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## Dorsal Horn Parvalbumin Neurons Are Gate-Keepers of Touch-Evoked Pain after Nerve Injury

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### SUMMARY

Neuropathic pain is a chronic debilitating disease that results from nerve damage, persists long after the injury has subsided, and is characterized by spontaneous pain and mechanical hypersensitivity. Although loss of inhibitory tone in the dorsal horn of the spinal cord is a major contributor to neuropathic pain, the molecular and cellular mechanisms underlying this disinhibition are unclear. Here, we combined pharmacogenetic activation and selective ablation approaches in mice to define the contribution of spinal cord parvalbumin (PV)-expressing inhibitory interneurons in naive and neuropathic pain conditions. Ablating PV neurons in naive mice produce neuropathic pain-like mechanical allodynia via disinhibition of PKC $\gamma$  excitatory interneurons. Conversely, activating PV neurons in nerve-injured mice alleviates mechanical hypersensitivity. These findings indicate that PV interneurons are modality-specific filters that gate mechanical but not thermal inputs to the dorsal horn and that increasing PV inter-neuron activity can ameliorate the mechanical hypersensitivity that develops following nerve injury.

### In Brief

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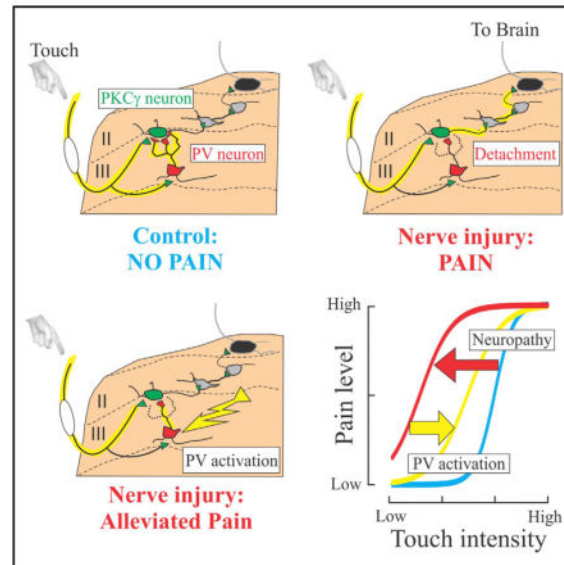
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### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.09.080>.

Improved therapy for neuropathic pain requires a better understanding of the spinal cord neuronal networks that process peripheral sensory inputs in health and disease. Petitjean et al. find that a subset of inhibitory interneurons, containing the marker parvalbumin (PV), prevent touch inputs from activating pain circuits. After nerve injury, a decrease is seen in the number of these synapses, and light touch can elicit pain.



## INTRODUCTION

Neuropathic pain is a chronic debilitating disease that follows nerve injury, persists long after the initial injury has subsided, and decreases the quality of life of the patients (Jensen et al., 2007). Despite a plethora of medications and treatment modalities, new treatment approaches for neuropathic pain are needed. Two hallmarks of neuropathic pain are spontaneous pain and mechanical allodynia, a painful response to an innocuous stimulus, such as gentle touch. These symptoms are due in part to a spinal cord dysfunction characterized by decreased inhibitory controls (Castro-Lopes et al., 1993; Moore et al., 2002; Lever et al., 2003; Coull et al., 2003). Enhancing inhibition by activating spinal GABA receptors is effective in some animal models of chronic pain, but the use of GABA agonists in patients is limited due to lack of efficacy and/or adverse side effects (Munro et al., 2009). These shortcomings are due in part to our limited understanding of the neuronal circuitry through which the loss of inhibitory control is manifested (Braz et al., 2014).

Particularly relevant to this question is that touch-sensitive A $\beta$  primary afferent fibers can access spinal cord nociceptive (pain) circuits through polysynaptic pathways (Torsney and MacDermott, 2006). Under normal conditions, these pathways are under tonic inhibition, likely derived from glycinergic inter-neurons located in deeper laminae (Takazawa and MacDermott, 2010). However, in the setting of nerve injury, this inhibitory input is reduced, thereby enabling A $\beta$  fiber-transmitted innocuous inputs to engage and activate nociceptive pathways (Torsney and MacDermott, 2006); see Figure 1. The particular circuits that are

disinhibited and whether the disinhibition involves the death of inhibitory interneurons are not clear (Polgár and Todd, 2008).

Here, we used genetic and functional approaches to examine the contribution of parvalbumin (PV)-expressing inhibitory interneurons as gate-keepers of the touch-pain circuitry in the dorsal horn. The location of these interneurons at the border between inner lamina II (Ii) and III is ideal for the modulation of mechanical inputs derived from myelinated primary afferent fibers. However, there is currently nothing known about their contribution to the processing of sensory information in the dorsal horn.

We report that PV interneurons act as modality-specific filters of sensory inputs in the dorsal horn. Increasing their activity significantly alleviates the mechanical allodynia in a mouse model of neuropathic pain. Conversely, selective ablation or silencing of PV interneurons induces mechanical allodynia in naive mice. Using electron microscopy, we identified synaptic contacts between PV<sup>+</sup> terminals and PKC $\gamma$ <sup>+</sup> excitatory interneurons. Nerve injury or ablation of PV neurons results in a significant decrease in the number of these contacts, which disinhibits PKC $\gamma$  interneurons and leads to mechanical allodynia. Inhibition of PKC $\gamma$  interneurons attenuates mechanical allodynia in both conditions.

## RESULTS

### Parvalbumin Interneurons Do Not Die after Nerve Injury

It has been suggested that nerve-injury-mediated disinhibition in the dorsal horn is due to the degeneration of GABAergic inhibitory interneurons (Scholz et al., 2005). Although this claim has been challenged (Polgár et al., 2004), we reexamined the question, focusing specifically on the survival of PV interneurons in the spared nerve injury (SNI) model of neuropathic pain (Shields et al., 2003). Because loss of PV immunostaining following nerve injury does not necessarily indicate loss of PV<sup>+</sup> neurons, we examined a marker that is independent of PV expression. To this end, we crossed a transgenic *Parvalbumin::Cre* (*PV::cre*) knockin mouse to a tdTomato (tdTom, red fluorescent protein) reporter mouse. In *PV::cre; tdTom* mice, tdTom is expressed in the cytosol and processes of PV neurons of the adult spinal cord, independently of the expression level of PV.

Given that the expression pattern of certain markers can change during development (Cavanaugh et al., 2011a), we first asked whether tdTom-expressing cells correspond to the PV-immunoreactive (IR) neurons in the adult. Our experiments (Figures 2A–2C) indicate that 83.7%  $\pm$  3.0% of the PV-IR neurons are tdTom positive, and all tdTom-positive neurons are positive for PV-IR. Thus, the expression of tdTom recapitulated the endogenous PV expression, and importantly there is no ectopic expression of tdTom in the spinal cord of the *PV::cre; tdTom* mice. Two previous reports indicated that, in the rat dorsal horn, not all PV-IR neurons are inhibitory (Antal et al., 1991; Laing et al., 1994). To address this question, we stained dorsal horn sections of *PV::cre; tdTom* mice with a mixture of GABA and glycine antibodies and demonstrate that 95.4% of tdTom-expressing neurons are GABA and/or glycinergic (Figure 2D). Furthermore, we observed less than 5% colocalization with Lmx1b (Figure 2E) or Tlx3 (four of 79 neurons, n = 2 mice, data not shown), two transcription factors expressed in excitatory interneurons (Szabo et al., 2015; Foster et al.,

2015). We next determined whether PV neurons die after nerve injury. In agreement with previous studies (Huang et al., 2010; Polgár et al., 2013a; Yamamoto et al., 1989), we observed that the dorsal horn of naive mice contains  $12.3 \pm 3.4$  PV neurons on each side of a 25- $\mu\text{m}$ -thick transverse section. Our results indicate, however, that there is no loss of tdTom<sup>+</sup> (i.e., PV) neurons between naive, sham, or nerve-injured mice, 3, 5, or 8 weeks post-nerve injury (Figures 2F and 2G). Given that ~16% of PV-immunoreactive neurons were negative for tdTom expression, it is not possible to know what happens to these neurons after SNI. However, staining with an anti-activated caspase 3 antibody failed to show any evidence of apoptosis in dorsal horns of nerve-injured mice 1, 3, and 8 weeks post injury (data not shown). We conclude that there is no significant loss of PV inter-neurons in the SNI model of neuropathic pain.

### Pharmacogenetic Activation of PV Interneurons Reveals Their Modality-Specific Filtering of Sensory Inputs

Although Celio and Heizmann described PV interneurons in the dorsal horn over 30 years ago (Celio and Heizmann, 1981), little is known about their contribution to the processing of sensory inputs. Reports monitoring Fos expression found that PV neurons are not activated by peripheral noxious stimuli (Polgár et al., 2013a; Neumann et al., 2008). Furthermore, approximately 50% of the nerve terminals of large diameter fibers (Hughes et al., 2012), presumably of the A $\beta$  type, receive inputs from PV<sup>+</sup> profiles in the spinal cord, which suggests that they may regulate the processing of innocuous inputs. To assess the function of dorsal horn PV interneurons, we modulated their activity using a pharmacogenetic approach (Alexander et al., 2009; Rogan and Roth, 2011; Dong et al., 2010). This approach uses a modified G<sub>q</sub>-coupled muscarinic receptor (M3D) that only responds to an exogenous, blood-brain-barrier-permeable, synthetic agonist: clozapine N-oxide (CNO) (Figure S1). CNO activation of cultured dorsal horn neurons infected with M3D induces membrane depolarization ( $8.3 \pm 3$  mV) and generates action potentials in PV interneurons (Figure S1D) (Alexander et al., 2009).

To activate PV neurons in vivo, we injected the lumbar spinal cord of *PV::cre; tdTom* mice with an adeno-associated viral (AAV) vector expressing Cre-dependent *M3D* and the reporter mCitrine (*mCit*). Although a subset of peripheral sensory neurons also expresses PV, we did not observe any M3D expression in dorsal root ganglia (DRG) (data not shown). Figure 3A shows that activation of PV neurons significantly increased baseline mechanical withdrawal thresholds compared to saline-injected mice ( $1.86 \pm 0.30$  versus  $1.27 \pm 0.10$  g), without affecting thermal withdrawal latencies (Figure 3B). Furthermore, CNO injection had no effect on mechanical withdrawal thresholds in control virus (mCit alone)-injected *PV::cre; tdTom* mice (baseline:  $1.35 \pm 0.37$ ; 5 mg/kg CNO:  $1.16 \pm 0.26$  g; data not shown). A subset of ventral horn interneurons expresses parvalbumin (Antal et al., 1990). To exclude their potential activation by CNO, we examined mice injected with the highest dose of CNO on the rotarod test. We found no motor impairment in these mice (Figure 3C). Taken together, our results indicate that dorsal horn PV interneurons function as modality-specific filters of somato-sensory information.

## PV Neuron Activation Reduces Nerve-Injury-Induced Mechanical Hypersensitivity

There is no evidence for a direct projection of large diameter, A $\beta$  afferents to pain-transmitting projection neurons of lamina I. In a model of neuropathic pain, however, A $\beta$  fiber stimulation evokes polysynaptic excitation of these projection neurons, an effect that can be reproduced by intracisternal administration of the glycine receptor antagonist, strychnine (Miraucourt et al., 2007). These findings suggest that loss of glycinergic inhibitory inputs contributes to the mechanical allodynia following nerve injury. In fact, PV interneurons co-express GABA and glycine (Laing et al., 1994) and are activated by A $\beta$  fibers (Hughes et al., 2012). Nevertheless, the consequence of decreased function of PV interneurons to mechanical allodynia in neuropathic pain models remains unknown. Here, we asked whether increasing the activity of PV interneurons with CNO can attenuate mechanical allodynia in the setting of nerve injury. We performed these studies in mice that received a unilateral injection of the Cre-dependent AAV-*M3D* 21 days prior to nerve injury.

Figures 3D and 3E demonstrates that activation of PV interneurons ipsilateral to the nerve injury significantly and dose-dependently attenuates the mechanical allodynia ( $0.58 \pm 0.08$  g in saline-injected versus  $1.00 \pm 0.12$  g in CNO (3 mg/kg)-injected mice). However, CNO had no effect on mechanical allodynia in control virus-injected *PV::cre; tdTom* mice ( $0.30 \pm 0.04$  g in saline-injected versus  $0.29 \pm 0.17$  g in CNO 3 mg/kg-injected mice). To determine whether this attenuation is selective for mechanical inputs, we used an inflammatory pain model associated with both mechanical allodynia and thermal hyperalgesia (Bonin and De Koninck, 2014). In this model, activation of PV interneurons selectively attenuated the mechanical allodynia without affecting the thermal hyperalgesia (Figure 3F). We conclude that increasing the activity of PV interneurons selectively attenuates mechanical allodynia without affecting thermal hypersensitivity.

## Parvalbumin Interneurons Target Excitatory PKC $\gamma$ Interneurons

Activity of PKC $\gamma$  excitatory interneurons is essential to the development of mechanical allodynia after nerve injury (Miraucourt et al., 2007; Malmberg et al., 1997). In normal conditions, these neurons are under tonic, strychnine-sensitive (Miraucourt et al., 2007, 2009), glycinergic (Lu et al., 2013) inhibition, which prevents the induction of mechanical allodynia (Miraucourt et al., 2007). To determine whether PV interneurons are anatomically positioned to modulate the activity of PKC $\gamma$  interneurons, we examined the presence of appositions between the terminals of PV interneurons, which express tdTom, and the cell bodies of PKC $\gamma$ -IR interneurons, which predominate in inner lamina II. Consistent with this hypothesis, we recorded a significant number of PV<sup>+</sup> terminals ( $3.87 \pm 0.41$  appositions per PKC $\gamma$  soma,  $n = 64$  neurons from six mice; Figures 4A–4C). Because a subset of large-diameter sensory afferents express PV (Ernfors et al., 1994; Antal et al., 1990), it is possible that they contribute to the tdTom-positive appositions onto PKC $\gamma$  interneurons instead of or in addition to the dorsal horn PV interneurons. To evaluate this possibility, we performed double immunohistochemical experiments to determine whether the tdTom-positive terminals colocalize with the vesicular glutamate transporter-1 (VGluT1), a marker of large-diameter myelinated sensory neurons (Hughes et al., 2012). We found that only  $0.2\% \pm 0.1\%$  of the VGluT1-positive terminals colocalized with tdTom in the dorsal horn lamina IIi and III, where the PV and PKC $\gamma$  interneurons are found (Figures S2A and S2B).

Sensory neurons that express PV are mainly proprioceptors, with axons terminating in deeper laminae of the spinal cord, mainly lamina IX (Zampieri et al., 2014). Not surprisingly, when we examined the colocalization of VGluT1<sup>+</sup> terminals with tdTom in lamina IX, we observed a significantly larger percentage of co-localization ( $55.6\% \pm 15.7\%$ ) than in laminae Iii-III. Additionally, we failed to see any colocalized VGluT1<sup>+</sup>/tdTom<sup>+</sup> terminals on the surface of PKC $\gamma$  interneurons (Figure S2D). We conclude that the tdTom signal detected on PKC $\gamma$  interneurons does not originate from peripheral sensory neurons.

To investigate the nature of the appositions, including whether they expressed specific synaptic markers, we immunostained dorsal horn sections from *PV::cre; tdTom* mice for PKC $\gamma$  and (1) VAMP2 or (2) GlyT2, a presynaptic and an inhibitory presynaptic marker, respectively (Figure 5A). We found significant coincident localization of tdTom<sup>+</sup> appositions with VAMP2 and GlyT2 (Figure 5B). We confirmed the inhibitory nature of the tdTom apposition by staining for gephyrin, a postsynaptic marker of inhibitory synapses (Majdi et al., 2007). Our data demonstrate that the terminals of PV neurons appose gephyrin clusters on the soma of PKC $\gamma$  neurons (Figures 5A and 5B). The average number of appositions per PKC $\gamma$  soma was similar in all three conditions compared to the situation without synaptic marker (Figure 5B), confirming that these are glycinergic appositions.

To determine whether the appositions constitute functional synapses, we performed electron microscopy on the dorsal horn of naive *PV::cre; tdTom* mice, immunostained for tdTomato and PKC $\gamma$ . In inner lamina II, we observed tdTom-IR dendrites and boutons (Figure 5C). PKC $\gamma$ -IR dendrites and somata occurred in the same layer. The tdTom-IR boutons established synapses onto PKC $\gamma$ -IR dendrites (Figure 5C). The boutons contained pleomorphic vesicles, and the synapses were of the symmetric type, suggestive of inhibitory contacts. As shown in the rat (Peirs et al., 2014), PKC $\gamma$  labeling was located at the plasma membrane and in the cytoplasm of neurons. Our results therefore indicate that the PKC $\gamma$  subset of excitatory interneurons is a target of the PV interneurons.

### **Nerve Injury Induces a Detachment of PV Interneuron Processes, which Disinhibits PKC $\gamma$ Interneurons**

We hypothesize that a decrease in the function of PV interneurons can lead to a disinhibition of the PKC $\gamma$  interneurons. To determine whether this reduction is caused by a detachment of PV nerve terminals from PKC $\gamma$  somata, we examined the connections between these two interneurons subtypes in naive and nerve-injured mice. Our data demonstrate that the mean number of PV appositions on PKC $\gamma$  somata is significantly lower in nerve-injured compared to naive mice (Figure 6A). There is a redistribution of the number of appositions on PKC $\gamma$  somata (Figures 6B and 6C), with the median shifting from 3.28 appositions in naive to 1.84 in nerve-injured mice.

We hypothesized that if this loss of PV interneurons inputs enables PKC $\gamma$  interneurons to drive mechanical allodynia after nerve injury, then blocking the activity of the PKC $\gamma$  interneurons should attenuate the allodynia. To test this possibility, we recorded the mechanical thresholds of naive and nerve-injured mice after intrathecal injection of the selective PKC $\gamma$  inhibitor  $\gamma$ V5-3. Figure 6D shows that blockade of PKC $\gamma$  has no effect in naive mice but significantly attenuates the mechanical allodynia in nerve-injured mice. In



related studies, we monitored stimulus-induced Fos expression in the superficial dorsal horn. In naive mice, gentle brushing of the hindpaw does not induce Fos expression in neurons of laminae I and III. However, the same innocuous stimulus induces Fos expression ipsilateral to a nerve injury; these mice were intrathecally injected with vehicle (TAT; Figures 6E and 6F). In contrast, pre-treatment with  $\gamma$ V5-3 significantly reduced the number of activated (Fos-positive) superficial laminae neurons (Figures 6E and 6F). The anti-allodynic effect of PKC $\gamma$  inhibition appears to be specific to mechanical inputs as testing  $\gamma$ V5-3 in an inflammatory pain model associated with mechanical allodynia and thermal hyperalgesia (Bonin and De Koninck, 2014) attenuated only mechanical allodynia (Figure S3).

### Selective Ablation of PV Interneurons in Naive Mice Induces Mechanical Allodynia

The loss of PV neuron-mediated inhibition of PKC $\gamma$  neuron activity is likely one of many events taking place in the dorsal horn following nerve injury. To examine the effects of this disinhibition in isolation from other mechanisms, we ablated PV interneurons in the dorsal horn of healthy, naive mice. We injected an AAV expressing Cre-dependent ribosome inactivating protein saporin (Ng et al., 2010) in the dorsal spinal cord of *PV::cre; tdTom* mice (Figure S4A). Saporin prevents protein synthesis, which leads to cell death (Stirpe et al., 1992). We validated the AAV-*Saporin* vector in vitro by infecting cultured DRG neurons obtained from a *TRPV1::cre; tdTom* mouse and recording neuronal death over time (Figures S4B and S4C). By 16 days post infection, we recorded a significant reduction in the number of tdTom-positive neurons infected with AAV-*Saporin* compared to control (culture media) ( $22.1 \pm 7.2$  versus  $70.6\% \pm 22.4\%$  of baseline in saporin and control, respectively; Figure S4B). The reduction was restricted to Cre-expressing neurons as the number of neurons immunostained with the neuronal marker  $\beta$ 3-tubulin, and tdTom-negative, did not significantly change after AAV infection (Figure S4C).

When we injected the AAV-*Saporin* into the dorsal horn of *PV::cre; tdTom* mice, we observed a significant decrease in the number of PV interneurons ipsilateral to the injection (ratio ipsi/contra:  $0.55 \pm 0.08$ ) 21 days post-infection. On the other hand, a GFP-expressing virus had no effect on PV interneurons number (ratio:  $0.98 \pm 0.08$ ; Figure 7A, right panel). No other visible neuronal loss was observed, as assessed by neuronal staining ( $215.1 \pm 1.1$  neurons ipsilateral to the saporin injection compared to  $211.2 \pm 0.6$  neurons contralateral to the saporin injection, Figure 7A). Because proprioceptor neurons also express PV (Zampieri et al., 2014), we also examined the ipsilateral and contralateral L3-L4 DRGs from the AAV-*Saporin*-treated mice. We found no loss of PV-positive DRG neurons after injection of saporin in the dorsal horn (Figures S5A and S5B), indicating that the loss of tdTom-positive neurons was restricted to the dorsal horn. Most importantly, the selective loss of PV interneurons was associated with a gradual development of mechanical allodynia ipsilateral to the injected side (Figure 7B), with no change in the sensitivity to thermal (heat) stimuli (Figure 7C). This modality-specific sensitization was reproduced in *PV::cre; tdTom* mice that had received an intra-spinal injection of a silencing DREADD (hM4D), which couples to inhibitory G proteins (Figure S5C). These results further confirm the modality-specific filtering function of PV interneurons.

## Mechanical Allodynia following PV Interneuron Ablation Occurs via a PKC $\gamma$ -Dependent Mechanism

Our findings indicate that the PKC $\gamma$  subset of excitatory inter-neurons is a target of PV interneurons. The kinase activity of PKC $\gamma$ , by enhancing excitatory (Lan et al., 2001; Lin et al., 2006) and reducing inhibitory (Yan and Surmeier, 1997; Deng et al., 2009) transmission, is critical to the development of mechanical allodynia (Malmberg et al., 1997; Li et al., 2005; Shumilla et al., 2005; Miraucourt et al., 2007). We therefore asked whether the mechanical allodynia observed after ablation of PV interneurons resulted from loss of inhibitory control of the PKC $\gamma$  interneurons. Potentially relevant to this scenario is the result of our immunofluorescence experiments indicating that the percentage of PKC $\gamma$  neurons without apposition from PV interneurons is significantly increased in the AAV-*Saporin*-injected mice compared to control or AAV-*GFP*-injected mice (Figure 7E). Additionally, the mean number of appositions is significantly reduced after ablation of PV neurons. (Figure 7D).

Taken together, these findings suggest that in comparison to control or AAV-*GFP*-injected mice, a greater number of PKC $\gamma$  interneurons are disinhibited in the AAV-*Saporin*-injected mice. We hypothesized that the loss of inhibitory control over PKC $\gamma$  interneuron activity would enable these neurons to transfer innocuous information to nociceptive circuits. Indeed, light brushing of the hindpaw ipsilateral to the site of saporin injection induced Fos expression in the superficial lamina of the dorsal horn. No induction was evoked in the superficial laminae of control or AAV-*GFP*-injected mice (Figure 7F). Finally, we speculated that if the mechanical allodynia observed in the AAV-*Saporin*-injected mice resulted from disinhibition of PKC $\gamma$  interneurons (Li et al., 2005; Shumilla et al., 2005), then blocking the activity of the PKC $\gamma$  interneurons should attenuate the allodynia. To test this possibility, we recorded mechanical thresholds 20–30 min after intrathecal injection of  $\gamma$ V5-3. Figure 7G illustrates that inhibition of PKC $\gamma$  activity, in fact, significantly reduced the mechanical allodynia produced in the saporin-injected mice (Figure 7G). In contrast, injection of TAT peptide alone did not attenuate the mechanical allodynia.

## DISCUSSION

Sensory inputs from the periphery are processed by a complex network of excitatory and inhibitory interneurons in the dorsal horn prior to transmission to projection neurons and from there to the brain (Figure 1). Our understanding of this processing is incomplete, due in part to the heterogeneity of the interneurons. For example, four non-overlapping subsets of inhibitory inter-neurons have been identified, based on their expression of the markers galanin, neuropeptide Y (NPY), neuronal nitric oxide synthase (nNOS), and PV (Tiong et al., 2011). Immunohistochemical experiments have established that noxious stimuli activate the NPY, nNOS, and galanin, subsets of interneurons, with the latter being also engaged by pruritic stimuli (Kardon et al., 2014). The PV neurons, however, appear not to be activated by noxious (and possibly not by pruritic) stimuli (Polgár et al., 2013a). Our results are the first to characterize the role of PV neurons to the processing of sensory inputs and thus advance our understanding of the organization of inhibitory circuits in the dorsal horn.



## Contribution of PV Interneurons to the Processing of Somatosensory Inputs

We used pharmacogenetic tools to control the activity of PV neurons in vivo. Our findings provide the first evidence of a highly selective functional contribution of the PV neurons to the processing of somatosensory information, and critical elements of the circuits that are engaged by these neurons. Activity of the PV neurons prevents innocuous mechanical inputs from activating nociceptive mechanical circuits but has no impact on the transmission of noxious thermal inputs, highlighting their function as modality-specific filters of sensory inputs. Increasing their activity reduces mechanical allodynia in a mouse model of neuropathic pain. The effect of PV neuron activation on mechanical sensitivity is more pronounced in nerve-injured mice compared to naive mice. This may be due to a near-saturation basal activity of PV neurons under control conditions, and a reduced activity after nerve injury. It follows that, in the setting of nerve injury, the net effect of activating PV interneurons with CNO would be more demonstrable. Thus, selectively increasing the activity of PV neurons may be beneficial in the treatment of neuropathic pain.

## Postsynaptic Targets of PV Interneurons

In the mouse dorsal horn, PV interneurons have an extensive dendritic arborization in the rostral-caudal axis (Hughes et al., 2012). Consistent with previous reports, we observed that PV axons are largely limited to lamina III, occasionally extending into lamina IIo, but they never reach lamina I. Furthermore, we found that PV interneurons form synapses onto PKC $\gamma$  excitatory interneurons, which populate lamina III and are implicated in mechanical allodynia (Malmberg et al., 1997; Miraucourt et al., 2007). The functional relevance of this synaptic connection remained unknown. We showed that selective ablation of PV neurons precipitated a mechanical allodynia comparable to that produced by nerve injury and that was correlated with a significant reduction in the number of appositions onto PKC $\gamma$  interneurons. The disinhibition of PKC $\gamma$  interneurons is central to the allodynia, as it was abolished by PKC $\gamma$  blockade. Taken together, our findings illustrate how a functional interaction between two distinct subsets of dorsal horn interneurons can influence the flow of non-nociceptive information to deeper laminae of the dorsal horn, while concurrently preventing access to pain transmission circuits in the superficial dorsal horn. It was recently reported that a newly identified subset of excitatory interneurons, located mainly in lamina III and expressing the vesicular glutamate transporter VGluT3, contribute to mechanical allodynia (Peirs et al., 2015). These neurons receive low-threshold mechanical inputs, and their activation with pharmacogenetic tools produces mechanical hypersensitivity (Peirs et al., 2015). Whether PV interneurons also target these VGluT3 excitatory interneurons remains to be determined.

## Contribution of PV Interneurons to Nerve-Injury-Induced Mechanical Allodynia

Although loss of glycinergic tone is reported to contribute to mechanical allodynia in neuropathic pain models (Lu et al., 2013; Miraucourt et al., 2007; Torsney and MacDermott, 2006; Foster et al., 2015), the mechanisms responsible for the decrease remain unclear. Previous studies reported that inhibitory interneurons undergo apoptotic cell death following nerve injury (Scholz et al., 2005). However, we found no change in the number of PV interneurons after nerve injury. On the other hand, the mean number of appositions from

PV nerve terminals onto PKC $\gamma$  neurons somata is significantly lower after nerve injury. Whether this detachment is enough to explain the mechanical allodynia is not known.

A decrease in excitatory inputs impinging on PV neurons can decrease their inhibitory output. Consistent with this scenario, Lu et al. (2013) showed that the amplitude of the A $\beta$  fiber-evoked excitatory post-synaptic potentials (EPSPs) in glycinergic neurons is significantly smaller in rats with nerve injury than in naive rats. Furthermore, Leitner and colleagues (2013) reported that peripheral nerve injury decreases the frequency of miniature EPSPs in inhibitory interneurons, with no visible change in spine density or morphology (Leitner et al., 2013). Changes in intrinsic membrane properties of PV neurons could decrease their inhibitory function. Parvalbumin neurons are characterized by a high-frequency firing rate and absence of spike frequency adaptation (Hughes et al., 2012, 2013), a process requiring the recruitment of small conductance, calcium-activated potassium (SK) channels (Bischof et al., 2012). By buffering the spike-mediated calcium influx and preventing the calcium from activating SK channels, PV could enable fast spiking (Franconville et al., 2011; Eggermann and Jonas, 2012). In contrast, a decrease in PV expression and a consequent buildup in intracellular calcium would reduce the fast spiking of these interneurons, thus reducing their inhibitory output. Either mechanism would lead to a reduced inhibitory tone on PKC $\gamma$  interneurons. In fact, Lu et al. demonstrated that peripheral nerve injury significantly reduces the amplitude of glycinergic IPSPs in PKC $\gamma$  interneurons compared to control rats, with a concomitant increase in transmission failure rate and a decrease in paired-pulse ratio, suggesting a presynaptic mechanism within the glycinergic neurons (Lu et al., 2013).

It was recently reported that dynorphin-expressing GABAergic interneurons, concentrated in lamina I and II, act as a gate to prevent innocuous and noxious inputs from activating somato-statin-expressing excitatory interneurons (Duan et al., 2014), located in lamina II. These inhibitory neurons contribute to the inhibition of itch signals from the periphery (Kardon et al., 2014). They receive mainly polysynaptic A $\beta$  inputs (Duan et al., 2014) as well as inputs from small-diameter afferents, evidenced by their expression of Fos in response to noxious heat, capsaicin, pinch, and formalin (Polgár et al., 2013b). Although the direct synaptic target of these dynorphin-neurons is unknown, they are linked to somatostatin interneurons. Given the morphology of these neurons (Duan et al., 2014) and their dorso-ventral location, they could be downstream targets of PKC $\gamma$  interneurons.

These putative circuits presumably occur upstream of the mechanism described by De Koninck and colleagues, which also contributes to decreased inhibition following peripheral nerve injury. These authors demonstrated that nerve injury triggers the synthesis and release of brain-derived neurotrophic factor (BDNF) from activated microglia. The latter binds its receptor on lamina I neurons, resulting in a downregulation of the expression of the potassium-chloride co-transporter KCC2. The result of this event is a shift in the chloride gradient of lamina I neurons, which reduces the hyperpolarization effect of inhibitory synapses (Cordero-Erausquin et al., 2005; Coull et al., 2003, 2005; Prescott et al., 2014).

In conclusion, we have identified a subset of inhibitory inter-neurons that normally prevents low threshold mechanoreceptive (tactile) inputs from engaging nociceptive circuits in the

dorsal horn of the spinal cord. In the absence of injury, these PV-containing inhibitory interneurons contact PKC $\gamma$ -expressing excitatory interneurons and prevent their activation by innocuous tactile stimuli. Importantly, our ablation data indicate that PV interneurons exert a tonic inhibitory control over their targets. By contrast, increasing the activity of PV interneurons alleviated the mechanical allodynia in a mouse model of neuropathic pain. Our results point to the PV interneurons as potential therapeutic targets for the treatment of mechanical allodynia following nerve injury.

## EXPERIMENTAL PROCEDURES

### Mouse Strains

All mouse strains used have previously been described. *PV::cre*, GFP, and tdTomato reporter mice were obtained from Jackson Labs (stock numbers 008069, 007906, and 007914, respectively). *TRPV1::cre* mice were made by A.I.B. (Cavanaugh et al., 2011b).

### Immunohistochemistry

Immunohistochemistry on tissue sections and cell cultures were performed using standard methods (see the Supplemental Experimental Procedures for details).

### Behavioral Assays

Experiments were conducted at room temperature. Mice were acclimatized to their testing environment prior to all experiments. Experimenters were blind to the genotype during testing. All experiments were performed on male 8- to 14-week-old mice, according to the Guidelines of the Animal Care Committee of McGill University (see the Supplemental Experimental Procedures for details).

See the Supplemental Experimental Procedures for details on virus injection procedures, detection of appositions, and electron microscopy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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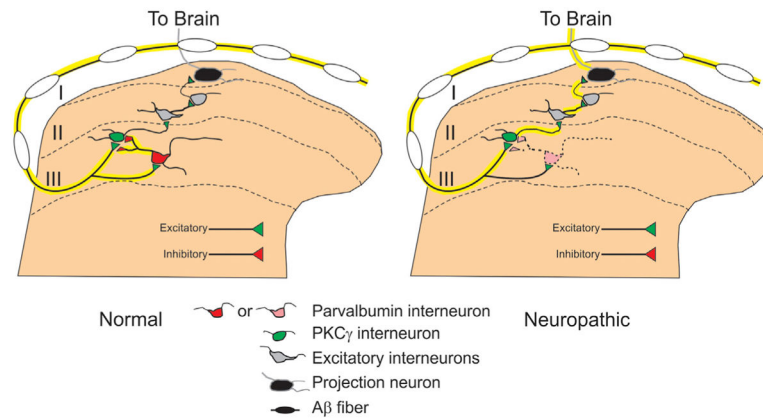
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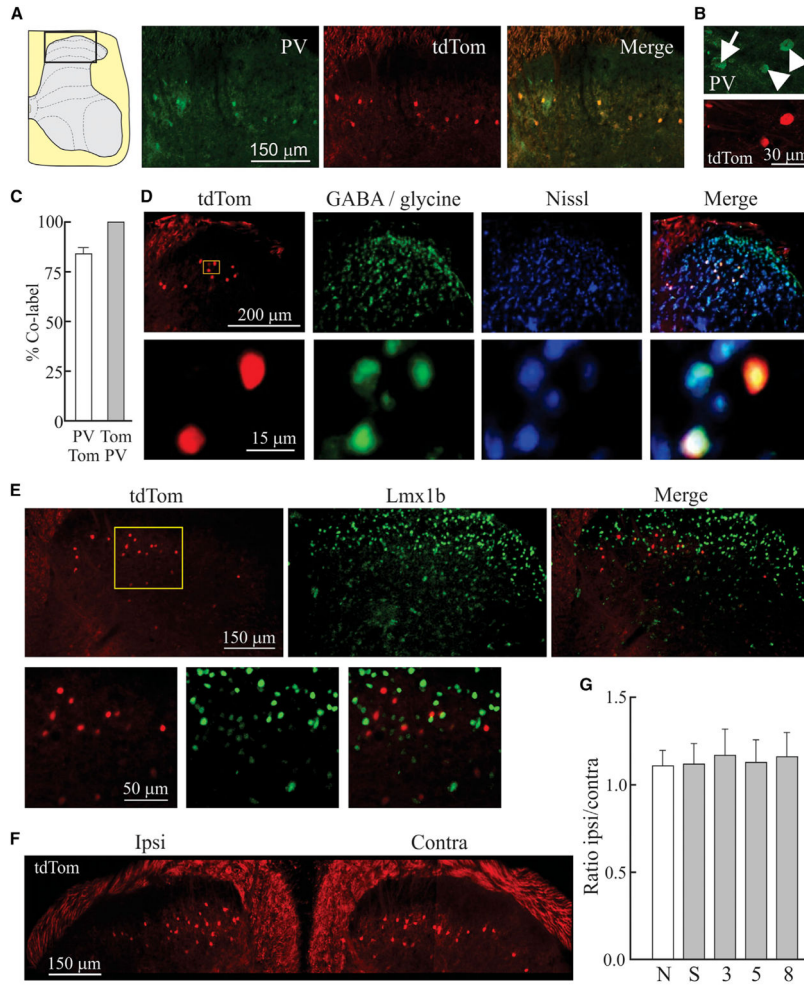
**Highlights**

- Parvalbumin (PV) neurons of the dorsal horn synapse onto PKC $\gamma$  excitatory neurons
- After nerve injury, many PV-PKC $\gamma$  synapses are lost and touch stimuli become painful
- Specific activation of PV neurons after nerve injury alleviates mechanical pain

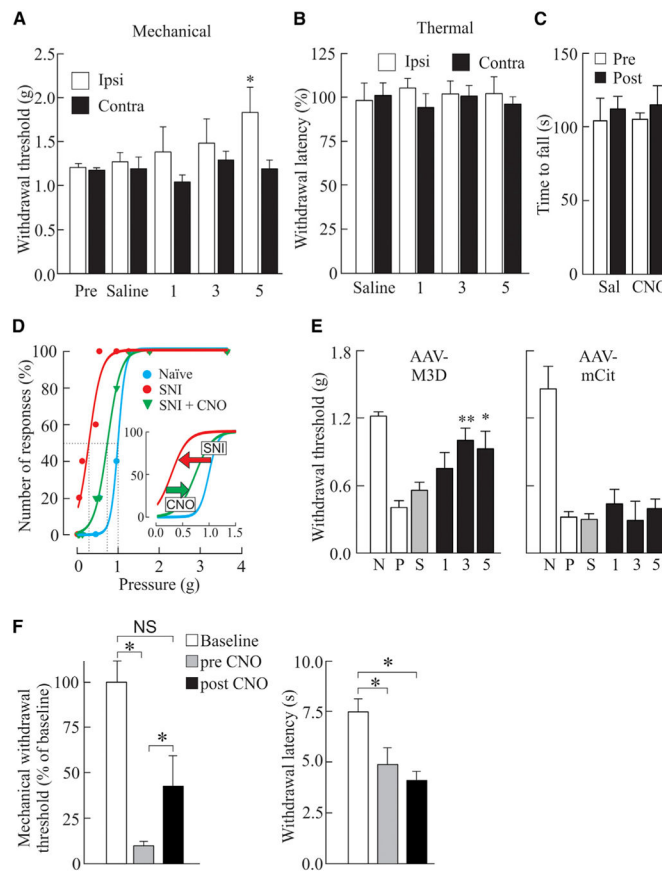


**Figure 1. Spinal Lamina I Output Neurons Do Not Receive Direct Input from Low-Threshold Aβ Primary Afferents**

Yet a polysynaptic pathway links myelinated Aβ fibers to lamina I neurons (Torsney and MacDermott, 2006). Under normal conditions (left panel), the link is repressed by inhibitory glycinergic interneurons (red, left panel). After nerve injury (right panel), impaired inhibition unmasks the connection, thus enabling low-threshold inputs to drive lamina I projection neurons. Yellow highlighting indicates flow of information. Adapted from Prescott et al. (2014).



**Figure 2. *PV::cre; tdTomato* Transgenic Mice Recapitulate Endogenous PV Expression**  
 (A) Transgenic *PV::cre; tdTomato* mice express tdTom (red) exclusively in PV (green)-expressing interneurons. Scale bar, 150  $\mu$ m.  
 (B and C) The vast majority of PV-immunoreactive (IR) neurons (~84%; 142 out of 168 neurons, n = 3) express tdTom, and all tdTom<sup>+</sup> neurons (142/142) express PV. Scale bar, 30  $\mu$ m.  
 (D) Top panels: transverse section of the dorsal horn stained with GABA and glycine antibodies (green) and Nissl (blue). Bottom panels: magnification of top panels. The majority of TdTom-positive PV neurons (~95%; 419 of 439 neurons, n = 5 mice) express GABA and/or glycine.  
 (E) Top panels: transverse section of the dorsal horn stained with an Lmx1b antibody (green). Bottom panels: magnification of top panels. The majority of PV neurons are Lmx1b negative (62 of 65 neurons, n = 2 mice).  
 (F) Composite image showing the presence of PV neurons 8 weeks after SNI. Scale bar, 150  $\mu$ m.  
 (G) Number of PV interneurons (ratio of ipsi/contra) at different time points after nerve injury. N, naive (n = 5); S, sham-operated (n = 5); 3, 5, and 8, the number of weeks post nerve injury, with n = 5, 7, and 8, respectively.



### Figure 3. Activation of PV Interneurons Elevates Mechanical Thresholds without Affecting Thermal Sensitivity in Awake Naive and Neuropathic Mice

(A) Activation of PV neurons in naive mice (CNO 5 mg/kg; i.p) significantly increases mechanical withdrawal thresholds ( $n = 12$ ) ipsilateral to the AAV-M3D injection side. Lower doses of CNO had no effect. \*Significant difference from the saline-injected group.

(B) Activation of PV neurons has no effect on thermal withdrawal latency ( $n = 12$ ).

(C) CNO-injected mice perform as well as saline-injected mice in the rotarod test ( $n = 3$ ), indicating that 5 mg/kg CNO does not cause any motor impairment.

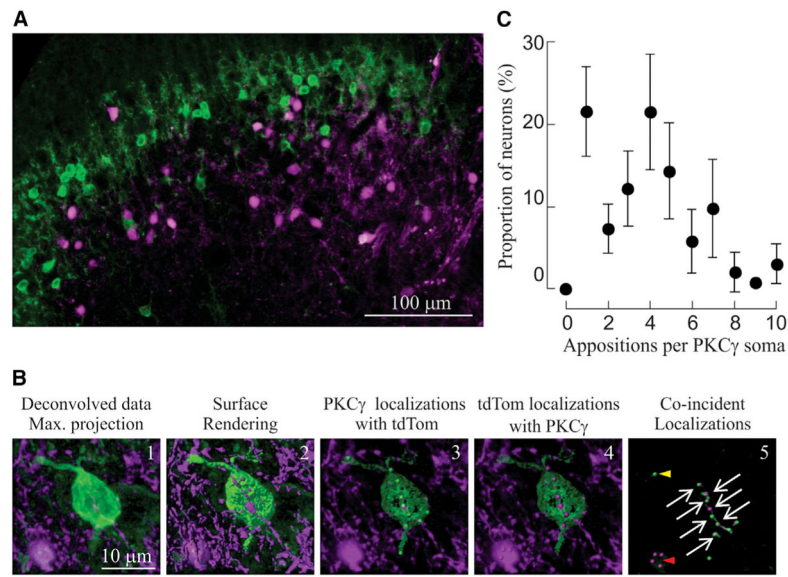
(D) Peripheral nerve injury (SNI) causes a leftward shift in the behavioral responses to mechanical stimulation (from blue to red curve), indicating mechanical allodynia (see inset). Activation of PV neurons (CNO 3 mg/kg) 20 min prior to testing attenuates the allodynia (green arrow in inset).

(E) Injection of CNO in nerve-injured *PV::cre; tdTomato* mice infected with AAV-M3D significantly attenuates the mechanical allodynia (left panel). No anti-allodynic effects were observed with a control AAV (*mCit* alone). Mechanical thresholds were recorded before and after systemic administration of CNO. N, naive; p, nerve-injured mice, baseline; S, saline injected, nerve-injured mice; 1, 3, and 5, doses of CNO in mg/kg ( $n = 8-10$  mice per group). \*, \*\*Significant difference from the saline-injected group.

(F) Activation of PV interneurons modulates mechanical thresholds without affecting thermal sensitivity in an inflammatory pain model. Subcutaneous injection of capsaicin into the heel of the hindpaw induces a significant reduction of mechanical threshold (left panel)

( $9.4\% \pm 2.3\%$ ,  $n = 4$ , gray bar) that is attenuated following administration of CNO (5 mg/kg; i.p.) in *AAV-M3D*-injected *PV::cre* mice ( $42.2\% \pm 16.7\%$ ,  $n = 4$ , black bar) ipsilateral to the AAV injection side. The capsaicin injection also produces a significant reduction of thermal withdrawal thresholds (right panel) ( $4.86 \pm 0.71$  sec,  $n = 4$ , gray bar) compared to baseline values ( $7.41 \pm 0.61$  sec,  $n = 4$ , white bar). CNO administration had no effect on thermal withdrawal thresholds, demonstrating the modality-specific PV interneuron-mediated inhibition in this model ( $4.09 \pm 0.39$  sec,  $n = 4$ , gray bar). \*Significant difference between groups compared, following a Kruskal-Wallis ANOVA on ranks with multiple comparisons (Student-Newman-Keuls Method). NS, non-significant; i.p., intraperitoneal.



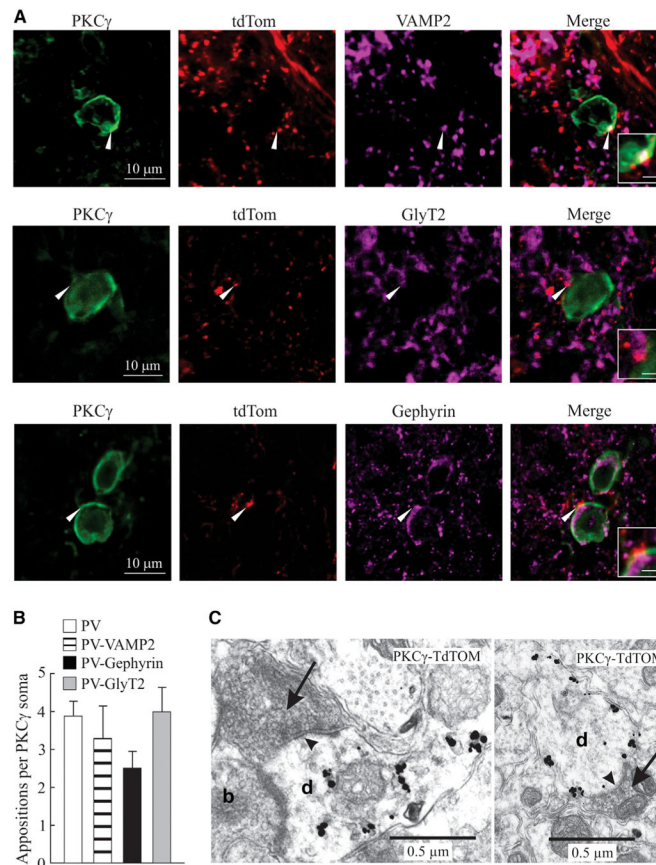


**Figure 4. Appositions between PV<sup>+</sup> Terminals and Lamina III PKC $\gamma$ -Expressing Inter-neurons**

(A) tdTom<sup>+</sup> interneurons (magenta) are located in the vicinity of excitatory PKC $\gamma$  (green) interneurons.

(B) Panels: (1) Maximal projection of a deconvolved z stack of confocal images taken at the 0.1- $\mu$ m interval and then processed through the Imaris software to define surface rendering (2) of the tdTom profiles surrounding the PKC $\gamma$  soma. (3) Areas of tdTom signals on the PKC $\gamma$  surface and of PKC $\gamma$  signal on tdTom surface (4). The “spot co-localization” feature identifies areas positive for both PKC $\gamma$  and tdTom. This process defines the number of appositions of tdTom terminals on PKC $\gamma$  soma, as co-incident localizations (white arrows), and excludes unspecific detections, as PKC $\gamma$  spots distant from tdTom processes (yellow arrowhead) or tdTom spots distant from the PKC $\gamma$  soma (red arrowhead). Scale bar, 10  $\mu$ m.

(C) Mean ( $\pm$ SEM) proportion (%) of PKC $\gamma$  somata in relation to the number of PV appositions (n = 64 somata from six mice).

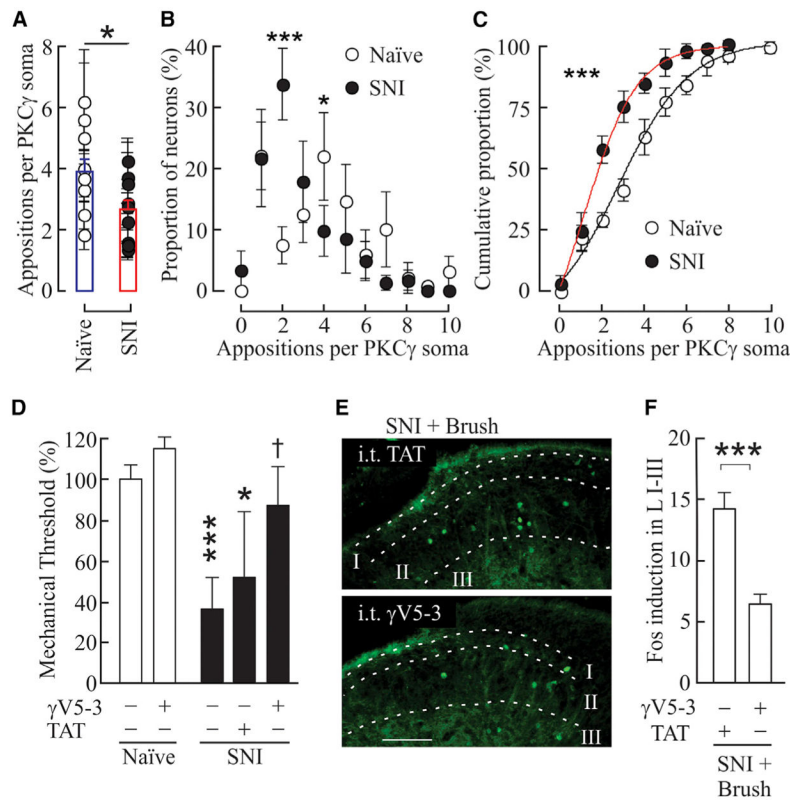


**Figure 5. Inhibitory Nature of the Synaptic Connection between PV and PKC $\gamma$  Inter-neurons**

(A) Confocal picture from the lamina Iii of the dorsal horn displaying PKC $\gamma$  neurons (green), PV neurons' processes (red), and in magenta: VAMP2 (top panel), GlyT2 (middle panel), or gephyrin (bottom panel). Arrowheads in top and middle panel, colocalization of the tdTom and VAMP2 or GlyT2 signals. Bottom panel, apposition of the TdTom and gephyrin signals. Scale bar of insets, 2  $\mu$ m.

(B) Mean ( $\pm$ SEM) appositions per PKC $\gamma$  neuron soma is not significantly different (Kruskal-Wallis test) in tdTom<sup>+</sup> alone ( $3.87 \pm 0.41$ ; white bar; n = 64 somata, six mice) compared to tdTom<sup>+</sup> colocalized with VAMP2 ( $3.31 \pm 0.86$ ; hatched bar; n = 10 somata, two mice) or tdTom<sup>+</sup> colocalized with GlyT2 ( $4.03 \pm 0.64$ ; gray bar; n = 13 somata, three mice) or tdTom<sup>+</sup> facing gephyrin clusters ( $2.51 \pm 0.45$ ; black bar; n = 10 somata, two mice).

(C) Electron micrographs of lamina Iii of the dorsal horn of a PV::cre; tdTomato mouse showing the association between PKC $\gamma$ -IR dendrites (d; note silver-gold particles) and tdTom-IR boutons (note dense precipitate) indicated by arrows; note the presence a symmetric synapse (arrowheads) between the tdTom-IR boutons and the PKC $\gamma$ -IR dendrites (d); b: unlabeled bouton.



**Figure 6. Nerve Injury Induces a Detachment of PV-PKC $\gamma$  Appositions that Disinhibits PKC $\gamma$  Interneurons**

(A) The mean ( $\pm$ SEM) number of somatic appositions is significantly lower in nerve-injured (SNI) compared to naive mice ( $3.87 \pm 0.41$  in naive mice;  $n = 64$  somata from six mice versus  $2.63 \pm 0.31$  in nerve-injured mice;  $n = 54$  somata from three mice;  $*p = 0.037$ ; t test for unpaired data).

(B) Mean ( $\pm$ SEM) proportion (%) of PKC $\gamma$  somata in relation to the number of PV appositions. ( $*p < 0.05$ ,  $***p < 0.001$ ; test of independence in contingency table, Fisher's exact probability).

(C) Conversion of data in (B) into cumulative proportion demonstrates a leftward shift of the fit after nerve injury (SNI) ( $***p < 0.001$ , Kolmogorov and Smirnov test).

(D) Inhibition of PKC $\gamma$  activity reduces mechanical allodynia produced after SNI. Naive mice were tested before (double minus) and 20–30 min after intrathecal (i.t.) injection of 100 pmol of  $\gamma$ V5-3. The PKC $\gamma$  inhibitor and its control (TAT peptide) were then tested on nerve-injured mice.  $*$ ,  $***$ Significant difference compared to naive baseline ( $n = 8$ ).

$\dagger$ Significant effect of  $\gamma$ V5-3 treatment compared to SNI baseline ( $n = 7$ ), Wilcoxon signed-rank test for paired data.

(E) Representative images of brush-induced Fos expression in the dorsal horn of a nerve-injured mouse pretreated i.t. with TAT (upper panel) or  $\gamma$ V5-3 (lower panel). Scale bar, 100  $\mu$ m; roman numbers indicate lamina.

(F) Mean ( $\pm$ SEM) number of Fos-IR neurons per 25- $\mu$ m section. In nerve-injured mice pretreated i.t. with TAT ( $n = 3$ ), gentle brushing evoked Fos induction in neurons of laminae I–III ipsilateral to the nerve injury. In nerve-injured mice pretreated with  $\gamma$ V5-3, brush-

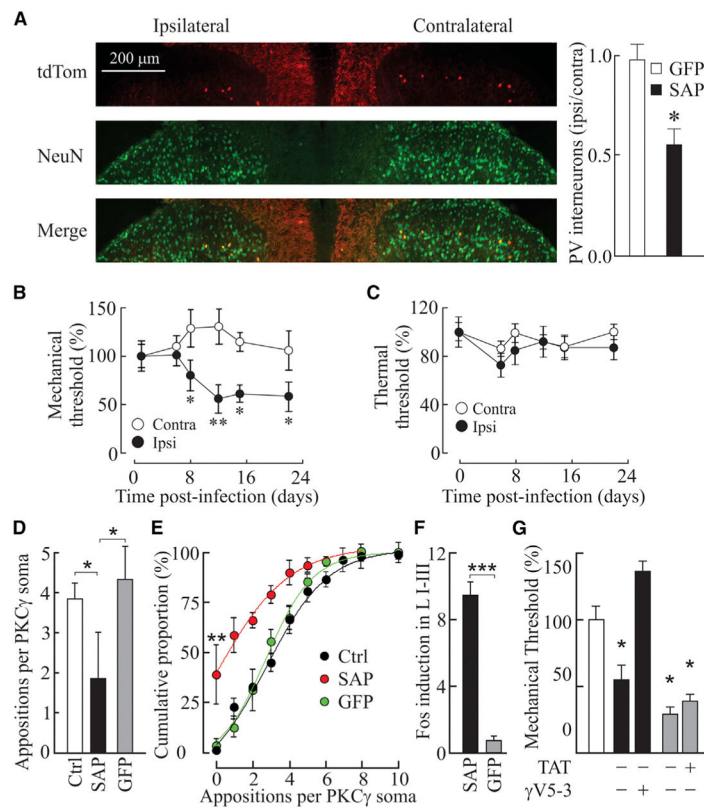
evoked FOS induction is significantly lower ( $n = 3$ ). \*\*\*Significant difference between the TAT and the  $\gamma$ V5-3 groups. Mann-Whitney test.

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**Figure 7. Saporin-Mediated Ablation of PV Neurons Induces a PKC $\gamma$ -Dependent Mechanical Allodynia**

(A) Composite image of a dorsal horn section of a PV::cre; tdTomato mouse injected with AAV-*Saporin* (SAP). There is a decrease in the number of tdTom<sup>+</sup> neurons on the ipsilateral side (top-left panel). No visible decrease in neuronal density is observed by neuronal staining (left, middle panel). Control mice were injected with a virus (same titer) expressing GFP (n = 4). The number of tdTom<sup>+</sup> neurons was counted on the ipsilateral and contralateral sides, and the ratio ( $\pm$ SEM) of ipsi/contra is plotted (right panel). \*p < 0.05, Student's t test.

(B) Ablation of PV neurons triggers mechanical hypersensitivity ipsilateral (black circles) to the AAV-*Saporin* injection side. \*Significant difference from contralateral side (white circles, n = 9).

(C) Ablation of PV neurons has no effect on paw withdrawal latencies in response to noxious thermal stimuli.

(D) The mean ( $\pm$ SEM) number of tdTom<sup>+</sup> processes apposing PKC $\gamma$  somata is significantly lower in AAV-*Saporin*-injected mice ( $1.87 \pm 1.18$ ; n = 38 somata from five mice) than in control mice ( $3.87 \pm 0.41$ ; n = 64 somata from six mice) or in AAV-GFP-injected mice ( $4.36 \pm 0.84$ ; n = 30 somata from three mice). ANOVA (p = 0.013) with Tukey, all pairwise multiple comparison (\*p < 0.05).

(E) The cumulative proportion of PKC $\gamma$  in-terneurons with varying numbers of PV appositions is significantly shifted to the left in AAV-*Saporin*-injected mice (p = 4.23 E-5 VS. AAV-GFP-injected mice; Kolmogorov-Smirnov test). The proportion of PKC $\gamma$  somata without tdTom<sup>+</sup> appositions is significantly higher in AAV-*Saporin*-injected mice ( $38.92\% \pm 14.6\%$ ; n = 38 somata; five mice) than in control mice (0%; n = 64 somata; six mice) or in

AAV-GFP-injected mice ( $6.36\% \pm 3.2\%$ ;  $n = 30$  somata; three mice).  $**p < 0.01$  AAV-*Saporin*- versus AAV-GFP-injected group. Test of independence in contingency table, Fisher's exact probability.

(F) Mean ( $\pm$ SEM) number of Fos-IR neurons in laminae I–III per 25- $\mu$ m-thick spinal cord section. A gentle brushing of the hindpaw induces Fos expression ipsilateral to AAV-*Saporin* injection ( $9.44 \pm 0.68$  neurons;  $n = 6$ ). It is not the case in AAV-GFP-injected mice ( $0.75 \pm 0.25$  neurons;  $n = 3$ ).  $***p < 0.001$  AAV-*Saporin*-versus AAV-GFP-injected group, Mann-Whitney test.

(G) Mean ( $\pm$ SEM) mechanical withdrawal threshold, expressed as percentage of baseline (white bar). Mice were tested before and 30 min after i.t. injection of 100 pmol of either  $\gamma$ V5-3 ( $n = 6$ ) or the TAT peptide ( $n = 3$ ).  $*p < 0.05$ ; Friedman repeated-measures ANOVA on ranks with Tukey's post hoc test.