Glycine at the Gate—from Model to Mechanism

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In this issue of *Neuron*, Foster et al. (2015) show that ablating, silencing, or activating spinal glycinergic inhibitory neurons with viral vectors all have dramatic effects on pain and itch. These results provide molecular insights into pain gate control and useful tools for the rapid analysis of other CNS circuits.

Gasser (1941), who had been measuring action potential velocities in peripheral nerves, demonstrated that there were fast and slower fibers that could both produce a sensation of pain. He also observed, "it (slow pain) can be relieved by rubbing the spot, that is, it may be inhibited by sending into the central nervous system a flood of impulses carried in rapidly conducting fibers."

This observation was the precursor of the gate control theory expounded by Melzack and Wall 50 years ago in *Science*, which has had a profound influence on the development of ideas about peripheral pain mechanisms and their central regulation. Simply put, the theory states that innocuous sensation can drive inhibitory circuits that tonically block potentially painful input into the dorsal horn of the spinal cord (Melzack and Wall, 1965). Although the original gate control proposals have been modified many times over the years (Mendell, 2014), the present paper by the Zeilhofer group provides a magisterial cell and molecular analysis of the role of glycinergic inhibitory neurons of the spinal cord in gating noxious input, and demonstrates that the gate control concept remains important and relevant to understanding pain - mechanisms (Figure 1). Perhaps as importantly, these studies describe broadly applicable rapid approaches to the analysis of neuronal circuitry using AAV virus-mediated transduction to activate, silence, or kill Cre-expressing neurons.

Inhibitory neurons in the spinal cord release GABA, glycine, or both neurotransmitters. A great deal of evidence that spinal inhibitory circuits play a key role in regulating pain perception in disease states has been reviewed recently (Zeilhofer et al., 2012). In the present study, Foster et al. (2015) generated a mouse expressing a BAC-Cre driven by the neuronal glycine transporter GlyT2 promoter and examined the pattern of expression of recombinase activity. Using Lac-Z reporters together with GFP present in GlyT2-positive neurons, an excellent match of functional Cre expression was found in spinal cord neuronal populations, predominantly in the deep dorsal horn.

Having genetically defined spinal cord glycinergic neurons, a range of AAV and rabies viruses were generated that exploit the FLEX system to tightly control Cre-dependent cell type-specific expression (Atasoy et al., 2008). The first question addressed was, what cells innervate the deep dorsal horn glycinergic neurons? A recently developed virus-mapping strategy was employed (Wickersham et al., 2007). Defective rabies virus coding for eGFP and pseudotyped with the avian sarcoma/leukosis virus envA glycoprotein was used to infect only glycinergic neurons. This selective infection of glycinergic neurons was made possible through an earlier injection of an AAV vector that expresses the avian envA binding receptor TVA in a Cre-dependent manner. The competent rabies virus was then observed in GFP+ neurons in the spinal cord, and progressed to neuronal cell bodies in adjacent dorsal root ganglion neurons that were typed with immunocytochemical markers to show they were myelinated and had properties consistent with a non–nociceptive role. In other words, inhibitory neurons in the spinal cord are indeed innervated by non-nociceptive presumptive touch-sensitive myelinated neurons consistent with gate control theory and Gasser's early observations. Other inputs to glycinergic neurons from supraspinal sites may also contribute to inhibitory activity, but this aspect was not addressed in the present study.

Next, the role of glycinergic neurons was investigated by cell ablation using diphtheria toxin or by neural silencing with tetanus toxin using AAV virus to deliver the toxins. Both approaches provided consistent data. As microglial activation is much more pronounced with cell death, rather than silencing, a critical role for microglia in regulating pain in these experiments could be ruled out. Diphtheria toxin delivery led to the loss of substantial numbers of deep dorsal horn inhibitory neurons as well as almost one-fifth of inhibitory neurons in laminae 1 or 2 where 80% of inhibitory neurons are GlyT2 negative.

What neurons are targeted by the GlyT2-Cre positive neurons? The consequence of GlyT2+ cell loss on inhibitory input into laminae 1 and 2 neurons was investigated optogenetically in spinal cord slices, where cell ablation caused more than 50% diminution of inhibitory postsynaptic currents in presumptive excitatory dorsal horn neurons. Here, channelrhodopsin was expressed in both GABAergic and glycinergic neurons using a vGAT promoter, while only the GlyT2 subset was ablated with diphtheria toxin. About 70% of the inhibitory input is blocked by strychnine supporting an important role for glycine in inhibitory input to dorsal horn neurons. A high level of expression of the glycine receptor GlyRα3 is observed in the superficial laminae of the spinal cord where GlyT2+ fibers are also observed. These observations fit with some aspects of the Melzack and Wall model in demonstrating a circuit that links innocuous input to inhibitory neurons that subsequently synapse on dorsal horn neurons receiving nociceptive input. Whether there may

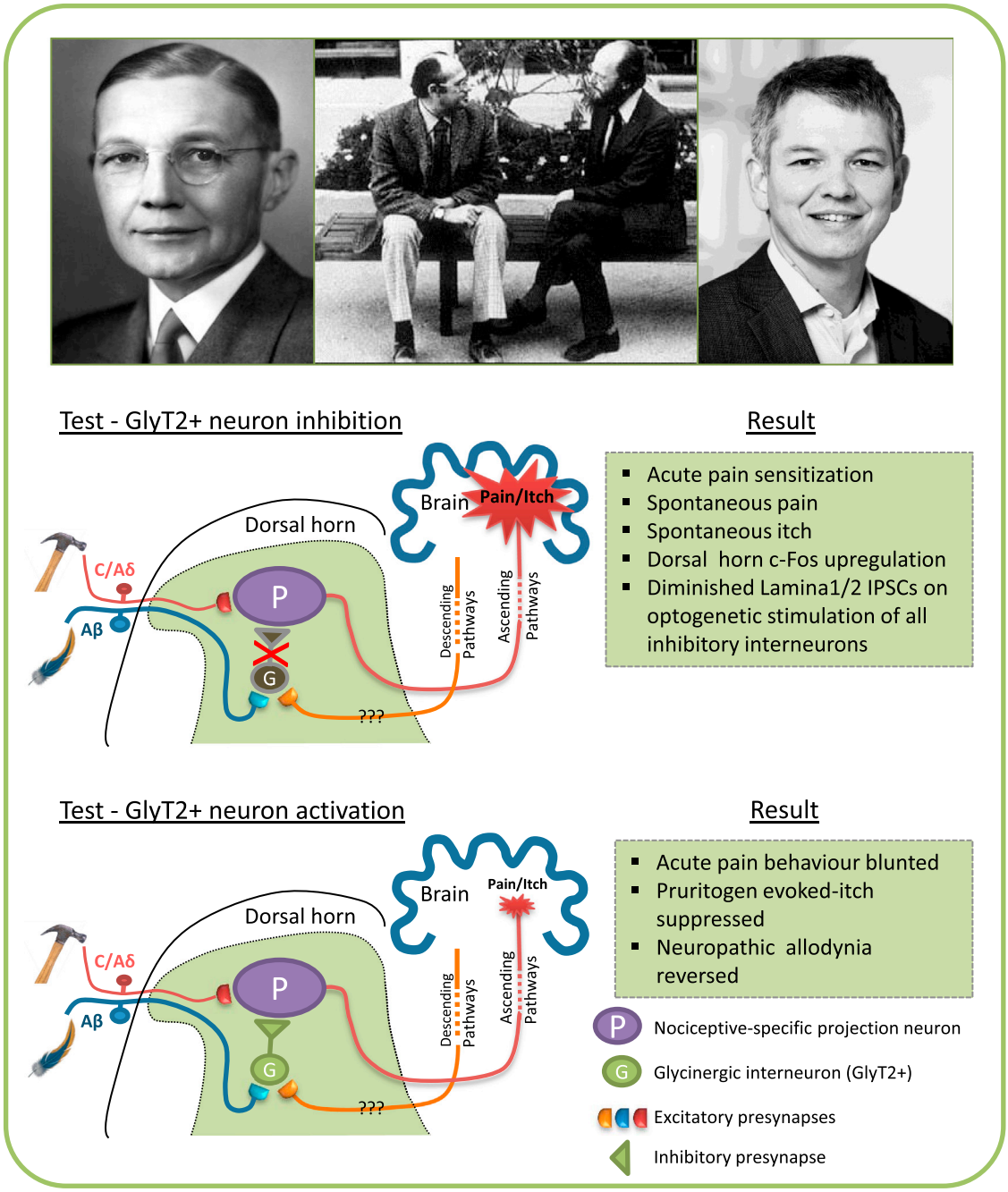

Figure 1. How Glycinergic Interneurons Mediate Gate Control of Pain Pathways in the Spinal Cord

Gasser (left) articulated the role of low-threshold mechanoreceptors in pain control in his Nobel Prize-winning studies of peripheral neuron excitability. A model of gate control theory by Melzack and Wall (center) was published 50 years ago in *Science*. In the present issue, Zeilhofer (right) and collaborators show the role of glycinergic neurons in controlling nociceptive input leading to sensations of pain and itch. The cartoon demonstrates the functional consequences of manipulating glycinergic inhibition on dorsal horn neurons involved in pain circuitry.

also be presynaptic effects on nociceptive input to the dorsal horn, an aspect of the original model, was not examined, but the significance of glycinergic inhibition on second-order sensory neurons thus seems clear.

Next, the behavioral consequences of manipulating GlyT2+ neuronal activity were assessed in classical pain tests for mechanical, heat, and cold sensation. Within 1–2 days, cell ablation with AAV-flex-diphtheria toxin lead to sensitization of withdrawal responses to von Frey filaments, heating with a Hargreaves apparatus, and cold withdrawal with minimal effects on motor behavior. Signs of spontaneous discomfort were also apparent in terms of flinching and limb guarding.

An intriguing observation that was made in these studies was the spontaneous reversal of ongoing pain activity with time in mice where glycinergic input was lowered. The mechanistic basis of this interesting effect remains mysterious, but C-fos expression in the dorsal horn mirrored the pain both in terms of its development and partial resolution, suggesting that plasticity in the spinal cord plays a role. Tetanus toxin-mediated silencing of GlyT2 cells gave effectively similar results to cell ablation.

Can exogenous activation of the GlyT2 cells produce useful analgesia? This question was addressed by delivering a modified muscarinic GPCR that is activated by the compound clozapine-N-oxide (CNO) to drive neuronal activity (Alexander et al., 2009). This Gq- and PLC-coupled receptor probably acts through regulation of potassium channels to enhance neuronal excitability. Acute pain thresholds were blunted with the increased inhibitory activity of GlyT2 neurons upon administration of CNO. More importantly, after a chronic constriction injury leading to mechanical allodynia, CNO reversed the sensitization within a few hours. Itching caused by pruritogens such as histamine was also reversed, demonstrating a role for glycinergic inhibition in the control of itch circuitry. Thus, spontaneous pain, neuropathic pain, and itch all can be inhibited by activating spinal GlyT2+ glycinergic neurons.

These observations raise a number of questions. If innocuous A-beta fiber activation is able to maximally activate glycinergic inhibition of nociceptive input, then should a good rub be an effective treatment for neuropathic pain or itch? It certainly seems to be useful for acute pain. If, however, innocuous sensation normally keeps nociceptor input close to the threshold for the induction of pain, as seems to be the case, one has to ask, what are the physiological advantages of such a system? One possibility is that innocuous input is as important as damage sensing in terms of organismal function and survival. Deficits in innocuous sensation might then lead to pain through the loss of glycinergic inhibition and cause subtle modifications in behavior to redress the loss of innocuous input (e.g., stretching or other movements).

In summary, the present study adds cell and molecular detail to a gate control model of spinal pain control that reinforces the importance of glycinergic inhibitory circuits in the control of pain. Whether this information will provide a route to clinically useful pharmacological control of chronic pain remains to be tested. However, the exploitation of AAV viruses in conjunction with mice expressing Cre-recombinase to map circuits with rabies virus, silence cells with tetanus toxin, kill cells with diphtheria toxin, or activate them optogenetically or pharmacologically provides experimental paradigms and reagents that should be useful for a myriad of questions about brain function. Dramatic advances in single-cell RNAseq technology (Usoskin et al., 2015) and combinatorial genetics (Yang et al., 2013) that help define distinct neuronal subsets, combined with the technology described by Zeilhofer's team, suggest that we have now entered an exciting new period of genetic insight into mammalian neuronal circuitry, sensation, and behavior.

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