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## Synaptic pathways and inhibitory gates in the spinal cord dorsal horn

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### Abstract

Disinhibition in the dorsal horn accompanies peripheral nerve injury and causes the development of hypersensitivity to mild stimuli. This demonstrates the critical importance of inhibition in the dorsal horn for maintaining normal sensory signaling. Here we show that disinhibition induces a novel polysynaptic low-threshold input onto lamina I output neurons, suggesting that inhibition normally suppresses a preexisting pathway that probably contribute to abnormal pain sensations such as allodynia. In addition, we show that a significant proportion of superficial dorsal horn inhibitory neurons are activated by low-threshold input. These neurons are well situated to contribute to suppressing low-threshold activation of pain output neurons in lamina I. We further discuss several aspects of inhibition in the dorsal horn that might contribute to suppressing pathological signaling.

### Keywords

inhibition; pain; tonic inhibition; dorsal horn

### Introduction

The spinal cord dorsal horn is a key region in the central nervous system (CNS) in which sensory information is received, integrated, and relayed to higher brain structures. In fact, the dorsal horn receives inputs from a wide variety of primary afferent fibers including nociceptors, chemoreceptors, and thermoreceptors that respond to stimuli from the skin, muscles, joints, and viscera. The patterns of termination of primary afferents within the spinal cord are related to axonal diameter, receptive field, and sensory modality. Most nociceptive primary afferents are small-diameter thinly myelinated A $\delta$  or unmyelinated C fibers. These terminate primarily in the superficial part of the dorsal horn, specifically in lamina I and II. Dorsal horn neurons in lamina III/IV receive low-threshold inputs from large-diameter, more heavily myelinated A $\beta$  fibers of mechanoreceptors. Thus roughly speaking, touch sensitive fibers terminate deeper and nociceptive fibers terminate more superficially within the dorsal horn.

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### Conflicts of interest

The authors declare no conflicts of interest.

The dorsal horn neurons themselves are a mixture of neuron types. Nociceptive projection neurons are found in lamina I. Excitatory and inhibitory interneurons are found throughout lamina I, II, and III/IV. Some indications are beginning to emerge that there are detectable activity patterns in the dorsal horn indicating a dorsally directed flow of information. For example, a recent study used laser-scanning photostimulation to uncage glutamate to activate individual presynaptic dorsal horn neurons while recording postsynaptic responses. Excitatory synaptic drive was observed to move ventral to dorsal across lamina II toward lamina I through neurons with long ventral dendrites called vertical neurons.<sup>1</sup> This pattern suggested the idea that there are polysynaptic circuits that drive up toward the output neurons in lamina I.<sup>1</sup> The presence of such polysynaptic, ventral to dorsal circuits has been demonstrated under conditions of disinhibition<sup>2-4</sup> and is believed to be associated with behavioral allodynia.<sup>3,5</sup> Details of actual circuitries within lamina I and II, however, mostly remain obscure. Similarly, the general issue of how sensory modalities are processed properly within dorsal horn is still unclear. In this paper, we discuss the role of inhibition in dorsal horn for maintaining separation of sensory modalities such as touch and pain.

## Results and discussion

### Disinhibition unmasks low-threshold input to pain output neurons

Chronic neuropathic pain often develops following peripheral nerve injury. One manifestation of that pain is allodynia, a painful response to a normally nonpainful stimulus. Behavioral studies have demonstrated that pharmacological disruption of dorsal horn inhibition *in vivo* using intrathecal antagonists for GABA<sub>A</sub> or glycine receptors transiently causes allodynia.<sup>6-8</sup> This suggests that inhibition is a critical element in maintaining separation between touch sensitive afferent input and projection neurons in lamina I that normally transmits information about noxious stimuli to higher centers in the CNS. Evidence that peripheral nerve injury causes a disruption of inhibition was subsequently demonstrated and shown to be, at least in part, due to a loss of Cl<sup>-</sup> gradient in lamina I neurons associated with accumulation of activated microglia.<sup>9,10</sup> Effective inhibition mediated by both GABA<sub>A</sub> and glycine receptors depends on a strong Cl<sup>-</sup> gradient and thus loss of the gradient diminishes inhibition.

Similarly, *in vitro* experiments have shown a strong impact of dorsal horn inhibition on excitatory synaptic drive from low-threshold peripheral fibers onto neurons in lamina I and II, a region where high-threshold fibers normally dominate. Disinhibition *in vitro* strongly enhances low-threshold activation of lamina II neurons,<sup>11</sup> demonstrating the importance of local inhibition for suppressing low-threshold drive. More recently, we have specifically tested whether pharmacological disinhibition is able to allow strong, low-threshold drive of lamina I output neurons.<sup>4</sup>

We took advantage of the fact that many projection neurons in lamina I express receptors for substance P, NK1 receptors.<sup>12</sup> Spinal cord slices were incubated with tetramethylrhodamine-conjugated substance P (TMR-SP) to label NK1 receptor positive (NK1R<sup>+</sup>) neurons before recording dorsal-root evoked excitatory postsynaptic currents (EPSCs). Under control conditions, lamina I NK1R<sup>+</sup> neurons were shown to receive predominantly high-threshold (A $\delta$ /C fiber) monosynaptic input. In the example shown in Figure 1A (left column), the NK1R<sup>+</sup> neuron received polysynaptic A $\delta$  fiber and monosynaptic C fiber input.<sup>4</sup> However, blockade of local GABAergic and glycinergic inhibition with bicuculline (10  $\mu$ M) and strychnine (300 nM) revealed significant A fiber input to the lamina I NK1R<sup>+</sup> neuron that was predominantly A $\beta$  fiber mediated (Fig. 1A, middle column). The total integrated current of EPSCs evoked using A $\beta$  (25  $\mu$ A) and A $\delta$  (100  $\mu$ A), but not C fiber (500  $\mu$ A) stimulation intensities were significantly increased after disinhibition (Fig. 1B). This novel A $\beta$  fiber input was identified as polysynaptic in nature because the input showed both failures and

substantial variability of latency when recorded at high-stimulation frequencies (not shown). These results suggest the presence of an excitatory, polysynaptic pathway between low-threshold afferents and nociceptive NK1R<sup>+</sup> projection neurons that is normally suppressed by inhibition.

The polysynaptic excitatory pathway revealed by disinhibition was critically dependent upon NMDA receptor activation as shown in Figure 1A (right column). All of the novel polysynaptic activity that was activated in the presence of bicuculline and strychnine was inhibited by the NMDA selective receptor antagonist, APV (30  $\mu$ M). In the presence of APV, the synaptic activity observed in the absence of bicuculline and strychnine are still apparent even though the newly revealed polysynaptic activity is blocked. These data indicate that disinhibition enhances the contribution of NMDA receptor activation to the polysynaptic low-threshold drive.

### Subset of inhibitory neurons in dorsal horn receive low-threshold input

Pharmacological disinhibition, mimicking disinhibition associated with nerve injury, allowed us to observe an underlying polysynaptic excitatory pathway in the dorsal horn between low-threshold afferents and lamina I output neurons. This raises the question of how inhibition suppresses this pathway under normal, nonpathological, or nonpharmacologically altered conditions. It is known that about 30% of the neurons in the superficial dorsal horn are immunoreactive for GABA, and glycine coexists in a subpopulation of these neurons, supporting the idea that inhibitory neurons have an important role of in local network activity.<sup>13</sup> Normal inhibition of the polysynaptic excitatory pathway acts as a gate, preventing the painful consequences. One way to control such a gate would be to simultaneously have low-threshold synaptic drive of inhibitory neurons that could suppress the existing polysynaptic excitatory pathway between low threshold afferents and NK1R<sup>+</sup> projection neurons.

To test for low-threshold synaptic excitation of lamina I and II inhibitory neurons, we used homozygotic transgenic mice that express EGFP under control of the *gad1* gene promoter to identify glutamic acid decarboxylase 67 (GAD67) GABAergic neurons. This results in fluorescent labeling of 30–70% of the GABAergic neurons in the dorsal horn.<sup>14–16</sup> Recording from these neurons and stimulating the dorsal root to activate low- and high-threshold sensory afferent fibers allowed us to investigate the excitatory synaptic inputs onto GABAergic inhibitory neurons.<sup>17</sup> A subclass of EGFP positive GABAergic neurons had low threshold, A $\beta$  fiber input as well as input from high-threshold fibers (A $\delta$  and/or C). For example, the data shown in Figure 2A were recorded from a neuron that received A $\beta$  fiber input identified as polysynaptic due to synaptic failures seen at high-frequency (20 Hz) stimulation (arrows). In addition, this neuron received both monosynaptic (arrowheads) and polysynaptic (open arrowheads) C fiber input.<sup>17</sup> Indeed, A $\beta$  fibers activate a significant proportion (~20%) of lamina I and II GABAergic neurons (Fig. 2B). This occurs with similar excitatory synaptic drive throughout postnatal maturation, but with a greater prevalence at younger ages.<sup>17</sup>

These GABAergic neurons receiving low-threshold, primary afferent, synaptic drive are well suited to contribute to suppressing low-threshold activation of output projection neurons. Low-threshold activation of inhibitory neurons is also consistent with the classical idea that nociceptive projection neurons are activated mainly by noxious afferent input that “opens” the gate while low-threshold, non-noxious fibers inhibit this signal and “close” the gate.<sup>18</sup> However, nearly all of the inhibitory neurons tested in our studies that received low-threshold input also received high-threshold excitatory drive (Fig. 2B), a combination that is not predicted by the gate theory of pain. It may be that more extensive information about

local circuitry considered together with sensory modality will be required to understand the impact these inhibitory neurons have on pain detection.

### **Glycinergic inhibition at the gate of polysynaptic pathway causing mechanical allodynia**

Recent evidence has been accumulating regarding a similar but different polysynaptic pathway that subserves a specific type of allodynia, dynamic mechanical allodynia. This pathway begins in inner lamina II (lamina Ii). In this region of the dorsal horn, there is a population of excitatory interneurons that express the enzyme, PKC $\gamma$  and that receive low-threshold afferent input.<sup>19</sup> These PKC $\gamma^+$  positive (PKC $\gamma^+$ ) neurons are a key element for activated circuits after disinhibition by intrathecal application of glycine receptor antagonist, unmasking normally blocked local excitatory circuits onto nociceptive output neurons.<sup>2,3</sup> However, this circuit does not involve lamina I NK1R $^+$  neurons.<sup>3</sup> This evidence suggests that dynamic mechanical allodynia is transmitted through a specific pathway that involves nonnociceptive myelinated primary afferents, PKC $\gamma^+$  neurons as a gateway, and NK1R negative (NK1R $^-$ ) lamina I projection neurons, as shown in Figure 3. Thus, glycinergic inhibition appears to be a key player to prevent dynamic mechanical allodynia.

What is the source of this glycinergic inhibition? A significant proportion of superficial dorsal horn neurons have immunoreactivity for glycine. In addition, a recent study reported that presynaptic inhibitory neurons, which send inhibitory output onto postsynaptic lamina I–III neurons, are located relatively close to postsynaptic neurons along the dorso-ventral axis, suggesting local inhibitory synaptic connections are mostly made within the same lamina.<sup>1</sup> Therefore, we predict that glycinergic inhibitory neurons located near the lamina II/III border may directly inhibit PKC $\gamma^+$  neurons to close the gate by blocking the pathway activated for dynamic mechanical allodynia. Figure 3 shows a schematic diagram for putative neuron networks causing mechanical allodynia and suppressing it under normal condition (Fig. 3).

### **Region- and cell type-specific inhibitory control of superficial dorsal horn neurons**

Given that the distinct physiological roles of different dorsal horn neurons depend on their location and their neurochemical properties, it is important to determine whether, in fact, dorsal horn neurons receive synaptic inhibition in a region- and/or cell type-specific manner. It has been shown that adult lamina I neurons receive only glycinergic synaptic inhibition, whereas about a half of lamina II neurons received pure GABAergic and the rest received mixed GABAergic and glycinergic inhibition.<sup>20</sup> In addition, other studies have focused on cell type-specific inhibition in the dorsal horn. For example, central cells, which are commonly situated in the mid-zone of the lamina II, receive GABAergic inhibition from islet cells.<sup>21</sup> Moreover, inhibitory synaptic connections have been demonstrated between lamina II inhibitory interneurons identified by endogenous EGFP under the control of the GAD65 promoter.<sup>22</sup> However, what drives the activity of identified neurons with specific physiological roles in the dorsal horn has still not been well studied. We expect that genetic and/or neurochemical approaches to neuron identification will facilitate investigations of cell type-specific inhibitory control of dorsal horn neurons. This in turn would provide insight into sensory information processing within spinal cord dorsal horn. Interestingly, some lamina II neurons receive tonic GABAergic and glycinergic inhibition, suggesting that superficial dorsal horn neurons are inhibited through two different modes, phasic, and tonic, similar to other CNS regions.<sup>23–25</sup> We predict that tonic inhibition may be important in regulating the inhibitory tone in the dorsal horn.

## Conclusions

There are several polysynaptic excitatory circuits in the dorsal horn that carry low threshold signals to lamina I output neurons. The inhibition of these pathways is critically important to avoid pathological pain. This focuses attention on the mechanisms by which inhibition functions within the dorsal horn.

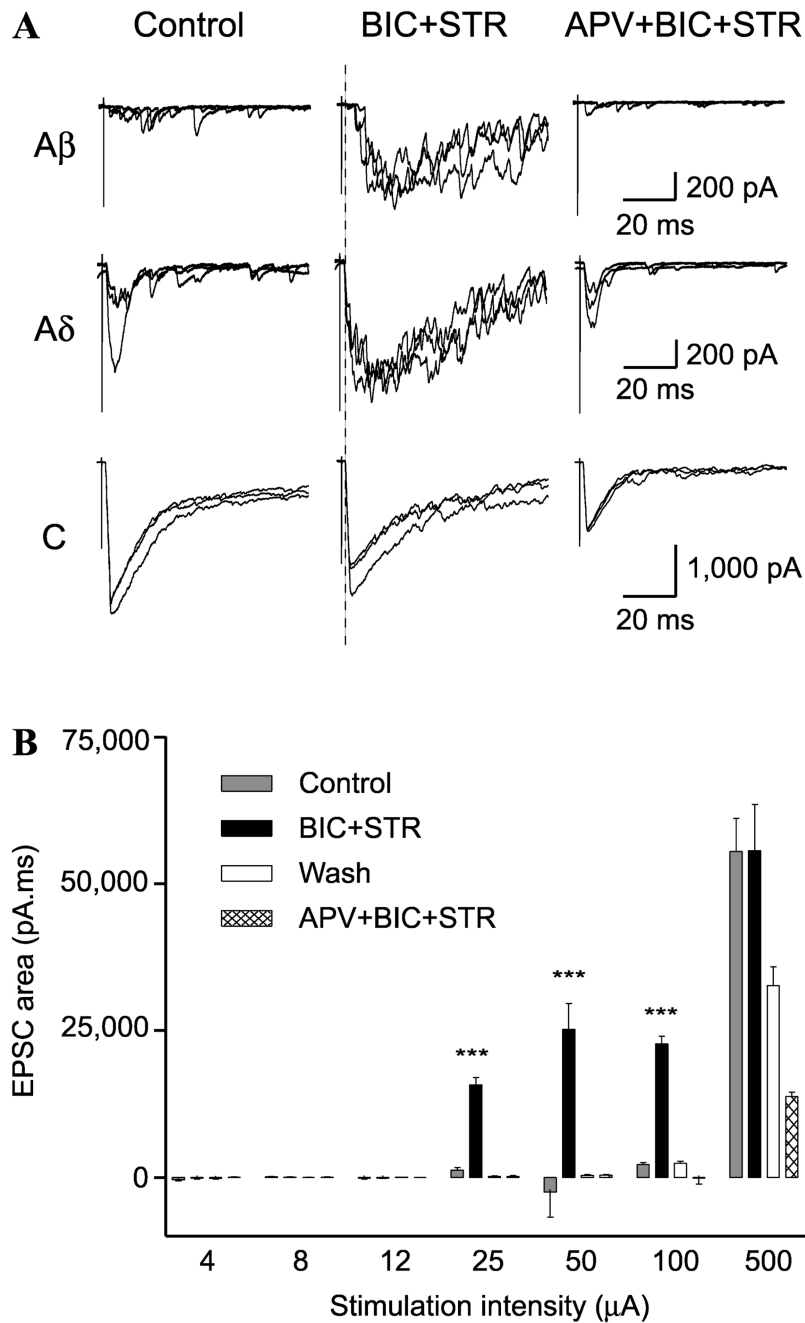
## Acknowledgments

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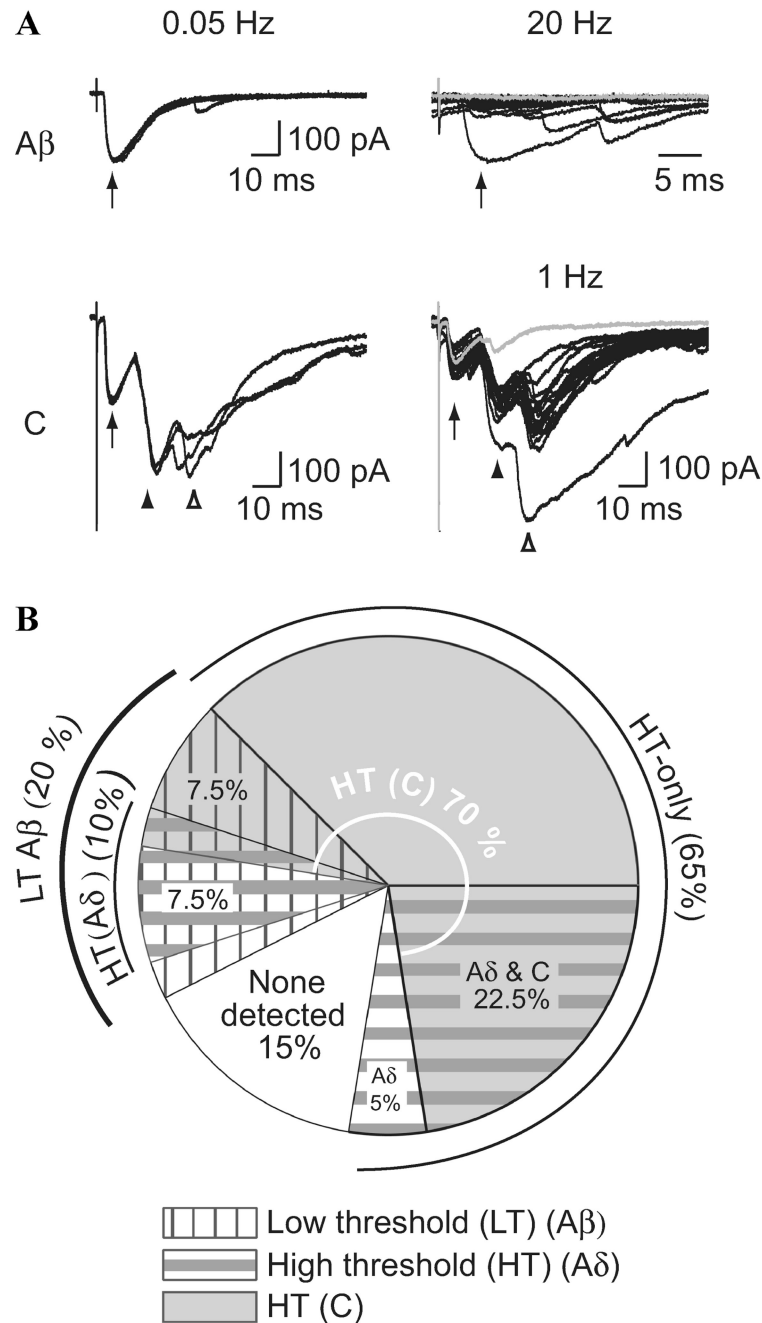
**Figure 1.**

Disinhibition reveals polysynaptic A $\beta$  fiber input to lamina I NK1R<sup>+</sup> neurons.<sup>4</sup> (A, B) Show data from a neuron with C fiber monosynaptic input that has polysynaptic A $\beta$  fiber input revealed during disinhibition. (A, left column) Example of EPSCs evoked by stimulation (0.1 ms) using A $\beta$  (25  $\mu$ A), A $\delta$  (100  $\mu$ A), and C fiber (500  $\mu$ A) stimulation intensities at low frequency under control conditions. Each trace comprises three superimposed traces evoked at 0.05 Hz. (Middle column) EPSCs evoked by the same stimulation protocol but in the presence of bicuculline (BIC; 10  $\mu$ M) and strychnine (STR; 300 nM). (Right column) In the presence of APV (30  $\mu$ M), BIC, and STR. B, the synaptic response stimulus-intensity profile generated by calculating the total EPSC area under the curve from the artifact to the



end of the recording (900 ms) for each of the three EPSCs at each intensity tested and for all conditions.

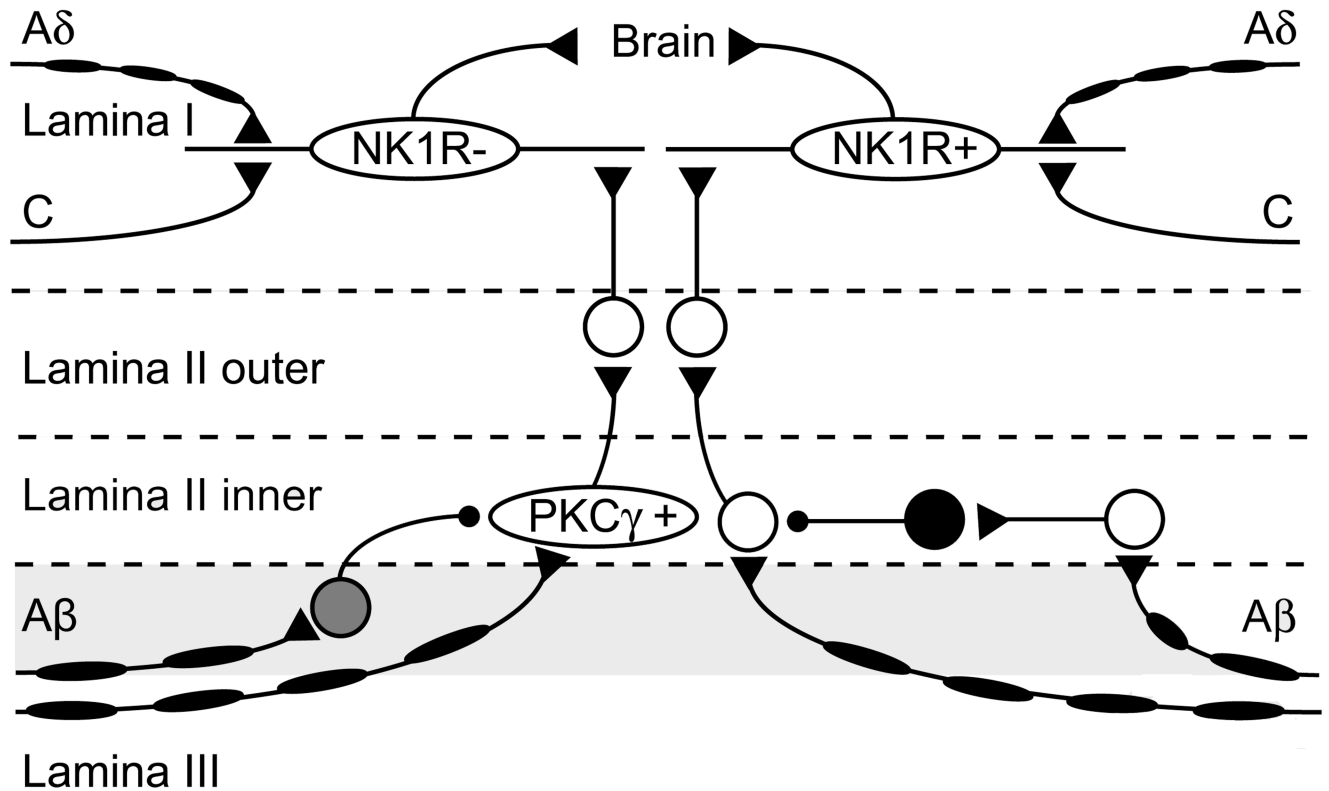




**Figure 2.**

Low-threshold (A $\beta$ ) fiber input as well as input from high-threshold fibers (A $\delta$  and/or C) to GABAergic neurons.<sup>17</sup> (A) Example recordings from a P35 GABAergic neuron with input from A $\beta$  and C fibers. (Upper panel) Three consecutive traces show responses to low-frequency A $\beta$  fiber stimulation (left, 0.05 Hz, 25  $\mu$ A, arrow). Twenty consecutive A $\beta$  fiber responses to high-frequency stimulation (right, 20 Hz, arrow, expanded timescale). (Lower panel) C fiber input was observed when the stimulation intensities were increased (left, 0.05 Hz, 500  $\mu$ A, arrowheads). The C fiber response had a monosynaptic component with no failures (but note the small amplitude in the gray trace) when tested at high frequency (right, 1 Hz, filled arrowhead). There was also a later, polysynaptic C fiber component with

failures (*open arrowhead*, illustrated by the gray trace). (B) The proportion of GABAergic neurons with input from different afferent fiber classes is summarized. For simplicity, this representation does not distinguish between monosynaptic and polysynaptic responses. There is considerable overlap between the subsets of GABAergic neurons receiving input from each class of afferent fiber type.



**Figure 3.**

Schematic diagram illustrating two putative dorsal horn neural networks involved in mechanical allodynia. The schematic diagram was drawn based on evidence reported by several groups.<sup>1-4,17,19</sup> NK1R<sup>+</sup> neurons that receive innocuous input through polysynaptic pathways seem to be part of a local excitatory circuit that mediates mechanical allodynia after loss of GABAergic and glycinergic inhibitory control.<sup>4</sup> Inhibitory interneurons in lamina II (*black*) receiving polysynaptic A $\beta$  fiber input may inhibit neurons that relay non-nociceptive information to NK1R<sup>+</sup> neurons.<sup>17</sup> Moreover, PKC  $\gamma$ <sup>+</sup> neurons that receive innocuous input via A $\beta$  fibers at the lamina II/III border (*shaded*) are part of a local excitatory circuit that mediates dynamic mechanical allodynia after loss of glycinergic inhibitory control.<sup>2</sup> We tentatively put a glycinergic inhibitory interneuron (*gray*) at the lamina II/III border. Given local inhibitory synaptic connection within same lamina,<sup>1</sup> glycinergic inhibitory interneurons may be at lamina II/III border, although their exact location is still unknown. The target of the PKC  $\gamma$ <sup>+</sup> neuron appears to be lamina I neurons lacking NK1 receptors.<sup>3</sup>