A craniofacial-specific monosynaptic circuit enables heightened affective pain

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Humans often rank craniofacial pain as more severe than body pain. Evidence suggests that a stimulus of the same intensity induces stronger pain in the face than in the body. However, the underlying neural circuitry for the differential processing of facial versus bodily pain remains unknown. Interestingly, the lateral parabrachial nucleus (PB), a critical node in the affective pain circuit, is activated more strongly by noxious stimulation of the face than of the hindpaw. Using a novel activity-dependent technology called CANE developed in our laboratory, we identified and selectively labeled noxious-stimulus-activated PB neurons and performed comprehensive anatomical input–output mapping. Surprisingly, we uncovered a hitherto uncharacterized monosynaptic connection between cranial sensory neurons and the PB-nociceptive neurons. Optogenetic activation of this monosynaptic craniofacial-to-PB projection induced robust escape and avoidance behaviors and stress calls, whereas optogenetic silencing specifically reduced facial nociception. The monosynaptic circuit revealed here provides a neural substrate for heightened craniofacial affective pain.

Noxious stimuli experienced by the head and facial region are detected and conveyed to the CNS by sensory neurons located in the trigeminal (TG) ganglia, whereas noxious stimuli affecting extracranial regions are sensed and relayed to the CNS via primary sensory neurons residing in the dorsal root ganglia (DRGs). Humans generally rank head and facial pain as much more severe and emotionally draining than body pain. For example, two of the arguably most severe chronic pain conditions are trigeminal neuralgia and cluster headaches1–3. Craniofacial pain sensation is qualitatively different from bodily nociception, as shown in human experiments in which repeated application of noxious heat to the face induces sensitization, yet similar stimulation applied to the hand induces habituation4. Fear induced by pain in human subjects was rated higher for face than for extremities, despite comparable ratings of the pain intensity4. fMRI studies further revealed that face pain resulted in higher levels of amygdala activation compared to the same intensity of stimulation applied to the hand5. Despite these studies, the neurobiological underpinning for heightened craniofacial pain remained enigmatic.

'Suffering' and 'fear of pain' are emotional aspects of pain that are not processed by the canonical discriminative pathway via the spinothalamic–cortical somatosensory circuits. Instead, these feelings are relayed by the less-studied affective pain pathway, where nociceptive afferent information is routed from second-order neurons to the lateral parabrachial nucleus (PB) on to various limbic regions, such as the central amygdala (CeA), the bed nucleus stria terminalis (BNST), the lateral hypothalamus (LHA), the anterior cingulate and the insular cortices (also known as the spino–parabrachial circuit)6–9. Interestingly, it has been suggested that subregions of the PB, a critical relay node in the affective pain circuit, might be differentially activated by noxious stimuli applied to the face versus the extremities in rats8,9,10.

In this study, we show that painful stimuli applied to the face activate more PB neurons, and do so more bilaterally, than those applied to the paw. We utilize our novel activity-dependent technology, called CANE12, to identify PBnociceptive neurons and their connections with the affective pain system. We further discover the circuit mechanism underlying the more robust activation of PB by noxious facial stimuli and show that activation of this circuit drives strong aversive behaviors, whereas its inhibition specifically reduces craniofacial nociception.

Results

Noxious facial stimuli activate PB more robustly and bilaterally than noxious bodily stimuli. We injected 4% formalin (a noxious chemical) unilaterally into either the whisker pad or one hindpaw and then immunostained for expression of the immediate-early gene Fos as a marker for activated neurons in the PB (Fig. 1a). Whisker-pad formalin injection activated the PB, resulting in significantly more Fos+ neurons than paw injection of an equivalent amount of formalin (Fig. 1c; whisker, 952 ± 143; and paw, 616 ± 75.1 total Fos+ neurons; P = 0.04; n = 7), especially in the external lateral sub-nucleus of the PB (PB-el) (Fig. 1b).

Furthermore, unilateral whisker-pad formalin injection induced Fos+ neurons in PB-el bilaterally, with a trend of more Fos+ cell on the ipsilateral side (Fig. 1b,d; contra: 213.8 ± 32.8, and ipsi: 281.5 ± 22.3 Fos+ neurons; P = 0.053; n = 4). By contrast, unilateral paw formalin injection preferentially activated the contralateral PB-el, with significantly more Fos+ neurons on the contralateral than on the ipsilateral side (Fig. 1b,d; contra: 253.3 ± 24.1, and ipsi: 129.7 ± 14.3 Fos+ neurons; P < 0.01; n = 3), which is consistent with the fact that spino–parabrachial projection neurons in dorsal spinal cord are known to predominantly send axons to the contralateral side8,11. Additionally, consistent with the fact that the affective pain circuit does not discriminate the types of pain, we found that capsaicin, formalin, and even the minor pain associated with control injection of saline unilaterally into the whisker pad all activated the PB neurons (including neurons in PB-el) as compared to the PB neuron activity seen in no-injection controls, with formalin being the most potent in evoking Fos+ neurons (Supplementary Fig. 1a,b; home cage: 73 ± 26, saline: 421 ± 94,

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Lateral parabrachial nucleus (PBl) is differentially activated by the same noxious stimulus applied to the face versus hindpaw. a. Schematic illustration of Fos induction protocol. Ninety minutes after 10 µL 4% formalin was injected, brainstem slices containing PBl were stained for Fos expression. TG, trigeminal ganglion; Sp5C, trigeminal nucleus, caudalis; DRG, dorsal root ganglion; S.C., spinal cord. b. Representative images of Fos+ neurons in PBl after formalin injection into right whisker pad (top) and right hindpaw (bottom). Large white dashed circle (left) indicates the entire structure of PBl, whereas small white dashed circle (right) indicates ventral region of PBl including PBl-el. Blue, DAPI stain. Scale bars, 200 µm. c. Total numbers of Fos+ neurons in PBl on both sides combined (n = 4, 3; two-tailed unpaired Student’s t test; *P = 0.0445; t_{16} = 2.674). d. Numbers of Fos+ neurons in ipsilateral (magenta) and contralateral (teal) PBl-el in mice unilaterally injected with formalin into one whisker pad (n = 4) or one hindpaw (n = 3 mice; two-way ANOVA; whisker: P = 0.0533; hindpaw: **P = 0.0090; F_{2,15} = 3.257). n.s., nonsignificant. Data are mean ± s.e.m.

capsaicin: 673 ± 72, and formalin: 952 ± 101 Fos+ neurons; n = 3, 3, 3, 3, 4). In the same animals, we also observed Fos+ neurons in spinal trigeminal nucleus caudalis (Sp5C), which was expected since Sp5C is a main relay in the trigeminal–thalamic–cortical pain pathway (Supplementary Fig. 1c; n = 3).13,14

PB1 neurons activated by noxious facial stimuli are molecularly heterogeneous. Two-color fluorescence in situ hybridization further showed that most Fos+ PB1 nociceptive neurons were Slc17a6+ (i.e., vGlut2+) (Supplementary Fig. 2a,b; glutamatergic: 80 ± 1%; n = 3), while only a minority of Fos+ cells were Gad1 Gad2+ (Supplementary Fig. 2a,b; GABAergic: 7 ± 2%, n = 3). A recent study showed that the gene Calca, encoding calcitonin-gene-related peptide (CGRP), is expressed in PBl-el15. These CGRP+ PBl-el neurons were activated by intense foot shock and transmitted affective pain signals to the CeA.14 We therefore decided to focus on CGRP expression, and we found that a subset of Fos+ PB1 nociceptive neurons in the ventral region indeed expressed CGRP (Supplementary Fig. 2c,d; 56 ± 5% of ventral, 2 ± 1% of dorsal, and 34 ± 3% of total Fos+ PB1 nociceptive pain neurons were CGPR+; n = 3). Another marker, the Forkhead box protein P2 (FoxP2), implicated in circuits related to vocal communication and sodium intake, has also been found to be expressed in the PB1.16,17 We found that, again, only a subset of Fos+ PB1 neurons in the dorsal region expressed FoxP2 (Supplementary Fig. 2c,d; 9 ± 4% of ventral, 46 ± 10% of dorsal, and 21 ± 5% of total Fos+ PB1 nociceptive neurons were FoxP2+; n = 3).

CANE is efficient and selective in activity-dependent capture of facial nociceptive relay PB1 neurons. How might noxious facial stimuli activate more neurons in the PB1, particularly in the PB1-el, compared to noxious bodily stimuli, especially on the ipsilateral side? To answer this question, we needed to identify neurons that provide presynaptic inputs to face-nociception-activated PB1 neurons. Previous studies using anterograde and retrograde tracer dyes labeled the general afferents to the entire PB1 region.15,17,18,19 However, the PB1 contains diverse populations of neurons in addition to neurons responsive to noxious stimuli, such as cells activated by innocuous warm and cool temperatures, as well as cells responsive
to various taste stimuli\textsuperscript{21,23}. The PB\textsubscript{L} is also known for its significant role in regulating instinctive behaviors, namely thirst for water, sodium appetite and hunger for food\textsuperscript{22-24}. Thus, tracer-based studies lack the resolution to identify specific inputs to the PB\textsubscript{L}-nociceptive neurons.

We first determined whether CANE could indeed selectively label PB\textsubscript{L}-nociceptive neurons. In a two-bout experimental paradigm, CANE was used to capture PB\textsubscript{L} neurons activated by a noxious stimulus (capsaicin or formalin injection) through co-injection of CANE-LV-Cre and AAV-flex-GFP into the PB\textsubscript{L}. Three weeks later, the same animal was given a second painful stimulus to induce Fos expression and was then anesthetized and killed to obtain samples for immunostaining (Fig. 2a). In the capsaicin–capsaicin and formalin–formalin conditions, 55 ± 2% (n = 9) and 55 ± 2% (n = 9) of CANE-captured PB\textsubscript{L} neurons, respectively, were Fos\textsuperscript{+} (Fig. 2d,f,h,i). This indicated that the second noxious injection reactivated many (~55%) of the same cells excited by the first stimulus. In the noxious–noxious condition, only 40 ± 2% (n = 9) of PB\textsubscript{L} neurons were Fos\textsuperscript{+} (Fig. 2d,f,i). All data shown are mean ± s.e.m.

Fig. 2 | Capturing and mapping the axonal projection targets of PB\textsubscript{L}-nociceptive neurons. a, Schematic illustration of strategy to express GFP in nociceptive relay PB\textsubscript{L} neurons in Fos\textsuperscript{TVA} mice using CANE. Examination of CANE-captured neurons activated by the first stimulus (magenta) versus Fos\textsuperscript{+} neurons activated by the second stimulus (green) in the PB\textsubscript{L}. In all six conditions, CANE method was used to capture neurons activated by stimulus/no stimulus, and 2 weeks later, Fos was induced by the second stimulus. Blue, DAPI. Scale bars, 10 μm. b–j, The percentages of Fos\textsuperscript{+} neurons among CANE\textsuperscript{+} neurons in the different conditions. Data are mean ± s.e.m. (from left to right: h, n = 4, 9, 7, 4; one-way ANOVA; ****P < 0.0001, **P = 0.0005, P = 0.3952, P = 0.3223; **P = 0.0005, P = 0.0047; F\textsubscript{1,20} = 12.49; i, n = 5, 5, 9; one-way ANOVA; ****P < 0.0001, ***P ≤ 0.0001, P ≤ 0.001, P = 0.6876; F\textsubscript{1,17} = 52.17; j, n = 3, 3; two-tailed unpaired Student’s t test; P = 0.2759; t\textsubscript{6,180} = 1.289). k–p, Representative images of axonal projections from captured formalin-activated PB\textsubscript{L} (magenta) in several brain nuclei expressing Fos (green) induced by formalin. Insets, schematics of coronal view of location (in red box) in brain. * in k denotes very large terminal boutons from labeled PB\textsubscript{L} axons in BNST; some of boutons surround the Fos\textsuperscript{+} neurons in the different conditions. Data are mean ± s.e.m.
in the absence of noxious stimuli), which had significantly less overlap with FoS neurons induced by noxious stimuli (Fig. 2b,c,h,i; 27 ± 3% CANE cells were FoS in the no stimulus–formalin condition (n = 5; P < 0.0001); 31 ± 5% CANE cells were FoS in the no stimulus–capsaicin condition (n = 4; P < 0.0001)). In the capsacin–saline condition, 36 ± 3% CANE-captured cells were FoS activated by saline injection (Fig. 2e,h; n = 4; P = 0.0005), consistent with the fact that saline injection caused only moderate PB-L activation. Previous electrophysiological studies reveal that the same PB neurons could be activated by different noxious modalities14, prompting us to ask whether CANE-captured capsacin-activated PB neurons overlapped with formalin-activated neurons and vice versa. Indeed, we observed a similar percentage of CANE+ neurons that were FoS regardless of whether the capsacin–formalin or the formalin–capsacin condition was used (capsacin–formalin: 51 ± 2%; n = 7; formalin–capsacin: 55 ± 2%; n = 6) (Fig. 2c,h,i). We also examined the overlap between CANE-captured face-activated PB-nociceptive neurons and FoS neurons induced by contralateral hindpaw nociception and vice versa. About 30% of CANE+ neurons were FoS in both whisker–hindpaw and hindpaw–whisker nociception paradigms (Fig. 2g,j; whisker–hindpaw: 26 ± 3%; hindpaw–whisker: 33 ± 4%, n = 6 for each condition). Our observations are consistent with the current concept that the PB mediated affective pain circuit plays a limited role in discriminating the types and locations of injury14,15,16. As an additional control for the specificity of CANE, we co-injected CANE-LV-Cre, AAV-flex-GFP (CANE::GFP), and AAV-tdTomato into the PB, after formalin injection into the whisker pad and compared the labeling resulting from the two AAV constructs. CANE::GFP labeled a specific subset of PB-nociceptive neurons, whereas AAV-tdTomato labeled a majority of neurons at the injection site (Supplementary Fig. 3; n = 4), thus further confirming the specificity of our method.

PB-nociceptive neurons project axons to multiple emotion- and instinct-related centers in the brain. We next traced the axonal projection patterns of CANE::GFP-captured PB-nociceptive neurons. The targets of PB-nociceptive neurons included the BNST (where PB axons form large axonal boutons surrounding BNST neuron cell bodies), the paraventricular thalamic nucleus, the paraventricular nucleus of the hypothalamus (PVH), the capsular division of CeA, the ventral tegmental area, the ventrolateral periaqueductal gray (PAGvl), the nucleus of the solitary tract (NST) and the intermediolateral reticular regions, NST and Sp5C were distributed bilaterally from ipsilateral trigeminal ganglion. Interestingly, trans-synthetically labeled mCherry+ neurons were also observed in the ipsilateral TG, but not in any of the DRGs on either side (n = 6; Fig. 3k,l), suggesting that TG sensory neurons innervating head and face provide direct monosynaptic inputs to ipsilateral PB-nociceptive neurons. A few previous anatomical studies hinted at the possibility of a direct TG→PB connection25,26. Interestingly, trans-synaptic tracing of inputs to hindpaw formalin-activated PB-nociceptive neurons also revealed labeled neurons in TG but not in any DRG (n = 4; Fig. 3n), suggesting that craniofacial but not body primary sensory neurons provide direct, monosynaptic inputs onto PB-nociceptive neurons. The result is also consistent with the idea that some PB-nociceptive neurons receive convergent inputs from both face and body. We examined the expression of IB4 (a marker for non-peptidergic C fibers), CGRP, TrpV1 (the receptor for capsaicin and a marker for a subset of C fibers and a small subset of Aδ fibers), and NF200 (a marker for both Aδ and Aβ fibers) among the trans-synthetically labeled TG neurons. The TG neurons directly presynaptic to the PB included NF200+ (45 ± 4%), TrpV1+ (38.5 ± 4%), CGRP+ (26.2 ± 7%), and IB4+ cells (12 ± 4%; n = 8; Fig. 3p, q). Taken together, the trans-synaptic tracing studies suggest that there are two separate pathways transmitting craniofacial nociception from TG to the PB: (i) the previously known indirect TG→Sp5C→PB, and (ii) the newly revealed direct TG→PB projection. By contrast, there is only one indirect pathway transmitting somatosensory body nociception from DRG to the PB: DRG→spinal dorsal horn→PB. Notably, a previous study using TrpV1+;PLAP mice observed that fibers from a possible primary afferent source of TrpV1-lineage neurons were present in the PB, especially in the PB-el. The authors speculated that the TrpV1+ fibers may have emerged from TG neurons, which could provide an alternative circuit contributing to craniofacial pain experience25,26. These previous findings, in addition to our finding that ~40% of trans-synthetically labeled TG neurons are TrpV1+, led us to postulate that TrpV1+ fibers may be a major source of nociceptive TG inputs to PB-el. Therefore, we performed neonatal intraperitoneal (IP) injection of AAV to selectively label periphery-derived TrpV1-Cre+ axons27,28. Briefly, Cre-dependent AAV9-flex-GFP was injected into TrpV1-Cre+ mouse pups at postnatal day 1–2. The IP injection resulted in selective labeling of TrpV1-Cre+ primary sensory neurons with GFP without labeling of TrpV1-Cre+ CNS neurons (Fig. 3r,s and Supplementary Fig. 4; n = 3). Furthermore, axonal terminals from labeled TrpV1+ primary sensory neurons were observed near nociceptive FoS+ neurons in PB-el and in Sp5C (Fig. 3t; FoS was induced by capsaicin injection into the ipsilateral whisker pad).

We further designed a TrpV1-Cre and retrograde-FloP O intersectional strategy (Supplementary Fig. 5a) to determine whether PB, projecting TG neurons also project to Sp5C. Briefly, retrograde lentivirus expressing either FloP O (RG-LV-hSyn-FloP O, n = 4) or Cre-dependent FloP O (RG-LV-hSyn-DIO-FloP O, n = 6) was injected into nigra pars compacta, the PAGvl, brainstem reticular regions, the NST, Sp5C and the dorsal horn of the spinal cord (Fig. 3c–j;m; quantification represents numbers of labeled presynaptic neurons/number of starter neurons; n = 6). Note that the labeled neurons in the reticular regions, NST and Sp5C were distributed bilaterally with an ipsilateral dominance (Fig. 3m; numbers of trans-synthetically labeled cells/number of starter cell were as follows: ipsilateral: IRt, 6.3 ± 1.3; PCRt, 6.4 ± 1.4; MRn, 1.3 ± 0.4; GRn, 3.1 ± 0.7; NST, 1.9 ± 0.7; Sp5C, 5.3 ± 1.6; and contralateral: IRt, 0.9 ± 0.2; PCRt, 1.4 ± 0.5; MRn, 3.8 ± 0.8; GRn, 2.1 ± 0.5; NST, 0.8 ± 0.2; Sp5C, 0.5 ± 0.2), which is consistent with previous dye tracing studies13,14,16,19. Additionally, there were a few labeled cells in the contralateral PB (Fig. 3m; 0.7 ± 0.2). A schematic summary of the projections is shown (Fig. 3o).

CANE-captured PB-nociceptive neurons receive direct inputs from ipsilateral trigeminal ganglion. Additionally, a specific subset of PBL neurons, whereas AAV-tdTomato labeled a majority of neurons at the injection site (Supplementary Fig. 3; n = 4), thus further confirming the specificity of our method.
**Fig. 3** Trans-synaptic labeling of presynaptic neurons for PBγ-nociceptive neurons reveals the direct TG→PB pathway. **a**, Schematic illustration for trans-synaptic tracing of presynaptic inputs to PBγ-nociceptive neurons. **b**, Representative image of CANE-RV-mCherry-infected PBγ-nociceptive neurons. Green, PBγ-nociceptive neurons expressing TVA and RG; red, RV-mCherry+; yellow, starter cells. Scale bar, 10 μm. **c–l**, Representative images of trans-synaptically labeled neurons in several brain regions. Scale bars: **c–h**, 50 μm; **i–j**, 100 μm; **j–l**, 20 μm. **m,n**, Quantification of trans-synaptically labeled neurons in each brain area contralateral (teal) and ipsilateral (magenta) to injected site (**m**) after whisker-pad formalin injection and (**n**) after hindpaw formalin injection. The value is normalized against the number of starter neurons and averaged across animals. Data are mean ± s.e.m. (n = 6; n = 3). **o**, Schematic summary for input sources for PBγ-nociceptive neurons. BNST, bed nucleus of the stria terminals; PVH, paraventricular hypothalamic nucleus; LHA, lateral hypothalamic nucleus; CeAγ, central amygdaloid nucleus, medial; SNγ, substantia nigra pars compacta; PAG, periaqueductal gray; DRn, dorsal raphe nucleus; DRnγ, ventrolateral DRn; NST, nucleus of the solitary tract; Sp5C, trigeminal nucleus, caudalis; Pr5, principal sensory trigeminal nucleus; TG, trigeminal ganglion; DRG, dorsal root ganglion; S.C., spinal cord (dorsal horn). Reticular (ret.) nuclei: PRn, pontine reticular nuclei; IRt, intermediate reticular tract; PCRt, parvicellular reticular tract; MRn, medullary reticular nucleus; GRn, gigantocellular reticular nucleus. **p**, Molecular characterization of trans-synaptically labeled TG neurons. Green, bottom to top: IB4+, CGRP+, NF200+, TrpV1+. Left, colocalized trans-synaptically labeled TG neurons. Right, non-colocalized labeled TG neurons. Scale bars, 20 μm. **q**, Percentage of trans-synaptically labeled trigeminal ganglion neurons expressing IB4, CGRP, NF200, or TrpV1 (n = 8; one-way ANOVA: *P* = 0.0135, **P** = 0.0008, ***P* = 0.0468, ****P = 0.0001, *****P = 0.0001, F1,7 = 22.7). Data are mean ± s.e.m. **r**, Schematic illustration and timeline of intraperitoneal injection in 1–2-day-old TrpV1-Cre pup with AAV9-CAG-flex-GFP. Four weeks after injection, TrpV1Cre::GFP mouse was injected with capsaicin in the whisker pad and sections stained for Fos (n = 4 mice). **s**, Representative image of trigeminal ganglion with TrpV1Cre::GFP+ neurons. Scale bar, 200 μm. **t**, Representative image of PBγ with TrpV1Cre::GFP+ axon terminals (green) and capsaicin-induced Fos+ neurons (magenta). Scale bar, 50 μm (high magnification).
Fig. 4 | Optogenetic activation of TrpV1-Cre* sensory axons activates PB1,-nociceptive neurons and elicits aversive behavior and stress calls in a real-time place escape/avoidance task. a, Schematic illustration of intraperitoneal injection of a 1-2-day-old TrpV1-Cre pup (n = 3), followed by optogenetic-assisted whole-cell patch-clamp recording from a PB neuron in acute brain slices. b, Representative traces from a cell showing no light-evoked IPSC at a holding potential of 10 mV, but observed to have light-evoked EPSC at a holding of –65 mV. Cell, held at –65 mV, was bath applied 1 μM TTX, followed by 100 μM 4-AP and 1 μM TTX, and showed a light-evoked monosynaptic EPSC. c, Averaged current amplitude. Data are mean ± s.e.m. (closed circles represent individual cells, n = 15). d, Representative high-magnification image of TrpV1Cre::ChR2* axon terminals and CANE-RV-mCherry captured PB1-pain neurons (n = 3 mice; scale bar, 50 μm). e, Representative example of an mCherry+ PB1-pain neuron recorded to have light-evoked EPSC at a holding of –65 mV. Cell was bath applied 1 μM TTX, followed by 100 μM 4-AP and 1 μM TTX, and showed a light-evoked monosynaptic EPSC. f, Averaged current amplitude. Data are mean ± s.e.m. (closed circles represent individual cells, n = 6). g, Schematic illustration of real-time place escape/avoidance (PEA) test. h, i, Representative spatial tracking maps showing the location of (h) an experimental mouse before, during, and after optogenetic stimulation of TrpV1Cre::ChR2* axon terminals and (i) a control mouse before, during, and after illumination of TrpV1Cre::GFP* axon terminals in the PB, in the preferred chamber. j, Percentage of preference (per 30 s) the experimental and control groups had before, during, and after optogenetic stimulation (n = 8, 3) shown across time (min). Data are mean ± s.e.m. k, Quantification of time the TrpV1Cre::ChR2 group spent in preferred chamber before, during, and after optogenetic stimulation (n = 8 one-way repeated measures ANOVA; ***P ≤ 0.0001, *P = 0.0128, **P ≤ 0.001; F(2,14) = 49.41). Data are mean ± s.e.m. l, Quantification of time the TrpV1Cre::GFP group spent in preferred chamber before, during, and after light illumination (n = 3; one-way repeated measures ANOVA; P = 0.8867, P = 0.6377, P = 0.8886; F(2,12) = 0.4412). Data are mean ± s.e.m. m, Schematic illustration of vocalization recording chamber. n, Quantification of frequency of pips induced by optogenetic stimulation of TrpV1Cre::ChR2 (experimental) or TrpV1Cre::GFP (control) axon terminals in the PB. Data are mean ± s.e.m. (ChR2, n = 8; GFP, n = 3; two-tailed unpaired Student’s t test; **P ≤ 0.0001; t; 10.13).
whether TG→PB axons form functional synaptic connections in PB, we injected Cre-dependent AAV9-flex-ChR2-YFP into Trpv1-Cre pups intraperitoneally to express channelrhodospin-YFP (ChR2-YFP) in peripheral Trpv1-Cre+ neurons (Trpv1Cre::ChR2) and performed whole-cell patch-clamp recording of PB neurons in slices from these animals (Fig. 4a). Photoactivation of...
TrpV1Cre::ChR2+ terminals elicited excitatory post-synaptic currents (EPSCs) in 15 out of 54 neurons (Fig. 4b,c and Supplementary Fig. 6). Furthermore, the EPSCs persisted in the presence of action potential blockade caused by administration of 1 μM tetrodotoxin (TTX) and 100 μM 4-aminopyridine (4-AP) (Fig. 4b). In a complementary set of experiments, we captured PB−, pain neurons using CANE-RV-mCherry in TrpV1Cre::ChR2 animals (Fig. 4d). In six CANE-captured mCherry+ PB−, pain neurons, photoactivation of TrpV1+ terminals elicited EPSCs that were not blocked by TTX (Fig. 4e,f). These results corroborate and extend the circuit-tracing findings that the inputs from TG TrpV1-Cre+ fibers to PB−, nociceptive neurons are monosynaptic and excitatory.

Activation of TrpV1-Cre+ axon terminals in PB− induces robust aversive behavior and audible vocalization. To address the behavioral impact of the direct TG→PB−, monosynaptic projection in awake behaving animals, we asked whether its activation would be sufficient to elicit aversive responses in a modified real-time place escape/avoidance (PEA) assay, which has been used in recent studies to assay affective components of pain. Optic fibers were implanted bilaterally above PB-el in either TrpV1Cre::ChR2 mice (n = 8) or control mice TrpV1Cre::GFP (n = 3) (Fig. 4g). Mice were habituated and placed in a two-chamber arena. Their behaviors were recorded under three conditions: (i) freely exploring with no stimulation for 10 min (baseline), followed by (ii) 10 min of conditioned photoactivation when the mouse is in its preferred chamber (stimulation), and followed again by (iii) 10 min without stimulation (post-stimulation). Upon photo-stimulation of TrpV1Cre+ axons in PB-el, TrpV1Cre::ChR2 mice immediately fled to the opposite chamber (Fig. 4h; Supplementary Video 1), and subsequently they moved less and spent significantly more time on the unstimulated side (Fig. 4h,j,k, Supplementary Video 1; P < 0.0001). In the post-stimulation period, some but not all mice still showed avoidance of the chamber in which they received photostimulation (Fig. 4j,k). Light illumination had no effect on movement and behavior of the control TrpV1Cre::GFP mice (Fig. 4i,j,k, Supplementary Video 2; P = 0.66). These results suggest that the optogenetic stimulation of the TG→PB−, monosynaptic projection caused a drastic aversive effect that is likely to be due to activation of the downstream affective pain pathway.

We further wanted to determine whether optogenetic activation would be sufficient to induce an aversive affective memory using the conventional conditioned place aversion (CPA) assay (Supplementary Fig. 7a). Mice were habituated first by placing them in the two-chamber arena and allowing free exploration. Subsequently, they were subjected to 2 d of conditioning: mice were placed in the two-chamber arena and allowing free exploration. By contrast, control TrpV1Cre::GFP mice show no preference for the paw, and both TrpV1Cre::eArch and TrpV1Cre::GFP mice drastically lowered the withdrawal threshold in responses to von Frey application to face or paw, respectively (Fig. 5c,d). Hence, capsaicin injection induced mechanical allodynia in both face and hindpaw as expected (Fig. 5c,d). Importantly, eArch-mediated photosilencing of TrpV1-Cre+ axons in PB-el partially alleviated the capsaicin induced allodynia in the face but had no effect on the mechanical hypersensitivity of the hindpaw (Fig. 5c,d; face P = 0.0046, paw P ≥ 0.9999). Light illumination had no effect on TrpV1Cre::GFP mice (Fig. 5c,d; P ≥ 0.9999). These results confirmed that the TG→PB−, direct pathway indeed specifically contributes to face nociception.

We further tested whether photosilencing of TrpV1-Cre+ axons in PB−, after facial capsaicin injection would elicit conditioned place preference for the light illuminated chamber. The effect of capsaicin only lasts about 20 min, and we therefore performed a real-time place preference (RTPP) assay (Fig. 5e; 10 min without light and 10 min with light illumination in the non-preferred chamber). After capsaicin injection into the whisker pad, TrpV1Cre::eArch mice spent significantly more time in the chamber with photosilencing of the TrpV1-Cre+ terminals in PB− (Fig. 5f; n = 6; P = 0.029). By contrast, control TrpV1Cre::GFP mice show no preference (Fig. 5g; n = 7; P = 0.6). Taken together, these data demonstrated that when mice are subjected to noxious facial stimulation, silencing the neural activity of the direct TG→PB−, pathway reduces facial allodynia and induces place preference, indicating that this pathway contributes significantly to the manifestation of facial-pain equivalents.

Silencing TrpV1-Cre+ axon terminals in PB− selectively reduces facial allodynia after capsaicin injection. We next asked whether silencing the direct TG→PB−, monosynaptic projection would affect pain-related behaviors. Previous studies showed that the optogenetic silencer archehodopsin (Arch) can effectively silence nociceptors including TrpV1+ neurons. We therefore used the neonatal IP injection strategy to express eArch+ or GFP in TrpV1-Cre+ sensory neurons. Optic fibers were implanted bilaterally above PB-el in TrpV1Cre::eArch mice (n = 9) or TrpV1Cre::GFP (n = 8) mice (Fig. 5a,h). A von Frey test was used to assess the mechanical threshold of face or paw withdrawal responses before and after capsaicin injections into the whisker pad or hindpaw and with or without photosilencing of TrpV1-Cre+ axons in PB− (Fig. 5a). After capsaicin injection into either the face or the paw, both TrpV1Cre::eArch and TrpV1Cre::GFP mice drastically lowered the withdrawal threshold in responses to von Frey application to face or paw, respectively (Fig. 5c,d). Hence, capsaicin injection induced mechanical allodynia in both face and hindpaw as expected (Fig. 5c,d). Importantly, eArch-mediated photosilencing of TrpV1-Cre+ axons in PB-el partially alleviated the capsaicin induced allodynia in the face but had no effect on the mechanical hypersensitivity of the hindpaw (Fig. 5c,d; face P = 0.0046, paw P ≥ 0.9999). Light illumination had no effect on TrpV1Cre::GFP mice (Fig. 5c,d; P ≥ 0.9999). These results confirmed that the TG→PB−, direct pathway indeed specifically contributes to face nociception.

Discussion

In this study, we discovered that nociceptive trigeminal afferents transmit painful signal to the affective pathway through both the direct monosynaptic TG→PB− and the indirect disynaptic TG→Sp5C→PB− projections. In a previous study, researchers injected neural tracer WGA-HRP into the peripheral anterior ethmoidal nerve (AEN), which originates from TG and innervates the nasal cavity, and observed labeled afferent fibers in regions near PB31. In a follow-up study, the authors showed that trigeminal rhizotomy results in loss of CGRP-expressing fibers innervating the PB30.
These and other studies have implied that a direct TG→PB pathway might exist but have not provided synaptic or behavior evidence to support this possibility. Here we used a combination of activity-dependent tagging, monosynaptic trans-synaptic tracing, intersegmental genetic labeling, optogenetic-assisted slice electrophysiology, and in vivo optogenetic activation and silencing experiments to definitely establish the monosynaptic connection between TG and PB-nociceptive neurons, and revealed the important functions of this pathway in craniofacial-pain-related aversive behaviors.

Our findings have several important implications. First, the dual and bilateral pain-transmitting pathways compared to the single indirect DRG→dorsal horn→PB pathway could explain why similar-intensity stimuli applied to face activate more PB neurons than when applied to limbs. This could in turn lead to heightened and bilateral activations of the affective pain responses, such as a higher level and more persistent activation of CeA, BNST, hypothalamus and insular cortex through the axonal projections from PB-nociceptive neurons (Fig. 2k–r). This projection pattern can provide a circuit basis for the perception of trigeminally mediated pain as more severe, fear inducing and emotionally draining than other body pain. The monosynaptic TG→PB connection also provides a mechanism for rapid, short-latency direct connections of nociceptive inputs from the head and face to brain centers involved in homeostatic regulation and emotional processing. Second, current palliative neurosurgical procedures aimed at alleviating refractory trigeminal pain target the descending spinal trigeminal tract (Supplementary Fig. 10), including making thermal lesions, referred to as ‘dorsal root entry zone coagulation’ (DREZ), to lesion pain-transmitting pathways in Sp5C, a contemporary adaptation of the classic trigeminal tractotomy. Based on our study, DREZ coagulation will lesion only the TG→Sp5C connection, while leaving the TG→PB connection intact (Supplementary Fig. 10). This may explain the lack of therapeutic response or post-operative pain relapse seen in some patients subjected to trigeminal DREZ surgery. Designers of future surgical procedures should consider severing both the TG→Sp5C and TG→PB connections as a means to provide invasive palliation of chronic, refractory orofacial pain, for example, for trigeminal neuralgia. Notably, our discovery presented here critically relied on the CANE methodology, although CANE does have qualifiers, namely that the 60–90-min waiting interval between the stimulus application and the surgery (in order for Fox3VA protein to reach peak levels) inevitably resulted in some background labeling. Nevertheless, CANE is still the best-validated tool to selectively label and trans-synaptically trace the presynaptic inputs to transiently activated neurons as shown here and in our previous studies. Our input–output circuit mapping of PB-nociceptive neurons revealed many limbic centers that are reciprocally connected with PB, providing a circuit basis for understanding comorbidities that are closely associated with and clinically highly relevant to pathologic trigeminal pain, namely anxiety, depression, disturbance of circadian rhythm and altered intake behavior. Future studies on mechanisms underlying chronic craniofacial pain disorders can now take advantage of this circuit diagram including the newly unveiled monosynaptic TG→PB pathway to identify specific maladaptive plasticity in each of the nodes in the circuit and, it can be hoped, to effectively revert them.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-017-0012-1.

Received: 9 August 2017; Accepted: 22 September 2017;
Published online: 13 November 2017

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**Acknowledgements**

We thank J. Takahits for helping with a method to quantify axon innervation densities, K. Tschida and T. Gibson for helping with vocalization quantification and analysis, and V. Prevosto for helping with statistics. We also thank T. Gibson, M. Fu, K. Tschida, T. Stanek, V. Prevosto, and R. R. Ji for providing input and support throughout the project, and S. Lisberger and R. Mooney for critical reading of this manuscript. E.R. is supported by a F31 DE025197-03 fellowship. Y.C. is supported by K12DE022793. W.L. is supported by DE018549. This work is supported by NIH Grant DP1MH103908 to F.W.

**Author contributions**

F.W. and E.R. conceived the idea and designed the experiments. E.R. performed the majority of the experiments and data analysis. K.S. performed some independent CANE capture experiments, bilateral fiber implantations and the place escape/avoidance (PEA) behavioral experiments. K.T. analyzed PEA results (blind to genotype). J.X. performed immunohistochemistry, quantified axon projections, and quantified cells in Fox and trans-synaptic experiments (blind to experimental conditions). Y.C. performed all the face and hindpaw frey assays (blind to genotypes). D.R. quantified cells in a subset of colocalization experiments. S.Z. produced all the CANE-LV and CANE-RV viruses. B.-X.H. took care of mouse husbandry and genotyping. H.Y. and W.L. provided critical equipment and reagents. F.W. and E.R. wrote the manuscript with help from W.L.

**Competing interests**

The authors declare no competing financial interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41593-017-0012-1.

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Methods

Animal statement. All experiments were conducted according to protocols approved by The Duke University Institutional Animal Care and Use Committee.

Animals. Adult (P30–P60) male and female C57BL/6 mice (Jackson Laboratory) were used for immunohistochemistry and in situ hybridization. Male and female Fox2+/- mice12 (Jackson Laboratory, stock 027831) were used for capturing PB-nociceptive neurons with the CANE technology, immunohistochemistry, electrophysiology, and input-output circuit mapping. Male and female Ai6512 mice expressing a Cre and Flip double-dependent STOP cassette in front of the tdTomato reporter (Jackson Laboratories, stock 024109) were used for electrophysiology experiments. All mice were housed in a vivarium with normal light/dark cycles in cages with 1–5 mice. A day before experiments, we singly housed mice. We used two exclusion criteria for our subjects: (1) poor recovery or other health concerns following surgical intervention or (2) missed injection or implantation target, as determined by histological analysis. Animals were randomly selected from each litter. Random group allocation was maintained throughout the study, within constraints set by availability of in-house, purpose-lined lines. Experimenter blinding was sufficient to control for selection bias. Furthermore, behavioral analysis relied on objective, automated measurements.

Viruses. CANE-LV-Cre (titer, 5 × 10^6 ifu/ml; plenti-hSynapns-Cre-WPRE [Addgene Plasmid #86641]; CANE-LV envelope [Addgene Plasmid #86666]) and CANE-RV-mCherry (titer, 5 × 10^6 ifu/ml) were produced as previously described11. FuG2-coated RG-LV-hSyn-FloPo and RG-LV-hSyn-DIO-FloPo were produced and concentrated as described previously. pAAV-Syn-DIO-TVA-EGFP-RG (pAAV-Syn-DIO-tvlpE8) was packaged in serotype AAV2/8 by the University of Pennsylvania Vector Core. AAV-CAG-flex-GFP, AAV-CAG-Chr2 (Hi34R)-eYFP and AAV-EF1a-DIO-eARCH-eYFP44 were purchased from the University of Pennsylvania Vector Core.

Surgery. Animals were anesthetized with isoflurane in a respiratory system (2% isoflurane in 100% O2) and then transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was collected and kept in 4% paraformaldehyde overnight. The sections were washed 3 times followed by secondary antibody treatment at 4°C for 2h. Sections were counter-stained NeuroTrace fluorescent Nissl stain (fluorescent Nissl stain) (Invitrogen, N-21479) or 4,6-diamidino-2-phenylindole (DAPI) (Sigma, D9564). After this incubation, sections were washed, mounted, and coverslipped. The primary antibodies used in this study were: goat anti-Fos11 (Santa Cruz Biotechnology, sc-52-1,300), rabbit anti-CGRP11 (Millipore, AB13536, 1:1,000), sheep anti-Fox2P22 (R&D Systems, AF5647, 1:5,000), rabbit anti-NF20023 (Sigma, N4142, 1:200), GS-IB4-Alexa 488–conjugated (Invitrogen, I4121, 1:1,000), rabbit anti-V1R(TvRv1)24 (Abcam, ab31895, 1:1,000), and rabbit anti-GFP25 (Abcam, ab290, 1:1,000). The secondary antibodies were: Alexa Fluor 488 donkey anti-goat (Jackson Immunoresearch, 705-454-171 1:1,000), Cy3 donkey anti-goat (Jackson Immunoresearch, 705-145-147 1:1,000), Alexa Fluor 488 donkey anti-rabbit (Jackson Immunoresearch, 703-545-155 1:1,000), Cy3 donkey anti-rabbit (Jackson Immunoresearch, 711-165-152 1:1,000), and Alexa Fluor 647 donkey anti-sheep (Abcam, ab150181, 1:1,000).

Behavioral experiments for Fox immunostaining. Adult male and female C57BL/6 mice at ages more than 6 weeks were singly housed at least one day before noxious stimulation. Single housed mice were directly perfused for staining background Fox expression. For visualizing Fox expression induced by noxious stimuli, mice were lightly anesthetized with isoflurane and unilaterally injected with 10 μl of saline, or 4% capsicain, or 4% formalin into either the whisker pad or the hindpaw and returned to their home cage. 90 min later, the animals were perfused (as described in the method for immunostaining above).

Behavioral experiments for capturing PB-nociceptive neurons with CANE virus. A brief description of CANE method: in Fox+/- mice, activated neurons transiently express Fox which induces expression of a destabilized TVA (dTVA) receptor. Lentivirus or deficient rabies virus pseudotyped with an engineered mutated envelope protein (CANE envelope) specifically binds cells expressing high-level TVA receptor, which are strongly Fox+ neurons. In this way, CANE-viruses selectively infect Fox+ neurons and deliver desired transgenes to be expressed in Fox+ neurons. Here, adult male and female Fox+/- mice at ages more than 6 weeks were singly housed at least one day, and then either handled without injection, or handled and then subjected to noxious stumulation. Briefly, mice were placed in a home cage, placed in the anesthesia chamber, lightly anesthetized with isoflurane, and injected unilaterally with 10 μl of saline or 4% capsicain or 4% formalin into either the whisker pad or the hindpaw, and returned to their home cage. 60–90 min later, mice were anesthetized and underwent stereotaxic surgery for CANE-virus injection. Note that PB is a relatively large area and formalin/capsaicin activated neurons were only partially included in the injection. To capture neurons, we injected a sufficient amount of virus into the PB and then lowered the virus to the target area. After 60–90 min, mice were then anesthetized and subjected to noxious stimulation. Briefly, mice were placed into a home cage, placed in the anesthesia chamber, lightly anesthetized with isoflurane, and injected unilaterally with 10 μl of saline or 4% capsicain or 4% formalin into either the whisker pad or the hindpaw. 1 week after injection. The data was normalized by dividing with the number of the number of starter neurons (GFP and mCherry double positive neurons in the PB) in each animal.

Electrophysiological recording in acute brainstem slices. Four weeks after intraperitoneal injection of AAV9-EF1a-flex-Chr2-eYFP into Trpv1-Cre P1-2 mice, or 3 days after injection of CANE-RV-mCherry into the PB of Trpv1-Cre::Ai6512 mice, mice were anesthetized with isoflurane, and transected
perfused in ice-cold NMDF artificial cerebrospinal fluid (NMDF-ACSF; containing 92 mM NaMDG, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 2 mM glucose, 5 mM sodium ascorbate, 2 mM sodium pyruvate, 10 mM MgSO4, 0.5 mM CaCl2) and bubbled with 95% O2. The brain was then extracted and sectioned into 250 µm thick sagittal slices using a vibratome (VT-1000S, Leica Microsystems) containing ice-cold oxygenated NMDF-ACSF. Sagittal sections including the PB were then bubbled in same solution at 37°C for 8 min, and transferred to bubbled, modified-HEPES ACSF at room temperature (20–25°C; 92 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 2 mM glucose, 5 mM sodium ascorbate, 2 mM sodium pyruvate, 10 mM MgSO4, 0.5 mM CaCl2) for at least 1 h before recording. Recordings were performed in a submerged chamber, superfused with continuously bubbled ACSF (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 20 mM glucose, 2 mM CaCl2, 1.3 mM MgSO4) at near-physiological temperature (34.5 °C). After placing the slice on the recording stage, it was impaled by infrared differential interference contrast and fluorescence video microscopy (Examiner D1, Zeiss). Whole-cell current clamp recordings were amplified with Multiclamp 700B (digitized at 2 kHz), digitized with Digidata 1440 A (5 kHz), and recorded using pClamp 10 software (Axon). The onset latency of the light-evoked EPSCs and IPSCs was defined as the time of photosilencing/illuminating of TrpV1Cre::eArch during von Frey tests, a continuous green light (561 nm) stimulation (~12 mW) was delivered through the optic fiber to silence the TrpV1Cre::eArch and TrpV1Cre::GFP mice were also subjected to a real-time place preference test (RTPP). An individual mouse was placed in the center of the box and allowed to explore both chambers without light stimulation (pre-stimulation) for 10 min. Generally, after exploration, the mouse shows a small preference for one of the two chambers. Subsequently, blue light stimulation (10 Hz, 20 ms pulse-width, ~3.5 mW) was delivered whenever the mouse entered or stayed in the preferred chamber, and light is turned OFF when the mouse moves to the other chamber (stimulation phase, total 10 min). Finally, the mouse can freely explore both chambers separately, after exploring the mouse shows a significant preference for one of the two chambers. After recording the baseline behavior, individual mouse was injected with 5 µl 4% capsicain into the left whisker pad and placed in the chamber again to freely explore both chambers without light stimulation (no stimulation) for 10 min again. Subsequently, a continuous green light stimulation (561 nm, ~12 mW) was delivered through the optic fiber to silence the TrpV1Cre::eArch fibers (or illuminate the control GFP fibers) in PB, whenever the mouse entered or stayed in the non-preferred chamber, and light was turned off when the mouse moved to the other chamber (total 10 min of real-time stimulation). We recorded behavioral data via a webcam (Logitech video camera, PN 960-000764) interfaced with Bonsai software. Real-time laser stimulation was controlled by Bonsai software through Arduino with a custom-made Arduino sketch (Arduino UNO, A00273). After 1 week, the same group of mice were subjected to another behavioral test, the mouse was placed in a circular field in a sound proof chamber. The mouse’s movements and audible vocalizations were recorded from the top of field using the webcam with audio control at a frame rate 30 fps. The experimental mouse was placed in the center of the circular field and allowed to explore freely. Blue light was delivered as described above. The duration of each light stimulation was 30 s and the interval between light stimulations was 2 × 2 s. The number of light stimulations was 4. The number of pigs was calculated for each interval and averaged offline. All the data were expressed as mean ± s.e.m. All data (with the exception of behavioral data in-vivo) are expressed as mean ± s.e.m. A two-way ANOVA was performed to determine the statistical significance of the effects of laser stimulation on behavioral measures. A Bonferroni post-hoc test was used for multiple comparisons. The experimental data were analyzed using SPSS statistical software (Version 17.0, SPSS Inc). All error bars represent the mean ± s.e.m. The number of light stimulation for each mouse in each experiment is indicated in parentheses (e.g., 3 trials).
for the real-time PEA test) were analyzed using two-tailed paired and unpaired Student's t test between 2 groups (experimental or control), or in the case of multiple groups, one-way or two-way ANOVA followed by Tukey's test. The criterion for statistical significance was P < 0.05. Regarding the assumption of normality for large/medium datasets, D'Agostino and Pearson normality test was used. When the sample size was less than four, Shapiro-Wilk normality test was used. We provide mean values with associated s.e.m. values. To determine whether the variance was similar between the groups that are being statistically compared, F test was used for t tests, and Brown-Forsythe was used for one-way ANOVA. The results showed that the variance was similar.

Behavioral data for real-time PEA and RTPP tests were analyzed using one-way repeated measures ANOVA with Matlab R2016a. The statistical test was used for ChR2 group and GFP group independently. For PEA, the preference of the stimulation side between PRE (no-stim), STIM, and POST (no-stim) periods was compared. For RTPP, the preference of the stimulation side between Baseline, No Stimulation, and Stimulation periods was compared. Tukey's test was used post-hoc. Behavioral data for von Frey tests were analyzed using two-way repeated measures ANOVA. The statistical test was used for face test and hindpaw test independently. For both tests, both between and within eArch and GFP groups across conditions were compared.

**Life Sciences Reporting Summary.** Further information on experimental design and reagents is available in the Life Sciences Reporting Summary.

**Data availability.** The data collected in this study are available from the corresponding author upon request.

**Code availability.** All custom-written MatLab code used in this study is available at https://github.com/wanglab-duke/craniofacial-specific-monosynaptic-circuit-for-affective-pain.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

1. Sample size
   Describe how sample size was determined. 
   No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications (reference 28).

2. Data exclusions
   Describe any data exclusions. 
   For behavioral experiments, we used two exclusion criteria for our subjects: (1) poor recovery or other health concerns following surgical intervention or (2) missed injection or implantation target, as determined by posthoc histological analysis. Animals were randomly selected from each litter.

   For axonal tracing experiments, ROI’s with densities in which the total pixel numbers of GFP-labeled axons divided by the area of the nuclei was less than 0.1 were not counted.

3. Replication
   Describe whether the experimental findings were reliably reproduced. 
   All experimental findings were reliably reproduced among all subjects in all experiments. This is reported throughout all the figure legends.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups. 
   Animals were randomly selected from each litter. Random group allocation was maintained throughout the study, within constraints set by availability of in-house, purpose-bred lines.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis. 
   Investigators were blinded to group allocation during data collection and during data analysis. Behavioral analysis relied on objective, automatized measurements.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑   |           |
| ☑   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☑   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | A statement indicating how many times each experiment was replicated |
| ☑   | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ☑   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☑   | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
| ☑   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☑   | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

7. Software
Describe the software used to analyze the data in this study.

We recorded all behavioral data via a webcam (Logitech web-camera, PN 960-000764). We recorded all place preference and avoidance data via a webcam interfaced with Bonsai software. Real-time laser stimulation was controlled by Bonsai software through Arduino with a custom-made Arduino sketch.

Matlab 2016a was used to analyze axonal projections and mouse movements for place preference and avoidance tests.

FIJI (image J) were used to count the number of cells in various experiments.

Igor pro was used to analyze electrophysiological data.

GraphPad Prism 7 was used for statistical analysis and graphing the results.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials and reagents
Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Goat anti-Fos (Santa Cruz Biotechnology, sc52-g, 1:300), rabbit anti-CGRP (Millipore, AB15360, 1:1000), sheep anti-FoxP2 (R&D Systems, AF5647, 1:5000), rabbit anti-NF200 (Sigma, N4142, 1:200), GS-IB4-Alexa 488–conjugated (Invitrogen, I21411, 1:1000), rabbit anti-VR1 (Abcam, ab31895, 1:1000), and rabbit anti-GFP (Abcam, ab290, 1:1000).

For each primary antibody, we cited a reference.
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. n/a
   b. Describe the method of cell line authentication used. n/a
   c. Report whether the cell lines were tested for mycoplasma contamination. n/a
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. n/a

- Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.
   Adult (p30-p60) male and female C57/BL6, FosTVA, TrpV1-Cre, Ai32 & Ai65D mice.

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.
   n/a