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Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli

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Behavioral responses to painful stimuli require peripheral sensory neurons called nociceptors. Electrophysiological studies show that most C-fiber nociceptors are polymodal (i.e., respond to multiple noxious stimulus modalities, such as mechanical and thermal); nevertheless, these stimuli are perceived as distinct. Therefore, it is believed that discrimination among these modalities only occurs at spinal or supraspinal levels of processing. Here, we provide evidence to the contrary. Genetic ablation in adulthood of unmyelinated sensory neurons expressing the G protein-coupled receptor Mrgprd reduces behavioral sensitivity to noxious mechanical stimuli but not to heat or cold stimuli. Conversely, pharmacological ablation of the central projections of neurons that express the heat-sensitive channel TRPV1 (15) caused a complete loss of heat pain sensitivity, without affecting responses to noxious mechanical or cold stimuli. Combined elimination of both populations yielded an additive phenotype with no further behavioral deficits. These data reveal the existence of distinct subsets of primary sensory neurons that selectively mediate behavioral responses to different noxious stimulus modalities.

Results

Conditional Ablation of Mrgprd+ Nociceptors. Mrgprd+ afferents exclusively innervate the epidermis and constitute >90% of all nonpeptidergic cutaneous C-fibers (2, 14, 16). These neurons bind isoelectin IB4 and terminate in inner lamina II of the spinal cord dorsal horn (4). In vitro, Mrgprd+ neurons exhibit electrophysiological properties characteristic of nociceptors (17) and behave as C-polymodal units in ex vivo recordings (K. K. Rau, S. L. McIlwrath, H. Wang, J. J. Lawson, M. P. Jankowski, M.J.Z., D.J.A., H. R. Koerber, unpublished data).

To determine the behavioral consequences of ablating Mrgprd+ neurons, we used a conditional strategy (18, 19), in which the human diphtheria toxin receptor (DTR) was inserted in the Mrgprd locus (Fig. 1A) by homologous recombination in murine embryonic stem cells. The human DTR binds diphtheria toxin (DTX) with 105-fold higher affinity than does the endogenous mouse receptor. Heterozygous Mrgprd+/DTR+ mice (hereafter referred to as Mrgprd/DTR mice) expressed DTR in an identical pattern as a GFP reporter expressed from a second targeted Mrgprd allele (Fig. 1 B–D). Injection of DTX into adult Mrgprd/DTR mice produced a virtually complete (>98%) loss of Mrgprd+ cell bodies in the DRG (Fig. 1 E and F) and in their central and peripheral fibers (Fig. 1 I–N). Consistent with Mrgprd expression in the nonpeptidergic afferents, DTX treatment caused an 82.4% reduction in labeling for IB4 but no change in labeling for calcitonin gene-related peptide (CGRP), a marker of peptidergic afferents [Fig. 1 E–N and supporting information (SI) Table S1]. The overall reduction in neuron number is commensurate with the size of the Mrgprd+ popula-

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tion, suggesting that no other population is affected. No loss of Mrgprd \(^{+}\) neurons in the DRG was observed in GT mice. Sections of the DRG (E–H), spinal cord (I–L), and glabrous skin (M and N) from DTX-treated Mrgprd\(^{EGFPf/+}\) mice (E, G, I, K, and M) and Mrgprd\(^{DTR}\) mice (F, H, J, L, and N) were stained for the indicated markers. (F, H, J, L, and N) Note selective loss of Mrgprd \(^{+}\) cells (green) and fibers. See Table S1 for quantification. (Scale bar in D, F, H, J, L: 50 \(\mu\)m; scale bar in N: 25 \(\mu\)m.)

Selecte Reduction of Behavioral Responses to Noxious Mechanical Stimuli Following Ablation of Mrgprd \(^{+}\) Neurons. To determine the behavioral consequences of ablating Mrgprd \(^{+}\) neurons, we measured paw withdrawal responses to calibrated von Frey filaments. Before DTX treatment, the mechanical threshold of Mrgprd\(^{DTR}\) mice was not different from that of WT littermates (Fig. 2A). Following DTX, however, the mechanical threshold of Mrgprd\(^{DTR}\) mice more than doubled (from 0.41 \(\pm\) 0.04 g to 0.99 \(\pm\) 0.15 g; Fig. 2A; \(P < 0.01\)). There was no significant change in WT mice after DTX treatment (Fig. 2A). The elevated mechanical threshold in Mrgprd\(^{DTR}\) mice persisted for at least 31 days after DTX (not shown). The response frequency to a series of von Frey filaments ranging from 0.16–1.4 g was also significantly reduced in DTX-treated Mrgprd\(^{DTR}\) mice relative to controls (Fig. S1). These data demonstrate that Mrgprd \(^{+}\) neurons are necessary for normal responsiveness to acute noxious mechanical stimuli. Importantly, no deficits in mechanical pain sensitivity were detectable in mice in which Mrgprd \(^{+}\) neurons were deleted from birth (Fig. S2 and Fig. S3), suggesting that other nociceptor populations can functionally compensate for the loss of Mrgprd \(^{+}\) neurons, but only if the loss occurs sufficiently early in development (18).

We next asked whether Mrgprd \(^{+}\) afferents also contribute to the mechanical hypersensitivity caused by hind paw injection of an inflammatory mediator, complete Freund’s adjuvant (CFA). The mechanical thresholds of DTX-treated Mrgprd\(^{DTR}\) mice were significantly higher than those of DTX-treated WT mice at all time points following CFA injection (Fig. 2B; \(P < 0.01\)). In control (DTX-treated WT) mice, mechanical thresholds dropped significantly 1 day following CFA, to 40% of baseline (Fig. 2C; \(P < 0.05\)) and recovered to pre-CFA baseline by day 3 (Fig. 2B; \(P < 0.01\)). In contrast, the mechanical threshold in DTX-treated Mrgprd\(^{DTR}\) mice at 1 day after CFA treatment was only reduced to 80% of pre-CFA values. Thus, Mrgprd \(^{+}\) neurons...
are required for full expression of mechanical hypersensitivity after tissue injury.

**Behavioral Responses to Noxious Heat and Cold Stimuli Are Normal in DTX-Treated Mrgrpdr mice.** Strikingly, DTX-treated MrgrpdrDTR mice exhibited no deficits in their behavioral sensitivity to noxious heat, as scored by tail withdrawal latency from a hot-water bath (Fig. 2D), latency to exhibit evidence of discomfort (paw shaking and licking) on a hot plate (Fig. 2E), or latency to paw withdrawal from radiant heat (Fig. 2F, BL). Following CFA injection, DTX-treated MrgrpdrDTR and WT mice developed equivalent heat hypersensitivity (Fig. 2F). Thus, Mrgrpdr+ neurons are dispensable for baseline heat pain sensitivity as well as for CFA-induced sensitization to heat pain in vivo.

We next evaluated cold sensitivity in the DTX-treated MrgrpdrDTR mice. In a test of temperature preference between a 32 °C chamber and a chamber at variable cold temperatures (20 to 5 °C), both DTX-treated WT and MrgrpdrDTR mice preferred the 32 °C chamber (Fig. S4A). Furthermore, DTX-treated WT and MrgrpdrDTR mice exhibited identical paw withdrawal latencies on a −5 °C plate (Fig. S4B). Taken together, these results indicate that Mrgrpdr+ neurons are not required for normal cold sensitivity.

The lack of a requirement for Mrgrpdr+ neurons in behavioral responses to noxious heat was surprising, given their polymodal properties. To investigate whether ablation of Mrgrpdr+ neurons might be compensated for by other heat-sensitive nociceptor populations, we sought to ablate a known heat-sensitive C-fiber population, while sparing Mrgrpdr+ neurons. We therefore focused on the large population of TRPV1+ nociceptors (20). Previous studies indicated that pharmacological ablation of TRPV1+ afferents causes at least a partial loss of heat pain sensitivity (21–23). Furthermore, less than 10% of Mrgrpdr+ neurons express TRPV1 in vivo (2, 14), and no more than 10% respond to the TRPV1 agonist capsaicin in vitro (17). TRPV1+ and Mrgrpdr+ neurons are therefore largely nonoverlapping populations.

**Ablation of the Central Terminals of TRPV1+ Afferents.** Because TRPV1 is also expressed in cells outside of the DRG (24), we could not use the DTR strategy to eliminate TRPV1+ nociceptors selectively. Instead, we exploited the fact that high doses of capsaicin destroy TRPV1+ fibers. Intrathecal injection of capsaicin eliminated TRPV1+ afferent fibers in the lumbar spinal cord (Fig. 3A and E); however, TRPV1 staining of DRG cell bodies was preserved (Fig. S5A–D). Importantly, capsaicin injection also eliminated retrograde transport of Fluorogold from the spinal cord to the cell bodies of TRPV1+ neurons (Fig. S5A–C), indicating that the loss of TRPV1 fiber staining reflected destruction of the central terminals of the TRPV1+ nociceptors and not simply downregulation of TRPV1 expression.

The pharmacological ablation of TRPV1+ afferents spared the Mrgrpdr+ afferent population, as revealed by the preserved expression of a GFP reporter included in the DTR targeting cassette (Figs. 1A and 3F). Consistent with this, IB4 binding was unchanged (Fig. 3C and E). In contrast, there was a significant reduction in the expression of CGRP, which is found in many TRPV1+ neurons, to 54.5 ± 5.0% of that of vehicle-treated controls ($P = 0.0004; \text{Fig. 3B and E}$). Immunoreactivity for other markers of TRPV1+ afferents was also reduced in the spinal cord [i.e., the 5HT1D subtype of serotonin receptor (25) and the water channel aquaporin 1 (26); Fig. S6A, B, and E]. Importantly, we found no change in immunoreactivity for the substance P receptor (NK1), a marker of spinal cord lamina I projection neurons that are postsynaptic targets of TRPV1+ afferents, or for calbindin, which marks a large population of spinal cord interneurons (Fig. S6C–E). These observations indicate that capsaicin treatment did not produce a generalized neurotoxic effect in the spinal cord.

**Intrathecal Capsaicin Treatment Eliminates Behavioral Responses to Noxious Heat.** Capsaicin-treated mice showed a complete and prolonged behavioral insensitivity to heat (Fig. 4A and B). When tested on a 55 °C hot plate, vehicle-treated mice licked their hind paw with a latency of 11.8 ± 0.8 s, whereas capsaicin-treated mice were unresponsive up to the 30-s cutoff ($P < 0.0001$). Consistent with these behavioral data, capsaicin treatment eliminated induction of Fos, a marker of neuronal activity, in the dorsal horn of the spinal cord following hind paw exposure to a 55 °C stimulus (Fig. 4E and F). Capsaicin-treated mice also showed no withdrawal of
the hind paw in response to radiant heating (P<0.0001, Student’s t test) (A) and at weekly intervals after injection of cap (n = 4) (B). (C) CFA-induced heat hypersensitivity (**, P < 0.01, P < 0.0001, two-way repeated measures ANOVA with Bonferroni posttest). (D) Temperature preference assay (**, P < 0.01, Student’s t test). (E) Fos-induced hind paw immersion in 55 °C water bath. (F) Fos+ neurons in laminae I/II and V per 40-μm section (**, P < 0.01, Student’s t test; n = 3 per group). (G) Mechanical threshold (von Frey test). (H) CFA-induced mechanical hypersensitivity (P<0.0001, two-way repeated measures ANOVA with Bonferroni posttest). Data represent mean ± SEM. Unless otherwise noted, n = 6–10.

TRPV1+ fiber ablation also affected both the induction and maintenance of behavioral heat hypersensitivity following CFA injection. In vehicle-treated mice, CFA injection produced a profound heat hypersensitivity that returned to baseline over the course of 3 days. In contrast, mice pretreated with capsaicin 1 week before CFA injection were not only unresponsive to heat before the inflammation was induced but showed no change in sensitivity following CFA injection (Fig. 4C). Even when mice were treated with capsaicin 1 day after CFA injection, they completely lost sensitivity to noxious heat (P<0.0001). Thus, ablation of TRPV1+ fibers abolishes all heat pain sensitivity under normal conditions and in the setting of injury.

The complete loss of heat responsiveness following intrathecal capsaicin indicates that Mrgprd+ neurons are unable to compensate for the absence of TRPV1+ afferents. These results also indicate that other heat-sensitive nociceptors, such as those that express the capsaicin-insensitive heat channel TRPV2 (27, 28), cannot compensate either. Capsaicin-treatment caused no change in TRPV2 staining (P = 0.21; Fig. 3D and E), confirming that TRPV2+ neurons are spared by this manipulation.

Behavioral Responses to Mechanical and Cold Stimuli Are Normal in Capsaicin-Treated Mice. Capsaicin treatment did not influence behavioral responses to either mechanical or cold stimuli. The mechanical withdrawal threshold in capsaicin-treated mice did not differ from that of vehicle-treated mice (P = 0.47; Fig. 4G). Furthermore, CFA-induced mechanical hypersensitivity persisted in mice treated with capsaicin either before or after CFA injection (Fig. 4H). Although treatment with capsaicin before CFA injection caused a slight reduction in mechanical hypersensitivity at 1 day after CFA injection (Fig. 4H; P = 0.0001), treatment with capsaicin after CFA injection had no effect on mechanical hypersensitivity. Thus, TRPV1+ afferents are not required for mechanical hypersensitivity following injury but may facilitate the initial development of hypersensitivity, perhaps by modulating other populations of mechanoreceptive neurons.

Capsaicin-treated mice also exhibited normal cold pain sensitivity, as assessed by hind paw withdrawal latency on a −5 °C plate (Fig. 5C) and discrimination between 30 and 20 °C in the temperature preference test (Fig. 4D). Thus, the perception of both moderate and intense cold as aversive/painful is preserved in the absence of input carried by TRPV1+ nociceptors. Because the TRPA1 channel is found in a subset of TRPV1 afferents (29), this result is consistent with our conclusion that TRPA1 is not required for acute cold-evoked pain behavior (30).

Combined Ablation of Mrgprd and TRPV1-Expressing Nociceptors Does Not Produce Further Deficits. TRPV1+ afferents were not sufficient to support full mechanical sensitivity in the absence of Mrgprd+ neurons. Nevertheless, the partial loss of mechanical pain sensitivity in DTX-treated MrgprdDTR mice left open the possibility that TRPV1+ afferents contribute to the residual mechanical sensitivity in these mice. To address this possibility, we compared mechanical sensitivity in DTX-treated MrgprdDTR mice before and after ablation of TRPV1+ afferents.

Importantly, capsaicin treatment in mice lacking Mrgprd+ neurons produced no further decrease in mechanosensitivity compared with that observed before capsaicin treatment (Fig. 5B; 1.05 ± 0.38 g before and 0.87 ± 0.22 g after capsaicin treatment; P = 0.315). As expected, the heat sensitivity of DTX-treated MrgprdDTR mice was fully eliminated following capsaicin treatment (Fig. 5A; P < 0.001), confirming the efficacy of TRPV1+ fiber ablation. Thus, TRPV1+ neurons do not contribute to the residual mechanical pain sensitivity in MrgprdDTR/DTX mice. This residual sensitivity must therefore reflect a contribution of the remaining TRPV1- and Mrgprd-cutaneous C fibers and/or of myelinated afferents (e.g., high-threshold mechanosensitive Aδ fibers).

Discussion

Genetic approaches to nociception have focused primarily on identifying individual molecules that transduce painful stimuli. However, the discrimination of different pain modalities by the brain depends not only on which molecules are activated but on which neurons are activated. Primary afferent nociceptors are heterogeneous; therefore, an understanding of the behavioral function of different subsets of these neurons is essential to deciphering the logic by which different types of painful stimuli are sensed and encoded. Here, we selectively ablated 2 nonoverlapping populations of nociceptors and observed a double dissociation between noxious mechanical and heat pain sensitivity. These data suggest that behavioral discrimination between different pain modalities can occur at the earliest stages of sensory processing.

A caveat is that our behavioral observations are constrained by the cut-off and intensity limitations that are necessary to prevent tissue damage. Therefore, we cannot exclude that these nociceptor classes contribute to behavioral responses to both heat and mechanical stimuli, but only at stimulus intensities greater than those we tested. Furthermore, because mechanical pain sensitivity is only partially reduced in mice lacking Mrgprd+ neurons, our results do not rule out the existence of Mrgprd- subpopulations of mechanosensitive nociceptors that also mediate behavioral responses to other noxious stimulus modalities.
Our data indicate that most IB4+ neurons, caused profound deficits in basal mechanical and cold pain sensitivity as well as inflammatory pain deficits. In contrast, ablation of Mrgprd+ neurons (which constitute \( \approx 90\% \) of IB4+ cutaneous afferents) impaired mechanical but not cold pain sensitivity. It is likely that elimination of Nav1.8+ DRG neurons leads to changes in the behavioral response to multiple modalities, because this channel is present in \( >85\% \) of small-diameter neurons, including both Mrgprd+ and TRPV1+ neurons. By contrast, the manipulations used in the present study target more specific populations of neurons and reveal more specific phenotypes.

**TRPV1+ Neurons Are Essential for Heat-Pain Sensitivity.** Ablation of TRPV1+ central afferent fibers by capsaicin injection abolished heat-pain sensitivity, without affecting the responses to noxious mechanical or cold stimulation. Previous studies using similar approaches reported less complete deficits in heat pain sensitivity than we observed here, but the effects were also modality specific (21–23, 33, 34). The partial behavioral deficits observed in earlier studies using capsaicin or resiniferatoxin treatment likely reflected incomplete ablation of the TRPV1+ afferent fibers, because these studies were performed before the availability of reagents to monitor TRPV1 expression.

It is surprising, given our results, that Abrahamsen et al. (10) observed only subtle deficits in heat pain sensitivity following ablation of Nav1.8+ neurons, because this manipulation destroyed a large fraction of TRPV1+ neurons. This may reflect compensation by the small fraction of TRPV1+ neurons that were spared. Alternatively, the fact that Nav1.8+ neurons were constitutively ablated from the embryonic stage at which this gene is first transcribed (10) could permit compensation by other neuronal populations during development and maturation, as we observed following constitutive ablation of Mrgprd+ neurons (Fig. S2 and Fig. S3). The complete heat pain insensitivity of mice lacking central TRPV1+ fibers contrasts with the phenotype of Trpv1 knockout mice, which exhibit only a partial reduction in heat sensitivity (35, 36). The residual heat pain behavior in these gene knockout mice must therefore reflect the existence of additional molecular heat transducers that act, either cell autonomously or nonautonomously, via TRPV1+ neurons.

**Physiology vs. Behavior.** How do we reconcile our behavioral observations with the fact that the majority of C fibers are polymodal by electrophysiological criteria (6)? It is possible that the response properties of these nociceptors, as determined by ex vivo electrophysiological recordings, differ from those exhibited by these neurons in vivo. Importantly perhaps, recordings from identified Mrgprd+ and TRPV1+ afferents have been performed on hairy skin, whereas our behavioral assays are performed using stimuli applied to glabrous skin. Conceivably, the properties of these C-fibers in glabrous skin more closely correlate with our behavioral finding of modality specificity. Alternatively, Mrgprd+ and TRPV1+ neurons may indeed be activated by both heat and mechanical stimuli in vivo, but these 2 types of stimulus modalities may evoke different spiking patterns within each class of nociceptors (e.g., ref. 37). If so, perhaps only a single “preferred” modality is able to activate each nociceptor subtype to a level sufficient to drive second-order spinal cord neurons above a threshold required to evoke nociceptive behavior. It is also possible that these nociceptor subtypes convey polymodal information to the spinal cord but that this information contributes to aspects of the pain experience that are not measurable by the behavioral assays we used. Electrophysiological recordings from spinal cord neurons of mice
lacking Mrgrp 

TRPV1 and MRgrp 

TRPV1 affere nts should help to resolve these questions. Finally, although polymodal afferents are putative ATP-sensing nociceptors. Whether these pathways exclusively mediate mechanical vs. heat pain discrimination or have additional functions is not clear. Whatever the case, our data suggest that this discrimination can be achieved at the earliest stages of nociceptive sensor y processing and does not, as previously believed, exclusively emerge at spinal or supraspinal levels. Thus, as in the mammalian (38) and invertebrate (39) gustatory systems, the cellular logic of information processing in the “pain” system incorporates distinct subsets of primary sensory cells that selectively mediate appropriate behavioral responses to different stimulus modalities.

Experimental Procedures

Animals and Injections. Animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the recommendations of the International Association for the Study of Pain. Two to 5 animals were housed per cage and maintained on a 12-h light/dark schedule with ad lib access to food and DTX (100 μg/kg) was injected i.p. on 2 days, separated by 72 h. Behavioral tests were performed 7 to 31 days after the initial DTX injection. For intrathecal capsaicin studies, adult male C57Bl6 mice (20–30 g; Charles River) were anesthetized with 1.5% isoflurane (vol/vol) and injected with DTX injection. For intrathecal capsaicin studies, adult male C57Bl6 mice (20–30 g; Charles River) were anesthetized with 1.5% isoflurane (vol/vol) and injected with DTA (100:10043–10048).

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Statistical Analysis. Behavioral, densitometry, and Fos data were analyzed by the Student’s t test, one- and two-way repeated measures ANOVA (Bonferroni posttest), or the Mann-Whitney U test, with P < 0.05 considered to be significant.