2589 ±254</td>
<td>84.3±11.2</td>
<td>-52.8±9.5</td>
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<td></td>
<td></td>
<td>SNI (21)</td>
<td>5348 ±701</td>
<td>245 ±27.5</td>
<td>-45.6±7.1</td>
<td>-2.3±0.4</td>
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<td><strong>LII inner</strong></td>
<td><strong>ExIN</strong></td>
<td>Naive (52)</td>
<td>1405 ±114</td>
<td>42.1±2.6</td>
<td>-330.6±25.6</td>
<td>-11.0±0.9</td>
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<td>Naive; Cs+Cd (28)</td>
<td>1670 ±155</td>
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<td>-67.3±9.2</td>
<td>-1.8±0.3</td>
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<td>SNI (32)</td>
<td>2365 ±171</td>
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<td>-5.8±1.2</td>
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<td>SNI; TAF4A (28)</td>
<td>1235 ±148</td>
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<td>-9.9±1.1</td>
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<td>SNI; TAF4A+RAP (18)</td>
<td>1657 ±202</td>
<td>72.5±10.4</td>
<td>-191.8±48.9</td>
<td>-7.5±1.7</td>
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(legend on next page)
With this mouse model, we found that \( I_A \) amplitudes were much larger in ExINs than in InhINs for L-Ilo and L-II (Figures 5B, SC, and SI). Consistent with our blind recording data, a large \( I_h \) (probably carried by HCN1 because it displays rapid activation with a time constant at \(-120 \, \text{mV} = 205.52 \pm 25.79 \, \text{ms}, n = 52\) was observed specifically in L-II InhINs (Figures 5B, 5D, and 5I). \( I_h \) was much smaller in L-Ilo InhINs (Figures 5B, 5D, and 5I). Similar low \( I_h \) amplitudes were found in L-Ilo and L-II ExINs (Figures 5D and 5I).

By subjecting \( \text{gad1}^{GFP} \) mice to SNI, we were able to identify two IN populations displaying alterations to low-threshold ion currents. First, SNI induced a significant decrease in \( I_A \) amplitudes in L-Ilo ExINs (Figures 5B, 5E, and 5I). Second, more surprisingly, SNI significantly increased \( I_A \) amplitudes in L-II InhINs (Figures 5B, 5F, and 5I). This increase in \( I_A \) amplitudes following SNI in L-II InhINs was accompanied by a decrease in \( I_h \) amplitude (Figures 5B, 5H, and 5I). We checked that the SNI-induced increase in \( I_A \) amplitudes in L-II InhINs persisted when the recordings were performed with CsCl (to block \( I_h \)) together with 20 \( \mu \text{M} \) CdCl (to block \( Ca^{2+} \) currents) (Figure 5I). SNI had no effect on \( I_A \) amplitudes in L-II ExINs or L-Ilo InhINs (Figures 5G and 5I). In addition, SNI did not modify \( I_h \) in all IN types in which this current was small (Figure 5I).

We then investigated whether TAF4 could reverse the SNI-mediated alterations in \( I_A \) and \( I_h \). Recombinant TAF4 totally reversed the SNI-induced decrease in \( I_A \) in L-Ilo ExINs (Figures 5E and 5I). TAF4 induced a small but significant increase in \( I_h \) in these same INs (Figures 5G and 5I). Recombinant TAF4 also reversed the SNI-mediated increase in \( I_h \) in L-II InhINs (Figures 5F and 5I) as well as the SNI-induced decrease in \( I_h \) in L-II InhIN (Figures 5H and 5I). Finally, consistent with our behavioral pharmacology results, the TAF4-mediated reversal of the SNI-induced \( I_A \) decrease in L-II ExINs and increase in L-II InhIN was prevented by incubating the spinal cord slices with a mixture of recombinant TAF4 and RAP (Figures 5E, 5F, 5H, and 5I). RAP addition had no significant effect on TAF4-mediated modulation of \( I_h \) in either of the subsets of INs (Figures 5G–5I).

**SNI downregulates TAF4 in injured DRG neurons and their central terminals**

Our findings demonstrate that TAF4, RAP-sensitive LRPs, HCN1, and A-type Kv channels are key players in modulation of SNI-induced mechanical hypersensitivity. We systematically investigated the effects of SNI on these components by performing a series of qualitative and quantitative analyses on DRGs and DHSC from naive mice and SNI mice 7 days after surgery. We found that SNI had no effect on the levels of transcripts encoding \( lrp1 \), HCN channels (\( hcn1–hcn4 \)) and all A-type Kv channels (\( KcnA4, Kcnc3 \) and 4, and \( Kcnf1–3 \)) (Figure S2A). We then performed TAF4 immunostaining with a newly generated rat anti-mouse-TAF4 antibody (Hoeffel et al., 2021) on DRG and spinal cord sections from WT and TAF4-null mice. Standard protocols were used for TAF4 immunostaining on DRG sections. In contrast, TAF4 immunostaining on SC sections required special conditions, as described in the STAR methods section. TAF4 is expressed in a subset of DRG neurons, and, as expected for C-LTMRs, TAF4+ central terminals project into LIIi, just beneath IB4+ fibers (Figures S3B and S3C). TAF4 labeling was abolished in both types of samples from TAF4-null mice, demonstrating the specificity of the anti-TAF4 antibody.

We used this tool in combination with qRT-PCR, to determine whether SNI affected TAF4 expression at the transcriptional, translational, or trafficking levels. We first identified the lumbar DRGs affected by the nerve lesion by injecting the cholera toxin B subunit (CTb) into the footpad of the hind paw. Under these conditions, CTb retrogradely labeled myelinated sensory neuron cell bodies located in lumbar 4 and 5 (L4 and L5) DRGs but not in L3, L2, or L1 DRGs (Figure 6A; data not shown). We then performed qRT-PCR on contralateral and ipsilateral L3, L4, and L5 DRGs 7 days after SNI. Consistent with our CTb retro-labeling results, qRT-PCR revealed a significant increase in the abundance of \( atf3 \) transcripts in the ipsilateral L4 and L5 DRGs but not in the L3 DRGs (Figure 6B). Consistent with this finding, SNI induced a significant decrease of tafa4 transcripts in the ipsilateral L4 and L5 DRGs (Figure 6C), whereas the total number of TAF4+ neurons in these DRGs was unaffected (Figure 6D). We investigated whether the decrease in tafa4 transcript levels was accompanied by a decrease in TAF4 protein levels within C-LTMR L-IIi spinal projections by performing double immunostaining on SC sections from lumbar segments with anti-TAF4 and anti-Iba1 antibodies. In contrast to what was observed on the contralateral side, SNI completely eliminated the TAF4 protein from the spinal region where high levels of microglial activation were demonstrated by Iba1 immunoreactivity (Figure 6E).

**DISCUSSION**

We show here that TAF4 is endowed with strong potential to reverse pathologically increased mechanical hypersensitivity.
and provide insights into its mechanism of action. We show that spinal (IT) or systemic (s.c.) administration of TAFA4 reverses inflammatory, postoperative, and nerve injury-induced mechanical hypersensitivity in male and female mice. We also show that the antihypersensitive actions of TAFA4 require functional RAP-sensitive LRPs. In parallel, we found that exogenous administration of recombinant TAFA4 reverses SNI-induced alterations of the electrophysiological properties of spinal cord INs, including a decrease in \( I_A \) in L-IIo ExINs and an increase in \( I_h \) together with a decrease in \( I_{\text{a}} \) in L-III InhINs, strongly suggesting that these changes underlie SNI-induced sensitization.

The decrease in \( I_A \) in L-IIo ExINs after SNI reported here has also been observed during inflammation following injection of capsaicin into the hind paw of mice (Zhang et al., 2018). In L-IIo ExINs, in particular L-IIo SOM-negative neurons, \( I_A \) is crucial for the timing mechanisms required for gating innocuous Aβ inputs that may activate pain pathways (Zhang et al., 2018). In the inflammation and SNI models, the decrease in \( I_A \) in this IN subtype opens the gate to innocuous inputs toward pain pathways. Targeting of this common mechanism by TAFA4 probably accounts for the effects of TAFA4 on inflammatory and SNI-induced mechanical hypersensitivity.

The SNI-induced alterations of subthreshold ion currents in L-III InhINs have not been described so far. Remarkably, SNI altered \( I_h \) and \( I_A \) in opposite directions (decreasing \( I_h \) amplitudes and increasing \( I_A \) amplitudes). These two currents have opposite influence on membrane excitability, with the depolarizing action of \( I_h \) promoting firing and \( I_A \) acting as a brake against firing (Marder and Goaillard, 2006). The SNI-related synergistic modulation of \( I_h \) and \( I_A \) demonstrated here should, therefore, lead to a strong decrease in the excitability of L-III InhINs. The changes in ion currents observed in L-IIo ExINs and in L-III InhINs in response to SNI (and reversed by TAFA4) should lead to a strong increase in the responsiveness of the network to incoming inputs because of the synergistic decrease in the excitability of InhINs and increase in that of ExINs.

The incubation of spinal cord slices with recombinant TAFA4 reversed each of the detected ion channel alterations caused by SNI. This reversal occurred after long incubations of the slices with TAFA4 (at least 40 minutes), suggesting the activation of as yet unidentified signaling pathways or cell behaviors restoring the normal function of L-IIo-ExIN and L-III-InhIN.

How can exogenous administration of TAFA4 lead to opposite modifications of \( I_A \) in L-IIo ExINs and L-III InhINs? Two scenarios are possible. (1) TAFA4 may activate different cellular and/or molecular pathways independently targeting \( I_A \) in L-IIo ExINs and in L-III InhINs, or (2) a single cellular and molecular pathway may be involved, with recombinant TAFA4 preferentially targeting L-III InhINs, whose activity modulates \( I_h \) levels in L-IIo ExINs. The second scenario was recently put forward by Zhang et al. (2018), who showed that ablation of preprodynorphin-derived neurons induces a significant decrease in \( I_h \) in L-IIo ExINs, leading to an increase in the number of L-IIo INs generating fast Aβ-evoked action potentials. Preprodynorphin-derived neurons are necessarily included in our large gad1-GFP-positive neuron sample, in which only PV neurons could be identified on the basis of their expression of large \( I_A \). It is also possible that PV neuron activity controls \( I_h \) size in L-IIo ExINs because these PV neurons target different excitatory neuron subpopulations in L-IIo (Boyle et al., 2019; Petitjean et al., 2015).

Finally, we show here that, in addition to altering spinal IN \( I_A \) and \( I_h \), SNI also decreases the levels of TAFA4 in L4 and L5 DRGs and in their corresponding C-LTMRs central terminals.

All of the effects of TAFA4 reported here (reversal of mechanical hypersensitivity and rescue of ion current alterations caused by SNI) require functional RAP-sensitive LRPs. Among these receptors, LRP1 has been shown to play a critical role in the somatosensory system (Gonias and Campana, 2014). Indeed, LRP1 is expressed in neurons (DRGs and SCs), astrocytes, activated microglia, and Schwann cells. LRP1 is a large (600 kDa) cell surface receptor with a broad spectrum of functions. In the context of pain, it may regulate the cellular microenvironment of the injured or inflamed nervous system through endocytosis of various extracellular mediators or by facilitating endocytosis of other transmembrane receptors involved in cell signaling (Gonias and Campana, 2014). Deletion of the LRP1 gene in Schwann cells leads to prolonged partial sciatic nerve ligation (PNL)-induced mechanical hypersensitivity (Orita et al., 2013), whereas deletion of this gene in microglia prevents development of PNL-induced mechanical allodynia by impairing microglial activation (Brifault et al., 2019). Consistent with these findings, we also found that deletion of microglial LRP1 prevents SNI-induced microglial activation and strongly decreases paw incision-induced mechanical hypersensitivity (data not shown).

There is a strong consensus that microglial mediators contribute to regulation of acute and persistent pain by modulating excitatory and inhibitory synaptic transmission in the spinal cord (Ji et al., 2013). For example, tumor necrosis factor alpha (TNF-α) increases the frequency of spontaneous EPSCs (sEPSCs), interleukin-6 (IL-6) decreases the frequency of...
spontaneous inhibitory postsynaptic currents (sIPSCs), and IL-1β acts on sEPSCs and sIPSCs by increasing (for sEPSCs) and decreasing (for sIPSCs) their frequency and amplitude (Kawasaki et al., 2006). Consistent with these findings, we have shown that bath applications of recombinant TAFA4 enhance inhibitory synaptic transmission within the spinal network by promoting microglial retraction and increasing the number of inhibitory synapses on L-II somata (Kambrun et al., 2018). We show here that inhibition of spinal RAP-sensitive LRPs completely abolishes the TAFA4-mediated reversal of mechanical hypersensitivity in the SNI and paw incision pain models. Given the crucial role of microglial LRPs in modulation of nerve injury (Brifault et al., 2019) and in the paw incision model (unpublished data), we favor the hypothesis that TAFA4 mediates its antihypersensitive actions by decreasing secretion of microglial mediators and restoring normal excitatory and inhibitory neurotransmission. In line with this, we recently showed that TAFA4 increases production of the anti-inflammatory cytokine IL-10 by dermal macrophages after UV radiation-induced skin damage, reducing skin inflammation and promoting tissue regeneration (Hoeffel et al., 2021). We thus propose a model where, under pathological conditions such as SNI (Figure 6F, right panel), exogenously administered TAFA4 restores the gating activity of L-II InhINs by reversing the SNI-mediated increase of Ia and decrease of Ih, mimicking TAFA4 restores the gating activity of L-II InhINs by reversing the SNI and paw incision pain models. Given the crucial role of mi-

SUPPLEMENTAL INFORMATION

We thank the members of the Moqrich laboratory for fruitful scientific discussions. This work was funded by the French National Research Agency (ANR-CE16-Myochronic to A.M.), a “Fondation pour la Recherche Médicale (FRM)” postdoctoral fellowship (to S.Y.), Equipe FRM-EQP202003010192 (to A.M.), and Biotrail PhD funding (to C.S.). This work was also supported by institutional funding from CNRS and Aix-Marseille-Université (to IBDM).

AUTHOR CONTRIBUTIONS

S.Y. generated all electrophysiology data and contributed to their analysis. C.S. performed all behavioral experiments. A.R. designed and performed qRT-PCR on DRGs and spinal cord and immunostaining for the quantification experiments. I.M. performed hFC-TAFA4 binding experiments. P.M. managed the mouse colonies used in this study and performed all qRT-PCR experiments. S.G. performed several behavioral experiments not presented here. A.C. contributed to unpublished data. S.U. and R.R. generated and validated the anti-TAFA4 antibody. A.E.K. contributed to validation of the TAFA4/LRP1 interaction. J.-Y.S. and M.P. designed and performed the BRET and calcium mobilization experiments. A.J.S. contributed to unpublished data. J.-M.G. participated in writing of the discussion of the electrophysiological data, designed the working model, and provided major input for the manuscript. F.C. designed and performed all biochemistry experiments and produced all figures. N.C. designed the electrophysiological experiments, analyzed all electrophysiological data, and participated in writing of the manuscript. A.M. conceived the project and wrote the manuscript.

DECLARATION OF INTERESTS

A.M. and S.G. are exploiting TAFA4 patent number DI 06104-01 to launch a start-up company, Tafalgie Therapeutics, with the aim of performing clinical trials on the use of TAFA4 to treat postoperative pain.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects.

REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Aziz Moqrich (aziz.moqrich@univ-amu.fr).

Materials availability
This study did not generate new unique reagents.
Data and code availability
All data reported in this paper will be shared by the lead contact upon request.
This paper does not report original code.
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
C57/Bl6J mice (4 to 12 weeks of age) were bought from Charles River Laboratories. Gad1<sup>GFP</sup>/+ mice (Tamamaki et al., 2003) were generated by crossing gad1<sup>GFP</sup>/+ males with C57/Bl6J females, and were used for experiments at four to five weeks of age. Mice of both sexes were used for all experiments. As no differences were noted between males and females, the data for the two sexes were then combined. Mice were maintained under standard housing conditions (22°C, 40% humidity, 12 h light cycles, and free access to food and water). Particular efforts were made to minimize the number of mice used in this study, and the stress and suffering to which they were subjected. All experiments were conducted in line with the European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC). All experimental procedures were approved by an independent ethics committee for animal experimentation (APAFIS), as required by the French law and in accordance with the relevant institutional regulations of French legislation on animal experimentation, under license number 2015070217242262-V5#1537. All experiments were performed in accordance with the ARRIVE guidelines.

METHOD DETAILS

Pain models
SNI
The spared nerve injury (SNI) model of peripheral nerve injury was implemented as previously described (Decosterd and Woolf, 2000). Briefly, mice were anesthetized with ketamine (40 mg/kg IP) and xylazine (5 mg/kg IP) and the left sciatic nerve was exposed under aseptic conditions. The distal trifurcation of the sciatic nerve was identified and the tibial and common peroneal branches were ligated with polypropylene nonabsorbable 6-0 sutures (Ethicon); 1 mm was cut out, leaving the sural branch intact. The wound was closed with sutures, and the animals were allowed to recover and returned to their cages.

Paw incision
Paw incision surgery was performed as described by Brennan (1999). Mice were anesthetized with ketamine (40 mg/kg IP) and xylazine (5 mg/kg IP) and a longitudinal incision was made through the skin and fascia of the right hind paw. Forceps were used to elevate the flexor digitorum brevis muscle longitudinally and an incision was made through the muscle with a scalpel, to cut it into two halves. The wound was closed with sutures, and the animals were allowed to recover and returned to their cages.

Carrageenan injection
We injected 20 µL of 1% λ-carrageenan (Sigma-Aldrich, 22049-5G-F) in 1 x PBS into the plantar surface of the left hind paw of the mouse with a Hamilton syringe.

Behavioral tests
All behavioral assays were conducted on 8- to 12-week-old C57/Bl6J mice. Animals were acclimated to their testing environment for 45-60 minutes before each experiment, and all experiments were performed at room temperature (~22°C). Experimenters were blind to the treatments used.

Von Frey’s test
Mice were placed in plastic chambers on a wire mesh grid and stimulated with von Frey filaments (Bioseb) by the up-down method (45) starting with a 1 g filament, and using 0.07 and 2 g filaments as the cutoffs.

Open Field test
The Open-field apparatus consists of an empty square arena (40X40X35 cm), surrounded by to prevent the animal from escaping. Light inside the arena was uniform and kept at approximately 100-lux throughout the tests. Mice were individually placed in the center of the arena and their behavior was recorded using the EthoVision XT16 video-tracking system (Noldus, Inc., Leesburg, VA) over a 60 minutes period. The total distance traveled and the total amount of time spent in the peripheral borders and in the center were recorded. This experiment was done on naive and SNI mice that received a subcutaneous injection of vehicle or 0.3 mg/kg of TAFA4.

Tail-flick test
The tail-flick examination was used to calculate analgesic activity according to the method defined by (Keyhanfar et al., 2013). A radiant heat automatic tail-flick analgesiometer (Bioseb) was used to measure reaction times. The basal reaction time of animals in response to radiant heat was determined by placing the tail on the radiant heat source and recording the time until the tail was removed. A cutoff time of 8 s was used, to prevent tail damage.
Drugs and in vivo administration methods
TATA4 (Biotechne) was dissolved in 0.1 M phosphate buffer (PB); baclofen (Sigma-Aldrich) was dissolved in H2O (pH 7.6), SNC80 (Tocris Bioscience) was dissolved in 100 mM HCl and DAMGO (Sigma Aldrich) was dissolved in 0.9% NaCl. RAP (Sigma-Aldrich) was dissolved in 0.1 M PB and N-formyl-Met-Leu-Phe (MLF, Sigma) was dissolved in 2.5% DMSO.

Drugs were administered intrathecally (IT) or systemically using subcutaneous (SC) injections in the neck of the mice, as indicated in the figure legends. For IT injections, 10 µL of each compound was injected into non-anesthetized mice. Successful placement of the needle was confirmed by a slight flick of the tail.

Cell lines and DNA constructs
RAW 264.7 macrophages and HEK293 cells lines were obtained from ATCC.

Nucleotide fragments encoding the LRP1 signal peptide (amino acids (aa) 1 to 21), domain II (aa 788 to 1185), domain III (aa 2467 to 2924) and domain IV (aa 3278 to 3779) were obtained by the RT-PCR amplification (Superscript III and Phusion Hi-Fi DNA polymerase, Thermo Fisher Scientific) of RNA extracted from C57/B16J mouse brain (RNAesy mini kit, QIAGEN) according to the manufacturer’s instructions. PCR products were inserted into pEGFP-C2 plasmids with In-Fusion Cloning technology (Addgene) to produce pLRP1-s-D-II-GFP, pLRP1-s-D-III-GFP and pLRP1-s-D-IV-GFP. These plasmids expressed secreted forms of domains II, III and IV of mouse LRP1 tagged with EGFP.

The mouse TATA4 nucleotide sequence devoid of signal peptide (coding aa 30 to 135) was amplified by PCR (Phusion Hi-Fi DNA polymerase, Thermo Fisher Scientific) and inserted between the BamHI and NotI sites of the pGEX-4T2 vector to produce GST-TATA4. All constructs were verified by DNA sequencing (Eurofins Genomics).

Recombinant protein production and extraction
GST-tagged proteins were produced in *Escherichia coli* strain BL21 cultured overnight in 2YT medium at 30°C. Bacterial pellets were resuspended in TBS-Triton buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and the Roche Complete EDTA-free protease inhibitor cocktail) and bacteria were lysed with a French press followed by sonication at 4°C. Soluble proteins were separated from cell debris by centrifugation and stored at −80°C until use.

HEK293 cells were transfected with pLRP1-sDomII-GFP, pLRP1-sDomIII-GFP and pLRP1-sDomIV-GFP in the presence of Lipofectamine 2000, according to the manufacturer’s instructions (Life Technologies). Two days after transfection, the supernatants of the cell cultures were collected, centrifuged to eliminate cells debris and stored at −80°C until use.

Pulldown assay
Bacterial lysates expressing GST, GST TATA4 or GST-RAP (kindly provided by Jérôme Terrand, CNRS UMR 7021, Strasbourg, France) were incubated for 2 h at 4°C with glutathione-Sepharose 4B beads (GE Healthcare). The beads were thoroughly washed and incubated overnight at 4°C with HEK293 cell culture supernatants containing sDomII-GFP, sDomIII-GFP or sDomIV-GFP. They were then thoroughly washed again, and the proteins retained on the beads were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (WB).

Intracellular calcium mobilization assay
Calcium mobilization was measured in CHO-K1 cells stably expressing apoaequorin, Gx16, and the receptor of interest, as previously described (El-Asmar et al., 2005). Cells were incubated for four hours in the dark in the presence of 5 µM coelenterazine h (Promega), then diluted to the appropriate cell density before use. The cell suspension (25,000 cells/well) was added to the wells

β-arrestin BRET assay
β-arrestin recruitment was measured in a BRET proximity assay, as previously described (Corbisier et al., 2015). Plasmids encoding *Renilla* luciferase-tagged β-arrestin 2 (RLuc-β-arrestin 2) and EYFP-tagged FPR1 were used for the cotransfection of HEK293T cells by using the calcium phosphate method. Twenty-four hours post-transfection, cells were collected and used to seed (25,000 cells/well) 96-well microplates (165306, Nunc). They were cultured for an additional 24 h. Cells were then rinsed once with PBS and incubated in PBS plus 0.1% (w/v) glucose. BRET between RLuc and EYFP was measured after the addition of 5 µM coelenterazine h (Promega). Ligands were added 5 min after coelenterazine h and BRET readings were recorded after 30 minutes, with a Mithras LB940 Multilabel Reader (Berthold Technologies). The BRET signal was calculated as the ratio of EYFP emission (520–570 nm) to RLuc emission (370–480 nm).

Antibodies and reagents
The primary antibodies used were: goat anti-Iba1 (1:500, Abcam, #ab5076); rabbit anti-ATF3 (1:200, Santa Cruz Biotechnology, #SC-188), rat anti-LRP1 (1:300, Abcam, #ab92544); guinea pig anti-VGLUT3 (1:500, Synaptic Systems, #135204); sheep anti-GFP (for WB, 1:4000, MRC PPU reagent, University of Dundee, UK, #S268), chicken anti-GFP (for immunostaining 1:1000, Thermo Fisher Scientific, #A10262), and rat anti-TATA4 (1:1000, clone 1D8, (Hoeffel et al., 2021). The secondary antibodies used for detection of the
primary antibodies were: goat anti-human Fc-Alexa488 (1:300, Jackson, 109-545-098), goat anti-human Fc-HRP (1:2000, Thermo Fisher Scientific, A 188-17), donkey anti-rabbit, anti-rat, anti-guinea pig, anti-chicken and anti-goat antibodies conjugated to Alexa Fluor 488, 555, or 647 (Thermo Fisher Scientific or Molecular Probes antibodies). Isoclectin binding 4 (IB4) conjugated to Alexa Fluor 488 was used at a dilution of 1:300 (Thermo Fisher Scientific, #I21411 and #I32450). Recombinant proteins were purchased from Interchim: FAM19A4-hFc Tag (Sino Biological, 11165-H01H), hFc (Sino Biological,10702-HNAC).

**Tissue processing for immunofluorescence (IF)**

Mice were deeply anesthetized with 100 mg/kg ketamine plus 10 mg/kg xylazine. They were intracardially perfused with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde (PFA; in PB). The spinal cord lumbar region and DRGs were dissected, fixed by incubation in the same fixative at 4°C until their use for IF experiments.

For TAF4a detection in the spinal cord, mice were deeply anesthetized with 100 mg/kg ketamine plus 10 mg/kg xylazine, intracardially perfused with ice-cold solution 1 (see Table S1; pH 7.4 through which O2 was bubbled. The spinal cord lumbar region was dissected in ice-cold solution 1 under continuous oxygenation and immediately frozen in OCT medium. We cut 20 μm-thick sections on a cryostat (Leica), and these sections were mounted on Superfrost slides maintained within the cryostat. Sections were immediately fixed by incubation in 4% PFA for 15 minutes and processed for immunostaining.

**Immunofluorescence**

Slides were washed for 5 minutes (min) in PBS, incubated for 1 h at room temperature with a blocking solution (10% normal donkey serum, 1% BSA, 0.4% Triton X-100, in PBS), and then overnight at 4°C with primary antibodies in the same blocking solution.

For TAF4a immunostaining, saturation, and incubations with the primary and secondary antibodies were performed in 3% BSA with no serum or detergents.

Images were acquired with a Zeiss AxioImager M2 fluorescence microscope or a Zeiss LSM 780 confocal microscope, with a 20x, 25x or 63x objective and appropriate excitation wavelengths and emission filters. Laser power and acquisition settings were kept constant between image acquisitions for different conditions. Images were analyzed with ImageJ software.

**Cell surface binding assay**

RAW264.7 cells were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2 mg/ml glutamine and 10% v/v heat-inactivated fetal calf serum (FCS) (Life Technologies) at 37°C, under an atmosphere containing 5% CO2. They were passaged enzymatically at a 1:1 ratio every five to six days. One day before the binding assay, the cells were plated at a density of 10^5 cells/ml on glass coverslips coated with 100 μg/ml poly-L-lysine (PLL, Sigma-Aldrich).

The surface labeling of TAF4a receptors was assessed by incubating cells for 1 h at 4°C for 1H with 50 nM TAFa-Fc (Interchim, 11165-H01H) or 50 nM Fc (Interchim, 10702-HNAC) recombinant proteins diluted in 10% FCS in PBS. Cells were then washed with PBS and fixed by incubation with 4% paraformaldehyde (PFA) in PBS for 20 minutes. Cells were incubated with the blocking solution (10% normal donkey serum, 1% BSA, 0.4% Triton X-100, in PBS) for 1 hour and then overnight at 4°C with anti-LRP1 antibodies (1:500, Abcam, ab92544) and Alexa Fluor-conjugated secondary antibody (1:500, Abcam, ab92544) in the same blocking solution. Cells were incubated with Alexa Fluor-conjugated secondary antibodies (1:500, Invitrogen, A32790) for 1 hour, washed with PBS (3 times, for 5 min each) and mounted on slides with Immuno-Mount (Thermo Fisher Scientific). Images were acquired with a Zeiss LSM 780 confocal microscope with a 63x objective and appropriate excitation wavelengths and emission filters. Laser power and acquisition settings were kept constant between image acquisitions for different conditions. Images were analyzed with ZEN and ImageJ software. The images shown are representative of cells from three independent experiments.

**Enzyme-linked binding assay**

RAW264.7 cells were cultured in a 96-well microplate at a density of 10^5 cells/ml. Cells were incubated for 1 h at 4°C with concentrations of recombinant TAFa-Fc or Fc proteins ranging from 0.74 nM to 500 nM, in PBS supplemented with 10% FCS. Cells were then washed with PBS, fixed by incubation with 4% paraformaldehyde (PFA) in PBS for 20 minutes and then incubated for 1 h at room temperature with goat anti-human Fc-HRP. The microplates were washed with PBS and peroxidase activity was measured by adding 100 μl/well 3,3',5,5'-tetramethylbenzidine (TMB) and 30% hydrogen peroxide (Sigma-Aldrich) and incubating for 30 minutes at room temperature. Optical density was measured at 450 nm on a TriStar® S LB 942, Multimode Microplate Reader (BertholdTech). Results were analyzed with MikroWin software.

**Slice preparation and treatment for electrophysiological recordings**

The experiments were performed with WT mice (Charles River) for the recording of unidentified neurons or with the glutamic acid decarboxylase–green fluorescence protein (GAD67-GFP) knock-in mice produced by Tamakaki, for recordings of GAD67-GFP+ or GAD67-GFP− neurons. In both cases, recordings were performed in the lumbar spinal cord Lii and Lilo. Adult WT and GAD-67-GFP+/− mice (5 to 6 weeks old) were either used in naive conditions or after being subjected to SNI, as described above. We chose
to study this neuropathic pain model because the DH ipsilateral to the lesion presents a dense accumulation of activated microglia (that can be used as a landmark), mostly in the L3-L5 segments (47).

Mice were anesthetized by an injection of 100 mg/kg ketamine plus 10 mg/kg xylazine. They were then perfused via the left ventricle with solution 1, pH 7.4, 0–2°C, through which O₂ was bubbled (Table S1). The lumbar region of the spinal cord was extracted, embedded in low-melting agarose and the L3 to L5 lumbar segments were then cut into 300 μm-thick coronal slices on a microtome (Leica VT1200, Leica Microsystems SAS). These steps were performed as quickly as possible. The slices were kept at least for 1 hour in solution 2 at 30°C (pH 7.4, 29°C, through which 95% CO₂-5% O₂ was bubbled; Table S1).

Slices obtained from mice subjected to SNL were incubated for 30 minutes in solution 2 to which Alexa Fluor 568-conjugated isolectin B4 (IB4; Invitrogen) was added, immediately before transfer to the recording chamber (see below) at room temperature. The IB4 labeling of activated living microglia (38) identified the area of the DHSC receiving the damaged primary afferent fibers and, thus, the site at which LII and LIII neurons should be targeted for recordings. When necessary, we added 40 nM TAFA4 with or without 40 nM RAP to the IB4-containing solution 2 and, the slices were incubated in this solution for 1 hour before recording.

**Patch-clamp recordings**

The borosilicate glass pipettes used had a resistance of 2.5 to 4 MΩ. Their tips were fire-polished with a Narishige microforge and coated with beeswax to reduce their capacitance. They were filled with the following intracellular solution: 125 mM potassium glutamate, 24 mM KCl, 1 mM EGTA, 10 mM HEPES, 0.1 mM CaCl₂, 2.5 mM MgCl₂, 0.3 mM GTP-Na, 2 mM ATP-Na₂.

The slices, immobilized with a nylon grid in an immersion-type recording chamber placed on the stage of an upright microscope (Olympus BX51WI, Olympus Fr., Rungis, France), were superfused at a flow rate of 2 ml/min with ACSF (solution 3, pH 7.4, room temperature, through which 95% CO₂-5% O₂ was bubbled; Table S1). In these conditions, the patch pipettes were guided to the neuron cell bodies, in the LIIo and LIIIi lamina, with the aid of differential interface contrast optics with infrared illumination (X40, Olympus BX51) and fluorescence optics. The location of the neuron was captured by taking photographs with a camera (VX55 TILL Photonics). The recording sessions were performed after solution 3 had been replaced with modified ACSF (solution 4; Table S1) containing tetrodotoxin (TTX, 0.5 mM; Bio-Teche (UK)) to block Na⁺ currents and tetracylammonium chloride (TEA-Cl; 36 mM) to block TEA-sensitive non-inactivating K⁺ currents. Kv4 channels are the only Kv channels carrying Iₐ preserved at this high concentration of TAE-Cl (39). In a small set of experiments, we added 2 mM CsCl to solution 4, to block hyperpolarization-activated depolarizing currents (Iₐ), and 20 μM CdCl₂ to block Ca currents (IₐCa), other than T- type IₐCa currents, which are known to interact with the Iₐ carried by Kv4 channels (48, 49). Iₐ was then isolated by exploiting its sensitivity to holding potential: from a hyperpolarized membrane potential (−120 mV), depolarizing voltage steps evoked both transient and sustained K⁺ current (total Iₐ), whereas, from a more depolarized membrane potential (−30 mV), depolarizing steps evoked only sustained K⁺ currents (IₐKDR). See protocol in Figures 4A and 5A. The conditioning prepulses at −120 or −30 mV lasted 750 ms and were immediately followed by 1 s depolarizing steps. Iₐ was revealed by the offline subtraction of IₐKDR from total Iₐ.

For the hyperpolarized prepulse, we chose a highly hyperpolarized value (−120 mV) because, in our preliminary exploration of Iₐ in the dorsal spinal cord, we found that some neurons had a very hyperpolarized inactivation midpoint. This hyperpolarized prepulse also made it possible to detect and measure Iₐ. For this purpose, the holding current was subtracted for the comparison of Iₐ amplitude despite the presence of cell-to-cell variations in holding current values.

In the course of our preliminary exploration, we observed that it was difficult to maintain a stable series resistance over long recordings in five- to six-week-old mice. This made it impossible to obtain reliable results for the two successive, relatively long protocols required to obtain incremented difference current values from −70 mV to +100 mV for the construction of activation curves. We thus simply used test pulses to +70 mV from a holding potential of −120 or −30 mV (see protocol in Figures 4A and 5A). These suprathreshold values were chosen because, in the case of the LII-InhIN, Iₐ could be accurately measured only at such strongly depolarized potentials (see results); comparability was ensured by also applying these values for the other interneuron types.

**Data acquisition and analysis**

Patchpipettes were connected to the head stage of an Axopatch 200B amplifier. A computer interfaced to a 12-bit A/D converter (Digidata 1322A using pClamp10 (all from Molecular Devices, Sunnyvale, USA) controlled the voltage-clamp protocols and data acquisition. Data were digitized at 20 kHz and filtered at 2 kHz. Series resistances, without compensation, to ensure rapid recording, ranged from 6 to 19 MΩ (recordings with Ra > 19 MΩ were excluded). The mean liquid junction potential between the pipette solution and extracellular solution 4 was 12 mV, and was not corrected for.

Analyses and curve fitting were conducted in Clampfit 10.3 (Molecular Devices) and SigmaPlot 12 (Systat Software Inc. San Jose, CA).

Iₐ activation (Iₐ(t)) was fitted for the traces obtained when stepping from −70 to −120 mV and was described as the sum of two exponential terms plus a constant according to the equation Iₐ(t) = Afast exp(−t/τfast)+Aslow exp(−t/τslow)+C, where Afast and Aslow are the amplitudes of the exponential terms and τfast and τslow are the corresponding time constants (τ). The weighted τ (τwact) was calculated as follows: τwact = (τfast × Afast + τslow × Aslow)/(Afast + Aslow).
Reverse transcription-quantitative polymerase chain reaction: RT-qPCR

Ipsilateral and contralateral L3, L4 and L5 DRGs and DHSCs (lumbar segments L3-L5) were dissected from P35 WT mice on day 7 post-SNI. RNA was extracted with the RNeasy Mini Kit (QIAGEN), in accordance with the manufacturer’s instructions. RNA quality was controlled with the RNA Agilent Pico 600 Kit (Agilent). We subjected 100 to 150 ng high-quality RNA (RIN > 9) to reverse transcription to generate cDNA (Superscript III, Thermo Fisher Scientific), which was then used as a template for quantitative PCR. Gene expression was quantified by qPCR in Sybr-Green master mix (Thermo Fisher Scientific). The relative abundances of the transcripts encoded by each gene were determined by normalization against β-actin by the standard ΔCt or ΔΔCt method.

The primers used for qPCR were:

- TAFA4-Fw: CAGTTACAAGGGACATTCACG
- TAFA4-Rse: AGCACAGACTCTCATCCTTG
- LRP1-Fw: GGTGAAAGAGATTGCCCCGA
- LRP1-Rse: CCCAGAACAACGTGTGCTCAT
- ATF3 Fw: GCTGGAGTCAATTACCGTCAA
- ATF3 Rse: CGCCTCCTTTTCTCCTCAT
- Knca4 Fw: CACCCCCAATGATTCTGC
- Knca4 Rse: TTCACAAGAGCACTTCACCA
- Knc1 Fw: ACTCAGAGTGAACATGCCC
- Knc1 Rse: GCCACCTCCCCATTCAGTTT
- Knc3 Fw: TATCCCCCTAGTGACAGGAC
- Knc3 Rse: CCACAATGCTGCTCAGGCT
- Knc4 Fw: CTCATGCTGAGGAATTATG
- Knc4 Rse: GGCAATGGTAGAGAGG
- Knd1 Fw: GCACAGAGCAAGAAGACCTC
- Knd1 Rse: GCCTGCAATGGGTCTAGGTA
- Knd2 Fw: ACCAAGAGCAGACAAAGGA
- Knd2 Rse: TTGCTCAGTGCCAATTCCG
- Knd3 Fw: TGATCCTTTCTGCAGACACCATAGT
- Knd3 Rse: GCTAAGAGTTGAGACTATCACACGG
- Hcn1 Fw: GCCAGAGCACTTCATGCTAT
- Hcn1 Rse: CACCTGGAGGTCATGAGGTC
- Hcn2 Fw: ATGGAGGAGACCTCCCAG
- Hcn2 Rse: GAAGAGCCGGAACAGATAGA
- Hcn3 Fw: AAGATCTTCAAGCTCTGCG
- Hcn3 Rse: GAAGATGCGAAACACACTGCAC
- Rse: GAAGATGCGAACCACCTGCAC
- Hcn4 Fw: CTCCCTAAGGATTTCCGAGA
- Hcn4 Rse: CACAGAAAGCATCCTGAGT

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The results are expressed as means ± SEM. Statistical analyses were performed with Prism 7 (Graphpad Software, La Jolla, CA, USA). The significance of differences was assessed in multiple t tests against BSA for behavioral experiments and in unpaired t tests for electrophysiology experiments.