Mapping Cortical Integration of Sensory and Affective Pain Pathways

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Pain is an integrated sensory and affective experience. Cortical mechanisms of sensory and affective integration, however, remain poorly defined. Here we combine optogenetics with *in vivo* electrophysiology and computational analysis in awake, freely behaving rats to investigate a direct projection from the primary somatosensory cortex (S1), which encodes the sensory information of pain, to the anterior cingulate cortex (ACC), a key area for processing the aversive component of pain. We show that whereas only a small fraction (<9%) of ACC neurons receives direct S1 inputs, these neurons contributes substantially to the overall ACC response to pain. Furthermore, activation of S1 axon terminals in the ACC can recruit new ACC neurons to respond to noxious stimuli, as well as increase the spiking rates of individual pain-responsive neurons. At the behavioral level, optogenetic modulation of this cortico-cortical projection bidirectionally regulates pain aversion. Importantly, in the chronic pain state, there is an increase in the connectivity between these two cortical areas, as manifested by a higher percentage of ACC neurons that respond to S1 inputs and the magnified contribution of these neurons to the nociceptive response in the ACC. This increased cortico-cortical connectivity in turn causes enhanced aversive behavior associated with chronic pain. These results define a novel cortical circuit for sensory and affective integration; this circuit undergoes plasticity in the chronic pain state and may be targeted by non-addictive neuromodulatory therapies.

Sensory processing requires the transmission of neural information from sensory cortices into higher order areas in the neocortex that mediate affective and cognitive responses. Such sensory and affective integration is particularly important for the experience of pain, as tissue injury or trauma elicits specific nociceptive signals, which in turn must trigger aversion or associated affective response to defend against physical harm[1]. On the other hand, whereas acute pain constitutes a normal response to physical harm, chronic pain represents a maladaptive experience in sensory and affective processing[2,3]. However, mechanisms of sensory and affective integration of nociception remain incompletely understood, and even less is known about how such mechanisms contribute to chronic pain.

Canonical ascending nociceptive pathways terminate in the primary somatosensory cortex (S1) and the anterior cingulate cortex (ACC) to process the sensory and affective aspects of pain, respectively[4-16]. The S1 carries important sensory information of pain, including the location,

timing, and intensity of pain. However, it remains unclear to which higher cortical areas the S1 projects its sensory pain signals. Meanwhile, the ACC has been shown to play a key role in the aversive reactions to pain in numerous studies of animal models as well as human subjects[4-14,17-20]. The ACC receives nociceptive information from the medial thalamus and has reciprocal projections to other subcortical regions such as the amygdala and nucleus accumbens[21-23]. However, it is not known whether the ACC receives direct nociceptive information from sensory cortices such as the S1. A direct nociceptive projection from the S1 to the ACC, however, would provide a simple mechanism for sensory and affective integration. Furthermore, both the S1 and ACC are known to undergo synaptic plasticity in the chronic pain state, raising the question if alterations in the S1→ACC circuit can contribute to chronic pain behaviors.

Here we investigated whether a direct projection from the S1 to the ACC could provide cortical integration of sensory and aversive pain information and how chronic pain alters such nociceptive information flow. Combining *in vivo* electrophysiological recordings of populations of S1 and ACC neurons in freely behaving rodents with targeted circuit disruption, we identified a subset of ACC neurons that received direct inputs from the S1, and found these neurons to be highly responsive to nociceptive stimuli. Chronic pain, meanwhile, strengthens this connection between the S1 and the ACC. At the behavioral level, we found that this S1→ACC projection could bidirectionally regulate the aversive response to pain. These results demonstrate a cortical circuit for the integration of sensory and affective pain signals, and indicate that enhanced cortico-cortical nociceptive information flow constitutes a key mechanism for chronic pain.

RESULTS

Nociceptive information flow from the S1 to the ACC

We first examined the anatomic link between the S1 and the ACC histologically. Retrograde beads injected in the ACC was found in the cell bodies of neurons in the S1 hind limb region (Fig. 1a, b). Likewise, anterograde expression of yellow fluorescent protein (YFP) injected in the S1 was found in the ACC (Fig. 1c-e). There was stronger anatomic connection between the ACC and the ipsilateral than the contralateral S1.

Having established the anatomic projection from the S1 to the ACC, we investigated if this projection plays a functional role in nociception. We injected channelrhodopsin (ChR2) in pyramidal neurons in the hind limb region of the S1, and inserted optrodes into the ACC to test if activation of the S1 axon terminals in the ACC could alter spiking activities of ACC neurons (Fig. 2a, b). We measured neural activities in the ACC in response to either a noxious (pin prick, or PP) or a non-noxious stimulus (von Frey filaments, or vF) (Fig. 2c, d). In the absence of S1 activation, we found that approximately 16% (98/623) of neurons in the ACC responded to the noxious stimulus, whereas only 7% (40/567) of neurons responded to the non-noxious stimulus (Fig. 2e-g). When we examined the neurons that responded to both PP and vF, PP triggered a higher spiking rate in these neurons (Fig. 2h). Discriminative statistical machine learning analysis (using a support vector machine) based on the firing rates of individual ACC neurons further validated the specificity of the nociceptive response in the ACC (Fig. 2i, j). We then optogenetically activated the axon terminals of S1 neurons that projected to the ACC (Fig. 2k). We found that the activation of presynaptic S1 inputs increased the firing rates of postsynaptic ACC neurons evoked by PP, but this activation did not change the ACC response to vF (Fig. 2l-n). These results suggest that S1 activation selectively enhances the nociceptive response in the ACC. Unbiased machine learning

analysis further demonstrated that activation of the S1→ACC projection enriched the specificity of the nociceptive neural codes in the ACC (Fig. 2o, p). Together, these findings indicate that the S1 constitutes an important source of nociceptive information in the ACC, and thus provide direct evidence for cortical integration of sensory and affective circuits for pain.

S1 projection recruits ACC neurons to respond to nociceptive inputs and increases the spiking rates of ACC pain-responsive neurons

Next, we investigated the mechanisms by which S1 activation could augment the nociceptive response in the ACC. First, we analyzed the proportion of ACC neurons that received S1 inputs. We found that less than 9% (54/623) of ACC neurons responded to S1 activation. However, 37% (20/54) of these neurons that received S1 inputs were capable of responding to noxious stimulations (Fig. 3a). In contrast, among ACC neurons that did not receive S1 inputs, only 14% (78/565) of them demonstrated pain-responsiveness (Fig. 3b, c). These results highlight the importance of the S1 pyramidal neurons as a source of nociceptive inputs to the ACC. Optogenetic activation of the presynaptic S1 inputs, however, was able to recruit an additional 30% of ACC neurons to respond to the pain stimulus (Fig. 3d, e). In addition to this population response, we also examined the firing rates of individual neurons. We found that among ACC neurons that received S1 inputs, S1 activation resulted in a further 69% increase in their firing rate responses to PP (Fig. 3f). Interestingly, activation of the presynaptic S1 inputs did not alter the baseline firing rates of the ACC neurons, suggesting that the effect of S1 activation on ACC is activity-dependent (Fig. 3g). Together, these results indicate that S1 inputs amplify the ACC nociceptive response at both population and single-cell levels.

S1 projection to the ACC bidirectionally regulates pain aversive response

The ACC is known to process the aversive component of pain, which can be tested by the well-established conditioned place aversion (CPA) assay[12,17-20,24]. Here we examined the effect of the S1→ACC projection on pain aversion using a two-chamber CPA assay. During the preconditioning phase, rats were allowed free access to both chambers. During conditioning, one of the chambers was paired with repeated noxious mechanical stimulations (PP) of the hind paw, whereas the opposite chamber was not paired with noxious stimulations (NS). Finally, during the test phase, rats were given free access to both chambers again without peripheral stimulations (Fig. 4a). As expected, rats preferred the non-noxious chamber during the test phase, demonstrating their aversive response to acute pain (Fig. 4b). We injected ChR2 into the pyramidal neurons in the S1 hind limb region, and inserted optic fibers into the ACC to directly activate the axonal projection of S1 neurons to the ACC (Fig. 4c). We paired one chamber with PP, and another chamber with PP as well as optogenetic activation of the direct S1→ACC projection (Fig. 4c, d, Supplementary Fig. 1). Activation of the direct S1→ACC projection did not have intrinsic aversive value (Supplementary Fig. 3). We found that rats avoided the chamber associated with the S1→ACC activation when presented with PP (Fig. 4e, f). This enhanced aversive response to PP in the presence of S1→ACC activation could be quantified by a CPA score, which was calculated by subtracting the time rats stayed in the PP chamber during the test phase from the preconditioning phase (Fig. 4g). In contrast, when we selectively inhibited this cortico-cortical projection using halorhodopsin (NpHR) (Fig 4c, h, Supplementary Fig. 2), rats preferred the chamber associated with S1→ACC inhibition (Fig. 4i, j). These data demonstrate that modulation of the S1→ACC circuit can bidirectionally regulate the aversive response to pain. Furthermore, these results indicate that the S1 projection plays an important role in the aversive response mediated by the

ACC, and this cortico-cortical connection likely confers additional specificity for the affective response to noxious inputs.

Enhanced connectivity between the S1 and the ACC in the chronic pain state

Previous studies have shown that chronic pain induces synaptic plasticity in both the S1 and the ACC[19,25-30]. Thus, we hypothesized that chronic pain could also increase the S1→ACC connectivity to further enhance the integration of sensory and affective nociceptive information. We injected Complete Freund's Adjuvant (CFA) to induce persistent inflammatory pain. To avoid confounding spinal and peripheral hypersensitivity, we introduced CFA in the opposite limb (Fig. 5a, b). We examined the firing rates of individual neurons in the ACC. We found that in CFA-treated rats, there was an increase in the peak ACC spiking rates both at baseline and in response to noxious stimulations (Fig. 5c-e), indicating that chronic pain increases the nociceptive response in the ACC, compatible with previous findings[19,20]. More importantly, activation of the S1 inputs resulted in an additional 10% increase in the peak ACC firing rates after PP (Fig. 5f, g). In contrast, S1 activation did not alter the basal firing rates of ACC neurons in CFA-treated rats (Fig. 5h).

Next, we examined the mechanism of this enhanced cortico-cortical connectivity in the chronic pain state. We found that the proportion of ACC neurons that received S1 inputs almost doubled in CFA-treated rats (Fig. 5i). Remarkably, in CFA treated rats, more than 50% of the ACC neurons that receive S1 inputs responded to noxious stimulations, substantially more than the ratio of pain-responsive neurons before the CFA treatment (Fig. 5j). These results suggest an increase in cortical connectivity at the population level. In addition, we found that S1 inputs further increased the spiking rates of the pain-responsive ACC neurons in the chronic pain state (Fig. 5k). Thus, chronic pain can also enhance S1→ACC connectivity at the single-cell level.

Enhanced S1 to ACC projection contributes to heightened aversive response in the chronic pain state

Finally, we examined the behavioral consequence of enhanced cortico-cortical connectivity in the chronic pain state. We have previously shown that similar to chronic pain patients, rats with persistent pain demonstrate elevated aversive response, as manifested by elevated CPA scores [19,20,31,32]. We compared the CPA scores for naïve rats that received S1→ACC activation paired with PP in one chamber, and NS in the opposite chamber, with CFA-treated rats that received PP in one chamber and NS in the opposite chamber (Fig. 6a-d). We found nearly identical CPA scores, indicating that activation of the S1→ACC circuit could reproduce the aversive phenotype of chronic pain. Next, we examined if the S1→ACC projection is involved in the tonic or spontaneous pain behavior associated with chronic pain. Previous studies have shown that CPA assays could be used to unmask the tonic-aversive behavior in rodent models of chronic pain[20,24,33]. Thus, during conditioning, we did not give additional noxious stimulations to CFA-treated rats, but we paired one chamber with S1→ACC, and another chamber without S1→ACC activation (Fig. 6e). After a prolonged period of conditioning to unmask tonic pain, CFA-treated rats avoided the chamber associated with S1→ACC activation (Fig. 6f-h). In contrast, when we paired one chamber with S1→ACC inhibition and another chamber without S1→ACC inhibition, rats with chronic pain preferred S1→ACC inhibition (Fig. 6i-k). These data indicate that the S1→ACC projection can bidirectionally regulate the aversive response to chronic pain. Together, our results from these CPA assays indicate that enhanced S1→ACC projection contributes to elevated evoked and tonic pain responses in the chronic inflammatory pain condition.

To validate these findings, we repeated the aversive behavioral tests in the spared-nerve injury (SNI) model of chronic neuropathic pain (Fig. 7a). First, we compared the CPA score for naïve rats that received S1→ACC activation paired with PP in one chamber, and NS in the opposite chamber, with SNI-treated rats that received PP in one chamber and NS in the opposite chamber (Fig. 7b). Similar CPA scores under these conditions indicate that activation of the S1→ACC circuit had the same effects on the aversive phenotype as neuropathic pain (Fig. 7c-e). Next, we examined the impact of S1→ACC circuit modulation on the tonic pain experience in SNI-treated rats (Fig. 7f, j). We found that whereas activation of the S1→ACC projection enhanced the aversive experience associated with peripheral neuropathy (Fig. 7g-i), the inhibition of this pathway decreased tonic pain-induced aversion (Fig. 7k, l).

DISCUSSION

Normal physiologic response to acute pain requires the integration of sensory and affective experiences. Whereas the S1 and the ACC are well-known brain regions for processing the sensory and affective components of pain, respectively, a direct cortico-cortical circuit linking these two areas has not been previously reported. In this study, we combined *in vivo* electrophysiology, machine learning, and targeted circuit disruption to demonstrate that a direct S1→ACC projection allows sensory pain information to be transmitted to a higher order cortical center that regulates the affective experience. Furthermore, we found that enhanced cortico-cortical connectivity occurs in the chronic pain state and contributes to aversive pain behaviors.

The ACC plays a key role in regulating the affective component of pain[4-14]. Our results show that neurons in the ACC can respond to noxious stimulations by increasing firing rates, consistent with previous findings[19,20,34-40]. In addition, our machine learning analysis demonstrates that ensemble activities in the ACC can provide a relatively specific neural code for pain. Previous human functional MRI data showed that the ACC encodes pain experience with similarly high specificity[6,41,42]. Thus, our results provide a cellular basis for these human imaging findings. Likewise, our data on the ability for ACC neurons to bidirectionally regulate the aversive response to acute pain are compatible with previous reports[19].

Most importantly, our study provides a simple and direct circuit mechanism for the relay of cortical pain sensory information into higher order cortical centers to drive appropriate affective responses. This conclusion is based on three independent lines of evidence. First, whereas less than 9% of the ACC neurons receive S1 inputs at baseline, these neurons contribute disproportionally to the neural and behavioral pain response. Second, activation of the S1 inputs further enhances the ACC firing rates in response to noxious stimulations, and unbiased machine learning analysis confirms that activation of the S1→ACC projection increases the accuracy of pain discriminative power in the ACC. Third, direct modulation of the S1 input to the ACC bidirectionally regulates the aversive response to pain at the behavioral level. Together, these results indicate that the S1 is an important source of nociceptive inputs to the ACC. These functional results are further supported by past and current anatomic studies linking the S1 hind limb region with the ACC[43,44]. While previous studies have suggested that alterations of S1 activities could regulate pain behavioral responses[28,29], the downstream targets that mediate such nociceptive responses have remained elusive. A recent study suggests the spinal cord to be a target for the S1 nociceptive regulation[45]. Our results here provide a cortical target that is critical for processing the aversive response to pain. Since the S1 encodes

specific somatotopic nociceptive information, the projection from the S1 can be expected to assign sensory-specific value to drive the aversive response in the ACC.

In contrast to cortico-subcortical projections, neural mechanisms for cortico-cortical connectivity in pain processing is not well established. Our study posits two potential mechanisms for the S1-ACC connection. First, we have previously reported a cortical gain control mechanism, whereby the responsiveness of a cortical neuron to noxious stimulations is dependent on its basal activity level[20,31]. Here, we found that activation of the S1 inputs to ACC neurons selectively increased the spiking rates of ACC neurons in response to noxious stimuli, without altering their basal firing rates or their response to non-noxious stimulations. This important finding suggests activity-dependent cellular plasticity that enables S1 inputs to enhance the cortical gain of the ACC neurons; enhanced cortical gain, in turn, allows cingulate neurons to respond more efficiently to noxious signals. A second mechanism for cortico-cortical nociceptive processing occurs at the population level, as activation of the S1 inputs recruits more ACC neurons to respond to pain stimuli.

Chronic pain induces synaptic plasticity in a number of cortical and subcortical regions[1,25,26,46]. Such maladaptive plasticity in turn contributes to both sensory hypersensitivity and increased aversion. Pyramidal neurons in the ACC demonstrate increased synaptic plasticity with persistent or chronic pain[19,20,25,26,47]. This increased plasticity likely contributes to enhanced S1→ACC projection observed in the current study. It is important to note that in the chronic pain state, we observed an increased number of ACC neurons that receive S1 nociceptive inputs, and at the same time, these neurons that receive S1 inputs also show higher firing rates. These results indicate that enhanced cortical connectivity in the chronic pain state occurs at both population and single-cell levels. Recent studies have suggested that chronic pain can also alter cortio-subcortical projections through similar dual-level mechanisms[48,49].

Our finding of enhanced cortical integration of sensory and affective nociceptive processing in the chronic pain state is compatible with studies in other sensory systems[50-53]. Thus, cortical integration may be a general mechanism for sensory processing in the mammalian brain. It should be noted, however, that the directed information flow from S1→ACC is likely not the only circuit mechanism for cortical nociceptive integration. Other cortical areas, such as the secondary somatosensory cortex (S2), insular, and prefrontal cortex (PFC) also play key roles in either sensory or affective pain response[54]. For example, the S1 can project to the S2, which in turn projects to the insular. While the insular can process aversive pain responses by itself, it also has anatomic connections to the ACC and the PFC. The PFC, meanwhile, can project to a number of subcortical structures to regulate both sensory and affective responses[48,55,56]. Thus, future efforts of circuit mapping will further enhance our understanding of cortical and subcortical connections for the integration of sensory and affective pain information, and how such integration is altered in the chronic pain state.

Our findings may have important translational impact. Optogenetic inhibition of the S1→ACC pathway effectively relieved the aversive component of both acute and chronic pain. In our model, the S1 provides sensory pain information to the ACC to enrich the pain-specific aversive experience. Inhibition of this pathway thus has the potential to specifically reduce pain-associated

affective symptoms, and as a result, the S1→ACC projection could be an important target for non-addictive neuromodulation therapy for pain.

In summary, we have discovered a direct projection from the S1 to the ACC that bidiretionally regulates the pain-aversive response. This projection is enhanced in the chronic pain state and thus may form a target for therapeutic neuromodulation.

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Methods

Animals
All procedures were performed in accordance with the guidelines of the New York University School of Medicine (NYUSOM) Institutional Animal Care and Use Committee (IACUC) to ensure minimal animal use and discomfort, as consistent with the NIH *Guide for the Care and Use of Laboratory Animals* . Male Sprague-Dawley rats were purchased from Taconic Farms and kept at the vivarium facility in the NYU Langone Science Building, with controlled humidity, temperature, and 12-hr (6:30 AM–6:30 PM) light-dark cycle. Food and water were available *ad libitum*. Animals weighed 250 to 300 g upon arrival to the facility and were given 10 days on average to adjust to the new environment before initiation of experiments.

Complete Freund's Adjuvant (CFA) Administration
0.1 mL of CFA (*Mycobacterium tuberculosis*, Sigma-Aldrich) was suspended in an oil:saline (1:1) emulsion and injected subcutaneously into the plantar aspect of the hind paw to induce chronic inflammatory pain. CFA was always injected contralateral to the paw that was stimulated by either pin prick (PP) or von Frey filament (vF), and ipsilateral to the recording electrodes. Control rats received an equal volume of saline injection.

Virus Construction and Packaging
Recombinant adeno-associated virus (AAV) vectors were serotyped with AAV1 coat proteins, packaged at Addgene viral vector manufacturing facilities. Viral titers were approximately 5×10^{12} particles per milliliter for AAV1.CaMKII. ChR2-eYFP.WPRE.hGH, AAV1.CAMKII.NpHR-eYFP.WPRE.hGH, and AAV1. CaMKII(1.3).eYFP.WPRE.hGH.

Stereotaxic Intracranial Injections and Optic Fiber Implantation
As described previously, rats were anesthetized with 1.5%–2% isoflurane[46]. Virus was delivered to the hind limb region of the primary somatosensory cortex (S1-HL) only in all of the experiments. Rats were unilaterally injected with 0.65 μL of viral particle solution at a rate of 0.1 μL/10 s with a 26G 1 μL Hamilton syringe at anteroposterior (AP) -1.5 mm, mediolateral (ML) ± 3.0 mm, and dorsoventral (DV) −1.5 mm. Rats were next implanted bilaterally with 200-μm optic fibers held in 1.25 mm ferrules (Thorlabs) in the ACC (AP +2.6 mm, ML ± 1.2 mm, DV −1.75 mm, angled 28° toward the midline). Fibers with ferrules were held in place by dental acrylic.

Chronic Optrode Implant and Intracranial Injections

After exposure of the skull, rats were injected with 0.65 μL of viral particle solution in the at a rate of 0.1 μL/10 s with a 26G 1 μL Hamilton syringe unilaterally in the S1 (AP -1.5 mm, ML ± 3.0 mm, DV −1.5 mm). After the intracranial injection, rats were allowed to recover for a period of three weeks before optrode implantation to allow for adequate viral expression.

Tetrodes were constructed from four twisted 12.7 μm polyimide-coated microwires (Sandvik) and subsequently mounted in an eight-tetrode VersaDrive Optical (NeuraLynx). A 200 μm optic fiber held in 1.25 mm ferrules (Thorlabs) was mounted in the VersaDrive Optical such that the end of the fiber was located approximately 0.5–1 mm above the mounted tetrodes. Electrode tips were gold plated to reduce electrode impedances to 100–500 kΩ at 1 kHz. Rats were anesthetized with 1.5%–2% isoflurane, and the skull was exposed. A 2 mm-diameter hole was drilled above the ACC target region. A durotomy was performed prior to lowering the optrodes slowly unilaterally into the ACC with the stereotaxic apparatus (AP +2.6 mm, ML +0.8 mm, DV −1.75 mm, with tetrode tips angled 15° toward the midline). The drive was secured to the skull screws with dental cement. After animal sacrifice, 20 μm brain sections were collected using a Leica CM3050S cryostat machine (Leica Biosystems) and analyzed for viral expression, optic fiber localization, and electrode localization with histological staining. Animals with improper fiber or electrode placements, low viral expression, or viral expression in cell bodies outside the S1-HL were excluded from further analysis.

In Vivo Electrophysiological Recordings

Prior to stimulation, animals with chronic optrode implants were allowed 30 min to habituate to a recording chamber over a mesh table, as described previously[19]. Noxious stimulation was applied by pricking the plantar surface of the hind paw contralateral to the brain recording site with pin prick by a 30G needle (PP) in free-moving rats. Noxious stimulation was terminated by withdrawal of the paw. Non-noxious stimulus was applied to the same hind paw using a 2 g vF, continuously for 3s or until paw withdrawal. There were no withdrawal responses to vF in the majority of cases. All of the recording sessions consisted of approximately 50 trials with variable inter-trial intervals. Approximately half of the recording sessions were conducted using either PP or vF filament stimulation for the entire session, with 25 trials either with or without constant 20 Hz optogenetic stimulation. The remaining recording sessions were conducted either with or without constant 20 Hz optogenetic stimulation for the entire session, with PP and vF filament stimulations applied randomly to the rat's hind paws (equal number of trials for each stimulation type). Recording conditions were counterbalanced by rats.

A video camera (HC-V550, Panasonic) was used to record the experiments. Long inter-trial intervals of approximately 60 seconds and the breaks between sessions were used to avoid sensitization. No behavioral sensitization or physical damage to the paws was observed.
Experiments involving the CFA were performed 7 days after CFA injection. Experiments involving the SNI were performed 14 days after surgery.

Neural Data Collection and Preprocessing

The neuronal activity and the onset of stimulation were simultaneously recorded with acquisition equipment (Open Ephys) via an RHD2132 amplifier board (Intan Technologies). Signals were monitored and recorded from 32 low-noise amplifier channels at 30 kHz, and band-pass filtered (0.3–7.5 kHz). In order to identify spike activity, the raw data were high-pass filtered at 300 Hz

with subsequent thresholding and offline sorting by commercial software (Offline Sorter, Plexon). The threshold was below the 3-sigma peak heights line and was manually optimzed based on the signal-to-noise ratio. The features of three valley electrodes were used for spike sorting. Trials were aligned to the initiation of the peripheral stimulus to compute the peristimulus time histogram (PSTH) for each single unit using MATLAB (MathWorks).

Immunohistochemistry

Rats were deeply anesthetized with isoflurane and transcardially perfused with ice-cold PBS and paraformaldehyde (PFA). After extraction, brains were fixed in PFA overnight and then cryoprotected in 30% sucrose in PBS for 48 hours or until sinking[55]. 20 μm coronal sections were washed in PBS and coverslipped with Vectashield mounting medium. Images containing tetrodes were stained with cresyl violet and imaged at 10x magnification with an Axio Zoom widefield microscope (Carl Zeiss). Sections also were made after viral transfer for opsin verification, and these sections were stained with anti-rabbit GFP (1:500, #AB290, Abcam), CaMKII-α (6G9) mouse monoclonal antibody (mAb) (1:200, #50049, Cell Signaling Technology), and DAPI (1:200, Vector Laboratories) antibodies. Secondary antibodies were anti-rabbit immunoglobulin G (IgG) conjugated to Alexa Fluor 488, and anti-mouse IgG conjugated to Alexa Fluor 647 (1:200, Life Technologies). Images were acquired with a Zeiss LSM 700 confocal microscope (Carl Zeiss).

Animal Behavioral Tests

Behavioral tests involving optogenetic stimulation were conducted approximately 2-4 weeks after viral injection of channelrhodopsin-2 (ChR2), and approximately 3-5 weeks after viral injection of halorhodopsin (NpHR). Prior to each experiment, optic fibers were connected to a laser diode (Shanghai Dream Lasers Technology) through a mating sleeve, as described previously[55]. Laser light was delivered using a transistor-transistor logic (TTL) pulse generator (Doric Lenses), and intensity was measured with a power meter (Thorlabs, Newton, NJ, USA). Laser diodes of wavelength 473 nm were used for channelrhodopsin-2, and laser diodes of wavelength 589 nm were used for halorhodopsin. Experiments involving the CFA were performed 7 days after CFA injection. Experiments involving the SNI were performed 14 days after surgery.

Conditioned place aversion (CPA) assay

CPA experiments were conducted similarly to those described previously[19], in a standard two-compartment apparatus (Stoelting Co., Wood Dale, IL, USA) consisting of two large compartments of equal size connected with an opening large enough for a rat to travel through freely. The CPA protocol included preconditioning (baseline), conditioning, and testing phases. The preconditioning phase was 10 min, and animals spending >500 s or <100 s of the total time in either chamber during the preconditioning phase were eliminated from further analysis. Immediately following the preconditioning phase, the rats underwent conditioning. During conditioning, at least one of the two chambers was paired with either peripheral stimulation (PP), or 20Hz optogenetic stimulation, or both. The PP stimulus was repeated every 10 s. Peripheral stimulation, optogenetic activation, and chamber pairings were counterbalanced. For experiments involving peripheral stimulation, the conditioning phase was also 10 minutes. For experiments without peripheral stimulation, the conditioning phase was 60 min, with the rats spending 30 min in each of the two treatment (optogenetic and control) chambers. During the test phase, the animals did not receive any treatment and had free access to both compartments for 10 min. Movements

of the rats in each chamber were recorded by a camera and analyzed with ANY-maze software. Decreased time spent in a chamber during the test phase as compared with the baseline indicated avoidance (aversion) of that chamber, whereas increased time spent in a chamber during the test phase as compared with the baseline indicated preference for that chamber.

Mechanical Allodynia Test

Mechanical allodynia was measured using a Dixon up-down method with vF filaments. Rats were placed individually into plexiglass chambers over a mesh table and allowed to acclimate for 20 min prior to testing. vF filaments were applied to the lateral one-third of the hind paw in the distrubition of the sural nerve with logarithmically incremental stiffness (0.45, 0.75, 1.20, 2.55, 4.40, 6.10, 10.50, and 15.10 g), beginning with 2.55 g, as described previously[55]. 50% withdrawal thresholds were calculated.

Statistical Analysis

Behavioral results were given as means ± SEM. For comparing mechanical allodynia withdrawal thresholds for CFA-treated, SNI-treated, and control rats, a two-way ANOVA with repeated-measures and post hoc multiple pairwise comparison Bonferroni tests or unpaired t tests were used whenever appropriate. During the CPA test, a paired Student's t test was used to compare the time spent in each treatment chamber before and after conditioning (i.e., baseline versus test phase for each chamber). Decreased time spent in a chamber during the test phase as compared with the baseline indicated avoidance (aversion) of that chamber. A CPA score was computed by subtracting the time spent in the more noxious chamber during the test phase from the time spent in that chamber at baseline[19]. A two-tailed unpaired Student's t test was used to compare differences in CPA scores under various testing conditions.

For neuronal spike analysis, we calculated PSTHs using a 5-s range before and after peripheral stimulus (i.e., PP or vF) and a bin size of 100 ms. The number of spikes in each stimulus-aligned bin was averaged across all of the trials to create the PSTH. We then calculated the basal spontaneous firing rate for each neuron to be the average of the PSTH bins before stimulus onset and the peak pain-evoked firing rate to be the maximum value of the PSTH after stimulus onset (within 5 s from the stimulus).

To define a neuron that altered its firing rate in response to a peripheral stimulus, we used the method described previously[31]. The baseline mean is the average of the PSTH bins before stimulus onset, and the standard deviation is the standard deviation of the PSTH bins before stimulus onset. To calculate the Z scored firing rate, we used the following equation: $Z = (FR -$ mean of $FR_b)$/standard deviation of FR_b, where FR indicates the firing rate for each bin and FR_b indicates the baseline firing rate before stimulus onset. To define a pain-responsive neuron, we used the following criteria: (1) the absolute value of the Z scored firing rate of least one time bin after stimulation must be ≥ 2.5, and (2) if the first criterion is passed, at least the next two bins must be >1.645. These criteria must be fulfilled within 3 s after the peripheral stimulus. Neuronal FRs had a non-Gaussian distribution, compatible with a previous report[57]. Thus, nonparametric tests were performed. For unpaired data, a Mann-Whitney U test was performed to test the equivalence of distributions. The Wilcoxon matched-pairs signed-rank test was used to test the equivalence of distributions for paired data. Fisher's exact test was used to analyze the population changes for pain response. In these studies, because of the negligible number of neurons

that decreased their FRs in response to stimulation (PP or vF filament), we included those neurons in the category of non-responders.

For all tests, a p value < 0.05 was considered statistically significant. All of the data were analyzed using GraphPad Prism version 7 software and MATLAB (MathWorks).

Population-Decoding Analysis Using Support Vector Machine (SVM)

After spike sorting, we obtained population spike trains from simultaneously recorded ACC neurons. For each single neuronal recording, we binned spikes into 50 ms to obtain spike count data in time. To simulate the online decoding, we used a 50-ms moving window to accumulate spike count statistics from the onset of the peripheral stimulus (time 0) up to 3 s (i.e., 60 bins). We assessed the decoding accuracy at each time bin based on the cumulative spike count statistics. Therefore, for a total of C neurons, the input dimensionality ranged from C (the first bin) to $60C$ (all bins). In these experiments in which we randomly mixed different stimulations (PP and vF filament), we assumed that we had $n1$ trials of PP and $n2$ trials of vF filament. We split the total ($n_1 + n_2$) trials into two groups: 80% used for training and 20% used for testing. The goal of population-decoding analysis was to classify the trial labels of different stimulations (PP versus vF filament) based on population spike data. We used an SVM classifier. The SVM is a discriminative supervised learning model that constructs the classification boundary by a separating hyperplane with maximum margin. Specifically, the SVM can map the input $\mathbf{x}$ into high-dimensional feature spaces, which allows nonlinear classification, as follows:

$$y=\sum i=1N\alpha iK(x,xi)+b$$

where y_i denotes the class label for the training sample $\mathbf{x}_i$ (some of which associated with nonzero α_i are called support vectors), b denotes the bias, and K ($\mathbf{x}$, $\mathbf{x}_i$) denotes the kernel function. We used a polynomial kernel and trained the nonlinear SVM with a sequential minimal optimization algorithm (MATLAB Machine Learning Toolbox ‘‘fitcsvm’’ function). Finally, the decoding accuracy was assessed by 2-fold cross-validation from 50 Monte Carlo simulations. We reported the means ± SEMs. In all of the population-decoding analyses, we used only the recording sessions with ≥5 simultaneously recorded ACC units, independent of the cell-firing properties.

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Fig. 1 Anatomic projection from the S1 to the ACC. a, Schematic showing the retrograde tracer injected into the ACC. **b**, Retrograde tracer expression in the hind limb region of S1. **c**, Schematic showing the YFP injected into the S1. **d**, Anterograde expression of YFP in the ACC. **e**, Higher magnification view of the expression of YFP in pyramidal neurons of the ACC.

Fig. 2 S1 projection enhances the nociceptive response in ACC neurons in freely behaving rodents. a, Schematic showing the ChR2 injection in the S1 hind limb region and optrode in the ACC. **b**, Histology showing tetrode locations in the ACC. **c**, Raster plots and peri-stimulus time histograms (PSTHs) of a representative ACC neuron. Time 0 indicates the onset of noxious pin prick (PP) stimulation. FR: firing rates. Inset shows representative single cell recordings. **d**, Raster plots and peri-stimulus time histograms (PSTHs) of a representative ACC neuron. Time 0 indicates the onset of non-noxious von Frey filament (vF) stimulation. **e**, 15.73% of recorded ACC neurons (n = 623 from 6 rats) responded to PP. **f**, 7.05% of recorded ACC neurons (n = 567 from 6 rats) responded to vF. **g**, The difference in the proportion of neurons that increased their firing rates in response to vF and PP is statistically significant. $p < 0.0001$, Fisher's exact test. See **Methods**. **h**, In neurons that responded to both stimuli, PP induced higher firing rates than vF. Data represented as median ± interquartile range. n1 = 40 for vF, n2 = 98 for PP; $p = 0.0009$, Wilcoxon paired signed rank test. See **Methods** for calculations of stimulus-evoked firing rates. **i**, SVM-based population-decoding analysis, based on ACC neuronal activity, demonstrated the ability to distinguish between painful and non-painful stimulation. n = 40 sessions from 5 rats. **j**, A representative session of unbiased support vector machine (SVM)-based population-decoding analysis to distinguish between painful and non-painful stimulations. Time zero denotes the stimulus (PP or vF) onset. The blue curve denotes the decoding accuracy (n1 = 25 trials for PP, n2 = 25 trials for vF; C= 40ACC neurons) derived from the data with true labels; the error bar denotes the SEM from 50 Monte Carlo simulations based on 2-fold cross-validation; the maximum decoding accuracy was 0.75. See **Methods** for details. **k**, Schematic for *in vivo* optrode recording experiments. **l**, Representative recording trace shows that optogenetic activation of the presynaptic S1 inputs increased the firing rates of a pyramidal neuron in the ACC, in response to PP. **m**, Representative recording trace shows that optogenetic activation of the S1 inputs did not change the firing rate response to vF in an ACC neuron. **n**, Activation of the presynaptic S1 inputs increased the firing rates of ACC neurons, in response to PP. n = 623; $p < 0.0001$, Wilcoxon matched-pairs signed rank test. In contrast, activation of the presynaptic S1 inputs had no impact on the firing rates of ACC neurons, in response to vF. n = 567; $p = 0.3299$, Wilcoxon matched-pairs signed rank test. **o**, SVM-based population-decoding analysis to distinguish between painful and non-painful stimulation, in the presence of S1 activation. **p**, S1 activation increases the decoding accuracy to distinguish between painful and non-painful stimulation. n1 = 40, n2 = 48; $p = 0.0406$, Wilcoxon matched-pairs signed rank test.

Fig. 3 S1 inputs increase nociceptive response in the ACC at both the population level and the level of single neuron firing rates. a, Proportion of ACC neurons that receive S1 inputs and their responsiveness to noxious stimulations. **b**, Proportion of ACC neurons that do not receive S1 inputs and their responsiveness to noxious stimulations. **c**, ACC neurons that receive S1 inputs are more responsive to noxious stimulations. $p < 0.0001$, Fisher's exact test. **d**, ACC response to noxious stimulations in the presence of S1 activation. **e**, Activation of the S1 inputs increase ACC response to noxious stimulations. $p = 0.0191$, Fisher's exact test. **f**, S1 inputs increased pain-

evoked firing rates of ACC neurons. Median ± interquartile range for evoked firing rates with and without activation of the S1 inputs. n = 54; $p < 0.0001$, Wilcoxon matched-pairs signed rank test. **g**, S1 inputs did not alter pain-evoked baseline firing rates of ACC neurons. n = 54; p = 0.1442, Wilcoxon matched-pairs signed rank test.

Fig. 4 S1→ACC projection bidirectionally regulates aversive pain behaviors. a, Schematic of the conditioned place preference (CPA) assay. **b**, Rats display aversive response to acute mechanical pain. One of the chambers was paired with PP, the other chamber was not paired with a painful stimulus (NS). n = 19; $p < 0.0001$, paired Student's t test. **c**, Schematic of injection of channelrhodopsin and halorhodopsin into the S1-HL, and insertion optic fibers into the ACC. **d**, Schematic of CPA assay with optogenetic activation. One of the chambers was paired with optogenetic activation of the S1→ACC projection and PP; the other chamber was paired with PP alone. **e**, Rats avoided the chamber associated with S1→ACC activation, when presented with PP. n = 10; p = 0.0114, paired Student's t test. **f**, YFP control rats did not demonstrate avoidance of the chamber associated with S1→ACC activation. n = 14; p = 0.8087, paired Student's t test. **g**, CPA score for S1→ACC activation in the presence of mechanical pain. n = 10-14; p = 0.0415, unpaired Student's t test. **h**, Schematic of CPA assay with optogenetic inhibition. One of the chambers was paired with optogenetic inhibition of the S1→ACC projection and PP; the other chamber was paired with PP alone. **i**, Rats preferred the chamber associated with S1→ACC inhibition, when presented with PP. n = 11; p = 0.0486, paired Student's t test. **j**, CPA score for S1→ACC inhibition in the presence of mechanical pain. n = 11-14; p = 0.0495, unpaired Student's t test.

Fig. 5 Persistent pain increases S1-ACC connectivity. a, Schematic of the CFA model. **b**, CFA treatment induces mechanical allodynia. **c**, Raster and PSTH of a representative ACC neuron in a CFA-treated rat. **d**, Chronic pain increased the basal firing rates of ACC neurons. Median ± interquartile range for basal firing rates of PFC neurons. n1 = 623, n2 = 294; $p < 0.0001$, Mann-Whitney U test. **e**, Chronic pain increased the peak firing rates of ACC neurons. Median ± interquartile range for peak firing rates of PFC neurons. n1 = 623, n2 = 294; $p < 0.0001$, Mann-Whitney U test. **f**, Representative recording trace shows that optogenetic activation of the presynaptic S1 inputs increased the firing rates of a pyramidal neuron in the ACC in response to PP, in a CFA-treated rat. **g,** Activation of the presynaptic S1 inputs increased the firing rates of ACC neurons, in response to PP in CFA-treated rats. n = 294; p = 0.0083, Wilcoxon matched-pairs signed rank test. **h**, Activation of the presynaptic S1 inputs did not alter the basal firing rates of ACC neurons in CFA-treated rats. n = 294; p = 0.4926, Wilcoxon matched-pairs signed rank test. **i**, Chronic pain increases the proportion of ACC neurons that received S1 inputs. p = 0.0021, Fisher's exact test. **j**, Chronic pain increases the number of pain-responsive ACC neurons that received S1 inputs. p = 0.0487, Fisher's exact test. **k**, Chronic pain increases the firing rates of ACC neurons that received S1 inputs, in response to PP. n = 98; $p < 0.0001$, Wilcoxon matched-pairs signed rank test.

Fig. 6 Enhanced S1-ACC connectivity contributes to chronic inflammatory pain. a, Schematic of the CPA assay in CFA-treated rats. One of the chambers was paired with optogenetic activation of the S1→ACC projection and PP; the other chamber was paired with NS alone. **b**, Rats spent significantly less time during the test phase than at baseline in the chamber paired with S1→ACC activation and PP. n = 15; p = < 0.0001, paired Student's t test. **c**, CFA treatment

increases the aversive value of PP. One of the chambers was paired with PP; the other chamber was paired with NS alone. n = 9; p = 0.0007, paired Student's t test. **d**, S1→ACC activation caused a similar increase in the aversive response to PP as chronic pain. paired Student's t test. A CPA score was calculated by subtracting the amount of time spent during the test phase from baseline in the chamber paired with simultaneous S1→ACC activation and PP in **b** or by subtracting the amount of time spent during the test phase from baseline in the chamber paired with PP in CFA-treated rats in **c**. n = 9-15; p = 0.4746, unpaired Student's t test. **e**, Schematic of the CPA assay for tonic pain in the inflammatory pain model. One of the chambers was paired with activation of the S1→ACC projection; the other chamber was not. No peripheral stimulus was given. **f**, CFA-treated rats avoided the chamber associated with S1→ACC activation. n = 7; p = 0.0017, paired Student's t test. **g**, CFA-treated rats with YFP control did not demonstrate any chamber preference. n = 7; p= 0.9502, paired Student's t test. **h**, CPA score for CFA-treated rats which received S1→ACC activation. n = 7; p= 0.0021, unpaired Student's t test. **i**, Schematic of the CPA assay for tonic pain in the inflammatory pain model. One of the chambers was paired with inactivation of the S1→ACC projection; the other chamber was not. No peripheral stimulus was given. **j**, CFA-treated rats preferred the chamber associated with S1→ACC inhibition. n = 9; p = 0.0128, paired Student's t test. **k**, CPA score for CFA-treated rats which received S1→ACC inhibition. n = 7-9; p = 0.0256, unpaired Student's t test.

Fig. 7 Enhanced S1-ACC connectivity contributes to chronic neuropathic pain. a, SNI treatment induces mechanical allodynia. **b**, Schematic of the CPA assay in SNI-treated rats. One of the chambers was paired with optogenetic activation of the S1→ACC projection and PP; the other chamber was paired with NS alone. **c**, Rats spent significantly less time during the test phase than at baseline in the chamber paired with S1→ACC activation and PP. n = 15; p < 0.0001, paired Student's t test. **d**, SNI treatment increases the aversive value of PP. One of the chambers was paired with PP; the other chamber was paired with NS alone. n = 7; p < 0.0001, paired Student's t test. **e**, S1→ACC activation caused a similar increase in the aversive response to PP as chronic pain. n = 7-15; p = 0.3012, unpaired Student's t test. **f**, Schematic of the CPA assay for tonic pain in the SNI neuropathic pain model. **g**, SNI-treated rats avoided the chamber associated with S1→ACC activation. n = 7; p < 0.0001, paired Student's t test. **h**, SNI-treated rats with YFP control did not demonstrate any chamber preference. n = 7; p = 0.9723, paired Student's t test. **i**, CPA score for SNI-treated rats which received S1→ACC activation. n = 7; p = 0.0010, unpaired Student's t test. **j**, Schematic of the CPA assay for tonic pain in the neuropathic pain model. One of the chambers was paired with inactivation of the S1→ACC projection; the other chamber was not. No peripheral stimulus was given. **k**, SNI-treated rats preferred the chamber associated with S1→ACC inhibition. n = 7; p = 0.0111, paired Student's t test. **l**, CPA score for SNI-treated rats which received S1→ACC inhibition. n = 7; p = 0.0260, unpaired Student's t test.

Supplementary Fig. 1 Expression of channelrhodopsin (ChR2) in the S1. **a**, Low magnification (10x) view of histologic expression of channelrhodopsin (YFP-ChR2) in the S1 hind limb region after injection. **b**, Higher magnification (63x) view of the expression of YFP-ChR2 in pyramidal neurons of the S1 demonstrated the co-staining of glutamatergic neuronal marker CaMKII with YFP. From left to right: CaMK II staining; ChR2-eYFP staining; DAPI staining and merged images.

Supplementary Fig. 2 Expression of Halorhodopsin (HR) in the ACC. a, Expression of Halorhodopsin (HR) in the ACC. **b**, Higher magnification view of the expression of HR in pyramidal neurons of the ACC.

Supplementary Fig. 3 Activation of the S1→ACC projection does not have intrinsic aversive value. a, Schematic of the conditioned place preference (CPA) assay, when rats are presented with vF stimulation to hind paws. **b**, Rats display no aversive response to non-noxious mechanical stimulus. One of the chambers was paired with vF, the other chamber was not paired with a painful stimulus (NS). n = 7; p = 0.8117, paired Student's t test. **c**, Rats did not avoid the chamber associated with S1→ACC activation, when presented with vF. n = 7; p = 0.8232, paired Student's t test. **d**, YFP control rats did not demonstrate avoidance of the chamber associated with S1→ACC activation. n= 4; p = 0.8154, paired Student's t test. **e**, CPA score for S1→ACC activation in the presence of vF stimulus.