Itch-specific neurons in the ventrolateral orbital cortex selectively modulate the itch processing

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Itch is a cutaneous sensation that is critical in driving scratching behavior. The long-standing question of whether there are specific neurons for itch modulation inside the brain remains unanswered. Here, we report a subpopulation of itch-specific neurons in the ventrolateral orbital cortex (VLO) that is distinct from the pain-related neurons. Using a Tet-Off cellular labeling system, we showed that local inhibition or activation of these itch-specific neurons in the VLO significantly suppressed or enhanced itch-induced scratching, respectively, whereas the intervention did not significantly affect pain. Conversely, suppression or activation of pain-specific neurons in the VLO significantly affected pain but not itch. Moreover, fiber photometry and immunofluorescence verified that these itch- and pain-specific neurons are distinct in their functional activity and histological location. In addition, the downstream targets of itch- and pain-specific neurons were different. Together, the present study uncovers an important subpopulation of neurons in the VLO that specifically modulates itch processing.

INTRODUCTION

Itch is an aversive cutaneous sensation that provokes the desire to scratch (1). However, repetitive scratching could lead to severe skin damage, which in turn intensifies the itch sensation and causes further scratching (2, 3). Therefore, itch can cause mental distress and reduce quality of life (4, 5). Beyond the understanding of the basic mechanisms of itch, exploring the itch-specific neurotransmitters and neurons may aid in the development of more effective therapies.

During the past decades, considerable progress has been made in identifying itch-specific neurotransmitters and neurons in both dorsal root ganglion (DRG) and spinal cord (6, 7). Previous studies suggested that gastrin-releasing peptide (GRP) is a crucial neurotransmitter for transmitting nonhistaminergic itch from primary sensory neurons in the DRG to GRP receptor (GRPR)–expressing neurons in the spinal cord (8, 9). In contrast, histaminergic itch is thought to be relayed by neurotransmitter neumedin B (NMB) from DRG neurons to the dorsal horn interneurons expressing NMB receptor (NMBR) (10, 11). A recent study demonstrated that B-type natriuretic peptide (BNP), which is co-released with NMB, is involved in facilitating NMB-encoded histaminergic itch via natriuretic peptide receptor C (NPRC)–NMBR cross-talk (12). Moreover, it was shown that Mas-related G protein–coupled receptor A3 (MrgprA3)–expressing neurons are considered the itch-specific neurons that transmit the sensation of itch elicited by chloroquine and other pruritogens from DRG to the spinal cord (13). At the spinal level, GRPR-expressing neurons are believed to be selectively involved in processing itch but not pain (6, 7, 14). This is evidenced by results indicating that ablation of the spinal GRPR+ neurons significantly disrupts the histaminergic and nonhistaminergic itch, but not pain, whereas selective activation of the spinal GRPR+ neurons directly induces itch-related scratching, but not pain-related behaviors (15, 16). Together, these studies provide converging evidence supporting the existence of itch-specific neurons and the selectivity theory of itch, suggesting that itch-inducing stimuli specifically activate itch-selective neurons to generate the corresponding sensations.

Although several subpopulations of neurons have been found to be specifically related to itch processing in DRG and spinal cord, the conclusion that itch and pain processing are separate is far from a consistent finding (8, 17, 18). For example, a recent study showed that a subpopulation of MrgprA3+ primary sensory neurons could convey pain and itch signals via different spinal GRPR or opioid pathways (19). In addition, a more recent study provided supportive evidence that glutamate is essential for MrgprA3+ primary afferents to transmit itch (20). However, glutamate was also used by almost all primary somatosensory afferents in nociceptive afferents (21), which argued that it is activation of specific combinations of postsynaptic neurons that might underlie itch specificity, rather than employment of modality-selective neurotransmitters (20). Meanwhile, a recent study also reported that spinal GRP interneurons received monosynaptic input from both pain and itch primary sensory neurons, and that ablation of these GRP neurons decreased itch but increased pain responses, arguing further against the selectivity theory at the spinal cord level (17). Thus, the intensity theory of itch–pain discrimination has also been proposed, claiming that the same group of polymodal neurons can be strongly or weakly stimulated to generate pain or itch sensations in the DRG and spinal cord, respectively (18, 22). Besides the intensity and selectivity theories, other theories also try to explain the relationship between itch and pain processing and to clarify mechanisms underlying itch (7, 23–25). Although different theories have been proposed to explain itch encoding in the DRG and spinal cord, they all targeted into one important direction—the difference between itch and pain encoding and transmission.

 Debates about differential encoding between pain and itch at the DRG and spinal cord levels have been ongoing for decades. The studies on brain mechanisms underlying itch may be helpful in addressing these disputes. It is encouraging that more detailed studies

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have increasingly focused on the brain mechanisms underlying itch processing (23). For example, a recent study has shown that GABAergic (γ-aminobutyric acid–releasing) neurons in the ventral tegmental area (VTA) play an important role in encoding the aversive component of itch, while VTA dopaminergic (DA) neurons modulate reward from itch relief (26). Selective activation of itch-responsive neurons in the amygdala enhanced itch- and anxiety-related behaviors (27). In addition, it has been shown that periaqueductal gray (PAG) GABAergic and glutamatergic neurons exhibited opposing effects on itch processing (28). Activation of PAG GABAergic neurons or inhibition of glutamatergic neurons suppressed itch, while inhibition of PAG GABAergic neurons or activation of glutamatergic neurons resulted in a significant increase in itch (28). Tac-1 neurons in the lateral and ventrolateral PAG have been shown to specifically modulate itch sensation via the rostral ventromedial medulla–dependent pathway (29). However, to date, there has been no specialized investigation into the distinct roles of the brain cortex between the processing of itch and pain. The ventrolateral orbital cortex (VLO), part of the limbic-thalamic-cortical circuit, is critical for pain (30–35). Neuroimaging studies have revealed that the orbital cortex is activated during itch processing (36–38). However, the functional importance of the VLO in itch modulation is largely unknown, and the question of whether there is a separate population of neurons that selectively modulates itch processing in VLO remains unexplored. In this study, we found that the VLO is essential for itch modulation, and we identified a well-defined subgroup of neurons in the VLO that is specifically involved in modulating itch but not pain.

RESULTS
Inhibition of VLO neurons significantly decreases itch and pain

First, we examined the activation of VLO neurons upon itch stimulation by measuring c-Fos expression. We found that the percentage of neurons expressing c-Fos in the VLO significantly increased after injection of 5-hydroxytryptamine (serotonin) (5-HT) into the napes of rats compared to treatment with saline (fig. S1B). To verify the function of VLO in the itch-scratching cycle, we inhibited VLO neurons by bilaterally injecting the VLO with a recombinant adeno-associated virus (rAAV) encoding hM4Di, an inhibitory designer receptor exclusively activated by a designer drug (DREADD), fused with mCherry under the control of the hSyn promoter (rAAV 2/9-hSyn-hM4Di-mCherry) (Fig. 1B). The efficacy of hM4Di-mediated inhibition was confirmed by slice recordings in this study (fig. S2A) and has also been examined in another recent study by our group (37). Itch-related scratching was evoked by intradermal injections of compound 48/80, 5-HT, chloroquine, or endothelin-1 into either the nape or the cheek 30 min after an intraperitoneal injection of clozapine N-oxide (CNO) (Fig. 1A). Our results showed that pharmacogenetic inhibition of VLO neurons significantly decreased the number (Fig. 1, C to J) and cumulative duration (fig. S2, B to I) of scratching bouts compared with the control group (bilateral injection of the rAAV 2/9-hSyn-mCherry into the VLO). Moreover, inhibition of the VLO also significantly suppressed acute pain–related wiping behavior induced by allyl isothiocyanate (AITC; fig. S2I). In addition, the evaluation of other types of pain by the electronic von Frey (EVF) test, formalin test, radiant heat test, and tail immersion test (TIT) showed similar results (fig. S2, K to O). Furthermore, the pharmacogenetic inhibition of VLO neurons did not affect rats’ motor function as measured by the balance beam test (BBT), open field test (OFT), and rotarod test (RT; fig. S2, P to T).

The effects of VLO inhibition on itch, pain, and motor function were further validated by an optogenetic manipulation. Rats were microinjected with rAAV 2/9-hSyn-eNpHR3.0-EYFP (or rAAV 2/9-hSyn-EYFP as a control) in the bilateral VLO (Fig. 2L). The efficacy of eNpHR3.0-mediated inhibition was confirmed by extracellular recordings (fig. S3, A and B). Scratching was induced in these rats by injections of compound 48/80, 5-HT, or chloroquine. During the 30-min test period for scratching behavior, we alternated between light-off and light-on periods, each of which lasted for 5 min. Thus, we cycled through these two states a total of three times (Fig. 1, K and M). Our results showed that optogenetic inactivation of the VLO also significantly reduced the number and cumulative duration of scratching bouts (Fig. 1, N to S, and fig. S3, C to H). During the light-off periods, however, the quantity of scratching did not significantly differ between the eNpHR3.0-expressing rats and the control rats (Fig. 1, N to S, and fig. S4). Moreover, optogenetic inhibition of the VLO significantly suppressed pain behaviors related to chemical, inflammatory, mechanical, and thermal pain (fig. S3, I to N). Similar to pharmacogenetic inhibition, the optogenetic inhibition of VLO neurons failed to influence rat motor function (fig. S3, O to S). These findings indicated that VLO neurons were critically involved in the modulation of both itch and pain processing.

Itch-specific neurons are labeled in the Tet-Off system

The above pharmacogenetic and optogenetic inhibition experiments have demonstrated that the VLO is involved in the cerebral processing of both itch and pain. Since the VLO contains different neuronal populations, whether they play different roles in itch and pain processing is worth investigating. Thus, we subsequently used the Tet-Off system to investigate whether itch-specific neurons participating in itch processing could be found in the VLO. We co-injected rAAV 2/9-c-Fos-tTA and rAAV 2/9-TRE-tight-mCherry into the bilateral VLO (Fig. 2A). To validate the Tet-Off system, we compared the expression of mCherry under various conditions (Fig. 2B). Under off-doxycycline (Dox) conditions, in which rats were fed a Dox-free diet for 34 days, 94.25% of VLO neurons expressed mCherry. However, only 0.59% of VLO neurons expressed mCherry when given Dox in their diet. These results supported the notion that Dox administration could steadily block the activation of the TRE promoter. Moreover, 7.46% of VLO neurons expressed mCherry 3 days after itch labeling, which was accomplished by injecting 5-HT into the cheek. Similarly, 6.89% of VLO neurons expressed mCherry 10 days after itch labeling. These data proved that mCherry expression lasted at least 10 days and that the expression level of mCherry did not significantly change during the behavioral experiments. In contrast, only 1.35% of neurons expressed mCherry 10 days after saline injection, which did not evoke scratching. At the level of virus expression, the results indicated that the Tet-Off system could label itch-specific neurons during the itch processing (Fig. 2, C and D).

Inhibition of VLO itch–specific neurons suppresses itch but not pain

We then investigated whether the labeled itch-specific neurons play a critical and specific role in itch as opposed to pain. Rats co-injected
with rAAV 2/9-c-Fos-tTA and rAAV 2/9-TRE-tight-hM4Di-mCherry (or rAAV 2/9-TRE-tight-mCherry as a control) in the bilateral VLO were fed a Dox-supplemented diet to prevent activation of the TRE promoter for 21 days (Fig. 2E). Next, the rats were given a Dox-free diet for 3 days, after which they were injected with 5-HT in the cheek to evoke itch and label itch-specific neurons (Fig. 2G). Thus, under the control of the c-Fos promoter and a tetracycline-controlled transactivator (tTA), VLO itch–specific neurons activated by intradermal injection of 5-HT in the absence of Dox expressed hM4Di-mCherry (or mCherry in the control group) (Fig. 2F). Moreover, double immunostaining showed that most itch-specific neurons (labeled with mCherry) were c-Fos–positive neurons (83.3 and 74.0%, respectively)
activated by 5-HT or chloroquine (fig. S5, A to H). The efficiency of hM4Di suppression was also verified by slice recordings (fig. S5I).

The pharmacogenetic inhibition of these itch-specific neurons decreased the number (Fig. 2, H to K) and cumulative duration (fig. S5, K to N) of scratching bouts evoked by injecting 5-HT (in the cheek and nape), compound 48/80 (in the nape), or chloroquine (in the nape). In contrast, the pharmacogenetic inhibition of itch-specific neurons did not affect the pain-related wiping induced by AITC (cheek; Fig. 2L) or motor function (fig. S5P). We also retested 5-HT–evoked
scratching without injections of CNO. As expected, there were no significant differences in the number (Fig. 2M) or cumulative duration (fig. S5O) of scratching bouts between the hM4Di-mCherry and control groups. Moreover, similar results were obtained in the pharmacogenetic inhibition of itch-specific neurons labeled with chloroquine (fig. S6).

In addition, we performed optogenetic inhibition of itch-specific neurons and found a similar inhibitory effect on itch processing (fig. S7, A to O). We further examined the possible role of VLO itch-specific neurons in pain using EVF, formalin, radiant heat, and TTT tests and found that optogenetic inhibition of VLO itch-specific neurons did not affect pain-related behaviors (fig. S7, P to T). Furthermore, motor function tested by BBT, OFT, and RT was not significantly affected by optogenetic suppression of VLO itch-specific neurons (fig. S7, U to Y). Together, these findings supported the notion that itch-specific neurons contributed only to modulating itch and not pain processing in the VLO.

**Activation of VLO itch–specific neurons selectively enhances itch but not pain**

To further investigate the possible neural mechanisms underlying the modulation of itch-specific neurons in itch scratching, we bilaterally injected rAAV 2/9-c-Fos-tTA mixed with rAAV 2/9-TRE-tight-Chr2-mCherry (or rAAV 2/9-TRE-tight-mCherry as a control) into the VLO. Similarly, 3 days after the withdrawal of Dox, rats’ cheeks were injected with 5-HT without laser stimulus to label itch-specific neurons with Chr2-mCherry (or mCherry in the control group) by 5-HT (Fig. 3, A to C, and fig. S8B). The efficacy of Chr2-mediated activation was confirmed by extracellular recordings (fig. S8A). Three days after labeling, rats were tested with no pruritogen or with pruritogens as flowchart sequence under the laser stimulus (Fig. 3A). Optogenetic activation of VLO itch-specific neurons failed to produce a significant increase in spontaneous scratching (Fig. 3D and fig. S8K) and spontaneous wiping (Fig. 3E and fig. S8L) in the absence of pruritogens and algogens.

**Fig. 3. Optogenetic activation of VLO itch–specific neurons increases itch-related scratching after pruritogen injection.** (A) Flow diagram of the basic behavioral experiment (above) and timetable of the whole optogenetic experiment (below). (B) Schematic showing bilateral injection of rAAV 2/9-c-Fos-tTA and rAAV 2/9-TRE-tight-Chr2-mCherry or rAAV 2/9-TRE-tight-mCherry into the VLO. Representative images showing Chr2-mCherry expression in the VLO 10 days after itch labeling. Scale bar, 200 μm. (C) Three days after the withdrawal of Dox, rats were injected with 5-HT in the cheek to label itch-specific neurons in the VLO. n = 7 to 9 rats in each group. (D to E) Optogenetic activation of itch-specific neurons did not affect the number of scratching bouts (D) or the quantity of wiping (E) in the absence of pruritogens and algogens. n = 7 to 9 rats in each group. (F to I) Optogenetic activation of itch-specific neurons significantly increased the number of scratching bouts induced by intradermal injection of 5-HT (cheek, F; nape, G), compound 48/80 (nape, H), or chloroquine (nape, I). n = 7 to 9 rats in each group. (J) Optogenetic activation of itch-specific neurons did not significantly affect AITC-induced wiping. n = 7 to 9 rats in each group. (K) The increase in itch processing was reversed when the same rats were restested in the absence of laser illumination. n = 7 to 9 rats in each group. Error bars represent the SEM. *P < 0.05, **P < 0.01, unpaired Student’s t test.
However, after the injection of pruritogens, optogenetic activation of VLO itch–specific neurons resulted in significant increases in the number (Fig. 3, F to I) and cumulative duration of scratching bouts (fig. S8, C to F and M to T). Nevertheless, optogenetic manipulation of itch-specific neurons did not significantly alter AITC-induced wiping (Fig. 3J and fig. S8U) or motor function (fig. S8H).

Last, we also restretched rats’ scratching after injection of 5-HT into cheeks without laser stimulus. There was no significant difference between two groups (Fig. 3K and fig. S8, G, V, and W). In addition, pharmacogenetic activation of itch-specific neurons showed similar results (fig. S9), which enhanced the reliability of our data. Activation of VLO itch–specific neurons enhanced scratching only in the presence of pruritogens, suggesting that these specific VLO neurons were involved in the modulation of itch processing rather than the direct transmission of itch sensation and scratching movement signals.

**Manipulation of pain-specific neurons selectively affects pain but not itch**

Combined with previous findings, the present data indicated that the VLO was involved in the modulation of both itch and pain and that itch-specific neurons of the VLO were implicated in itch but not pain processing. We thus asked whether the VLO also contained pain-specific neurons, i.e., the subpopulation of neurons that was specifically involved in the modulation of pain but not itch. We bilaterally injected rAAV 2/9-c-Fos-tTA mixed with rAAV 2/9-TRE-tight-hM4Di-mCherry (or rAAV 2/9-TRE-tight-mCherry as a control) into the VLO and labeled pain-specific neurons by injection of AITC into rats’ cheeks (Fig. 4, A and B). Double immunostaining showed that most (78.0%) pain-specific neurons (mCherry) were c-Fos–positive neurons activated by AITC (fig. S10, A to D). The pharmacogenetic inhibition of VLO pain-specific neurons significantly impaired pain (Fig. 4D) but not itch (Fig. 4, E to H, and fig. S10, E to I). Consistently, optogenetic suppression of VLO pain-specific neurons significantly inhibited pain-related behaviors in the AITC and capsaicin tests of chemical pain, EVF test of mechanical pain, formalin test of inflammatory pain, radiant heat, and TIT tests of thermal pain (fig. S11, C to I), while itch-induced scratching (fig. S11, J to Q) and motor function were not significantly influenced (fig. S11, R to V).

To further detect the influence of activating pain-specific neurons on pain and itch, we bilaterally injected rAAV 2/9-c-Fos-tTA mixed with rAAV 2/9-TRE-tight-ChR2-mCherry (or rAAV 2/9-TRE-tight-mCherry as a control) into the VLO. Pain-specific neurons were labeled by injection of AITC into the cheeks of the rats (Fig. 4, I and J). The optogenetic activation of pain-specific neurons did not significantly affect wiping or scratching in the absence of algogenes or pruritogens (Fig. 4, L and M) but increased AITC-induced (Fig. 4N) and capsaicin-induced (Fig. 4O) wiping. Correspondingly, the activation of pain-specific neurons did not significantly affect itch (Fig. 4, P to R, and fig. S12, A to D). Similarly, the pharmacogenetic activation of pain-specific neurons significantly enhanced the pain-related behaviors (fig. S13, D to J), while itch-induced scratching (fig. S13, L to T) and motor function (fig. S13, U to Y) were not influenced. Together, these results excluded the possibility that itch-specific neurons were a subset of pain-specific neurons and indicated that the neuronal subpopulations of modulation of pain and itch in the VLO were different.

The VLO is composed of inhibitory GABAergic neurons and excitatory pyramidal neurons, which are nonoverlapping populations.

We next examined whether there was a percentage difference between pyramidal neurons and GABAergic neurons among the subpopulations of itch-specific and VLO pain-specific neurons. Double immunofluorescence staining results showed that 70.43% of itch-specific neurons in the VLO were CaMKIIα+ neurons (pyramidal neurons), 9.57% were GAD 67+ neurons (GABAergic neurons), and 1.46% were parvalbumin+ neurons (fig. S14). Correspondingly, 78.44% of pain-specific neurons were pyramidal neurons, 8.02% were GABAergic neurons, and 2.08% were parvalbumin+ neurons (fig. S15).

Next, to investigate the differential roles of VLO pyramidal and GABAergic neurons in the regulation of itch and pain behaviors, we used fiber photometry to measure the activity of VLO pyramidal and GABAergic neurons during itch and pain processing in freely moving rats. We found that VLO pyramidal and GABAergic neurons showed opposing calcium responses during both itch-evoked scratching and pain-induced wiping. VLO pyramidal neurons showed significant increases in calcium signals during both itch-evoked scratching and pain-induced wiping. These increases in calcium signals began before the onset of scratching and wiping (fig. S16, A to C). In contrast, VLO GABAergic neurons showed the opposite calcium responses, wherein itch-induced scratching and pain-induced wiping resulted in slight and long-lasting decreases in calcium signals before the onset of scratching and wiping. Increased magnitudes in the calcium signal of VLO pyramidal neurons were significantly higher than decreased magnitudes in the calcium signal of VLO GABAergic neurons during itch-induced scratching and pain-induced wiping (fig. S17, A to C).

To further confirm the functional role of VLO pyramidal neurons in itch and pain processing, we injected rAAV 2/9-CaMKIIα-hM4Di-mCherry (or rAAV 2/9-CaMKIIα-mCherry as a control) into the bilateral VLO (fig. S16, E and F) and found that pharmacogenetic inhibition of VLO pyramidal neurons significantly suppressed both itch (fig. S16, G to R) and pain (fig. S16S) but did not significantly affect motor function (fig. S16T). In addition, to detect the role of VLO GABAergic neurons in itch and pain processing, rAAV 2/9-VGAT1-Cre, together with rAAV–EF1α-DIO-hM4Di-mCherry or rAAV–EF1α-DIO-mCherry, was injected into the VLO (fig. S17, D to E). Both itch and pain were significantly increased following the pharmacogenetic inhibition of VLO GABAergic neurons (fig. S17, F to M). However, pharmacogenetic inhibition had no significant effect on motor function (fig. S17N). Together, these results indicated that pyramidal neurons and GABAergic neurons in the VLO participated in the itch and pain processing.

**Itch- and pain-specific neurons are selectively activated during itch and pain, respectively**

Since the manipulation of VLO itch– and pain-specific neurons independently affected itch and pain processing, we further used the Tet-Off system combined with fiber photometry to examine the activities of VLO itch– and pain-specific neurons during itch and pain processing. We co-injected rAAV 2/9-c-Fos-tTA mixed with rAAV 2/9-TRE-tight-jGCaMP7s or rAAV 2/9-TRE-tight-EGFP into the unilateral VLO and then implanted optical fiber at the same place (Fig. 5, B and H). Three days after the removal of the Dox drug, we activated and labeled VLO itch– and pain-specific neurons by injection of 5-HT (cheek) and AITC (cheek), respectively (Fig. 5, A and G). We found that calcium signals of itch-specific neurons significantly increased only during itch processing and not during pain.
processing (Fig. 5, C to F). Accordingly, the calcium signals of pain-specific neurons significantly increased only during pain processing and not during itch processing (Fig. 5, I to L). Moreover, we also examined the calcium signals of itch- and pain-specific neurons with half concentrations of pruritogen and algogen. The respective activation patterns of the itch- and pain-specific neurons were consistent, but the amplitudes of calcium signals were lower at half the initial pruritogen and algogen concentrations than at the initial concentrations (fig. S18). The fiber photometry results were in accordance with the behavioral experiments, further indicating that there are different neuronal populations for the modulation of itch and pain processing in the VLO.
The itch- and pain-specific neurons are nonoverlapping subpopulations in the VLO

We co-injected rAAV 2/9-c-Fos-tTA mixed with rAAV 2/9-TRE-tight-mCherry into the VLO and then separately labeled itch- and pain-specific neurons with mCherry in different rats. Ninety minutes before perfusion for endogenous c-Fos staining, the two groups were relabeled, with the labels for itch and pain swapped (Fig. 6, A and B). Thus, we could simultaneously observe the distribution of itch- and pain-specific neurons by mCherry expression and endogenous c-Fos staining. For the Itch::mCherry and Pain::c-Fos groups, in which VLO neurons were labeled itch with mCherry and then labeled pain with endogenous c-Fos, the percentage of VLO neurons expressing both itch-induced mCherry and pain-activated c-Fos was 1.71% (Fig. 6, C and G). The ratio of mCherry and c-Fos double-positive neurons among mCherry + neurons (mCherry + c-Fos + / mCherry + ) was significantly lower than that of mCherry + -only neurons.
Fig. 6. The itch- and pain-specific neurons are nonoverlapping subpopulations in the VLO. (A) Experimental protocol for labeling of itch-specific (top) or pain-specific (bottom) neurons and detection of pain-activated (top) or itch-activated (bottom) endogenous c-Fos–positive neurons. (B) Schematic showing virus injection and representative images showing the general distribution of itch-specific neurons, pain-specific neurons, and c-Fos–positive neurons. (C) In the itch::mCherry and pain::c-Fos expression group, the overlapping ratios of itch-specific neurons (mCherry⁺) and pain-activated c-Fos–positive neurons were analyzed. Overlapping ratio = no. of (mCherry⁺ c-Fos⁺ neurons)/no. of (mCherry⁺ neurons + c-Fos⁺ neurons – mCherry⁺ c-Fos⁺ neurons). (D) Percentage of mCherry⁺ or c-Fos⁺ neurons in NeuN⁺ neurons. (E and F) Percentage of double-positive neurons among mCherry⁺ or c-Fos⁺ neurons was significantly lower than that of single-positive neurons. (G) Representative images of endogenous c-Fos activated by pain in the VLO of rats expressing mCherry⁺ itch-specific neurons. Scale bar, 100 μm. (H) In the pain::mCherry and itch::c-Fos expression group, the overlapping ratios of pain-specific neurons (mCherry⁺) and itch-activated c-Fos–positive neurons were analyzed. (I) Percentage of mCherry⁺ or c-Fos⁺ neurons among NeuN⁺ neurons. (J and K) Percentage of double-positive neurons among mCherry⁺ or c-Fos⁺ neurons was significantly lower than that of single-positive neurons. (L) Representative images of endogenous c-Fos activated by itch in the VLO of rats expressing mCherry in pain-specific neurons. Scale bar, 100 μm. n = 6 rats (five sections per animal) in each group. Error bars represent the SEM. ***P < 0.001, unpaired Student’s t test.
neurons among mCherry+ neurons (mCherry+ c-Fos-/mCherry+; Fig. 6E). Moreover, the proportion of mCherry and c-Fos double-positive neurons among c-Fos+ neurons (mCherry+ c-Fos+/c-Fos+) was also significantly lower than that of c-Fos+ single-positive neurons among c-Fos+ neurons (mCherry- c-Fos+/c-Fos-; Fig. 6F). In addition, following a similar protocol for the Pain:mCherry and Itch::c-Fos groups, the overlapping ratio was 2.86% (Fig. 6H). Representative immunofluorescence images and statistical analysis also showed a lower overlapping ratio between pain-specific neurons (mCherry+ neurons) and itch-specific neurons (c-Fos+ neurons; Fig. 6, J to L). Together, from a histological perspective, we verified that the subpopulations of itch- and pain-specific neurons in the VLO were also distinct.

In addition, we used the Tet-Off system combined with the HSVΔTK virus to evaluate whether the downstream targets of VLO itch- and pain-specific neurons were different. We injected rAAV 2/9-c-Fos-tTA mixed with rAAV 2/9-PTRE-tight-Cre and rAAV 2/9-EF1α-DIO-EGFP-T2A-TK into the unilateral VLO and fed rats Dox for 21 days. Rats were injected with 5-HT or AITC to activate and label itch- or pain-specific neurons in the VLO 3 days after removing the Dox. Three days later, we injected HSVATK-hUbc-tdTomato into the same brain region. Ten days after HSVATK virus injection, the rats were perfused to detect the downstream targets (fig. S19A) (39, 40). We found that VLO itch–specific neurons mainly projected to the piriform cortex, layer II (fig. S19, B to E), while more pain-specific neurons projected to the agranular insular cortex post (fig. S19, F to I). The difference in downstream projections showed obvious otherness of itch- and pain-specific neurons in the VLO.

**DISCUSSION**

Itch and pain are two closely related but functionally distinct forms of sensation that evoke different behaviors. In this study, we labeled both itch- and pain-specific neurons in the VLO by using the Tet-Off system. The fiber photometry results showed that itch- and pain-specific neurons responded only to itch–induced scratching and pain–induced wiping, respectively. By using pharmacogenetic and optogenetic manipulations, we verified that VLO itch–specific neurons modulate only itch but not pain. Correspondingly, the inhibition and activation of VLO pain–specific neurons affected pain but not itch. Furthermore, the immunofluorescence staining results showed that most itch–specific neurons did not overlap with pain–specific neurons in the VLO. Anatomical studies also indicated that the downstream targets of itch- and pain-specific neurons were also different. Together, our results strongly support the hypothesis that there is a specific population of neurons in the VLO that selectively modulates itch processing but not pain processing.

Fiber photometry studies showed a unique phenomenon in which the rising points of calcium signals were significant before the onset of scratching and wiping, which is significantly different from previous reports. For example, VTA GABA neurons and PAG glutamatergic neurons increased their activities at approximately the same time when the animal started scratching, while VTA DA neurons showed delayed activity to the onset of scratching (26, 29). The differential temporal patterns of VTA GABA and DA neuron activation were not affected by concentrations of pruritogens (26), suggesting that these neuronal subpopulations may play distinct roles in itch processing. The study has also demonstrated that VTA GABAergic neurons play a critical role in encoding the aversive component of itch sensation, while VTA DA neurons modulate the reward response to scratch-induced relief of itch (26). In contrast, our results showed that the rising points of VLO itch– and pain-specific neuron activation occurred before the onset of scratching and wiping movement, respectively, indicating that two specific neuronal subpopulations might be important for the motivational aspect of scratching and wiping and/or for transmitting sensory components of itch and pain, rather than being involved in directly modulating and participating in the scratching and wiping motor network. In addition, direct activation of both itch- and pain-specific neurons failed to produce a significant increase in either spontaneous scratching behavior or spontaneous wiping behavior, and manipulation of the VLO neurons did not affect the motor functions. These results could support the possibility that VLO neurons were not involved in triggering and controlling the sensory and movement network at the behavioral level. Thus, all of the results can be verified by one another without any inconsistency and support our conclusion. Given that itch and pain processing are regulated by different neural networks that encode sensory, emotional, attentional, evaluation, and motivational information (36, 41), it will be exciting for future studies to explore the precise roles of VLO itch– and pain-specific neurons in itch and pain processing.

Notably, the pruritogen 5-HT has been reported to induce hyperalgesia (42). However, our current findings can exclude the possibility that the subpopulation of VLO neurons activated and labeled by injection of 5-HT into the cheek contains pain-related neurons. First, consistent with a previous study (43), our behavioral results showed that injection of 5-HT into the cheek elicited a significant amount of itch–related scratching with little pain–related wiping. Moreover, manipulation of VLO itch–specific neurons labeled with 5-HT selectively affects itch but not pain processing. At the level of neuronal activity, fiber photometry results showed that the calcium signals of VLO itch–specific neurons did not change during AITC-induced pain. Furthermore, histological results indicated that the VLO itch–specific neurons labeled with 5-HT were highly overlapped with neurons activated by injection of the pruritogen chloroquine into the cheek. However, the VLO itch–specific neurons labeled with 5-HT did not conspicuously overlap with the neurons activated by pain. In addition, the downstream targets of VLO itch–specific neurons labeled with 5-HT were different from those of pain–related neurons labeled with AITC. Therefore, it is unlikely that the VLO itch–specific neurons labeled with 5-HT comprise pain–responsive neurons.

Given that neurons can be classified into different neuronal subpopulations according to distinct neurotransmitters and that the VLO comprises pyramidal and GABAergic neurons, we subsequently examined the possible role of distinct neuronal subpopulations in itch and pain processing. Our current findings indicated that the majority of both itch- and pain-specific neurons in the VLO were pyramidal neurons and that inhibition of pyramidal neurons significantly suppressed itch and pain processing, consistent with the effect of VLO neuronal global inhibition. These results strongly suggest that VLO pyramidal neurons play a positive regulatory role in itch and pain processing. On the other hand, although our data showed that only small portions of both itch- and pain-specific neurons in the VLO were GABAergic neurons, the role of GABAergic neurons in itch and pain processing should not be overlooked. As expected, we found that inhibition of GABAergic neurons significantly increased...
Itch and pain, suggesting that VLO GABAergic neurons might negatively modulate itch and pain processing. Consistently, the fiber photometry data also demonstrated that VLO pyramidal and GABAergic neurons showed opposing responses during both itch and pain processing. Thus, these results demonstrate that VLO pyramidal and GABAergic neurons have opposite functions in the modulation of pain and itch processing. Recent studies have also identified the differential roles of distinct neuronal subpopulations in the modulation of itch processing. Activation of PAG GABAergic neurons or inhibition of PAG glutamatergic neurons attenuated itch, while inhibition of PAG GABAergic neurons or activation of PAG glutamatergic neurons led to increases in itch, suggesting that PAG GABAergic and glutamatergic neurons exhibit opposing effects on the regulation of itch processing. (28). Moreover, VTA DA and GABAergic neurons are also shown to be differentially involved in hedonic and aversive aspects, respectively, of the itch-scratching cycle (26).

It has been widely recognized that chemical itch can be classified into two subtypes, histaminergic and nonhistaminergic. The central mechanisms underlying histaminergic and nonhistaminergic itching might not be identical. For example, projections from the anterior cingulate cortex to the dorsal medial striatum selectively modulate histaminergic but not nonhistaminergic itch-related behavior (44). Consistently, our recent study also showed that inhibition of the caudal anterior cingulate cortex reduced histaminergic but not nonhistaminergic itch-induced behavior (37). Thus, to investigate the potential different roles of VLO in histaminergic and nonhistaminergic itch processing, we used compound 48/80 as a histaminergic pruritogen and 5-HT and chloroquine as nonhistaminergic chemical pruritogens. All of these pruritogens have been widely used in previous studies (45), and the chosen concentration could steadily induce itch-related scratching without causing pain-related wiping or severe skin lesions in rats. Notably, histamine, by far the most widely pruritogen for mice, was not suitable for rats because it failed to elicit notable scratching in rats (43, 45). On the other hand, for labeling pain-specific neurons, AITC and capsaicin are both classic chemical algogens that induce wiping behavior in mice. However, we preferred AITC rather than capsaicin to label pain-specific neurons in rats because injection of capsaicin into the cheek could induce both pain-related wiping and itch-related scratching at different concentrations in rats (fig. S20), while injection of AITC into the cheek elicited only pain-related wiping, consistent with previous studies (43, 46, 47).

Notably, the specificity of itch and pain processing was measured only in the VLO region. The present findings may not be suitable for generalization to other brain regions, especially to the spinal and peripheral regions. Therefore, more studies will be required to uncover whether itch and pain are transmitted and modulated either by separate modality-specific neuronal populations or by a common population of nonspecific neurons in other brain regions.

In summary, we identified a subpopulation of itch-related neurons in the VLO that play specific and essential roles in the modulation of itch processing. Our results may advance the understanding of the central neural mechanisms underlying itching processing. In addition to labeling two specific neuronal subpopulations that modulate itch and pain processing in the present study, the application of the Tet-Off system may enrich our technological means for investigating central mechanisms underlying similar forms of somatosensation.

**MATERIALS AND METHODS**

**Animals**

Adult male Sprague-Dawley rats aged 3 to 4 months weighing 350 to 400 g (except for slice electrophysiology experiments, where rats aged 8 to 10 days were used for virus injection, and the recording was performed 20 to 25 days after the virus injection) were housed in standard laboratory cages on a 12:12 light/dark cycle with ad libitum access to food and water. The room temperature was maintained at 23° to 25°C. All behavioral experiments were performed during the light circle. All rat procedures were approved by the Animal Care Committee of Army Medical University and were performed in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Stereotaxic surgeries and injections**

Rats were anesthetized with 3% pentobarbital sodium [40 mg/kg, intraperitoneally (ip)] and then fixed in a stereotaxic apparatus (model 942, David Kopf Instruments, USA). The viruses were injected at a rate of 50 or 60 nl/min using a glass pipette with a tip diameter of approximately 20 μm, controlled by a 5-μl Hamilton microsyringe (51189, Stoelting, USA) and stereotaxic microsyringe pump (53311, Stoelting, USA), or directly controlled by a programmable nanoliter injector (Nanoject III, Drummond Scientific, USA). After the injection, the needle was left in place for an additional 5 min and then slowly withdrawn.

For global pharmacogenetic inhibition of VLO neurons, rats were bilaterally microinjected with 300 nl of rAAV 2/9-hSyn-hM4Di-mCherry [titer: 6.96 × 10^{12} genome copies (GC)/ml] or rAAV 2/9-hSyn-mCherry as a control (titer: 6.25 × 10^{12} GC/ml) into the VLO [anteroposterior (AP), +4.20 mm from bregma; mediolateral (ML), ±2.00 mm; dorsoventral (DV), −4.30 mm] (Fig. 1B). Moreover, to confirm the efficacy of hM4Di-mediated inhibition, rats aged 8 to 10 days were bilaterally microinjected with 300 nl of rAAV 2/9-hSyn-hM4Di-mCherry (titer: 6.96 × 10^{12} GC/ml) or rAAV 2/9-hSyn-mCherry as a control (titer: 6.25 × 10^{12} GC/ml) into the VLO. Slice electrophysiology recording was performed 20 to 25 days after virus injection.

For global optogenetic inhibition of VLO neurons, rats were bilaterally microinjected with 300 nl of rAAV 2/9-hSyn-EhNpHR3.0-EYFP (titer: 1.73 × 10^{12} GC/ml) or rAAV 2/9-hSyn-EYFP as a control (titer: 4.89 × 10^{12} GC/ml) into the VLO (AP, +4.20 mm; ML, ±2.00 mm; DV, −4.30 mm; Fig. 1L). For pharmacogenetic inhibition of VLO pyramidal neurons, rats were bilaterally microinjected with 300 nl of rAAV 2/9-CaMKIIα-hM4Di-mCherry (titer: 5.89 × 10^{12} GC/ml) or rAAV 2/9-CaMKIIα-mCherry as a control (titer: 5.04 × 10^{12} GC/ml) into the VLO (AP, +4.20 mm; ML, ±2.00 mm; DV, −4.30 mm; Fig. S17C). For pharmacogenetic inhibition of VLO GABAergic neurons, rats were bilaterally microinjected with 300 nl of rAAV 2/9-VGAT1-Cre (titer: 2.28 × 10^{12} GC/ml) mixed with rAAV-EF1a-DIO-hM4Di-mCherry (titer: 2.42 × 10^{12} GC/ml) or rAAV-EF1a-DIO-mCherry as a control (titer: 2.71 × 10^{12} GC/ml) into the VLO (AP, +4.20 mm; ML, ±2.00 mm; DV, −4.30 mm; Fig. S18B).

For labeling and pharmacogenetic inhibition of itch- or pain-specific neurons in the VLO, rats were microinjected with 300 nl of rAAV 2/9-c-Fos-tTA (titer: 2.58 × 10^{12} GC/ml) mixed with rAAV 2/9-TRE-tight-hM4Di-mCherry (titer: 2.25 × 10^{12} GC/ml) or rAAV 2/9-TRE-tight-mCherry (titer: 2.39 × 10^{12} GC/ml) as a control into the bilateral VLO (AP, +4.20 mm; ML, ±2.00 mm; DV, −4.30 mm;
Electrophysiological verification of pharmacogenetics

Electrophysiological verification of pharmacogenetics was performed as previously described (37, 48–52). Twenty to 25 days after virus injection, the rats were anesthetized with 3% pentobarbital sodium (40 mg/kg, ip) and decapitated. The brains were quickly removed into ice-cold cutting solution (220 mM sucrose, 2.5 mM KCl, 1.25 mM NaH2PO4, 6 mM MgCl2, 26 mM NaHCO3, 1 mM CaCl2, and 10 mM glucose) bubbled with 95% O2 and 5% CO2. Coronal slices containing the VLO (300 µm thick) were prepared by using a VT1200 vibratome (Leica, Germany) in ice-cold cutting solution. The slices were incubated for at least 1 hour in 95% O2 and 5% CO2 oxygenated artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 1.3 mM MgSO4, 26 mM NaHCO3, 2 mM CaCl2, and 10 mM glucose). During recording sessions, the slices were transferred to a submersion chamber (Warner, USA) in which oxygenated ACSF was continuously superfused at a rate of 2 ml/min at room temperature.

Borosilicate glass pipettes (3 to 5 meghohms) filled with an internal solution (130 mM K-gluconate, 5 mM KCl, 2 mM MgCl2, 10 mM HEPES, 0.1 mM EGTA, 2 mM Na–adenosine triphosphate, 0.2 mM Na–guanosine triphosphate, adjusted to pH 7.25 with 1 M KOH) were used for whole-cell patch-clamp recordings. hM4Di-mCherry+ neurons in the VLO were visually identified using a BX51WI fluorescence microscope (Olympus, Japan). Whole-cell patch-clamp recordings were then acquired on these neurons with a MultiClamp-700B amplifier (Molecular Devices, USA). Using the Digidata-1440 A interface (Molecular Devices, USA), the signals were low-pass-filtered at 2 kHz and then digitized at 10 kHz for data capture. Recording was performed in current-clamp mode. CNO (3 µM; HY-17366, MCE, USA) was bath-applied to confirm the efficacy of hM4Di-mediated inhibition (figs. S2, S5, and S17).

Electrophysiological verification of optogenetics

Three to 4 weeks after virus injection, rats were anesthetized with 3% pentobarbital sodium (40 mg/kg, ip) and their heads were placed in a stereotaxic apparatus (model 942, David Kopf). An optrode consisting of a multi-wire electrode tightly attached to an optical fiber cannula [ceramic ferrule: 2.50 mm diameter; optical fiber: 200 µm core diameter, 0.39 numerical aperture (NA), FT200EMT, Thorlabs, USA], with the tip of the electrode extending approximately 300 µm beyond the tip of the optical fiber, was used for laser illumination and extracellular recordings. The multi-wire electrode was made of 16 individually insulated nichrome wires (17.78 µm inner diameter, 761000, A-M Systems, USA) attached to a 20-pin connector. Extracellular signals were band-pass-filtered (0.3 to 5 kHz), amplified (1000×) using a 16-channel microelectrode amplifier (model 3600, A-M Systems, USA), and acquired with a data acquisition system (Powerlab 16/35, ADInstruments, Australia) with a sampling rate of 20 kHz. Data were analyzed using NeuroExplorer 4 (MicroBrightField, USA) and neurophysiological data analysis software.

To confirm the efficacy of eNpHR3.0-mediated inhibition, the optrode was slowly lowered to the left VLO using a micromanipulator (IVM-1000, Scientifica, UK) in anesthetized rats expressing eNpHR3.0. The optical fiber was connected to a 593-nm laser (YL593T6, SLOC, China) controlled by a pulse stimulator (Master-9, A.M.P.I., Israel). The power density of light emitted from the optrode was calibrated to approximately 5 mW. After spontaneous firing of neurons was detected, 593-nm laser illumination (10 epochs of 500 ms, separated by a variable interval of 20 to 40 s) reliably suppressed neural activities in a temporally precise, stable, and reversible manner (fig. S3).

Similarly, for the rats expressing ChR2, 473-nm laser (BL473T3, SLOC, China) illumination (5 mW, 20 Hz, 15-ms pulse duration,
Optical fiber implantation
The surgical protocol was performed as previously described and was similar to the protocol described above for virus injection. Briefly, for optogenetic manipulations, optical fiber cannulas (ceramic ferrule: 2.50 mm diameter; optical fiber: 200 μm core diameter, 0.39 NA, FT200EMT, Thorlabs) were implanted 300 μm above the virus injection site in the bilateral VLO (AP, +4.20 mm; ML, ±2.00 mm; DV, −4.00 mm; Figs. 1L and 4B). For fiber photometry recording, the same optical fiber cannula was placed 50 μm above the virus injection site in the right VLO (AP, +4.20 mm; ML, +2.00 mm; DV, −4.25 mm; Fig. 5, B and H). Following surgery, the rats recovered and were housed at least 1 week before the behavioral and recording experiments.

Labeling of itch-specific neurons
To label itch-specific neurons in the VLO, rats were raised on food with Dox (100 mg/kg) after virus injection for 3 to 4 weeks. Three to 4 weeks later, the Dox diet was replaced with regular food without Dox for 3 days (~72 hours) to open a window of activity-dependent labeling. Three days after removal of Dox, intradermal injection of 5-HT or chloroquine into the cheek was performed by using an injector with 30-gauge (0.3 mm external diameter) hypodermic needle to induce chemical itch and neural activity. Thirty minutes after 5-HT and chloroquine injection, the rats were returned to their home cages with a Dox diet (e.g., Figs. 2E and 3A).

Labeling of pain-specific neurons
To label pain-specific neurons in the VLO, rats were raised on food with Dox (100 mg/kg) after virus injection for 3 to 4 weeks. Three to 4 weeks later, the Dox diet was replaced with regular food without Dox for 3 days (~72 hours) to open a window of activity-dependent labeling. Three days after removal of Dox, intradermal injection of AITC into the cheek was performed by using an injector with 30-gauge (0.3 mm external diameter) hypodermic needle to induce pain-related wiping and neural activity. Thirty minutes after AITC injection, the rats were returned to their home cages with a Dox diet (e.g., Figs. 4A and 5A).

Pharmacogenetic manipulations
For in vivo pharmacogenetic inhibition, rats expressing hM4Di-mCherry or mCherry as a control were injected (intraperitoneally) with CNO (4 mg/kg; diluted with 5% dimethyl sulfoxide and saline), and their behaviors were measured 30 min after CNO injection.

Optogenetic manipulations
For in vivo optogenetic suppression, rats expressing eNpHR3.0 or EYFP/mCherry received 30-min rest-stimulation cycles of illumination (5 min laser off–5 min laser on, repeated three times) with a 593-nm laser (YL593T6) controlled by a pulse stimulator (Master-9) immediately after pruritogens or algogen injection. A constant laser (593 nm, ~5 mW) was bilaterally delivered during the laser-on period.

For in vivo optogenetic activation, rats expressing ChR2-mCherry or mCherry also received 30-min rest-stimulation cycles of illumination (5 min laser off–5 min laser on, repeated three times) with a 473-nm laser (BL473T3) controlled by a pulse stimulator (Master-9) immediately after pruritogens or algogen injection. A blue laser (473 nm, ~5 mW, 20 Hz, 15 ms) was bilaterally delivered during the laser-on period. In addition, for in vivo optogenetic activation during the BBT, the rats received laser illumination (473 nm, ~5 mW, 20 Hz, 15 ms) immediately after the start of the test.

Itch test
On the basis of the experimental design, 2 or 5 days before the behavioral tests, each rat was shaved on the nape and/or cheek. For the automatic assessment of scratching, a small rare earth magnet ring (outside diameter, 11 to 12 mm; inside diameter, 7.8 to 8.8 mm; height, 2 mm) was attached to the hindlimb of each rat, which was well tolerated by awake rats. The magnet ring was placed immediately before daily habituation and behavioral tests and removed immediately after the daily test. In addition, rats were habituated for 30 min for two consecutive days in a plastic behavioral recording box (30 cm by 30 cm by 35 cm) housed within a sound-attenuating chamber before experimentation and data collection. The recording box was surrounded by an induction coil connected to a differential amplifier (model 3500, A-M Systems).

During behavioral experiments, the baseline was recorded for 15 min in the recording box. Then, the rats were briefly removed from the recording box and intradermally injected with different pruritogens into the cheek or nape by using an injector with 30-gauge (0.3 mm external diameter) hypodermic needle. In the cheek itch model, rats received a unilateral intradermal injection of compound 48/80 (65.36 mM in sterile saline; C2313, Sigma-Aldrich), 5-HT (20.00 mM in sterile saline; H9523, Sigma-Aldrich), chloroquine (77.50 mM in sterile saline; C6628, Sigma-Aldrich), or endothelin-1 (20.00 μM in sterile saline; HY-P0202, MCE) into the cheek with a total volume of 50 μl. In the nape itch model, rats received a unilateral intradermal injection of compound 48/80 (65.36 mM in sterile saline), 5-HT (5.00 mM in sterile saline), chloroquine (77.50 mM in sterile saline), or endothelin-1 (20.00 μM in sterile saline) in the nape, with a total injection volume of 50 μl. Scratching was recorded for 30 min after injection. We also used half the baseline concentrations of 5-HT (nape, 2.5 mM, 50 μl), compound 48/80 (nape, 32.68 mM, 50 μl), and chloroquine (cheek, 38.75 mM, 50 μl) to induce itch in the fiber photometry experiments (fig. S18). The scratching was recorded by the magnetic induction method and analyzed with custom-written software in MATLAB.

In some experiments, the scratching was also simultaneously recorded with a digital video camera. The scratching was manually analyzed for comparison with the results obtained with the magnetic induction method. One bout of scratching was defined as a lifting of hindquarter to scratch the injected region of the body and then placing it on the floor or to the mouth.

Pain test
The rats underwent a protocol similar to that described above. Rats were shaved at the unilateral cheek and then habituated for 30 min for two consecutive days in the recording box before algogen injection. For the AITC pain model, rats received a unilateral intradermal injection of AITC (1007.50 mM in 7% Tween 80/saline; W203408, Sigma-Aldrich) into the cheek with a total volume of 25 μl immediately after a 15-min baseline recording. For the capsaicin pain model, rats received a unilateral intradermal injection of capsaicin (2, 4, and 8 μg/μl in 7% Tween 80/saline; HY-10448,
tested three times at 20-min intervals (55).

The cutoff time was set to 15 s to avoid tissue damage. Each rat was Dichuang, China). The latency to paw withdrawal was recorded.

The hindpaw was marked as the stimulating site, and radiant heat stimu-

For the EVF test, each rat was placed on a metallic grid floor in a plexiglass chamber 15 min before the test. Then the rat was calm, the left hindpaw was stimulated by applying increasingly strong per-

To measure radiant heat pain, the rat was placed in a plexiglass chamber 15 min before the test. Then, the midplantar area of the hindpaw was marked as the stimulating site, and radiant heat stimulation was applied with a radiant heat source (ZS-RECT-200, Zhongshi Dichuang, China). The latency to paw withdrawal was recorded. The cutoff time was set to 15 s to avoid tissue damage. Each rat was tested three times at 20-min intervals (55).

For the TTT, the tail of the rat was positioned horizontally over a basin of hot water (46° or 50°C) and held 5 cm above the surface. The tail was then released and allowed to drop naturally into the water. The tail-flick latency was recorded, with a cutoff time of 15 s to avoid tissue damage. Each rat was tested three times, with an inter-

For the formalin test, 5% formalin (25 μl; F8775, Sigma-Aldrich) was injected into the left hindpaw. Licking and flinching behaviors were recorded for 1 hour. The results in the acute pain phase (0 to 10 min; phase 1) and inflammatory pain phase (10 to 60 min; phase 2) were measured (44).

Balance beam test

The BBT was used to assess sensorimotor function. The beam appa-

The test was conducted over three consecutive days: 2 days for training and 1 day for testing. In the first 2 days, each rat was enc-

On the test day, the time of three successful trials in which the rat crossed the center 1.8 m of the beam without pausing was averaged.

Open field test

We assessed the locomotor activity of the rats by performing the OFT over a 10-min period using a 60 cm by 60 cm by 60 cm box made of black acrylic boards. Rats were initially placed in the center of the box and were recorded by camera individually. The center area was defined as centric 30 cm by 30 cm. The paths of the rats were auto-

Rotarod test

Rats were placed on a rotarod instrument (ZS-RDM-DS, Zhongshi Dichuang, China) and trained for the first 2 days to maintain their balance. Then, on the third day, the rod accelerated to 5 to 40 rpm, testing the rats with a maximum time of 300 s. The latencies of the rats to fall off were recorded by the rotarod instrument. This pro-

Analysis of scratching

To measure scratching, we recorded movement signals of the hind-

Fiber photometry recording

For recording of VLO neuron activity, the rat received an intrader-

The fluorescence data were analyzed using built-in software. The fluorescence value during each scratching bout with an interval of <2.0 s (fig. S1A). For the behavioral experiments, the number of scratching episodes and the cumulative duration of scratching bouts were calculated.

MCE) into the cheek with a total volume of 25 μl immediately after a 15-min baseline recording. We used an injector with 30-gauge (0.3 mm external diameter) hypodermic needle to inject the algol-

gens. The wiping behaviors induced by cheek injections of algogen were recorded for 30 min using a digital video camera. The total number of wipes was manually counted (fig. S19). One wiping behavior was defined as a single stroke with the forelimb at the cheek in a caudal-to-rostral direction. We also used half the baseline concent-

The withdrawal threshold was defined as the average force (g) required to cause withdrawal of the stimulated paw in three trials (53, 54).

To measure radiant heat pain, the rat was placed in a plexiglass chamber 15 min before the test. Once the rat was calm, the left hindpaw was marked as the stimulating site, and radiant heat stimula-

The latency to paw withdrawal was recorded. The latency was defined as centric 30 cm by 30 cm. The paths of the rats were auto-

The fluorescence value during each scratching train or wiping train was recorded by the magnetic induction method, and pain-

The induction coil voltage signals were bandpass-filtered (3 to 100 Hz), amplified (100×) using a 16-channel differential amplifier (model 3500, A-M Systems), and acquired with a data acquisition system (Powerlab 16/35) with a sampling rate of 1000 Hz.

After comparatively analyzing the same scratching recorded by the digital video camera and by the magnetic induction method simultaneously, we found that inductive coil voltage fluctuation could be induced by both scratching and hindlimb motion (e.g., walking). However, scratching induced a cluster of voltage fluctuations, ex-

Signals in the ipsilateral VLO were recorded by a fiber photometry recording system for 30 min after injection of the pruritogen or

The wiping behaviors induced by cheek injections of algogen were recorded for 30 min using a digital video camera. The total number of wipes was manually counted (fig. S19). One wiping behavior was defined as a single stroke with the forelimb at the cheek in a caudal-to-rostral direction. We also used half the baseline concentration of AITC (cheek, 503.75 mM, 25 μl) to induce pain in the fiber photometry experiments (fig. S18).

For the TTT, the tail of the rat was positioned horizontally over a basin of hot water (46° or 50°C) and held 5 cm above the surface. The tail was then released and allowed to drop naturally into the water. The tail-flick latency was recorded, with a cutoff time of 15 s to avoid tissue damage. Each rat was tested three times, with an inter-

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The fiber photometry data were analyzed using built-in software. The fluorescence value during each scratching train or wiping train was averaged.
values at each time point (−8 to 10 s relative to the onset of the scratching train or wiping train) and F0 refers to the median of the fluorescence values during the baseline period (−7 to −5 s relative to the onset of the scratching train or wiping train). To visualize the fluorescence change, the ΔF/F values are presented with heatmaps or plots with shaded areas indicating SEM. To statistically quantify the change in fluorescence values across the scratching train or wiping train, the average amplitude of ΔF/F was defined as the average fluorescence amplitude change from the baseline during the peak period (−1 to 5 s relative to the onset of the scratching train or wiping train; e.g., Fig. 5, C to F and I to L).

Immunofluorescence

For the detection of endogenous c-Fos expression induced by itch in the VLO, the rats received a unilateral intradural injection of 5-HT (5 or 20 mM), chloroquine (77.50 mM), or saline in the nape or cheek, with a total injection volume of 50 µl, and their brains were collected 90 min later. Moreover, for the examination of endogenous c-Fos expression in the VLO as a result of pain, rats received a unilateral intradural injection of AITC (1007.50 mM in 7% Tween 80/saline) in the cheek, with a total injection volume of 25 µl, and brains were collected 90 min later.

The immunofluorescence staining protocol was performed as previously described (37, 48–51). Briefly, rats were anesthetized with 3% pentobarbital sodium (40 mg/kg, ip) and transcardially perfused with physiological saline followed by cold 4% paraformaldehyde (PFA; prepared in 0.1 M phosphate buffer, pH 7.4). The brains were removed from the skull and stored in 4% PFA at 4°C for 24 hours and then transferred to a 30% sucrose solution at 4°C for 48 hours. Coronal sections (30 µm thick) were cut on a freezing microtome (CM3050 S, Leica, Germany) and collected in cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4). For immunostaining, each slice was placed in PBST (PBS + 0.3% Triton X-100) with 2% normal bovine serum albumin for 1 hour and then incubated with primary antibody at 4°C for 24 hours (rabbit anti-c-Fos: 1:500, 226003, Synaptic Systems; mouse anti-CaMKIIα, 1:100, MA1-048, Thermo Fisher Scientific; mouse anti-GAD67, 1:500, MAB5406, Millipore; mouse anti-parvalbumin, 1:500, MAB1572, Millipore; mouse anti-NeuN, 1:500, MAB377, Millipore). Slices then underwent three wash steps of 10 min each in PBST, followed by 2-hour incubation with secondary antibody (goat anti-rabbit conjugated to Alexa Fluor 488, 1:500, A11034 or A32731, Invitrogen; goat anti-mouse conjugated to Alexa Fluor 488, 1:500, A11001 or A32723, Invitrogen; goat anti-mouse conjugated to Alexa Fluor 647, 1:500, A21235, Invitrogen). Slices were washed with PBST (once, 10 min), incubated for 10 min with 4',6-diamidino-2-phenylindole (DAPI, 1:2000; D9542, Sigma-Aldrich), and then subjected to three more wash steps of 10 min each in PBST, followed by mounting and coverslipping on microscope slides. The images were acquired using a Carl Zeiss LSM 780/LSM 800 scanning laser microscope (Germany) or Olympus VS120-S5/VS200 virtual slide system (Japan).

Following publishing policy, the NeuN+ cells in VLO (Fig. 2C, green originally), which were stained with anti-mouse conjugated to Alexa Fluor 488, were adjusted into blue color channel using ZEN lite 2012 software (Carl Zeiss).

Histology

After the behavioral or fiber photometry experiments, rats were anesthetized with 3% pentobarbital sodium (40 mg/kg, ip) and transcardially perfused with physiological saline followed by cold 4% PFA (prepared in 0.1 M phosphate buffer, pH 7.4). The brains were removed from the skull and stored in 4% PFA at 4°C for 24 hours and then transferred to a 30% sucrose solution at 4°C for 48 hours. Coronal sections (30 µm thick) were cut on a freezing microtome (CM3050 S, Leica) and collected in cold PBS (0.01 M, pH 7.4). Slices were washed with PBST (once, 10 min), incubated for 10 min with DAPI (1:2000, D9542, Sigma-Aldrich), and then subjected to three more wash steps of 10 min each in PBST, followed by mounting and coverslipping on microscope slides. The extent of virus expression and the placement of the optical fiber were imaged using an Olympus BX53F fluorescence microscope (Japan) or an Olympus VS200 virtual slide system (Japan). We chose the section of maximum virus expression density as the injection center location. The criterion was consistent during the experiments.

Statistical analysis

All the data are expressed as the mean ± SEM. Statistical significance was determined by a two-tailed unpaired Student’s t test or by one-way analysis of variance (ANOVA) followed by a least significant difference post hoc test using the SPSS software package for Windows (v. 25.0). A value of P < 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.aba4408

View/request a protocol for this paper from Bio-protocol.
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Author contributions: G.-Y.W., J.-F.S., and H.-L.L. designed the experiments and interpreted the results. S.J., Y.-S.W., and Y.-G.W. performed most of the experiments, with assistance from X.-X.Z., S.-L.Z., and Y.W.S.J., G.-Y.W., Y.-S.W., and S.L. analyzed the data and generated the figures. P.-H.C. performed the slice electrophysiological experiments. Y.Z. wrote the code for the scratching analysis software. S.J., G.-Y.W., J.-F.S., and C.T. wrote the manuscript. All the authors commented on the manuscript. Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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