

Parallel “Pain” Pathways Arise from Subpopulations of Primary Afferent Nociceptor

Report

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Summary

A major unanswered question concerning “pain” circuitry is the extent to which different populations of primary afferent nociceptor engage the same or different ascending pathways. In the present study, we followed the transneuronal transport of a genetically expressed lectin tracer, wheat germ agglutinin, in Na_v1.8-expressing nociceptors of the nonpeptide class. We found that interneurons of lamina II are at the origin of the major ascending circuits targeted by the nonpeptide nociceptors. These interneurons contact lamina V projection neurons, which in turn predominantly target fourth-order neurons in the amygdala, hypothalamus, bed nucleus of the stria terminalis, and to a remarkable extent, the globus pallidus. These circuits differ greatly from the lamina I-based projection that is targeted by the peptide class of nociceptors. Our results indicate that parallel, perhaps independent pain pathways arise from different nociceptor classes and that motor as well as limbic targets predominate in the circuits that originate from the nonpeptide population.

Introduction

Neurochemical and electrophysiological studies have identified two major classes of unmyelinated primary afferent nociceptor. These two classes of nociceptor target different regions of the superficial dorsal horn of the spinal cord, which suggests that they engage different CNS “pain” transmission circuits (Craig, 2003; Hunt and Rossi, 1985; Snider and McMahon, 1998; Todd et al., 2000). The peptide population expresses substance P (SP) and calcitonin gene-related peptide (CGRP) and terminates almost exclusively in the most superficial laminae of the dorsal horn, directly contacting lamina I projection neurons that transmit nociceptive messages to brainstem and/or thalamus as well as interneurons in the outer part of lamina II. By contrast, the nonpeptide population, which binds the lectin IB4, primarily targets interneurons of inner lamina II.

These very fundamental differences in the patterns

of nociceptor termination argue that there is a multiplicity of circuits that are engaged by primary afferent nociceptors. Traditional tract tracing studies focused on the connections of spinal cord nociceptive neurons with different brainstem, thalamic, and limbic targets (Basbaum and Jessel, 2000; Burstein et al., 1987; Burstein and Potrebic, 1993), but the manner in which the primary afferent nociceptor feeds into these different projection systems is not fully understood. One exception is a study that defined a circuit that includes connections between peptide-containing nociceptors and SP/neurokinin-1 (NK1) receptor-expressing neurons of lamina I of the dorsal horn. The latter, in turn, project to the amygdala via a connection in the parabrachial nucleus of the dorsolateral pons (Jasmin et al., 1997).

Whether the nonpeptide population of nociceptors also engages ascending pathways that arise from lamina I neurons or whether there are segregated pathways from these nociceptors to the spinal cord and to higher centers is not known. In the present study, we addressed this question using a tract tracing method in transgenic mice that express the transneuronal tracer wheat germ agglutinin (WGA) in defined subsets of CNS neurons. Because synthesis of the tracer lasts for the life of the animal, it is possible to anatomically label multineuronal circuits after transneuronal transport of the WGA. Two key studies that adopted this approach used neuronally specific promoters to drive expression of the WGA or an equivalent barley lectin tracer and transneuronally labeled multineuronal circuits that arise from Purkinje cells and olfactory epithelium, respectively (Horowitz et al., 1999; Yoshihara et al., 1999).

The utility of those transgenic mice is limited to the CNS circuit influenced by the particular promoter that drives expression of the lectin transgene. This prompted us to generate a different transgenic mouse line in which transneuronal labeling of circuits originating from any region of the central and peripheral nervous system can be induced and followed (Braz et al., 2002). By expressing Cre recombinase in subsets of neurons, so as to excise an intervening lacZ sequence, we can selectively induce WGA in any subpopulation of neurons and then study the circuits engaged by those neurons. In the present analysis, we induced the WGA tracer in dorsal root ganglion (DRG) neurons that express the voltage-gated, tetrodotoxin-resistant Na⁺ channel (Na_v1.8). Because of a fortuitous mosaic expression of the WGA transgene, our analysis revealed the CNS circuits engaged by small-diameter Na_v1.8-positive neurons of the nonpeptide class of primary afferent nociceptors.

Results

WGA Localization within Primary Sensory Neurons

To trigger WGA synthesis in sensory neurons, we crossed the ZW mice with a heterozygous Na_v1.8-Cre mouse line that expresses Cre exclusively in Na_v1.8 DRG and trigeminal neurons (Stirling et al., 2005). Fig-

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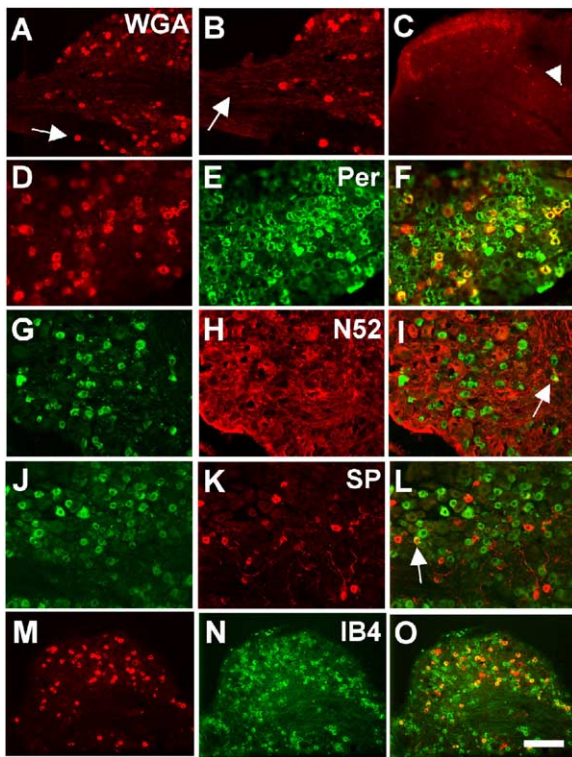


Figure 1. WGA Expression in Sensory Neurons of Na_v1.8-ZW Mice
WGA is expressed in small- and medium-diameter DRG neurons (column 1) and in axons (arrows in [A] and [B]) that course to the spinal cord and to the periphery. The densest accumulation of transganglionically transported WGA is in terminals and cell bodies in lamina II of the superficial dorsal horn. Labeled neurons are also found in all laminae of the dorsal horn and around the central canal (arrowhead in [C]). Double labeling (column 2) for markers of unmyelinated or myelinated afferents (peripherin; Per and N52, respectively) and for substance P (SP) or the isolectin IB4 illustrates the preferential expression of WGA in the nonpeptidergic population of unmyelinated nociceptors. Arrowheads point to rare double-labeled cells in myelinated (I) or peptidergic neurons (L). Calibration bar, 100 μ m in (A), (C), and (M)–(O); 50 μ m in (B) and (D)–(L).

Figure 1 shows the expression pattern of WGA in the L5 lumbar DRG of a 3-week-old double transgenic mouse (Na_v1.8-ZW) in which there was successful Cre recombination. Although most WGA-positive neurons were intensely immunoreactive (Figure 1A), some positive neurons showed weak labeling. The WGA labeling was restricted to the cytoplasm of small- to medium-sized sensory neurons, and the majority of these (~82%) were positive for the peripherin marker of unmyelinated axons (Figures 1D–1F). We also found that 10% of WGA-positive DRG neurons coexpressed N52, a marker of neurons with myelinated axons (23 of 234 neurons [9.8%]; Figures 1G–1I). This pattern did not change in Na_v1.8-ZW mice that were studied at 0, 6, or 11 weeks of age. Based on cell body size, we presume that the WGA was induced in lightly myelinated A delta and unmyelinated C fibers, the majority of which are nociceptors.

Neurochemistry of Neurons that Synthesized WGA

To better characterize the neurochemical makeup of the WGA-expressing neurons, we double labeled for SP

and CGRP, which mark the peptide class of primary afferent nociceptors, and for IB4 and P2X₃, which mark the nonpeptide class. Within the subset of small WGA-positive DRG neurons, we found that ~80% bound IB4 (Figures 1M–1O, 1201 of 1502 WGA-positive neurons), ~75% were P2X₃ immunoreactive (data not shown), ~21% were CGRP immunoreactive, and the great majority of these were unmyelinated. (See Figure S1 in the Supplemental Data online.) On the other hand, only ~8% were labeled with antisera to SP (Figures 1J–1L; 58 of 721 WGA-positive neurons). These data indicate that there is mosaic insertion of the ZW transgene, resulting in preferential induction of WGA in the Na_v1.8/IB4 population of neurons.

WGA Transport and Transneuronal Transfer to the Spinal Cord Dorsal Horn

We observed WGA-positive fibers in both peripheral and central branches of the DRG (Figure 1B), indicating that there is transport of the lectin tracer to both peripheral and central terminals of Na_v1.8 sensory neurons. Because detection of the lectin tracer in second-order neurons is concentration and time dependent, we examined the distribution of labeled cell bodies and axons in sections of the spinal cord of Na_v1.8-ZW animals at different ages (P0 to 11 weeks). We performed most of the analysis on sagittal sections of the lumbar spinal cord. Similar results were obtained at cervical segments. Figure 2 illustrates the typical pattern of transneuronal label in the spinal cord. WGA immunoreactivity appeared as a dense band of presumed terminal staining in the superficial dorsal horn, in a region corresponding to lamina II (Figures 2A and 2B and 2D and 2E). This band consisted mainly of terminal-like staining with interspersed small, immunoreactive cells, 100% of which contained NeuN, a neuronal marker (see Figure S2). The latter were difficult to quantify because they were embedded in the dense WGA terminal immunoreactivity, which overlapped with IB4 labeling (Figure S3). This pattern is, of course, entirely consistent with the extensive overlap of WGA and IB4 staining in the DRG.

We occasionally observed isolated WGA-positive axons that penetrated deeper regions of the dorsal horn, primarily in lamina III. We observed immunoreactive neuronal cell bodies in these regions as well as in more superficial (I) and deeper laminae (IV–V, Figures 2A and 2C) and around the central canal (Figure 1C).

The pattern of transneuronal labeling that we observed in the spinal cord did not differ significantly in Na_v1.8-ZW mice that were studied at 4, 6, or 11 weeks of age, although the intensity of positive terminals in lamina II decreased with time. To some extent, this may have resulted from an immunological response to the WGA. Specifically, we observed scattered round, WGA-immunoreactive microglial/macrophage-like cells that had invaded the territory of lamina II (data not shown).

With a view to identifying the primary target of the Na_v1.8/WGA-positive afferents, we also examined the pattern of transneuronal transport in three P0 animals. In these mice, the WGA labeling was restricted to terminals and cell bodies in the superficial dorsal horn (Figures 2F–2H). WGA immunolabeling of deeper laminae was only detected in animals older than 3 weeks, suggesting that the pattern of labeling observed in deeper

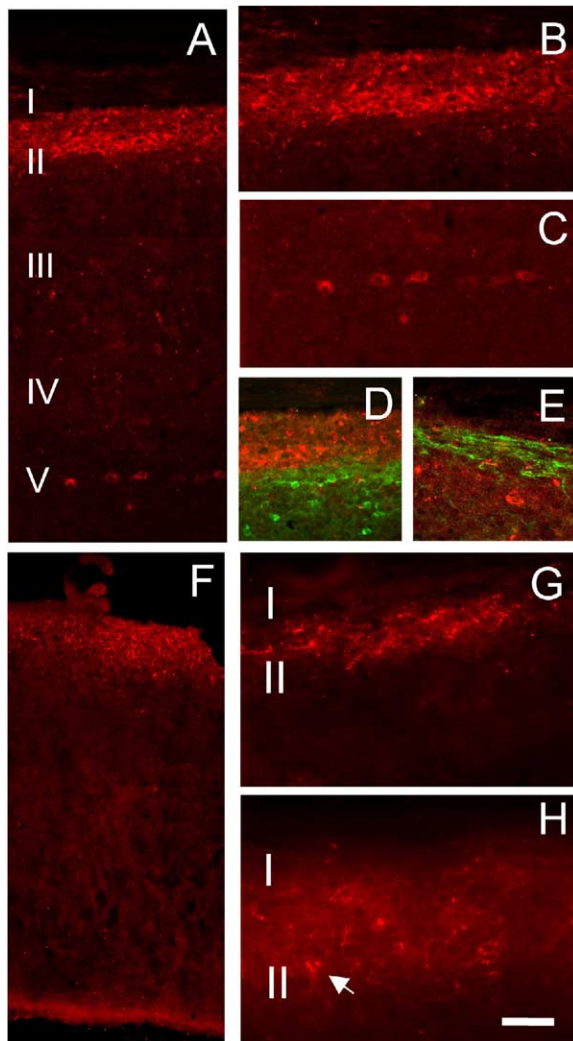


Figure 2. Transneuronal Transport of WGA in Lumbar Spinal Cord of Adult and P0 Na_v1.8-ZW Mice

(A) In adult Na_v1.8-ZW mice, labeled neurons are found throughout the dorsal horn, most abundantly in interneurons of lamina II (B) and in large, presumed projection neurons of lamina V (C). Transneuronal labeled interneurons in lamina II are located dorsal to the lamina III band of PKC γ interneurons (green in [D]) and ventral to NK1 receptor-positive neurons in lamina I (green in [E]).

(F and G) In P0 Na_v1.8-ZW mice, WGA immunoreactivity is restricted to terminals and cell bodies (arrow in [H]) in the superficial dorsal horn, which suggests that the labeling of deep dorsal horn neurons in the adult results from transport via lamina II interneurons. Calibration bar, 200 μ m in (A) and (F); 100 μ m in (B)–(E) and (G) and (H).

laminae resulted from transneuronal transport of the WGA from second-order interneurons in the superficial dorsal horn. Double labeling for the γ isoform of protein kinase C (PKC γ), which defines a population of interneurons in the inner part of lamina II, revealed that the WGA terminals were, in fact, concentrated in the more dorsal part of inner lamina II. As shown in Figure 2D, the WGA-immunoreactive terminals and PKC γ neurons clearly do not overlap.

To determine if the postsynaptic WGA-positive neurons expressed the NK1 receptor, a marker of spino-

parabrachial projection neurons of lamina I, we also double labeled Na_v1.8-ZW dorsal horn sections for this receptor. Surprisingly, even though \sim 80% of projection neurons of lamina I express NK1 receptors, we only found a rare WGA/NK1 receptor-positive neuron in lamina I. NK1 receptor-positive neurons in the deep dorsal horn were never double labeled after transneuronal transport of the WGA (Figure 2E).

Taken together, the double-labeling experiments suggest that a wide variety of spinal neurons receive inputs from Na_v1.8 nonpeptidergic/IB4 sensory neurons. But these do not include NK1 receptor-expressing projection neurons or PKC γ -positive interneurons. Although the phenotype of those postsynaptic spinal neurons is still under investigation, we noted that a small number of WGA-positive spinal neurons immunolabeled for parvalbumin, a calcium binding protein expressed in a large population of GABAergic interneurons (Celio, 1986). These neurons were concentrated in laminae III and V (data not shown).

WGA Transport and Transneuronal Transfer to Supraspinal Sites

There were no WGA-immunoreactive neurons in the brains of the P0 mice, despite the presence of significant label in primary afferents and in the superficial dorsal horn. However, in all 3 week (and older) old animals, we observed a consistent pattern of transneuronal transport. With some important exceptions (see below), the areas in which we recorded WGA-immunoreactive neurons corresponded well with regions previously recognized as major targets of spinal cord nociceptive neurons. All animals contained labeled neurons in the hypothalamus, amygdala, and the bed nucleus of the stria terminalis (Figure 3). The distribution of transneuronal labeling was always bilateral. In some animals, we observed isolated cells in the periaqueductal gray of the midbrain, the medial thalamus, and, very occasionally, in the cerebral cortex. The presence of neurons in these latter regions did not correlate with the age of the animal.

Surprisingly, the most extensive transneuronal transport was to neurons in the lateral aspect of the globus pallidus (GP; Figure 3C). These neurons were uniformly of small size. With a view to determining whether these GP neurons constituted a uniform subset, we immunostained for parvalbumin, which defines a population of pallidal neurons that project to the subthalamic nucleus and substantia nigra pars reticulata (Ruskin and Marshall, 1997). As Figure 3D illustrates, we found no overlap of immunolabeling with parvalbumin.

To conclude that the pattern of labeling in forebrain regions indeed resulted from transneuronal transfer of the lectin from spinal neurons, it is essential to show that synthesis of the WGA only occurs in the primary afferent neurons, after Cre recombination. To address this question, we crossed the Na_v1.8-Cre mice with the ROSA26 Cre reporter mouse (Soriano, 1999) and surveyed the spinal cord and brain for expression of the lacZ gene. In these mice, we only found β -galactosidase activity in DRG neurons (data not shown). There was absolutely no staining in the brain, confirming that the WGA immunoreactivity detected in supraspinal neurons of the Na_v1.8-ZW mice was the result of trans-

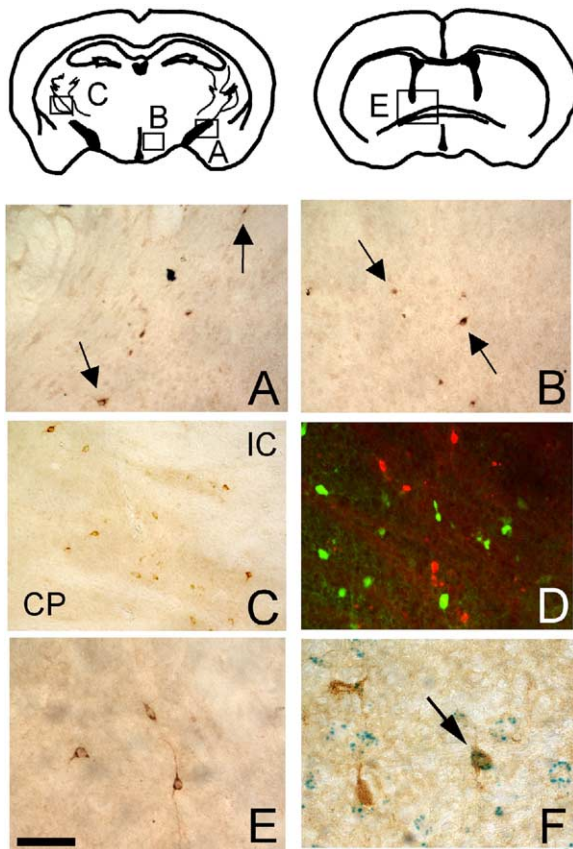


Figure 3. Transneuronal Transport of WGA in the Brain of Adult $Na_v1.8$ -ZW Mice

The great majority of labeled neurons (arrows) was found in the amygdala ([A]; Amg), the ventromedial hypothalamus ([B]; VMH), the globus pallidus ([C]; GP), and in the bed nucleus of the stria terminalis ([E]; BNST). Double immunofluorescence (D) shows that the WGA-positive GP neurons (red) do not colocalize with neurons that express parvalbumin (green). As at least 50% of WGA-immunoreactive neurons also expressed the lacZ gene ([F], arrow), the WGA label must have arisen from transneuronal transport of the tracer, rather than from local synthesis after Cre-mediated recombination. IC, internal capsule; CP, caudate putamen. Calibration bar, 100 μ m in (A)–(D); 50 μ m in (E) and (F).

neuronal transfer of the lectin tracer from the spinal cord.

Of course, the fact that we found no brain labeling in the P0 mouse provides additional evidence that there is no transient $Na_v1.8$ expression in brain regions during development. As a final confirmation, we also double labeled $Na_v1.8$ -ZW forebrain sections for both immunoreactive WGA and β -galactosidase activity (using the X-gal reaction). As the lacZ and WGA cDNAs in the ZW transgene are under the control of the same promoter (chick β -actin), neurons in ZW mice will express either lacZ or WGA, after Cre recombination, but never both, unless the WGA was incorporated by the neuron after its transneuronal transport. Figure 3F demonstrates X-gal staining of neurons from an $Na_v1.8$ -ZW double transgenic mouse. As expected, we detected lacZ expression throughout the brain, in about 50% of neu-

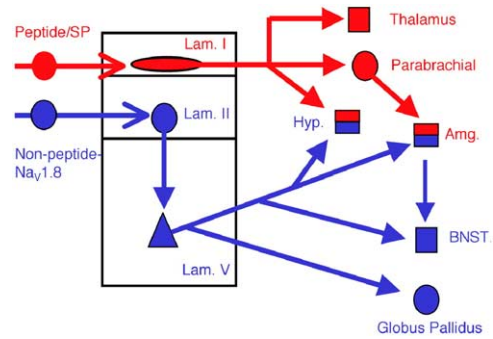


Figure 4. Parallel, and Largely Independent, Circuits Are Engaged by the Two Major Primary Afferent Nociceptor Populations

In contrast to the peptide population of nociceptors, which is at the origin of a pain pathway that involves projection neurons of lamina I, the major output from the $Na_v1.8$ contingent of nonpeptidergic nociceptors is via connections with second-order interneurons in lamina II. Some transneuronal label in lamina V neurons (third-order) could arise from monosynaptic primary afferent input to deep dorsal horn neurons with dendrites that penetrate lamina II. These may not be sufficiently developed at P0. These deep dorsal horn neurons project, in part directly, to several limbic and striatal regions (fourth-order neurons), including the globus pallidus. Lamina I neurons that receive a peptidergic input project heavily to brainstem (parabrachial nuclei) and thalamus, but these latter regions were not labeled in the $Na_v1.8$ -ZW mice. Inputs from the two nociceptor populations may converge at supraspinal levels (e.g., hypothalamus and amygdala), but the routes to these sites differ, in effect constituting parallel pathways for the transmission of pain messages. Amg, amygdala; BNST, bed nucleus of the stria terminalis; Hyp, hypothalamus.

rons, reflecting the mosaic expression of the transgene. Importantly, at least half of the WGA-immunoreactive neurons were also lacZ positive, indicating that there indeed was transneuronal transfer of the lectin tracer to these regions of the brain.

Discussion

The results of this genetic transneuronal tracing analysis provide information on the central circuits engaged by the “nonpeptide” population of primary afferent nociceptors. In distinct contrast to the peptide containing nociceptors, which communicate with projection neurons of lamina I, the $Na_v1.8$ -expressing subset of the IB4 population of nociceptors predominantly engages limbic regions of the brain, via projection neurons of the deep dorsal horn. We also uncovered a remarkably concentrated projection to the globus pallidus, an area rarely included in discussions of ascending nociceptive circuits. Taken together, we conclude that the two classes of primary afferent nociceptor are at the origin of parallel pathways that process nociceptive information. Although we cannot rule out convergence of the inputs at higher levels of the neuraxis, as is illustrated in Figure 4, the circuits engaged at the level of the spinal cord appear to be remarkably segregated.

What Are the Circuit Elements between the Nociceptor and the Forebrain?

There are some inherent limitations of this tract tracing method that should be mentioned. First, because there

is dilution of the tracer after its transneuronal transport, only a finite number of circuit elements will be labeled downstream of the neuron in which the tracer is synthesized. Second, when there is concurrent labeling of different cell populations, for example, interneurons in lamina II and projection neurons in lamina V, it may be difficult to dissect the circuit map from the pattern of labeling. The lamina V labeling could arise after transport from lamina II interneurons or there may be a monosynaptic connection from the nociceptor to both populations of dorsal horn neurons. For reasons described below, we believe that the supraspinal targets that were transneuronally labeled are part of a trisynaptic circuit that includes the primary afferent nociceptor, interneurons of lamina II, lamina V projection neurons, and the different limbic, hypothalamic, and striatal regions. We presume that dilution of the tracer prevented our detecting higher-order elements of this nociceptive circuit.

Output of the Substantia Gelatinosa Interneuron

There is far from complete agreement as to the direction of impulse flow from interneurons of the substantia gelatinosa. Golgi-impregnated lamina II neurons and anatomical analysis of neurons intracellularly filled with horseradish peroxidase provided evidence that axons of stalked cell interneurons, located at the lamina I–II border, target neurons in lamina I. Electrophysiological recordings of adjacent neurons in laminae I and II of the primate led to the same conclusion. Other studies, however, reported that the axonal arbor of some substantia gelatinosa interneurons extends ventrally into the deep dorsal horn (Gobel and Falls, 1979; Light and Kavookjian, 1988), possibly contributing to the relatively large, polymodal receptor field of wide dynamic range neurons in deep dorsal horn (Rethelyi and Szentagothai, 1969; Szentagothai, 1964). Although we previously suggested that PKC γ interneurons are a major target of the IB4 population of nociceptors, this is not the case for the Na $_v$ 1.8 subset, which clearly terminates dorsal to the band of PKC γ interneurons. This observation agrees with a recent study (Zylka et al. 2005) that showed, in the mouse, that a subset of IB4-positive DRG neurons that express the G protein-coupled receptor MrgD terminates exclusively in lamina III, but clearly *dorsal* to the neurons that express PKC γ . The present analysis shows that it is not just this subset that terminates dorsal to the PKC γ band, but the entire IB4 population.

Nociceptor Inputs to Projection Neurons of the Superficial and Deep Dorsal Horn

Previous anterograde and retrograde tracing studies reported significant projections from the spinal cord to the amygdala and hypothalamus (Burstein et al., 1987; Burstein and Potrebic, 1993). The present results demonstrate that inputs to this system arise, at least in part, from the Na $_v$ 1.8-expressing subset of IB4-positive nociceptors, but for several reasons, we conclude that lamina I neurons are not involved. First and most importantly, we only recorded a few neurons in lamina I. Second, we found almost no labeling in the parabrachial nucleus, a known relay to the amygdala from lamina I

cells. Third, in some animals we injected a retrograde tracer (Fluorogold) into the parabrachial nucleus. As expected, we found large numbers of retrogradely labeled neurons in lamina I, but only occasionally did we find cells that were WGA positive. It is likely that the latter correspond to neurons that received input from the small contingent of Na $_v$ 1.8-expressing, peptide class of nociceptors that carry the transgene.

A corollary of this conclusion is that there must be a very limited, direct projection from the IB4 population of nociceptors to lamina I neurons. A recent electron microscopic analysis supports this conclusion (Gerke and Plenderleith, 2004). We suggest that the predominant input to lamina I neurons, and to the supraspinal loci that they target, derives largely from the Trk-A-expressing, peptide population of primary afferent nociceptors. Consistent with this hypothesis, we only rarely observed WGA-positive neurons that expressed the NK1 receptor. The latter is not only targeted by substance P-containing nociceptors (Todd et al., 2002), but is also expressed in a large percentage of the dorsal horn neurons that project to the parabrachial nuclei (Todd et al., 2000). The direct amygdala projection from deep dorsal horn that receives inputs from the nonpeptide population of nociceptors is thus paralleled by the spino-parabrachial-amygdala projection from NK-1 receptor-expressing dorsal horn neurons that receive input from the peptide population of nociceptors. To what extent this parallel anatomical framework is maintained at higher levels of the neuroaxis and the extent to which these parallel pathways represent functionally distinct nociceptive circuits remains to be determined.

Spinopallidal Connections

Traditional anterograde tracing studies in rat (Cliffer et al., 1991; Gauriau and Bernard, 2004) and primate (Newman et al., 1996) observed isolated spinal cord axons that penetrate the globus pallidus. Although these studies left the impression that the spinopallidal projection is a minor one, our results using transneuronal labeling indicate that it is, in fact, significant. Importantly, we never found transneuronal labeling in subcortical regions that might link the spinal cord and the globus pallidus, e.g., the parabrachial nuclei (Bernard and Besson, 1990), the pedunclopontine nucleus (Nakano, 2000), or midline thalamic nuclei, parafascicularis, and submedius (Groenewegen et al., 1990). Therefore, we believe that the spinopallidal projection that we observed is direct. Furthermore, because the transneuronally labeled pallidal neurons were parvalbumin negative, they likely correspond to the enkephalin-containing population that projects to the striatum (Ruskin and Marshall, 1997) or to interneurons. We assume that the large number of neurons that were transneuronally labeled in the GP resulted from convergence of spinal afferents arising from all levels of the cord. This result illustrates a particularly powerful advantage of this genetic transneuronal tracing procedure; despite there being dilution of the tracer after it crosses several synapses, convergence of inputs to a higher-order cell could effectively “reconcentrate” the tracer so that it is detectable. In contrast, traditional anterograde tract tracing methods generally only reveal the output of a limited number

of neurons, e.g., those arising from a single spinal cord segment, which in the case of the spinopallidal projection is probably small.

There are two possible explanations for the lack of labeling of the very traditional targets of spinal cord neurons, including the ventroposterolateral nucleus of the thalamus. One is that this region may, in fact, not be part of the nociceptive circuit engaged by lamina V neurons that receive an input from the nonpeptide, Na_v1.8-expressing population of primary afferent nociceptors. If true, this provides further evidence that there is a significant segregation of the ascending nociceptive pathways that carry information arising from the two major classes of primary afferent nociceptor. Alternatively, the thalamic projection may be so topographically organized that the absolute amount of WGA transferred to any given neuron may be too low to be detected by traditional immunocytochemical methods. It will be of interest to determine the extent to which tracing of circuits triggered from the peptide population of nociceptors, which clearly target projection neurons in lamina I (Carlton et al., 1988), will result in transneuronal labeling of parabrachial and thalamic neurons.

Functional Implications

Studies of the differential functional contribution of the peptide and nonpeptide classes of nociceptors to pain transmission have generally relied on pharmacological blockade of subsets of these neurons (e.g., using NK1 or CGRP receptor antagonists) or deletion of genes that encode neurotransmitters and receptors uniquely expressed by different subsets, for example, the P2X₃ subset of IB4-positive nociceptors. In distinct contrast, the present analysis provides insights more relevant to the behavioral/perceptual consequences of activity of the circuits engaged by these afferents. Based on the preferential transneuronal labeling of hypothalamus, amygdala, and bed nucleus of the stria terminalis, we suggest that the information relayed by the Na_v1.8 subset of the nonpeptide nociceptors likely contributes more to the affective component of the pain experience than to the sensory discriminative component. Our failure to find evidence for connections to topographically organized regions of the thalamus supports this hypothesis. The functional difference between the spino-amygdala circuits that we defined and those engaged indirectly, via connections in the parabrachial nuclei (Bernard and Besson, 1990; Jasmin et al., 1997), is unclear and an important question for future study.

Finally, our studies underscore the fact that noxious input does not just evoke an experience of pain, with affective components, but that behavior is almost always induced. Conceivably, there are innate motor patterns that are triggered by noxious stimuli. There is, in fact, considerable evidence for nociceptive processing by pallidal neurons (Chudler et al., 1993). Interestingly, some patients with nerve injury-induced persistent pain conditions have profound abnormalities of posture and motor control. Indeed, the positions that injured limbs assume are reminiscent of those occurring in patients with extrapyramidal lesions (Schwartzman and Kerrigan, 1990). Whether there are conscious consequences of activity of nociceptive neurons in the globus palli-

cus is unclear, but this region is significantly activated in patients that experience noxious stimuli (Pukall et al., 2005; Tracey et al., 2000; Zambreau et al., 2005). Our results indicate that spinopallidal connections that are engaged by noxious stimuli carried by the nonpeptide population of nociceptors that express Na_v1.8 may be critical in driving that activity.

Experimental Procedures

Mouse Lines

To generate double-transgenic Na_v1.8-ZW mice, we crossed the ZW line (Braz et al., 2002) with mice that express Cre recombinase in Na_v1.8 DRG neurons (Stirling et al., 2005).

Immunohistochemistry

Antibodies used were as follows: polyclonal anti-WGA (1:50,000 for fluorescence, 1:200,000 using diaminobenzidine [DAB; Sigma] histochemistry), guinea-pig anti-substance P (1:10,000, a generous gift from Dr. J. Maggio), mouse anti-N52 (1:10,000, Sigma), guinea-pig anti-P2X₃ (1:7000, Chemicon), FITC-coupled IB4 (10 μg/ml, Sigma), guinea-pig anti-PKCγ (1:10,000, Strategic Biosolutions), mouse anti-parvalbumin (1:10,000, Sigma), guinea-pig anti-NK1 (1:10,000; generous gift from Dr. J. Marvizon), and goat anti-peripherin (1:1000, Santa Cruz).

Na_v1.8-ZW mice were killed at different ages: P0, n = 3; 3 weeks, n = 2; 6 weeks, n = 2; 11 weeks, n = 4; and 15 weeks, n = 1. Fourteen (DRG), 20 (spinal cord), and 40 μm (brain) cryostat sections were processed for immunofluorescence or using a diaminobenzidine protocol, as previously described (Braz et al., 2002; Abbadie et al., 1999).

Sections were viewed with a Nikon Eclipse fluorescence microscope. Fluorescent images were collected with a Spot Camera and processed with Adobe Photoshop, version 6.0. In all cases, labeled DRG neurons were counted from every fourth section (total of eight sections) from one lumbar DRG. The analysis was performed in two adult and one P0 animal. DAB-labeled neurons in the brain were observed with a microscope equipped with a camera lucida drawing attachment and mapped according to the mouse atlas of Franklin and Paxinos (1997).

Supplemental Data

The Supplemental Data for this article can be found online at <http://www.neuron.org/cgi/content/full/47/6/787/DC1/>.

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References

- Abbadie, C., Skinner, K., Mitrovic, I., and Basbaum, A.I. (1999). Neurons in the dorsal column white matter of the spinal cord: complex neuropil in an unexpected location. *Proc. Natl. Acad. Sci. USA* 96, 260–265.
- Basbaum, A.I., and Jessel, T. (2000). The perception of pain. In *Principles of Neuroscience*, E.R. Kandel, J. Schwartz, and T. Jessel, eds. (New York: Appleton and Lange), pp. 472–491.
- Bernard, J.F., and Besson, J.M. (1990). The spino(trigemino)pon-toamygdaloid pathway: electrophysiological evidence for an involvement in pain processes. *J. Neurophysiol.* 63, 473–490.
- Braz, J.M., Rico, B., and Basbaum, A.I. (2002). Transneuronal tracing of diverse CNS circuits by Cre-mediated induction of wheat

- germ agglutinin in transgenic mice. *Proc. Natl. Acad. Sci. USA* 99, 15148–15153.
- Burstein, R., Cliffer, K.D., and Giesler, G.J., Jr. (1987). Direct somatosensory projections from the spinal cord to the hypothalamus and telencephalon. *J. Neurosci.* 7, 4159–4164.
- Burstein, R., and Potrebic, S. (1993). Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. *J. Comp. Neurol.* 335, 469–485.
- Carlton, S.M., McNeill, D.L., Chung, K., and Coggeshall, R.E. (1988). Organization of calcitonin gene-related peptide-immunoreactive terminals in the primate dorsal horn. *J. Comp. Neurol.* 276, 527–536.
- Celio, M.R. (1986). Parvalbumin in most gamma-aminobutyric acid-containing neurons of the rat cerebral cortex. *Science* 237, 995–997.
- Chudler, E.H., Sugiyama, K., and Dong, W.K. (1993). Nociceptive responses in the neostriatum and globus pallidus of the anesthetized rat. *J. Neurophysiol.* 69, 1890–1903.
- Cliffer, K.D., Burstein, R., and Giesler, G.J., Jr. (1991). Distributions of spinothalamic, spinohypothalamic, and spinothalamic fibers revealed by anterograde transport of PHA-L in rats. *J. Neurosci.* 11, 852–868.
- Craig, A.D. (2003). Pain mechanisms: labeled lines versus convergence in central processing. *Annu. Rev. Neurosci.* 26, 1–30.
- Franklin, K.B.J., and Paxinos, G. (1997). *The Mouse Brain in Stereotaxic Coordinates* (San Diego: Academic Press).
- Gauriau, C., and Bernard, J.F. (2004). A comparative reappraisal of projections from the superficial laminae of the dorsal horn in the rat: the forebrain. *J. Comp. Neurol.* 468, 24–56.
- Gerke, M.B., and Plenderleith, M.B. (2004). Ultrastructural analysis of the central terminals of primary sensory neurons labeled by transganglionic transport of *bandeiraea simplicifolia* I-isolectin B4. *Neuroscience* 127, 165–175.
- Gobel, S., and Falls, W.M. (1979). Anatomical observations of horseradish peroxidase-filled terminal primary axonal arborizations in layer II of the substantia gelatinosa of Rolando. *Brain Res.* 175, 335–340.
- Groenewegen, H.J., Berendse, H.W., Wolters, J.G., and Lohman, A.H. (1990). The anatomical relationship of the prefrontal cortex with the striatopallidal system, the thalamus and the amygdala: evidence for a parallel organization. *Prog. Brain Res.* 85, 95–116.
- Horowitz, L.F., Montmayeur, J.P., Echelard, Y., and Buck, L.B. (1999). A genetic approach to trace neural circuits. *Proc. Natl. Acad. Sci. USA* 96, 3194–3199.
- Hunt, S.P., and Rossi, J. (1985). Peptide- and non-peptide-containing unmyelinated primary afferents: the parallel processing of nociceptive information. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 308, 283–289.
- Jasmin, L., Burkey, A.R., Card, J.P., and Basbaum, A.I. (1997). Transneuronal labeling of a nociceptive pathway, the spino-(trigemino-)parabrachio-amygdaloid, in the rat. *J. Neurosci.* 17, 3751–3765.
- Light, A.R., and Kavookjian, A.M. (1988). Morphology and ultrastructure of physiologically identified substantia gelatinosa (lamina II) neurons with axons that terminate in deeper dorsal horn laminae (III–V). *J. Comp. Neurol.* 267, 172–189.
- Nakano, K. (2000). Neural circuits and topographic organization of the basal ganglia and related regions. *Brain Dev. Suppl.* 22, S5–S16.
- Newman, H.M., Stevens, R.T., and Apkarian, A.V. (1996). Direct spinal projections to limbic and striatal areas: anterograde transport studies from the upper cervical spinal cord and the cervical enlargement in squirrel monkey and rat. *J. Comp. Neurol.* 365, 640–658.
- Pukall, C.F., Strigo, I.A., Binik, Y.M., Amsel, R., Khalifé, S., and Bushnell, M.C. (2005). Neural correlates of painful genital touch in women with vulvar vestibulitis syndrome. *Pain* 115, 118–127.
- Rethelyi, M., and Szentagothai, J. (1969). The large synaptic complexes of the substantia gelatinosa. *Exp. Brain Res.* 7, 258–274.
- Ruskin, D.N., and Marshall, J.F. (1997). Differing influences of dopamine agonists and antagonists on Fos expression in identified populations of globus pallidus neurons. *Neuroscience* 87, 79–92.
- Schwartzman, R.J., and Kerrigan, J. (1990). The movement disorder of reflex sympathetic dystrophy. *Neurology* 40, 57–61.
- Snider, W.D., and McMahon, S.B. (1998). Tackling pain at the source: new ideas about nociceptors. *Neuron* 20, 629–632.
- Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* 21, 70–71.
- Stirling, L.C., Forlani, G., Baker, M.D., Wood, J.N., Matthews, E.A., Dickenson, A.H., and Nassar, M.A. (2005). Nociceptor-specific gene deletion using heterozygous Nav1.8-Cre recombinase mice. *Pain* 113, 27–36.
- Szentagothai, J. (1964). Propriospinal pathways and their synapses. *Prog. Brain Res.* 11, 155–177.
- Todd, A.J., McGill, M.M., and Shehab, S.A. (2000). Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. *Eur. J. Neurosci.* 12, 689–700.
- Todd, A.J., Puskar, Z., Spike, R.C., Hughes, C., Watt, C., and Forrest, L. (2002). Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance P-containing afferents and respond to noxious stimulation. *J. Neurosci.* 22, 4103–4113.
- Tracey, I., Becerra, L., Chang, I., Breiter, H., Jenkins, L., Borsook, D., and Gonzalez, R.G. (2000). Noxious hot and cold stimulation produce common patterns of brain activation in humans: a functional magnetic resonance imaging study. *Neurosci. Lett.* 288, 159–162.
- Yoshihara, Y., Mizuno, T., Nakahira, M., Kawasaki, M., Watanabe, Y., Kagamiyama, H., Jishage, K., Ueda, O., Suzuki, H., Tabuchi, K., et al. (1999). A genetic approach to visualization of multisynaptic neural pathways using plant lectin transgene. *Neuron* 22, 33–41.
- Zambreanu, L., Wise, R.G., Brooks, J.C., Iannetti, G.D., and Tracey, I. (2005). A role for the brainstem in central sensitization in humans. Evidence from functional magnetic resonance imaging. *Pain* 114, 397–407.
- Zylka, M.J., Rice, F.L., and Anderson, D.J. (2005). Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to MrgprD. *Neuron* 45, 17–25.