Mrgprd<sup>Cre</sup> lineage neurons mediate optogenetic allodynia through an emergent polysynaptic circuit

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Abstract

Most cutaneous C fibers, including both peptidergic and nonpeptidergic subtypes, are presumed to be nociceptors and respond to noxious input in a graded manner. However, mechanically sensitive, nonpeptidergic C fibers also respond to mechanical input in the innocuous range, so the degree to which they contribute to nociception remains unclear. To address this gap, we investigated the function of nonpeptidergic afferents using the Mrgprd<sup>Cre</sup> allele. In real-time place aversion studies, we found that low-frequency optogenetic activation of Mrgprd<sup>Cre</sup> lineage neurons was not aversive in naive mice but became aversive after spared nerve injury (SNI). To address the underlying mechanisms of this allodynia, we recorded responses from lamina I spinoparabrachial (SPB) neurons using the semi-intact ex vivo preparation. After SNI, innocuous brushing of the skin gave rise to abnormal activity in lamina I SPB neurons, consisting of an increase in the proportion of recorded neurons that responded with excitatory postsynaptic potentials or action potentials. This increase was likely due, at least in part, to an increase in the proportion of lamina I SPB neurons that received input on optogenetic activation of Mrgprd<sup>Cre</sup> lineage neurons. Intriguingly, in SPB neurons, there was a significant increase in the excitatory postsynaptic current latency from Mrgprd<sup>Cre</sup> lineage input after SNI, consistent with the possibility that the greater activation post-SNI could be due to the recruitment of a new polysynaptic circuit. Together, our findings suggest that Mrgprd<sup>Cre</sup> lineage neurons can provide mechanical input to the dorsal horn that is nonnoxious before injury but becomes noxious afterwards because of the engagement of a previously silent polysynaptic circuit in the dorsal horn.

Keywords: Mrgd, SNI, Electrophysiology, Optogenetics, Dorsal horn, Spinoparabrachial, IB4, Pain, Allodynia

1. Introduction

Traditionally, cutaneous C fibers have been categorized into 2 major classes: peptidergic afferents that generally express substance P or calcitonin gene related peptide (CGRP), as well as the so-called nonpeptidergic afferents that express Mrgprd or bind the isolectin B4 (IB4).<sup>3,4</sup> Because both populations respond vigorously to noxious stimuli, it was generally assumed that both types function mainly as nociceptors. Consistent with this idea, ablation studies have suggested that peptidergic afferents are responsible for thermal pain, whereas nonpeptidergic afferents are responsible for mechanical pain.<sup>1,2</sup> Moreover, because these nociceptive-responsive neurons collectively make up most of C fibers, it was not uncommon to generally equate C-fiber activity with nociception and, by extension, pain.

However, other findings have challenged the presumption that these populations are exclusively pain-inducing nociceptors. In particular, it remains unclear whether the nonpeptidergic subset of IB4-binding afferents signal nociception or whether their function is more nuanced. For instance, although nonpeptidergic, IB4-binding neurons respond to noxious stimuli, they typically show a graded response over a wide range of mechanical forces and, indeed, have the capacity to detect even low-threshold input that is clearly nonnoxious.<sup>23</sup> In support of this idea, human microneurography studies have shown that most mechanically sensitive C fibers are activated by stimulus intensities that are reported as nonpainful.<sup>12,29</sup> Finally, although transient optogenetic activation of Mrgprd neurons in mice was found to cause withdrawal, prolonged exposure was not sufficient to elicit conditioned place aversion.<sup>7</sup> Thus, the degree to which activity in the nonpeptidergic, IB4-binding population is sufficient to drive aversion or pain remains ambiguous.

A second major gap in our understanding is the role of nonpeptidergic afferents in chronic pain. Injuries that give rise to chronic pain, such as spared nerve injury (SNI), cause central changes that alter the way sensory information is integrated by the nervous system.<sup>2,26</sup> One of the most common consequences of these injury-induced changes is allodynia: a phenomenon in
which innocuous mechanical stimuli, such as light brushing of the skin, are perceived as noxious. It is generally assumed that the afferents responsible for allodynia are those that are most responsive to innocuous mechanical input, i.e., low-threshold mechanoreceptors (LTMRs). However, many nonpeptidergic, IB4-binding neurons frequently respond vigorously to innocuous stimuli, such as brushing, raising the possibility of their involvement in allodynia. Thus, there remains significant uncertainty about both theafferent subtype(s) and the spinal circuits downstream thereof that give rise to allodynia.

MrgprdGt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze lineage neurons mediate optogenetic allodynia through an emergent polysynaptic circuit, which is not averse in naive mice. However, in the context of SNI-induced neuropathic pain, activation of MrgprdGt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze lineage neurons became averse. We subsequently show that SNI increases the proportion of lamina I spinoparabrachial (SPB) neurons that respond to innocuous brush input and that this increase is likely to be mediated, at least in part, by the activity of MrgprdGt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze lineage neurons, which seem to gain access to lamina I SPB neurons by way of an emergent polysynaptic circuit after SNI.

2. Methods

2.1. Animals

The mice used in these experiments were MrgprdGt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze, which were obtained from the Mutant Mouse Resource & Research Center at Chapel Hill (Stock No: 036118-UNC); Trpv1tm1(cre)Bom, which were obtained from Jax labs (Bar Harbor, ME) (Stock No: 017769); and Gt(ROSA)26SorCAG-CreERT2(CAG-IVYFP)NvoGtm1.1(cre)An, also known as Ai32 mice, which were obtained from Jax labs (Stock No: 024109). Additional crosses were made to obtain MrgprdGt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze; Ai32 and Trpv1tm1(cre); Ai32 mice for these experiments. Mice were given free access to food and water and housed under standard laboratory conditions. All experiments were performed using approximately equal number of male and female animals. We did not observe any trends that were suggestive of an effect of sex, so data were pooled; however, we acknowledge that we were not powered to look at sex differences. The use of animals was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.2. Ex vivo preparation

The ex vivo somatosensory system preparation has been previously described in detail. In brief, adult mice (aged >4 weeks) were anesthetized with a mixture of ketamine and xylazine (90 and 10 mg/kg, respectively) and perfused transcardially with room temperature, oxygenated (95% O2–5% CO2) potassium acetate). An electric search stimulus was applied at 1.5 Hz through a glass suction electrode applied to the saphenous nerve but had no mechanical RF, a thermal search was performed by gently applying hot (52˚C) and cold (0˚C) saline to the surface of skin using a syringe with a 20-gauge needle. If a thermal RF was located, the absence of mechanical sensitivity was confirmed by searching the identified RF using a glass probe. In most cases, the response characteristics of the DRG cell were then determined by applying computer-controlled mechanical and thermal stimuli. The mechanical stimulator consisted of a constant-force controller (Aurora Scientific, Aurora, ON, Canada) attached to a 1-mm diameter plastic disk. Computer-controlled 5-second square waves of 5, 10, 25, 50, and 100 mN were applied to the RF of the cell. After the functional characterization, responsiveness to laser stimulation was determined using an 80-mW, 473-nm wavelength laser and a 200-μm fiber optic cable affixed to a micromanipulator (Laserglow Technologies, Toronto, ON, Canada). The opening of the fiber optic cable was positioned approximately 5 mm from the skin surface.

2.3. Dorsal root ganglia recordings and peripheral stimuli

Intracellular recordings from L3 DRG cells in the chamber were made using quartz microelectrodes (>100 MΩ filled with 1 mol/L potassium acetate). An electric search stimulus was applied at 1.5 Hz through a glass suction electrode applied to the saphenous nerve to locate cells with axons in the saphenous nerve. Cutaneous receptive fields (RFs) were located with a fine paint brush, blunt glass probe, and von Frey hairs. When cells were driven by the nerve but had no mechanical RF, a thermal search was performed by gently applying hot (52˚C) and cold (0˚C) saline to the surface of skin using a syringe with a 20-gauge needle. If a thermal RF was located, the absence of mechanical sensitivity was confirmed by searching the identified RF using a glass probe. In most cases, the response characteristics of the DRG cell were then determined by applying computer-controlled mechanical and thermal stimuli. The mechanical stimulator consisted of a constant-force controller (Aurora Scientific, Aurora, ON, Canada) attached to a 1-mm diameter plastic disk. Computer-controlled 5-second square waves of 5, 10, 25, 50, and 100 mN were applied to the RF of the cell. After the functional characterization, responsiveness to laser stimulation was determined using an 80-mW, 473-nm wavelength laser and a 200-μm fiber optic cable affixed to a micromanipulator (Laserglow Technologies, Toronto, ON, Canada). The opening of the fiber optic cable was positioned approximately 5 mm from the skin surface.

2.4. Parabrachial injections

Four- to 6-week-old mice were anesthetized with isoflurane and placed in a stereotaxic apparatus. An incision was made to expose the bone, and a small hole was made in the skull with a dental drill. A glass pipette was used to inject 100 nL of FAST DiI oil (2.5 mg/mL; Invitrogen, Carlsbad, CA) into the left lateral parabrachial area at the following coordinates: from lambda, 1.3 mm lateral; from lambdoid suture, 0.5 mm posterior; and from surface, –2.4 mm. The incision was closed with sutures, and the mice were allowed to recover and returned to their home cages. These injections were made at least 5 days before electrophysiological recordings.

2.5. Whole-cell spinal neuron recordings

Neurons were visualized using a fixed-stage upright Olympus microscope equipped with a 40x water immersion objective, a CCD camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu City, Japan), and a monitor. A narrow-beam infrared light-emitting diode (LED) (L850D-06; Marubeni, Tokyo, Japan, emission peak, 850 nm) was positioned outside the bath, as previously described. Projection neurons in lamina I were identified by DiI fluorescence after retrograde labeling in the parabrachial nucleus. Whole-cell patch clamp recordings were made using borosilicate glass microelectrodes pulled using a PC-10 puller (Narishige International, East Meadow, NY). Pipette resistances ranged from 6 to 12 MΩ. Electrodes were filled with a solution containing the following (in mM): 135 potassium gluconate, 5 KCl, 0.5 CaCl2, 5 EGTA, 5 HEPES, and 5 MgATP; pH 7.2. Alexa Fluor 488 (Invitrogen; 25 mM) was added to confirm recording from the targeted cell. Recordings were acquired using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The data were low-pass filtered at 2 kHz and digitized at 10 kHz using a Digidata 1322A (Molecular Devices) and stored using Clampex version 10 (Molecular Devices).
2.6. Cutaneous stimulation

The cell’s receptive field was determined using a paint brush and 1- to 4-g von Frey filaments. Once the cell’s receptive field was located, subsequent stimuli were applied directly to the receptive field for 1 second to determine the cell’s response properties. Mini Analysis (Synapsoft, Decatur, GA) was used for detecting excitatory postsynaptic currents (EPSCs) and action potentials (APs).

2.7. Cell dissociation and pickup for reverse transcription polymerase chain reaction

Dorsal root ganglia—containing labeled cells from Mrgprd<sup>Cre</sup>; Ai32 mice were removed and dissociated as previously described. In brief, DRG were treated with papain (30 U) followed by collagenase CLS2 (10 U) or Dispase type II (7 U), centrifuged (1 minute at 1000 r/min), triturated in minimal essential medium, plated onto laminin-coated coverslips in 30-mm diameter dishes, and incubated at 37°C for 45 minutes. Dishes were removed and flooded with collection buffer (140 mM NaCl, 10 mM glucose, 10 mM HEPES, 5 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂). Single, labeled cells were identified using fluorescence microscopy, picked up using glass capillaries (World Precision Instruments, Sarasota, FL) held by a 4-axis micromanipulator under bright-field optics, and transferred to tubes containing 3 μL of lysis buffer (Epicentre, MessageBOOSTER kit). Cells were collected within 1 hour of removal from the incubator and within 4 hours of removal from the animals.

2.8. Fluorescence in situ hybridization

Mice were anesthetized with isoflurane and rapidly decapitated. The ipsilateral L2 and L3 DRG of mice were removed within 7 minutes, placed into optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura, Torrance, CA), and stored at −80°C. Samples were removed from the OCT cryostat and cryosectioned. Dorsal root ganglia samples were fixed for 15 minutes in ice-cold 4% paraformaldehyde and dehydrated with ethanol. Sections were then treated with protease IV for 15 minutes at room temperature. Probes for Sst, Mrgpra3, Mrgprd, and Sst were prepared according to the protocol for fresh frozen sections. A positive cell was defined as a cell with a clearly defined nucleus and fluorescent signal forming a ring around the nucleus. For determining the absolute number of Mrgprd<sup>Cre</sup> neurons in naive or SNI animals, the area of each counted DRG section was calculated using ImageJ (National Institutes of Health), and the number of cells per square area was then calculated for each slice. Once all image acquisition, counting, and analysis were completed, the treatment groups and probe identity were unblinded.

2.9. RNAscope imaging and quantification

All DRG sections were imaged and analyzed blinded to both treatment and probe identity. Dorsal root ganglia sections were imaged on an upright epifluorescence microscope (Olympus BX63) at ×20 magnification. Quantification of probes was performed by manual counting of positive cells in 4 to 5 DRG slices per mouse for each combination of probes. A positive cell was defined as a cell with a clearly defined nucleus and fluorescent signal forming a ring around the nucleus. For determining the absolute number of Mrgprd<sup>Cre</sup> neurons in naive or SNI animals, the area of each counted DRG section was calculated using ImageJ (National Institutes of Health), and the number of cells per square area was then calculated for each slice. Once all image acquisition, counting, and analysis were completed, the treatment groups and probe identity were unblinded.

2.10. Single-cell amplification and quantitative polymerase chain reaction

The RNA isolated from each cell was reverse transcribed and amplified using T7 linear amplification (Epicentre, MessageBOOSTER kit for cell lysate), run through RNA Clean & Concentrator-5 columns (Zymo Research, Irvine, CA), and analyzed using quantitative polymerase chain reaction, as previously described, using optimized primers and SsoAdvanced SYBR Green Master Mix (Bio-Rad, Hercules, CA). Threshold cycle time (Ct) values were determined for each well.

2.11. Real-time place aversion

Behavioral boxes were constructed from plexiglass that were 12 (l) × 5 (w) × 10 (h) inches with guides for dividing the boxes into 3 chambers: 2 chambers measuring 5 × 5 inches and 1 measuring 2 × 5 inches in the middle. For optical stimulation, we used blue LED strip light, 120/20, 10 mm wide, by the 5 m reel, 12 VDC, 8.9 W/m, 742 mA/m (centered at 460 nM) and amber LED strip light, 120/20, 10 mm wide, by the 5 m reel, 12 VDC, 8.9 W/m, 742 mA/m (centered at 595 nM) (Environmental Lights, San Diego, CA). Five-inch strips of LEDs were affixed to a 5- × 12-inch metal box (heat sink) covering a 5- × 5-inch area on the ends of the box (one area blue and the other amber), leaving a 2-inch area in the middle free of lights. This box could then be placed under the plexiglass box to illuminate the floor. The LEDs were controlled using a CED 1401 interface. The LEDs were positioned 10 mm below the surface of the plexiglass floor because we found that at this distance, there was no heating of the plexiglass floor over the 15-minute period of observation.

Mice used in the real-time place aversion (RTPA) were first acclimated to the behavioral boxes for 3 days (15 minutes/day) before testing. On the day of testing, the mice were placed in the center compartment and the LEDs turned on and the sliding doors removed, so the mice could move freely throughout the box for 15 minutes. The mice were recorded during this period using a video camera. The videos were then scored offline. All experimenters were blinded to genotype and experimental condition of the mice.

2.12. Spared nerve injury

Mice were anesthetized with isoflurane, and the posterior right hind limb was shaved and cleaned using an aseptic solution. An incision was made over the popliteal fossa and the underlying muscle incised to expose the fossa. The tibial and peroneal nerves were isolated. The 2 nerves were tightly ligated and cut just distal to the ligature. The muscle and skin were sutured and the animals allowed to recover. For sham surgeries, the procedures were the same except for the ligation and transection. Electro-physiological recordings and FISH experiments were performed 1 week after surgery.
2.13. Immunohistochemistry

Deeply anesthetized mice were perfused with 4% paraformaldehyde, and tissue sections of the L3 spinal cord, DRG, and glabrous skin were embedded in OCT and cut on a cryostat at 20 μm. The sections were stained for IB4, CGRP, and eYFP using the following antibodies or probes: anti-GFP (chicken 1:1250; Aves Labs, Tigard, OR), anti-CGRPh (rabbit 1:1000; Chemicon, Temecula, CA), and IB4 (1:250; IB4-conjugated Alexa Fluor 647; Molecular Probes, Eugene, OR). After incubation in primary antiserum for at least 2 hours, tissue was washed and incubated in appropriate fluorescently tagged secondary antibodies (1:500; Jackson ImmunoResearch) for 1 hour, followed by Hoechst staining (1:10,000) for 30 minutes. After washing and mounting in Fluoromount (Sigma), sections were viewed and imaged on an Olympus BX53 fluorescence microscope with UPanSApo 10x or 20x objectives.

2.14. Statistical analyses

Data are expressed as the mean ± SEM or as a Superplot18 that shows both the technical replicates and mean value for each biological replicate. For electrophysiological experiments, dots represent data points from individual neurons combined from all animals. The following tests were used for statistical analyses: Student t test for continuous normal data (comparison of 2 groups), χ² test or Fisher exact test for categorical data, ordinary 1-way analysis of variance (ANOVA) with Tukey multiple comparisons post hoc test if indicated by the main effect (comparison of >2 groups), 2-way repeated measures ANOVA with Tukey multiple comparisons post hoc test if indicated by the main effect (time course with comparison of 2 or more groups), or a 1-way ANOVA with Šidák multiple comparisons post hoc. All tests were 2 tailed (unless otherwise indicated), and a value of P < 0.05 was considered statistically significant in all cases. All statistical analyses were performed using GraphPad Prism 9 software.

3. Results

3.1. Histological and molecular characterization of MrgprdCre lineage neurons

To investigate the functional role of IB4-binding neurons, we targeted these neurons genetically using the MrgprdCre knockin allele that was originally developed by David Anderson.23 Because Mrgprd shows some developmental expression,17 we carefully characterized the neurons that are captured by this genetic tool, as assessed using Ai32, a Cre-dependent reporter carefully characterized the neurons that are captured by this allele that was originally developed by David Anderson.23

A number of recent single-cell sequencing studies have suggested that nonpeptidergic afferents can be further subdivided into more refined subtypes characterized by expression of Mrgprd, Mrgpra3, or Sst/Nppb.22,25,28 To assess whether the MrgprdCre lineage captures these populations, we performed multiplex FISH of lumbar DRG from Mrgpra3Cre;Ai32 mice (Figs. 1N–P). These experiments revealed that Mrgprd-expressing afferents make up 77% of Eyfp-labeled neurons, whereas Mrgpra3-expressing neurons make up 17% and Sst-expressing neurons make up 7% (Fig. 1Q). Together, these 3 populations accounted for 99% of Eyfp-labeled cells. Virtually, all Mrgprd-expressing neurons expressed Eyfp. By contrast, only about half of the Mrgpra3 and Sst populations were recombined with the MrgprdCre allele. To complement the FISH experiments, we also performed quantitative real-time PCR of individual eYFP-labeled DRG neurons that were freshly dissociated from adult Mrgpra3Cre;Ai32 mice. These experiments similarly revealed that all genetically marked neurons fell into 1 of 3 apparent cell types: those with high Mrgprd (and lacking Mrgpra3, Sst, Calca, and Tac1); those with high levels of Mrgpra3 (as well as Calca but little or no Mrgprd, Sst, and Tac1); and those with high levels of Sst (and low levels of Mrgpra3 and Tac1 but lacking Mrgprd and Calca). Importantly, this single-cell analysis of eYFP-labeled cells confirmed that Mrgprd is no longer detected in the Mrgpra3 or Sst populations in the adult, suggestive of developmental expression (Fig. 1R). Thus, the Mrgpra3Cre lineage faithfully captures most afferents that have traditionally been termed “nonpeptidergic” nociceptors, which include most IB4-binding neurons, but not peptidergic nociceptors, C-LTMRs, or Trpm8-expressing cold or cool detectors. For purposes of comparison, we also visualized the recombination mediated by the Trpv1Cre allele. Consistent with previous reports,5 we observed that Trpv1Cre lineage neurons include most cutaneous C fibers, although not C-LTMRs or Trpm8-expressing afferents (shown in Fig. 1S).

3.2. Optogenetic manipulation of Mrgpra3Cre

Lineage neurons We next characterized the responses of Mrgpra3Cre lineage neurons to optogenetic stimulation of the skin with Mrgpra3Cre;Ai32 mice and the ex vivo preparation, performing intracellular recordings of L3 DRG neurons (Fig. 2A). Afferents were categorized as cutaneous C fibers if they responded to electrical stimulation of the saphenous nerve with a conduction velocity of 1.2 m/second or less. Next, the receptive field of a given cutaneous C fiber was identified manually through application of mechanical or thermal stimuli. Thereafter, quantitative phenotyping to natural and optogenetic stimuli was performed using a Peltier thermode, a mechanical stimulator, and a 473-nm laser applied to the skin within the identified receptive field. Typically, Mrgpra3Cre lineage neurons responded weakly to cooling and vigorously to noxious heat; they also responded to both low-threshold (10 mN) and high-threshold (50 mN) mechanical inputs in a graded manner, consistent with previous characterizations of nonpeptidergic nociceptors.15,23 Both optogenetic and natural stimulation of the skin elicited APs (Figs. 2B and C). We found that a 5-ms pulse of light was sufficient to drive a single action potential, whereas a 300-ms pulse of light was required to observe a doublet. Finally, Mrgpra3Cre lineage neurons could follow optogenetic stimulation of at least 5 Hz (Fig. 2C).

Overall, of 21 cutaneous C fibers that were recorded from Mrgpra3Cre;Ai32 mice, 15 showed responses to optogenetic stimulation (~70%), consistent with the idea that Mrgpra3Cre lineage neurons represent most cutaneous C fibers. For comparison, we found that 11 of the 12 (~90%) cutaneous C fibers from Trpv1Cre;Ai32 mice could be opto-tagged in this...
Figure 1. Mrgrd<sup>Cre</sup> lineage afferents, which comprise Mrgrd, Mrgrpra3, and Sst populations, target lamina IIi in the dorsal horn and the superficial aspect of the skin. (A–E) Representative images of a lumbar dorsal root ganglion from a Mrgrd<sup>Cre</sup>; Ai32 mouse that is costained for eYFP and IB4 or CGRP. (F–J) Representative images of the superficial dorsal horn, lumbar level from a Mrgrd<sup>Cre</sup>; Ai32 mouse that is costained for eYFP and IB4 or CGRP. (K–M) Representative images of the glabrous skin from a Mrgrd<sup>Cre</sup>; Ai32 mouse that is costained for eYFP and PGP9.5. Dotted line demarcates the epidermal–dermal junction. (N–P) Representative RNA FISH images of lumbar dorsal root ganglion from a Mrgrd<sup>Cre</sup>; Ai32 mouse that is costained with probes against Eyfp, Mrgrd, Mrgrpra3, and Sst. (Q) The relative proportion of primary afferent populations captured by the Mrgrd<sup>Cre</sup> lineage as determined by FISH. Data are from 3 mice, with 4 to 5 slices imaged per mouse for a total of 692 Mrgrd<sup>Cre</sup> lineage neurons counted per probe combination. (R) Expression of Mrgrd, Mrgrpra3, and Sst mRNA relative to Gapdh in individual eYFP-marked Mrgrd<sup>Cre</sup> lineage neurons. Data are presented as the log<sub>2</sub> ΔCT expression relative to Gapdh expression within the same cell such that smaller numbers represent higher mRNA expression. Colored dots represent data points from individual cells from a single, representative mouse. (S) Schematic to illustrate the different subtypes of cutaneous C fibers that are captured by the Mrgrd<sup>Cre</sup> and Trpv1<sup>Cre</sup> alleles with developmental (lineage) or adult recombination. FISH, fluorescence in situ hybridization.
3.3. Behavioral responses to optogenetic manipulation of Mrgprd<sup>Cre</sup> lineage neurons

Withdrawal in animals has traditionally been interpreted to represent a pain behavior, but the percept associated with this response is difficult to infer. Previous work has shown that selective optogenetic activation of Mrgprd neurons elicits withdrawal, but not flinching, licking, or guarding. We anticipated that optogenetic stimulation of Mrgprd<sup>Cre</sup> lineage neurons would be at least as aversive, if not more so, because the genetic tool we used captures a larger number of C fibers than that used by Beaudry et al. Although both alleles target the same genetic locus, the degree of recombination using the Mrgprd<sup>Cre</sup> allele is more extensive than the Mrgprd<sup>CremER</sup> allele for 2 reasons: the constitutive Cre allele captures more than the adult Mrgprd population due to developmental expression (ie, Sst and Mrgpra3 populations), whereas the Mrgprd<sup>CremER</sup> allele likely captures less than the adult Mrgprd population due to incomplete recombination. Contrary to our expectations, however, we found that the responses of Mrgprd<sup>Cre</sup> mice to optogenetic stimulation of the hind paws appeared quite modest and not qualitatively different than those described for the Mrgprd<sup>CremER</sup> mice. Resting mice generally withdrew their hind paws for a brief moment, as if startled, and then replaced them on the floor, and alert mice rarely responded at all. By sharp contrast, activation of Trpv1<sup>Crem</sup> lineage neurons invariably caused rapid, robust withdrawal that was frequently accompanied by flinching or licking (Fig. 3A), just as previously described. These observations raised the possibility that low-frequency optogenetic activation of nonpeptidergic “nociceptors” might not actually be aversive.

Because reflexive behaviors can be hard to interpret and do not readily assess aversion, we turned to nonreflexive behaviors for quantitative analysis. When a stimulus is quite noxious, such as intraplantar formalin, mice will develop conditioned place aversion to the side of the chamber that is associated with the aversive experience. Intriguingly, Beaudry et al. showed that optogenetic activation of Trpv1<sup>Crem</sup> afferents was sufficient for conditioned place aversion, whereas activation of Mrgprd<sup>CremER</sup> afferents was not. However, conditioned place aversion can be difficult to achieve unless the paired stimulus is fairly injurious. We, therefore, developed an RTPA assay to enable the detection of aversive stimuli with higher sensitivity.

Our previous optogenetic characterization had been performed by laser, a coherent light source that is ideal for focal stimulation. However, for the RTPA studies, we were planning to use LEDs, an incoherent light source that is more suitable for widespread activation. To compare the efficacy of optogenetic stimulation by laser and LED, we performed ex vivo skin nerve recordings (Fig. 3B). Importantly, stimulation of the skin with either light source gave rise to a similar number of APs in ChR2-expressing Mrgprd<sup>Cre</sup> lineage neurons, indicating that optogenetic stimulation by laser or LED was equally effective at driving activity in ChR2-expressing cells (Fig. 3C). With this knowledge, we built a custom RTPA box comprising a stimulation side in which the floor was lined by an array of amber LED lights, which were separated by a small, unlit middle chamber. These lights were set to run in either the sustained mode, in which the lights were constantly on, or the wind-up mode, in which the lights would flash at 2 Hz for 30 ms per flash. Mice were placed in the RTPA box for 15 minutes, during which time they were allowed to move freely from 1 chamber to another (Fig. 3D).

![Figure 2. Response of Mrgprd<sup>Cre</sup> lineage afferents to optogenetic stimulation of the skin. (A) Schematic of cutaneous stimulation and intracellular DRG recordings using the ex vivo preparation. (B) Representative responses of Mrgprd<sup>Cre</sup> lineage afferents to cooling, heating, mechanical, and optogenetic stimulation. (C) Representative responses to optogenetic stimulation of Mrgprd<sup>Cre</sup> lineage afferents of varying duration and frequency. (D) Proportion of C fibers that respond to optogenetic stimulation of the skin in Mrgprd<sup>Cre</sup>; Ai32 and Trpv1<sup>Cre</sup>; Ai32 mice, as indicated. (E) Representative recording and quantification of the number of action potentials observed in a given cell on optogenetic stimulation of the skin in Mrgprd<sup>Cre</sup>; Ai32 and Trpv1<sup>Cre</sup>; Ai32 mice, as indicated. Data are mean ± SEM with dots representing data from individual neurons; n = 8 and 5 neurons, respectively, from at least 3 mice. N.S. indicates P > 0.05 (unpaired Student t test). DRG, dorsal root ganglia.](image-url)
Next, we performed RTPA assays to quantify the aversiveness of optogenetic stimulation. Just as expected, we found that Trpv1Cre<sup>AI32</sup> mice showed significant avoidance of the blue-light side, consistent with the idea that optogenetic activation of nociceptors is unpleasant. By sharp contrast, the time that naive mice spent on the blue-light side was not different than control littermates harboring AI32 alone (Fig. 3E). Similar findings were observed when the LED lights were flashing at 2 Hz to simulate wind-up (Fig. 3F). These striking observations suggest that optogenetic activation of MrgprdCre<sup>AI32</sup> fibers was not aversive, at least not in naive mice.

3.4. Effect of injury: behavioral responses to optogenetic manipulation of MrgprdCre<sup>lineage</sup> neurons in the context of neuropathic pain

Although our data suggested that MrgprdCre<sup>lineage</sup> neurons can be activated without causing acute pain, a key remaining question was whether they contribute to chronic pain. Allodynia is one of the hallmarks of chronic pain, particularly neuropathic pain. For people who experience allodynia, innocuous everyday experiences, such as fabric moving across the skin, can give rise to shooting pain. Because many nonpeptidergic afferents can respond robustly to dynamic low-threshold stimulation, we hypothesized that MrgprdCre<sup>lineage</sup> neurons might contribute to this allodynia. To address this question, we selected to use the SNI model of chronic neuropathic pain (Fig. 4A). Although the saphenous nerve remains intact in this model, there is, nevertheless, the potential for the function of afferents to be affected by the injury. We, therefore, measured the responses of MrgprdCre<sup>lineage</sup> afferents to optogenetic stimulation of the skin, comparing naive and SNI mice (Fig. 4B). Importantly, the number of APs that were elicited was not affected by SNI (Fig. 4C). Thus, neuropathic injury did not cause sensitization of MrgprdCre<sup>lineage</sup> afferents to optogenetic stimulation.

To address whether SNI altered how the input from MrgprdCre<sup>lineage</sup> lineages was integrated in the central nervous system, we performed RTPA assays. Once again, at baseline, the amount of time that naive MrgprdCre<sup>lineage</sup> mice spent on the blue-light side was not different than that of control mice, confirming that activity in MrgprdCre<sup>lineage</sup> neurons is not aversive in the absence of injury. By contrast, however, optogenetic activation of MrgprdCre<sup>lineage</sup> lineages in mice with SNI caused significantly more avoidance than activity in control mice with SNI (Figs. 4D and F). Importantly, this optogenetic allodynia was not due to an increase in the number of ChR2-expressing neurons; FISH experiments of L2-L3 revealed that the total number of afferents that express ChR2 (Fig. 4E and F) and the proportion thereof that express Mr3prdag (Figs. 4G and H) were unchanged after SNI. Taken together, these findings suggest that activity in nonpeptidergic afferents becomes aversive after nerve injury. Moreover, the lack of sensitization postinjury in MrgprdCre<sup>lineage</sup> lineage afferents to optogenetic stimulation implied that central mechanisms must mediate optogenetic allodynia due to SNI.

3.5. Modulation of spinoparabrachial neurons by MrgprdCre<sup>lineage</sup> afferent input

To investigate the spinal circuitry underlying this phenomenon, we performed whole-cell patch clamp recordings of lamina I
neurons using the ex vivo somatosensory preparation (Fig. 5A). Optogenetic stimulation of either the peripheral or central terminals gave rise to EPSCs onto recorded neurons in lamina I, albeit with different latencies (Fig. 5B). We also noted that the EPSC magnitude was larger when the optogenetic stimulation occurred at the skin rather than at the dorsal horn (Fig. 5C). However, there were several instances in which optogenetic stimulation at the dorsal horn was efficacious, whereas stimulation at the skin was not, likely reflecting the fact that not all recorded neurons had RFs in the skin of the dorsal hind paw. For this reason, we selected central stimulation for subsequent experiments.

We found that optogenetic activation of the central terminals of MrgprdCre lineage neurons gave rise to EPSCs in only 6 of 27 random lamina I neurons (Figs. 5D and E). By contrast, optogenetic activation of the central terminals of Trpv1Cre lineage neurons gave rise to EPSCs in 6 of 8 random lamina I neurons, which represented a significantly higher proportion relative to MrgprdCre (Fisher exact test; P < 0.05). Moreover, optogenetic activation of afferents in
Trpv1Cre; Ai32 mice was sufficient to generate APs in 5 of 6 neurons that exhibited EPSCs, whereas the optogenetic activation in MrgprdCre; Ai32 mice was insufficient to generate APs in lamina I cells (Figs. 5F and G), which again represented a significantly lower proportion than that observed on activation of Trpv1Cre lineage neurons (Fisher exact test; $P < 0.05$). Because randomly targeted neurons are predominantly interneurons, these observations suggest that MrgprdCre lineage neurons do not provide strong excitatory input, either directly or indirectly, onto lamina I interneurons.

### 3.6. Contribution of MrgprdCre lineage afferents to aversion after spared nerve injury

Our behavioral experiments suggested that MrgprdCre lineage input only became aversive after SNI. To investigate mechanisms that may contribute to this injury-induced change, we performed whole-cell recordings from lamina I SPB neurons in naïve or SNI animals (Fig. 6A). In naïve mice, less than half of lamina I SPB neurons displayed EPSCs in response to brushing of the skin with a paint brush, and this input led to APs in only 2 of the 12 cells (Fig. 6B). After nerve injury, however, 100% (13 of 13) lamina I SPB neurons showed EPSCs in response to the same dynamic, low-threshold input. Moreover, brushing of the skin from SNI mice resulted in APs in approximately 70% of LI SPB neurons in SNI mice, representing a 4-fold increase compared with that in naïve mice. These results raise the possibility that allodynia in SNI mice may be caused by increased LI spinal output to low-threshold stimuli, such as brushing.

Next, we began investigating the contribution of MrgprdCre lineage neurons to this abnormal spinal output. In naïve mice, 7 of the 12 lamina I SPB neurons showed optogenetically induced EPSCs on stimulation of MrgprdCre lineage afferents at 2 Hz. After SNI, however, this proportion increased...
significantly, with 12 of the 13 lamina I SPB neurons now responding to optogenetic stimulation (Fig. 6C). It should be noted, however, that this emergent MrgprdCre lineage neuron input was still rarely sufficient, in itself, to elicit an action potential in the recorded output neuron. Thus, although MrgprdCre lineage neurons are unlikely to be the only afferents involved in allodynia, they may, nevertheless, provide an important contribution. To gain insight into the underlying mechanism, we analyzed the EPSCs in more detail. Although a higher fraction of lamina I neurons showed light-induced EPSCs after SNI, the responses in individual neurons to optogenetic stimulation were similar, and we saw no evidence of synaptic sensitization. Instead, we found that the average response latency to optogenetic stimulation was significantly increased after SNI (Fig. 6D). This elevated latency implies that the enhanced number of responders to MrgprdCre lineage stimulation occurs through an emergent polysynaptic pathway that is normally silent in naive mice (Fig. 6E).

4. Discussion

Our study provides evidence that activation of MrgprdCre lineage afferents in a naive state is not aversive but becomes aversive in the context of chronic neuropathic pain caused by SNI. At the circuit level, we found that lamina I SPB neurons show significantly more brush-induced activity after nerve injury, which is an attractive mechanism to account for the phenomenon of mechanical allodynia. Moreover, MrgprdCre lineage afferents may contribute to this abnormal spinal output after SNI because more lamina I SPB neurons receive MrgprdCre lineage afferent input after optogenetic activation relative to naive controls. Finally, this increased input seems to be due to an emergent polysynaptic pathway, as evidenced by an increase in the average response latency to optogenetic stimulation of MrgprdCre lineage afferents. Altogether, our study suggests that SNI gives rise to changes in spinal circuitry that enables increased brush-evoked activity in lamina I SPB neurons and that this effect is likely due, at least in part, to increased input from MrgprdCre lineage afferents through a polysynaptic neural pathway that is normally silent in naive mice.

Previous studies have identified many mechanisms of injury-induced plasticity in the dorsal spinal cord.2,30 Our data suggest that one of these mechanisms involves the engagement of a polysynaptic circuit that emerges after SNI, as evidenced by the increased average latency of input from nonpeptidergic afferents. These findings are consistent with the idea that allogdynia after nerve injury is caused by disinhibition, which results in low-threshold stimuli abnormally reaching nociceptive pathways in the superficial dorsal horn.25

The results presented in this study show that low-frequency activation of cutaneous MrgprdCre lineage afferents does not induce aversive behaviors. This lineage represents most C fibers innervating the skin. Thus, an important question is, what information is being conveyed during low-frequency activation of these fibers? One potential answer to this question is found in a recent study looking at parallel ascending SPB pathways projecting to distinct subsets of lateral parabrachial subnuclei.7 They found that activation of one of these SPB pathways (GPR83, which is associated with cutaneous mechanosensation) could have either a positive or negative valence depending on the intensity of the optogenetic stimulation. Therefore, low-intensity activation of these fibers could possibly evoke a sensation of innocuous touch.

One of the limitations of this type of study is that optogenetic stimulation does not perfectly mimic natural stimulation. In this case, we found that light-mediated activation gave rise to APs in MrgprdCre lineage afferents that was similar in frequency to that observed on stimulation with low-threshold stimuli (eg, 10 mN), but we did not achieve the instantaneous firing frequencies that are typically observed on stimulation with high-threshold stimuli (eg, 50 mN). For this reason, we cannot exclude the possibility that increasing the frequency of APs generated by optogenetic stimulation would alter its valence. Nevertheless, activation of this population at low frequency (2 Hz) is clearly not aversive in naive mice, whereas activation of afferents that include the cutaneous peptidergic C fibers is aversive. This important distinction raises the possibility that the bona fide nociceptors—those whose main function is to warn the
organism of tissue damage—are primarily represented by peptidergic C fibers and that the function of nonpeptidergic C fibers is likely to be much more nuanced and context dependent. An unexpected finding of our work was that control mice lacking ChR2 expression, nevertheless, showed a modest degree of avoidance for the blue-light side of the RTPA apparatus that emerged after repeated exposure. Because both sides of the chamber produce equally low levels of thermal radiation, it is likely that this avoidance was in response to visual, rather than cutaneous, input. Moreover, mice avoided blue over amber light despite similar lux (illuminance), suggesting that it is the wavelength of light that contributes to the avoidance. We found that the avoidance observed in control mice was most pronounced when light was on continuously. By contrast, the optogenetically induced aversion in Trpv1<sup>Cre</sup> mice at 2 Hz. Because optogenetic stimulation in the wind-up mode gave rise to the highest degree of aversion and, thus, seemed to be the most effective, we selected this stimulation paradigm for the behavioral experiments involving SNI. However, we note that it will be important to investigate a wide range of stimulation frequencies, including ones that we were not able to achieve with the current tools, to get a more complete picture of the relationship between stimulation frequency and perceptual percept. Despite the caveat that mice are able to see cutaneous optogenetic stimulation through the floor in RTPA studies, we believe that this approach is still preferable to the implantation of fibers or LEDs through a surgical manipulation because the activation of cutaneous afferents with light does not involve tissue damage, which is likely to be confounding in pain studies. In this regard, it is noteworthy that sham-operated mice that expressed ChR2 in Mrgpr<sup>Dcre</sup> lineage afferents also showed a trend toward avoidance of the blue side of the RTPA chamber that was greater than that observed in naive mice, although not as pronounced as that observed in SNI mice. This intermediate phenotype of sham treatment suggests that the surgical procedure involving the cutting of skin and muscle is in itself an injury model that may be sufficient for ongoing changes in spinal circuitry. Just as previously reported,<sup>10</sup> we found that Mrgpr3-expressing afferents and Sst-expressing afferents are derived from the Mrgpr<sup>Dcre</sup> lineage. Intriguingly, all 3 of these afferent subtypes show predominant (although not exclusive) cutaneous targeting, and all 3 have been implicated in itch. We did not observe itch behaviors—scratching or biting —on optogenetic activation of Mrgpr<sup>Dcre</sup> lineage afferents in our study. Curiously, itch behavior seems to be more dramatic on chemogenetic (rather than optogenetic) activation of these populations. We speculate that it is not simply the type of afferent input but also the pattern and the frequency of activation that are interpreted by the nervous system to differentially represent itch, pain, and some aspects of touch. Understanding this integration is of fundamental importance to our basic understanding of somatosensation and may one day lead to the development of improved strategies for the treatment of pain.

Conflict of interest statement
The authors have no conflicts of interest to declare.

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