INTRODUCTION

Both itch and pain are unpleasant sub-modalities of somatic sensation but are essential in protecting our bodies from surrounding dangers. These two sensations share anatomically similar pathways: from the periphery to the brain. Although many attempts have been made to determine how itch and pain are differently encoded and processed over several decades, whether these sensations are processed in separate or the same cell populations remains controversial [1]. In the peripheral and spinal cord, several itch-specific neural pathways and molecular markers have been discovered, such as the gastrin-releasing peptide (GRP) receptor-expressing neuron [2]. Meanwhile, spinal GRP+ neurons can be activated by both itch and pain and show higher activity for pain and lower activity for itch [3]. In addition, pruriceptive neurons at the peripheral level co-express pain-mediating Transient receptor potential (TRP) channels and are activated by both itch and pain [4, 5]. Although several encoding mechanisms between itch and pain have been discovered in the peripheral and spinal cord, how their
information is processed differently in the supraspinal sensory area is unclear. The interaction between itch and pain sensation further supports the existence of shared circuits in the spinal cord and brain [6]. Through daily experience, we know that scratching can relieve itch, indicating that itch is suppressed by pain. This interaction has been experimentally explained for several noxious stimulations, including thermal, mechanical, and chemical pain [7-9]. Inhibitory neuronal population expressing Bhlhb5 is activated by painful stimulation and acts on itch-transmitting neurons [10, 11]. There is another antagonistic effect between itch and pain: the inhibition of pain processing can generate itch. Opioids can simultaneously induce analgesia and itch by acting on different subtypes of µ-opioid receptors in the spinal cord [12]. However, the brain mechanism of the interaction between itch and pain remains poorly understood, especially in areas related to perception.

Perception of sensation is generated at the brain level, and processing of somatosensory information involves multiple brain regions [13, 14]. Primary somatosensory cortex (S1) plays a crucial role in the perception and discrimination of somatosensory information, including touch, itch, and pain [15-17]. Recent studies have shown that S1 encodes the sub-modality of somatic sensations using multiplexed strategies [18, 19]. In other reports, the activation of the S1 layer 5 neurons projecting to the spinal cord decreased itch; meanwhile, activation of these neurons could facilitate mechanical allodynia. These results suggest that S1 can antagonistically modulate itch and pain [20, 21]. In our previous study, we showed that itch and pain could be distinguished by a deep-learning algorithm based on S1 neuronal activity, implying that S1 neurons have a distinct response pattern for itch and pain [22]. However, how S1 neurons differentially encode these two distinct sensations and whether S1 represents the interaction between two sensations remains unexplored at the cellular level.

MATERIALS AND METHODS

Experimental animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Kyung Hee University (KHUASP[SE]-20-133). C57BL/6 male mice, aged 5–6 weeks, were used for all experiments (Koatech, Seoul, Korea). Mice were housed in a room maintained at a constant temperature of 23±2°C under a 12-h light/dark cycle.

Mouse model for acute itch and pain

Both itch and pain were induced on the skin on the lateral side of the neck. To establish an acute itch model, 5-hydroxytryptamine (5-HT, 10 μg in 10 μl) or 10 μl PBS vehicle was administered intradermally. To establish the acute pain model, capsaicin (CAP, 10 μl of 1% capsaicin) or 10 μl saline solution containing 5% ethanol (EtOH) vehicle was intradermally administered.

Behavioral assay

Before behavioral assessments, the mice were acclimated to the experimental chamber. At least a week before observation, the hair of the neck was removed under isoflurane anesthesia (1%–1.5%). For all assays, the mice were filmed for 10 min immediately after treatment. Experimenters who were blinded to the treatment counted the behaviors. For itch-related behavior, scratching of the hind paw on the ipsilateral side of the neck was counted [23]. For pain-related behavior on the neck, we counted the wiping-like behaviors that stretched the ipsilateral forelimb to the injected site.

Cranial window surgery and virus injection

Before surgery, mice were anesthetized with an intraperitoneal injection of a zoletil/xylazine mixture (30 mg/10 mg/kg). First, we made an incision on the mouse head and attached a metal ring for fixation with dental cement. Then, the skull right above the left side of the S1 region, corresponding to the right side of the neck (center relative to Bregma: lateral, 2.5 mm, posterior 0.5 mm), was removed with a #11 surgical blade. An adeno-associated virus expressing GCaMP6s (AAV1-I-lys GcaMP6s, produced by the University of Pennsylvania Gene Therapy Program Vector Core) was injected into the cortical layer 2/3 using a glass pipette. After the viral injection, we placed a thin transparent glass for neuronal observation of the exposed cortex and sealed it with Vetbond (3M) and dental cement.

In vivo two-photon calcium imaging of layer 2/3 neurons in the S1 cortex

A two-photon microscope (FVMPE-RS, Olympus) equipped with a water immersion objective lens (XPLan N 25, NA=1.05, Olympus, Tokyo, Japan) was used for calcium imaging. To excite GcaMP6s, a Ti: sapphire laser (Chameleon, Coherent, USA) was tuned to 900 nm. All time-lapse images (512×512 pixels, 0.99 μm/pixel) were acquired at 5.11 Hz using FLUOVIEW (Olympus, Tokyo, Japan). The brain surface was imaged after the first session. By matching the blood vessel patterns on the brain surface, relocation of the previously imaged region was possible across different days.

To identify the region corresponding to the right side of the neck on S1, tactile stimulation was applied using a brush during in vivo calcium imaging (Fig. 1E–G). During imaging, mice were lightly anesthetized with isoflurane. Mechanical stimulation was applied to the hind paws and neck.

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To record spontaneous activities in awake condition, the mice were acclimated under head-fixed conditions on a treadmill for 30 min per day for 1 week (Fig. 2A). Imaging for each treatment was performed on different days for four consecutive days. The order of treatment was randomly selected. Data were acquired 6~7 min after PBS or 5-HT injection and 1~2 min after 5% EtOH or CAP injection. Before imaging, we manually corrected the imaged region to minimize location differences caused by mouse movement.

**Extracting calcium traces and event detection**

Motion correction was performed on all the acquired imaging data using the Turboreg algorithm (Biomedical Imaging Group, Swiss Federal Institute of Technology, Lausanne, Switzerland). To designate the soma as ROIs, we first used the CNMF-E algorithm. Then, the spatial information of the automatically detected ROIs was imported to ImageJ (https://imagej.nih.gov/ij/), and the ROIs were manually reviewed for undetected or wrongly detected neurons. The average fluorescence of the ROI was calculated along with the frame. We normalized the calcium signal by applying a Gaussian filter and calculated the baseline fluorescence signal (F0) by averaging the signal below the 70th percentile in each ROI and normalizing all the signals.

To detect events from the calcium traces, we used customized codes for Python. We first designated the peak at over 40% dF/F0 as an event. When the lowest value between two adjacent peaks was lower than 70th percentile, a peak was considered a 5-HT injection or 5% EtOH or CAP injection. Before imaging, we manually corrected the imaged region to minimize location differences caused by mouse movement.

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within 10 frames did not decline below 35% from the former peak, we considered the two peaks the same event [24].

**Calculation of response properties of calcium activities and classification of neurons**

To calculate the response properties of the calcium activities and
classify the cells, we used a customized Python code. In this study, three response properties were calculated. First, the frequency of events indicated the number of events in a session and the total event amplitude indicated the sum of the dF/F0 of all events that occurred in a session. Finally, the total integrated calcium indicated the sum of the dF/F0 of all frames in a session.

To compare the calcium activities in the four sessions, event frequency was used as a criterion. For the classification of both-responsive cells, we selected cells in which the event frequency exceeded the average of all imaged cells in both itch and pain sessions and was 20% higher than in vehicle sessions. For the classification of itch- or pain-preferred cells, we selected cells in which the activity of the itch or pain session exceeded the average of all imaged cells and showed higher activities than all other sessions. Among these cells, those that belonged to both-responsive neurons were excluded. If the cells were classified into both-responsive, itch-preferred, or pain-preferred neurons, we defined the neurons as responsive neurons.

**Statistics**

Statistical analyses were performed using Prism (Graph Pad Software, USA) and custom-written Python codes. We have described the detailed statistical methods for each dataset in the legends.

**RESULTS AND DISCUSSION**

To establish a mouse model of spontaneous itch and pain, animals were intradermally treated with 5-HT, CAP, or their vehicles, and spontaneous behaviors related to each somatic sensation were observed for 10 min. Instead of the nape area that was commonly used in previous itch studies, we selected the area on the lateral neck as the induction region. This was because we needed to observe distinct itch and pain behaviors. Previous studies have reported that behaviors related to itch or pain can vary according to the induction region [25, 26]. Behavioral observation of the nape cannot distinguish between itch and pain, since both sensations evoke the same scratching behavior on the nape of the neck [23, 27]. Instead, we used the lateral neck as the induction region of itch and pain, as we discovered that the mice showed clearly distinguished behaviors for itch and pain when treated in this region: 5-HT significantly increased ‘scratching behaviors’ using a hind paw, whereas these behaviors were rarely detected after treatment with PBS, 5% EtOH, or CAP (Fig. 1A). We observed that ‘wiping-like behaviors’ using a fore paw increased after treatment with CAP whereas these behaviors were rarely detected after treatment with PBS, 5% EtOH, or 5-HT (Fig. 1A). Our results suggest that the lateral neck is an appropriate region to observe the distinct behaviors of itch and pain. Based on the behavioral observations, we regarded 6–7 min after 5-HT as an itch session and 1–2 min after CAP as a pain session (Fig. 1B, C).

Next, we attempted to detect the corresponding region of S1 for the lateral neck. GCaMP6s were expressed in layer 2/3 neurons of the left S1ShNc, which is the receptive field of the neck and shoulder in S1 (Fig. 1D). In lightly anesthetized mice, the calcium activity of S1ShNc neurons was recorded during sensory stimulation with a brush on the right neck or hind paw (Fig. 1E). For the imaged S1 region, we semi-automatically designated the soma as ROIs and calculated the change in calcium fluorescence of each ROI (Fig. 1F). S1ShNc neurons were activated in response to the right neck stimulation but were not activated during sensory stimulation of the right hind paw (Fig. 1G). These results indicate that S1ShNc is a receptive field for the lateral neck, as expected.

Following the craniotomy, GCaMP6s virus was injected into the left S1ShNc region to observe the S1 neuronal calcium responses to itch and pain (Fig. 2A). One week after the surgery, we shaved the hair in the right neck region. Mice were acclimated under head-fixed conditions on a treadmill for 30 min/day over a week prior to awake imaging. Imaging for each treatment was performed on one day for four consecutive days. Before imaging, we manually corrected the imaged region to minimize location differences caused by mouse movement. A total of 1271 neurons in S1 were recorded, while 5-HT or CAP was applied to the right neck of mice. Total 236 responsive cells for itch or pain sessions were found (Fig. 2C, D). The responsive cells were further classified into three types: both-responsive, itch-preferred, or pain-preferred cells (see also Materials and Methods). Cells that showed preference for both itch and pain compared with vehicle-treated sessions were named both-responsive. Only 11.0% of the cells were both-responsive (26/236). For cells that exhibited significantly increased activity only in the itch or pain session compared to other sessions, we named them itch- or pain-preferred. 28.4% (67/236) were itch-preferred and 60.6% (143/236) were pain-preferred. Subsequently, we analyzed the response properties of the classified cell populations. Here, we defined the frequency of events as the number of events in a session, the total event amplitude as the sum of the dF/F0 of all events that occurred in a session, and the total integrated calcium as the sum of the dF/F0 of all frames in a session (Fig. 2E–M). Although we used the event frequency as a criterion for classification, other properties, such as the total event amplitude and the total integrated calcium, showed similar patterns.

We found that the both-responsive neurons accounted for a relatively small portion, occupying 11% among all the responsive cells in S1. The both-responsive neurons showed relatively higher...
activities for pain than itch, which was significant in the total event amplitude (Fig. 2E–G). In terms of other responsive cells, the pain-preferred cells (60.6%) accounted for much more portion than the itch-preferred cells (28.4%). In addition, the responsive activities were higher in the pain-preferred cells compared to the itch-preferred cells (Fig. 2H–M). These results indicate that the sensory-responsive cells in the S1 region are more reactive to painful stimulation than pruritic stimulation. This might be due to the property of the neurons participating in itch or pain sensation in the periphery and spinal cord. In the sensory nervous system, itch- or pain-processing cells share common ascending pathway. Primary sensory neurons that transmit itch information to the spinal cord co-express TRP channels, which are also related to pain sensation [5]. In case of the serotonergic itch, the primary sensory neurons that express serotonin receptors such as HTR2 or HTR7 [28, 29] are affected by TRP channels, such as TRPV4 and TRPA1. The other type of itch sensation, such as histaminergic itch, involves the activation of TRPV1, which is also a direct target of CAP [4, 5]. In contrast, TRPV1 did not mediate serotonergic itch [4, 29]. In future studies, it will be necessary to investigate whether the proportion of both-responsive neurons for itch and pain in S1 changed depending on whether the mediators share TRP channels at the peripheral level.

Another intriguing result is that itch-preferred neurons showed decreased activity in the pain session (Fig. 2H–I) and pain-preferred neurons showed decreased activity in the itch session (Fig. 2K–M). The functional role and neuronal circuits related to the phenomenon are currently unknown. One hypothesis related to the role of these opposing activities for counter sessions is that these might be related to the direct top-down modulation of itch and pain. The activities of S1 layer 2/3 neurons were recorded in this study, and these layer 2/3 neurons play a major role in amplifying sensory responses in layer 5 neurons [30]. Some S1 layer 5 neurons directly project to the spinal cord and show opposing actions to itch and pain [20, 21]. Activation of S1-spinal projection neurons decreases both histaminergic and non-histaminergic itch while facilitating mechanical allodynia. In terms of the circuit level mechanism, we speculate that the local inhibition is involved in the opposing activity of the preferred cells. S1 Layer 2/3 pyramidal neurons are affected by several types of local inhibitory circuit [31, 32]. The different inhibitory populations may be activated by itch and pain in the S1 cortex. To test these hypotheses, we further need to investigate cell type-specific responses and behavioral changes by modulation of the specific cell type in future studies.

We propose that the S1 neurons adopt a multiplexed coding strategy for itch and pain sensations. The coding strategy explains that the sensory system processes distinct sensations by both specific cell populations and converged cell populations [19, 33]. These coding strategies were found at the supraspinal level, including S1 [18, 19, 34]. When we classified the preferred cells, we disregarded the activities of counter pair. In other word, pain-preferred cells considered only the activity difference in PBS and 5HT, whereas itch preferred cells considered only the activity difference in 5% EtOH and CAP. In this study, as well as the both-responsive neurons, most of the preferred neurons showed polymodal activity although they exhibited a weighted preference for each type of sensation. The presence of these ‘converged’ cell populations suggests that pain or itch sensations are processed in S1 using a converged strategy, similar to intensity coding [1, 3]. In contrast, a small portion of the preferred neurons showed more selective response to itch or pain. These cell population is directly indicative of the presence of labelled lines as suggested by specific coding theory [1, 2]. Both the converged and specific cell populations seemed to process itch and pain in S1, indicating a multiplexed coding strategy. Another feature of our results is that the preferred neurons for itch or pain sensation showed decreased activity when their counter sensation was evoked. These results are also consistent with the explanation of the multiplexed, population coding hypothesis that neurons responding to each sensation crosstalk with one another [33].

In conclusion, we showed how S1 neurons represent itch and pain at the cellular level using in vivo two-photon calcium imaging. Prior to imaging, we established a new behavioral model for spontaneous itch and pain. The lateral neck model could distinguish specific behaviors between the two. Cell classification based on event frequency revealed interesting response properties under different conditions. First, both-responsive neurons showed relatively higher activity for pain than for itch. Second, itch- and pain-preferred neurons showed the decreased activity in their counter condition. This implies that S1 layer 2/3 neurons represent the opposing interaction between itch and pain. Our findings provide an important insight into how S1 neurons encode itch and pain, which are counter somatic sensations.

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