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Investigating roles of a novel spinal ascending pathway in transmitting mechanical itch sensation

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by

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The Dissertation of Xiangyu Ren is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE DISSERTATION

Investigating roles of a novel ascending pathway in transmitting mechanical itch sensation

by

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Itch is an unpleasant sensation that provokes the urge to scratch. The sensation of itch can be induced by distinct triggers including pruritogen (chemical itch) or tactile stimuli (mechanical itch). Chemical and mechanical itch, due to distinct nature of the stimuli, are mediated by remarkably different peripheral and central neural circuitries. A cohort of molecularly defined sensory and spinal neuron populations that relay chemical itch has been characterized, while very little is known about those that relay mechanical itch.

Scratching is usually the immediate response induced by itch in human and animals to remove dangerous pruritic agents. However, when itch becomes prolonged or chronic, affective motivational components of itch, such as negative valence and aversive memory

induced by itch, form and more complicated protective behavior is recruited to start itch-relief, which require the involvement of the supraspinal itch-relaying regions through ascending and descending itch pathways. It has been reported that chemical itch sensation is potentially relayed by spinal projection neurons that express Tacr1 (as known as NK1R) to the parabrachial nuclei (Carsten et al. 2010; Acton et al. 2019). However, it is still not known what supraspinal structures encode mechanical itch responses and how mechanical itch information is transmitted to these supraspinal regions through ascending pathways. By focusing on these questions in my thesis project, I have tried to answer these questions by addressing three more specific issues: 1. What is the identity of supraspinal region that relays mechanical itch? 2. What is the molecular identity of the spinal projection neurons that relay mechanical itch information and who do they connect to? 3. What is the molecular identity of the neurons in the supraspinal region that are contacted by the spinal projection neurons that transmit mechanical itch and are they also necessary for itch-induced scratching?

In the first part of my thesis, I identified the parabrachial nucleus (PBN), located in the dorsolateral pons area of the brainstem, as a crucial relay hub for mechanical itch. Chemogenetic silencing of the PBN neurons suppresses mechanical itch sensitivities under both acute and chronic conditions in mice. In the second part of my thesis, I have characterized a spinal neuron population that is selectively targeted by $Calcrl^{Cre}$ and $Lbx1^{FlpO}$ (hereafter referred to as $Calcrl^{Lbx1}$ neurons) that have a dedicated projection to the PBN. Genetic ablation or chemogenetic silencing of this neuron population in the spinal cord suppresses mechanical itch sensitivities under baseline, acute as well as chronic itch conditions. By contrast, chemogenetic activation of the $Calcrl^{Lbx1}$ population dramatically sensitizes mechanical itch in mice. A similar but lesser extensive suppression of mechanical

itch sensitivity was observed when the spinoparabrachial projections of $Calcrl^{Lbx1}$ neurons were selectively silenced. In the third part of my thesis, I have shown that PBN neurons that express FoxP2 function as a postsynaptic partner of spinal $Calcrl^{Lbx1}$ neurons and are necessary for relaying and expressing mechanical itch. In vivo calcium imaging by fiber photometry showed $FoxP2^{PBN}$ neurons are tuned to respond to mechanical itch stimulation under baseline, acute and chronic itch conditions. Chemogenetic silencing of these neurons in the PBN suppresses mechanical itch sensitivity in all conditions. In summary, my thesis project identified and characterized a novel mechanical itch-relaying spinoparabrachial pathway featured by the Calcrl-FoxP2 functional connection.

Chapter 1: Introduction

The somatosensory system processes a number of distinct cutaneous sensory modalities, like touch, vibration, itch, pain and temperature, to generate the appropriate motor responses, discriminate sensory modalities and determine the affective-motivational valence. A number of neural populations in the periphery, spinal cord and supraspinal regions function as crucial drivers or modulators for those sensory modalities. In my thesis, the first chapter will give a brief summary of the field, and the current knowledge on the heterogeneous neuron populations that underlie pain, itch and touch processing.

Itch is an unpleasant sensation that drives scratching. The pruritic agent can either be a chemical, like histamine and chloroquine (chemical itch) or light touch (mechanical itch). However, very little is known about the molecular, cellular and circuit mechanisms that underlie the transmission and modulation of these itch subtypes. In the second chapter, the neural populations and circuitries that relay and encode chemical and mechanical itch, from the periphery to the central nervous system will be discussed in detail.

The parabrachial nucleus (PBN), located in the dorsolateral pons of the brainstem, is a well-known sensory relay center, which integrates signals from a wild spectrum of sensory modalities, including gustatory, exteroceptive and interoceptive signals. Recently, the PBN has also been implicated in the modulation of the chemical itch sensation (Mu et al. 2017). In the last chapter of my introduction, I will summarize the current knowledge on the functional roles of the PBN, with a particular focus on the neuron populations that contribute to the itch perception and modulation.

1.1 Neural basis of Somatosensation

1.1.1 Peripheral circuits for cutaneous sensory processing

The somatosensory system processes a number of distinct cutaneous sensory modalities. Primary sensory neurons relay somatosensory information from the periphery to the central nervous system (CNS). Their cell bodies reside in dorsal root ganglia (DRG) and trigeminal ganglia (TG) and their pseudo-unipolar axons extend towards both the periphery, where they innervate the skins and its specialized sensory corpuscles, and towards the spinal cord or brainstem where they connect to the second-order neurons. The pseudo-unipolar morphology of the primary sensory neurons facilitates the sensory information flow from the periphery to the central nervous system (Abraira et al. 2013).

DRG neurons are highly heterogeneous and can be classified into distinct subgroups based on their anatomical, electrophysiological and molecular features. According to the soma size, and the myelination and conduction velocity of their axons, DRG neurons can be grouped in Aβ (large-sized soma, heavily myelinated fibers and fast-conducting), Aδ (medium-sized soma, thinly myelinated fibers and medium-conducting) and C (small-sized soma, unmyelinated fibers and slow-conducting) neurons. An oversimplified summary is that unmyelinated or thinly myelinated C/Aδ neurons predominantly detect and relay noxious cutaneous stimuli (like itch, pain and temperature), whereas heavily myelinated Aβ neurons primarily convey innocuous cutaneous stimuli (like touch, stroking, and vibration). However, a number of exceptions to this classification have been reported. For example, pleasant touch, has been shown to be relayed by a subset of mechanosensitive C-fibers (Löken et al. 2009; McGlone et al. 2007), and touch-evoked pain under chronic pain conditions (like chronic

inflammation or neuropathy) is also conveyed by Aβ fibers (Duan et al. 2014; Lu et al. 2013).

Differentiation of DRG neurons in terms of morphology or conductance oversimplifies their diversity. Nevertheless, combining these two features with the type of sensory modalities these fibers relay or the skin organelles they innervate allows a more accurate description of the heterogeneity. According to the stimulus properties and consequent modal selectivity the cutaneous stimuli are generally classified as mechanoreceptive (touch-sensing), pruriceptive (itch-sensing) and nociceptive (pain-sensing). Previous studies have shown that DRG neurons that respond to pruriceptive and nociceptive stimuli, named pruriceptors and nociceptors receptively, are predominantly C and Aδ neurons, whereas those that respond to mechanoreceptive stimuli, termed mechanoreceptors, comprise a broader variety of fiber types. Two categories of mechanoreceptors have been identified based on the intensity of mechanoreceptive stimuli they respond to: C/Aδ high-threshold mechanoreceptors (HTMR) and Aβ/Aδ/C low-threshold mechanoreceptors (LTMRs).

In addition to the sensory modalities, DRG neurons can be divided according to the associated skin organelles. Most of C/Aδ nociceptors and pruriceptors terminate as free nerve endings within the epidermis, whereas the LTMR family includes sensory fibers associated with a number of specialized skin sensory organelles, located in the glabrous or hairy skin. In the glabrous skin, there are four subclasses of Aβ LTMRs that innervate four major types of cutaneous mechanosensory organelles: Aβ SAI-LTMR the Merkel cells, Aβ SAII-LTMR the Ruffini endings, Aβ RAI-LTMR the Meissner corpuscles and Aβ RAII-LTMR the Pacinian corpuscles. These Aβ-LTMR afferents also display unique electrophysiological response

properties to low-threshold mechanical stimuli. Aβ SA-LTMRs show maintained firing during sustained indentation of the glabrous skin, whereas Aβ RA-LTMRs respond with initial and terminal firing to vibration. Aβ SA-LTMRs can be further divided into two small subgroups, Aβ SAI- and SAII-LTMRs, based on the features of their interspike intervals. Likewise, Aβ RA-LTMRs can also be divided into two subsets, Aβ RAI- and RAII-LTMRs, according to the receptive fields and responsive frequency to vibration. In contrast, much simpler organization was found in the hairy skin, with most Aβ/Aδ/C-LTMRs form longitudinal lanceolate endings that surround hair follicles (Abraira et al, 2013).

In the last two decades, the application of novel molecular and genetic techniques significantly broadened our knowledge of the molecular profile and population diversity of DRG neurons (Basbaum et al. 2009; Julius 2013). Two non-overlapping subclasses of C/Aδ nociceptors were initially found based on neuropeptide expression: CGRP+/SP+ peptidergic and IB4+ non-peptidergic neurons (Basbaum et al. 2009; Julius 2013). Recently, this classification has been further refined with new molecular markers that identify more functionally specialized subsets. For example, the DRG neurons that express TRPM8 channel sense cooling and cold sensation (Bautista et al. 2007). A subset of peptidergic nociceptors expressing the Mas-related G-protein coupled receptor member A3 (MrgprA3) has been shown to exclusively relay itch but not pain or temperature (Han et al. 2013). Another nociceptor subclass expressing MrgprD constitutes around 90% of IB4+ non-peptidergic nociceptors, and selectively function as mechanoreceptor (Balci et al. 2009). Additionally, C/Aδ nociceptors express other markers including cation ion channels of the TRP family members TRPV1 and TRPA1, and voltage-gated sodium channel Nav1.8 (Akopian et al.

1996; Basbaum et al. 2009; Julius 2013). Likewise, a cohort of molecular markers has also been discovered for functionally distinct subsets of Aβ LTMRs, including TrkA, TrkB, TrkC, TH, cRet, Npy2r and TLR5 etc. (Abraira et al. 2013; Li et al. 2011; Pan et al. 2019; Xu et al. 2015).

In recent years, the molecular landscape of DRG heterogeneity has been further clarified by single cell RNA-sequencing (scRNAseq) studies. Usoskin *et al.* applied large-scale scRNAseq technique and dissect the heterogeneous DRG family into 11 subclasses, which include 5 nociceptor (NP1, NP2, NP3, PEP1 and PEP2), 4 LTMR (NF1, NF2, NF3 and TH) and 2 proprioceptors subclasses (Usoskin et al. 2015). The scRNAseq data correlates well with previous histological results, although with some discrepancies, like the NP2 subtypes also includes CGRP+ neurons (Usoskin et al. 2015). This classification was also supported by additional scRNAseq studies on DRG heterogeneity (Li et al. 2018; Zeisel et al. 2018).

The identification of modality-specific DRG neurons suggests they may act as labeled-line in conveying somatosensation, although the presence of polymodal neurons confounds this view. For example, activation of MrgprA3+ DRG neurons with optogenetic stimulation induces nocifensive whereas it triggers pruriceptive responses when achieved using chemogenetics, suggesting this neuron population may be polymodal, and its function being under a context- or state-dependent modulation (Sharif et al. 2020).

1.1.2 Spinal processing of cutaneous sensory modalities

The spinal cord plays a central role in sensorimotor transformation. Neurons in the spinal dorsal horn have been recognized as the main players for receiving, encoding and

integrating the different sensory modalities, including itch, touch, pain, temperature and proprioception (Braz et al. 2014; Todd 2010; Abraira et al. 2013; Koch et al 2018; Gatto et al. 2019). These neurons relay somatosensory information from primary afferent neurons, located in the DRG or TG, to different downstream targets, including neurons in the spinal ventral horn to control movement (spinal reflex pathways), or to supraspinal structures to generate more complex behaviors and affective responses (spinal ascending pathways) (Basbaum et al. 2009; Todd 2010). Spinal dorsal neurons are highly heterogeneous with respects to laminar organization, developmental origin, morphology, molecular profile and function (Gatto et al. 2019).

Neurons in the spinal dorsal horn are organized in five laminae along the dorso-ventral axis. Each of these spinal laminae is preferentially innervated by selective sensory afferents that convey distinct sensory modalities. Laminae I and II primarily receive innervations from unmyelinated C fibers and/or thinly myelinated Aδ fibers, which relay noxious pain and itch sensations, whereas laminae III and IV are predominantly innervated by heavily myelinated Aβ LTMRs, which convey innocuous touch information. Finally, lamina V neurons, most of which are wide dynamic range (WDR) polymodal neurons, receive a combination of Aβ, Aδ, C fiber and proprioceptive innervation (Basbaum et al. 2009).

In addition to the organization of peripheral innervation, the developmental origin of spinal dorsal neurons is another key determinant of their molecular heterogeneity. During spinal cord development, the majority of dorsal horn neurons originates from six cardinal dorsal interneuron progenitor groups (dI1, dI2, dI3, dI4/dIL$_A$, dI5/dIL$_B$ and dI6) (Alaynick et al. 2011). At early developmental stages, these six cardinal interneuron progenitors are initially specified by BMP/Wnt signaling, with dorsally derived dI1, dI2 and dI3 populations

being BMP/Wnt-dependent and ventrally derived dI4, dI5 and dI6 populations being BMP/Wnts-independent. Then each cardinal dorsal interneuron population goes through next-step specification by expressing a unique cohort of transcription factors. Lbx1, a key transcription factor, specifies a number of cardinal dorsal interneuron populations, including the dI4, dI5 (contain late-born dIL_A and dIL_B), and dI6 populations (Gross et al. 2002; Müller et al. 2002). Within the Lbx1-lineage populations, transcription factors, Tlx1/3 and Lmx1b, further specifies dI5 and dIL_B populations into spinal glutamatergic excitatory interneurons (Cheng et al. 2004; Cheng et al. 2005). By contrast, dI4 and dIL_A populations that specified by the transcription factors Ptf1α and Pax2 differentiate into GABAergic and/or Glycinergic inhibitory interneurons (Cheng et al. 2004; Glasgow 2005). While the excitatory subsets mostly act to convert sensory feedback into actions or perceived sensations, the inhibitory populations mostly perform modulatory roles. The dI6 neuron population migrates into the intermediate zone of the spinal cord and coordinates the activity of ventral premotor interneurons and motor neurons (Lanuza et al. 2004). Additionally, the dI1, dI2 and dI3 populations also contribute to relay sensory information to neurons in the dorsal horn that project to supraspinal nuclei and/or to ventral motor pools (Lu et al. 2015).

Our current understanding of spinal dorsal neuron heterogeneity has been largely promoted by the recent application of refined molecular and genetic techniques, including scRNAseq and intersectional genetic approach. The work of many groups has identified multiple molecular markers, which are expressed during post-natal development and subdivide the dorsal neurons in more discrete subsets (Häring et al. 2018). These markers include neuropeptide, membrane receptors or calcium binding proteins. For example, the excitatory dI5/dIL_B lineages express a diversity of neurochemical markers including

somatostatin (SST), cholecystokinin (CCK), protein kinase C γ isotype (PKCγ), calbindin (CB), calretinin (CR), gastrin-releasing peptide (GRP), GRP receptor (GRPR), and RAR-related orphan receptor α (RORα). Similarly, selective expression of neuropeptide Y (NPY), dynorphin (Dyn) and parvalbumin (PV) has been shown in dI4/dIL$_A$ inhibitory populations (Gatto et al. 2019).

In parallel, the advances of intersectional genetics using the dual Cre/Flp recombination strategy and of viral approaches, provide powerful tools to interrogate more restricted cell types within the dorsal horn and better assess to investigate their functions. Among the excitatory neuron populations, SST-, CCK- and PKCγ-lineage neurons have been shown as important modulators of tactile sensation and touch-evoked pain (mechanical allodynia), which makes innocuous touch being felt as painful (Duan et al. 2014; Peirs et al. 2021). The GRP- and GRPR-expressing neurons selectively encode chemical itch (Mishra et al. 2013; Sun et al. 2009; Sun et al. 2007). Finally RORα-lineage neurons located in lamina III are specialized in relaying innocuous touch (Bourane et al. 2015). Among the inhibitory populations, NPY-lineage neurons selectively modulate the mechanical itch transmission (Bourane et al. 2015), whereas the Dynorphin-lineage neurons regulate the mechanical pain pathway (Duan et al. 2014).

The recent application of scRNAseq technology boosted our understanding of the molecular heterogeneity of the spinal dorsal neurons. Unbiased scRNAseq analysis of spinal dorsal horn neurons identified 15 glutamatergic excitatory (Glut 1-15) and 15 GABAergic inhibitory (Gaba 1-15) clusters (Häring et al. 2018). These neuron types correlated well with the previously identified functionally distinct neuron populations of spinal dorsal horn interneurons. For example, the itch-specific GRPR+ neuron population (Glut12-13 clusters)

were largely separated from the touch-tuned CCK-expressing neuron (Glut1-3, Glut5 and Glut13-14 clusters) (Häring et al. 2018). Also, some of cell types predicted by his study were later on shown by other groups to have specific functions in somatosensation. Tac1+ neurons that constitute Glut10 and Glut 11 clusters have been identified as a key ascending projection neuron population that drive pain transmission to the PBN and elicit nocifensive responses (Huang et al. 2019).

Although molecular diversity has been used as the "gold standard" for characterizing neuronal heterogeneity and a major predictor of function of the spinal dorsal neurons. It is still an open question that how these molecularly distinct cell types correlate with function and whether they act as bona fide labeled-lines. Gatto *et al.* used intersectional genetic manipulations to target specific subpopulations of spinal dorsal neurons, showing that the location of the neurons may also be another key determinant of spinal neuron function (Gatto et al. 2021). In future, a comprehensive view combining anatomical, transcriptomic and connectivity as well as positional information of the spinal dorsal neurons will be needed to address the functional contribution of dorsal horn heterogeneity to somatosensation.

1.1.3 Ascending sensory pathways and supraspinal processing

Somatosensory signals, after being processed by the spinal dorsal interneuron networks, are conveyed to the spinal ventral motor network or the supraspinal regions. The sensory signals transmitted to the spinal ventral horn are for driving motor/premotor neuron network to generate reflexive motor output, while those signals relayed to the supraspinal regions by projection neurons convey the information for sensory discrimination, affective motivation or offer sensory feedback for descending modulation (Basbaum et al. 2009; Chen

et al. 2020; Lay et al. 2020; Todd 2010).

The projection neurons form a relatively small population in the spinal cord. Retrograde tracing from downstream targets in the supraspinal regions showed projection neurons constitute around 5% of lamina I neurons in rat lumbar spinal cord (Todd 2010). Projection neurons are intermingled with interneurons and are distributed throughout the dorsal horn, with a smaller percentage also present with the ventral spinal cord. In the dorsal horn, they are particularly abundant in the superficial (laminae I-IIo) and deep (laminae III-V) laminae. Interestingly, projection neurons in the spinal dorsal and ventral horn demonstrate involvement in separate ascending pathways and functions in distinct sensorimotor processes. For example, projection neurons in the ventral horn project to motor-related area in the brainstem, including the lateral reticular nucleus (LRN), conveying efferent copies of skilled forelimb behavior like reaching (Azim et al. 2014; Pivetta et al. 2014). In contrast, dorsal horn-located projection neurons, especially located in laminae I and V, project to the brain nuclei including PBN, PAG and the thalamus transmitting sensory feedback of noxious pain and itch as well as non-discriminative touch information (Todd 2010).

The three major ascending pathways that relay somatosensory information to the supraspinal regions are the anterolateral system (ALS), the postsynaptic dorsal column (PSDC) pathway and the spinocerebellar pathway. These three pathways are remarkably distinct in terms of their supraspinal targets and the underlying sensory modalities (Al-Khater et al. 2009; Baek et al. 2019; Choi et al. 2020; Davidson et al. 2014; Davidson et al. 2010; Giesler et al. 1984; Paixão et al. 2019; Yuengert et al. 2015). Since the spinocerebellar tract is dedicated to the transmission of proprioceptive information, here I will focus on the ALS and PSDC pathways, introducing them in more detail.

1.1.3.1 The anterolateral system

The ALS is the one of the most thoroughly described ascending pathways. The ALS includes multiple ascending tracts, including the spinothalamic and spinoparabrachial pathways, which play significant roles in relaying pain, temperature and non-discriminative touch information from the spinal cord to specific brain regions. The following two subsections will discuss the anatomical, electrophysiological, molecular and functional characteristics of these two major pathways of the ALS.

1.1.3.1.1 The spinothalamic pathway

The spinothalamic pathway has long been recognized as the major ascending tract to relay noxious information from the spinal cord to the brain (Martin et al. 1990). However, this pathway also relays other sensory modalities, including temperature and non-discriminative touch (Willis et al. 1973). Multiple thalamic nuclei receive direct spinal inputs including the ventral posterolateral nucleus (VPL), the ventral posteromedial nucleus (VPM), the mediodorsal nucleus (MD), the intralaminar nuclei (Choi et al. 2020; Cliffer et al. 1991; Huang et al. 2019). These thalamic nuclei further relay the somatosensory information to cortical or other subcortical regions and eventually form perception towards the sensory stimuli or modulation to the motor outputs (Haber 2016; Hunnicutt et al. 2014). Interestingly, separate spinothalamic tracts have been found to convey distinct aspects of the somatosensation such as pain, with the STT projections to the VPL and VPM conveying the sensory-discriminative pain while those to the intrathalamic nuclei relaying the motivational-affective pain(Aziz et al. 2006). This functional segregation may largely result from the postsynaptic regions that thalamic neurons project to.

In the spinal cord across different species, cell bodies of spinothalamic neurons are distributed across all segments and are primarily located in laminae I and V of the dorsal horn, the white matter (the lateral cervical nucleus (LCN) and the lateral spinal nucleus (LSN)) as well as the ventral horn (Burstein et al. 1990; Davidson et al. 2010; Dilly et al. 1968; Trevino et al. 1972; Yezierski et al. 1991). Numerous studies have investigated the functional roles of spinothalamic neurons in nociceptive transmission. In primates, electrophysiological recording from spinothalamic neurons revealed their tuning to diverse natural stimuli including brush, pinch, squeeze and temperature applied on the skin (Willis et al. 1974). Interestingly, the spinothalamic neurons located in the superficial and deep dorsal horn differentially responded to distinct natural stimuli, substantially due to distinct types of sensory innervation they receive from sensory neurons. Lamina I spinothalamic neurons receive direct innervation from C/Aδ fibers that relay noxious sensory inputs and function as high threshold cells spiking in response to high-threshold mechanical squeezing but not to relatively low-threshold brushing or pressing. By contrast, lamina V spinothalamic neurons integrate C/Aδ/Aβ fiber inputs and function as wide-dynamic-range (WDR) neurons spiking in response to both low-threshold and high-threshold mechanical stimuli (Albe-Fessar et al. 1985; Craig 2003). Moreover, spinothalamic neurons in lamina I tend to respond more than the ones in lamina V to changes in temperature (Albe-Fessar et al. 1985).

The spinothalamic pathway has also been shown as a critical tract for itch transmission (Davidson et al. 2007; Davidson et al. 2014). Electrophysiological recordings in monkey showed that 32% of the recorded spinothalamic neurons spikes following stimulation with pruritogens like histamine or cowhage, suggesting a subpopulation of the spinothalamic

neurons is pruriceptive (Davidson et al. 2007). Interestingly, this subpopulation seems to include non-overlapping subsets of itch-responsive neurons, since the spinothalamic neuron subset that spikes in response to histamine, is not activated by cowhage and vice versa (Davidson et al. 2007).

Recently, the generation of genetic tools in mice (*Cre* lines), using specialized molecular markers has facilitated the functional study of the spinothalamic neurons. Novel markers of spinothalamic neurons include Tac1, Tacr1 and Gpr83 (Choi et al. 2020; Huang et al. 2019). Behavioral studies showed distinct roles of these spinal neuron populations in different process of sensorimotor transformation, with Tac1+ and Tacr1+ populations as key processor for affective pain while Gpr83+ population as crucial affective touch neurons (Choi et al. 2020; Huang et al. 2019). However, anterograde tracing by intersectional genetic labeling demonstrates that the three spinal neuron populations project to distinct but overlapping thalamic nuclei but also to other brain regions, including the parabrachial nuclei (PBN) and the periaqueductal grey (PAG). Further gain- or loss-of-function studies that selectively manipulates only the thalamic projections will be needed to confirm the functional contribution of each of these populations to the spinothalamic pathway.

1.1.3.1.2 The spinoparabrachial pathway

Another tract within the ALS is the spinoparabrachial pathway, which relay somatosensory information to the parabrachial nucleus (PBN) located in the dorsolateral pons of the brainstem. The PBN has been recognized as a key hub for integrating signals of multiple sensory modalities, including visual, auditory, gustatory, visceral and cutaneous sensory information etc., and generating appropriate behavioral responses and emotional

valence (Palmiter 2018).

Similar to the spinothalamic tract, the spinoparabrachial tract ascends through the lateral and ventral funiculus of the spinal cord and innervates the predominantly the contralateral but also the ipsilateral PBN. The terminals of spinoparabrachial projections predominantly innervate the dorsal part of the lateral PBN (lPBN), with very few projections innervating the ventral part of the lPBN and the Kölliker-Fuse subnucleus (Blomqvist et al. 1989; Mu et al. 2017; Choi et al. 2020). However, no topographic organization was found among the ascending projections arising from different rostrocaudal segments of the spinal cord. Sensory information from the PBN is further relayed to other brain nuclei, including the PAG, the hypothalamic and the medullary nuclei to generate or modulate protective responses, and the CeA and the BNST to form affective motivation (Chiang et al. 2020; Han et al. 2015).

The spinoparabrachial neurons, showed similar anatomical features to the spinothalamic neurons. Retrograde tracing data showed the majority of the spinoparabrachial neurons are located in laminae I and III-V of the rat spinal cord. Remarkably, 50% of the spinoparabrachial neurons contribute also to the spinothalamic tracts, suggesting that there is a subset of dual projecting neurons which send collaterals to both the PBN and the thalamus (Al-Khater et al. 2009). On the other hand, the majority of the spinothalamic neurons (90%) innervate the PBN and (Al-Khater et al. 2009). Additionally, it has also been found that a subset of the spinoparabrachial neurons also send collaterals to the periaqueductal grey (PAG), another nucleus part of the ALS that involved in the processing of noxious stimuli (Al-Khater et al. 2009).

The spinoparabrachial neurons are a heterogeneous population, which consists of a number of morphologically, electrophysiologically, genetically and functionally distinct subpopulations. Multiple cell morphologies have been found in the spinoparabrachial neuron population, including multipolar, pyramidal, fusiform etc. (Browne et al. 2019; Han et al. 1998). In the rat lumbar spinal cord, around 60% of the spinoparabrachial neurons that also send collaterals to the thalamus are multipolar. However, of those without thalamic collaterals, only 30% is multipolar, and the remaining neurons are either pyramidal or fusiform (Al-Khater et al. 2009). Electrophysiological recordings from lamina I spinoparabrachial neurons also showed functionally distinct subpopulations, including nociceptive-tuned and wide dynamic range (WDR) neurons. Nociceptive-tuned spinoparabrachial neurons respond exclusively to noxious mechanical and thermal stimuli, while WDR spinoparabrachial neurons respond to both innocuous and noxious stimuli (Allard 2019).

In rodents, 70-80% of the spinoparabrachial neurons in the spinal dorsal horn express NK1R (Cameron et al. 2015; Al-Khater et al. 2009). The Neurokinin 1 receptor (NK1R), also known as *Tacr1*, is the receptor for the neuropeptide substance P (SP) and plays a significant role in the sensory and affective pain transmission (Barik et al. 2021; Choi et al. 2020). Chemogenetic activation of NK1R+ spinal neurons drives nocifensive response including shaking and licking in mice (Barik et al. 2021). Likewise, optogenetic activation of their terminals in the PBN evokes similar nocifensive behaviors such as running, jumping and pupil dilation (Choi et al. 2020). Tac1, the gene that encodes SP, also target a spinoparabrachial neuron population. This spinoparabrachial population partially overlaps the NK1R+ population and has been shown crucial for relaying affective pain (Huang et al. 2018;

Barik et al. 2021; Polgar et al. 2020). Recently, a novel spinoparabrachial neuron population was described, as marked by their expression of G protein-coupled receptor 83(GPR83). Remarkably, this spinoparabrachial population substantially consists of NK1R-negative neurons, contributing to affective touch but not pain transmission (Choi et al. 2020). In addition to relaying pain and touch, Mu *et al.* demonstrated that the spinoparabrachial pathway is also important for itch transmission. Silencing of the spinal ascending projections in the PBN dramatically reduced pruritogen-induced and chronic itch sensitivities (Mu et al. 2017). In the future, transcriptome profiling by scRNAseq and intensive cell-type specific manipulation with behavioral assays will be needed for further dissecting the molecular and functional diversity of the spinoparabrachial neuronal ensemble, in order to clarify how this ascending sensory pathway integrates and processes distinct sensory modalities.

1.1.3.2 The postsynaptic dorsal column pathway

The dorsal column nuclei (DCN) are key relay centers for mechanoreceptive and proprioceptive information in the dorsal medulla of the brainstem. Two ascending tracts, the dorsal column (DC) pathway and the postsynaptic dorsal column (PSDC) pathway, transmit tactile, vibration and proprioceptive sensory information from the skin or the muscles to the brainstem. In the DC pathway, the first-order sensory neurons that detect mechanoreceptive and proprioceptive information directly innervate the DCN and form synaptic connection with the DCN neurons through the ipsilateral dorsal fasciculus in the cord (Niu et al. 2013). By contrast, the PSDC pathway requires the participation of the second-order neurons in the spinal cord, which are called the PSDC neurons, to transmit sensory information from the periphery to the brainstem (Cliffer et al. 1989; Giesler et al. 1984). The PSDC neurons, as key

mediators of mechanoreception and proprioception, primarily receive sensory inputs from LTMRs and proprioceptors, and innervate the DCN through the ipsilateral dorsal fasciculus. The mechanoreceptive and proprioceptive information delivered by the DCN and the PSDC pathways is integrated and further relayed by the DCN neurons to the contralateral thalamus and cortex through the medial lemniscal pathway. Here, I will assess the role of the PSDC pathway in relaying touch and mechanical itch information. Furthermore, I will compare the differential contribution that the ALS and the PSDC pathway make to the transmission and coding of mechanical itch.

This group of neurons are primarily located in the laminae III-IV of the dorsal horn, where they receive strong synaptic input from LTMRs, but a smaller number is also found in laminae I, V-VI and X (Condés-Lara et al. 2018; Giesler et al. 1984). A recent anatomical study showed there are ~11000 PSDC neurons in the rat spinal cord, and the majority of them (80%) are located on the ipsilateral side (Condés-Lara et al. 2018). Interestingly, double retrograde tracing from the DCN and the thalamus showed that the PSDC and the spinothalamic neurons are partially overlapping, especially in laminae I and III-IV on both ipsilateral and contralateral sides (Condés-Lara et al. 2018).

The majority of PSDC neurons located in the lamina III-IV, or called LTMR-recipient zone (RZ), receive substantial cutaneous inputs from Aβ-LTMRs and convey touch and vibration. Of the inputs that PSDC neurons receive, the majority comes from local spinal interneurons (60%) and sensory afferents (34%), while significantly fewer are from cortical neurons (6%) (Arbraira et al. 2017). This indicates that the PSDC neurons are key converging point for integrating cutaneous and cortical inputs to drive or modulate mechanosensation and proprioception.

Electrophysiological recordings identified functionally distinct PSDC populations: 64% of PSDC neurons selectively respond to low-threshold mechanical stimuli (e.g. brush) but not to noxious mechanical stimuli (e.g. pinch), while the rest responds to only to noxious stimuli. None of the PSDC neurons respond to thermal stimuli. These data suggest that sensory inputs that the PSDC neuron receives primarily come from low-threshold and high-threshold A-mechanoreceptors but not polymodal C-nociceptors (Giesler and Cliffer 1985).

The PSDC neurons play a crucial role in transmitting and processing touch and touch-evoked pain (allodynia) (Paixão et al. 2019; Sun et al. 2001). A subpopulation of PSDC neurons in lamina V of the spinal cord is marked by the expression of the transcription factor Zic2. Ablation of this neurons in mice causes an aberrant processing of tactile information and the inability of mice to discriminate diverse textures (Paixao et al. 2019). Lesion studies, which damaged the ipsilateral dorsal column nuclei, reversed the touch-evoked pain sensitization by spared nerve ligation in rats, suggesting that the PSDC tract is required for the generation of mechanical allodynia (Sun et al. 2001). Similarly, silencing DCN neurons by locally infusing lidocaine reversed the touch-evoked pain in sensitized animals (Sun et al. 2001).

1.2 Neural Circuits of Itch

Itch is an unpleasant sensation that generate an irresistible urge for scratching. Itch can be evoked by pruritogens, like histamine and chloroquine (chemical itch) (Lamotte et al. 2014), or by light mechanical stimuli, like insects stroking the animal's skin (mechanical itch) (Akiyama et al. 2012; Bourane et al. 2015). Chemical and mechanical itch seem mediated by

remarkably distinct peripheral and central neural circuitries (Acton et al. 2019; Bourane et al. 2015; Chen et al. 2020b; Chen et al. 2020; Pan et al. 2019).

1.2.1 Sensory neurons of itch

Pruriceptive sensory neurons constitute a subset of primary sensory neurons that resides in the DRG and TG (Han et al. 2013; Liu et al. 2012). Electrophysiological studies demonstrated that sensory neurons that sense chemical itch in the DRGs are primarily C/Aδ thinly myelinated neurons (Ringkamp et al. 2011). Identification of the molecular profiles and genetic manipulation have largely expanded our understanding of the identities and functionalities of the itch sensory neurons. MrgprA3, a member of the Mas-related G protein-coupled receptor (Mrgpr) family, is exclusively expressed in a subset of C fibers in the DRG and TG (Han et al. 2013). Genetic ablation or activation of MrgprA3+ neurons selectively reduce or sensitize itch sensitivity induced by a variety of pruritogens (except β-alanine), respectively (Han et al. 2013). Similarly, ablation of CGRP-expressing sensory neurons also impairs histamine- and chloroquine- but not β-alanine-induced itch (McCoy et al. 2013). All the results indicate that β-alanine-induced itch may be sensed by a separated sensory neuron population. Interestingly, MrgprD, another member of Mrgpr family, targets a non-overlapping itch-tuned sensory neuron population that exclusively mediates β-alanine-induced itch (Liu et al. 2012). In addition, more DRG or TG sensory neuron populations marked by their expression of GRP, natriuretic polypeptide b (Nppb), Sst and MrgprC11, respectively, have also been characterized as chemical itch sensors (Barry et al. 2020; Huang et al. 2018; Mishra et al. 2013; Steele et al. 2021). Recently, multiple groups have applied RNA sequencing technology and assessed transcriptomic profile on both bulk or single-cell

level (Li et al. 2018; Nguyen et al. 2017; Usoskin et al. 2015; Zeisel et al. 2018). Here, cell types and nomenclature are adopted from one of the unbiased single-cell RNA sequencing studies (Usoskin et al. 2015). In this study, 11 subclasses of mouse DTG neurons were grouped by principal component analysis, including NF1, NF2 and NF3 as LTMRs, NF4 and NF5 as proprioceptors, TH as C-LTMR, PEP1 and PEP2 as peptidergic nociceptors, as well as NP1, NP2 and NP3 as non-peptidergic nociceptors and potential pruriceptors. Particularly, for those subgroups as potential pruriceptors, NP1 is related to MrgprD+ neurons, and NP3 is marked by its expression of MrgprA3+ and MrgprC11 that detect most pruritus (Usoskin et al. 2015).

Different from chemical itch that sensed by C/Ad pruriceptors, mechanical itch (as known as touch-evoked itch) is detected by Aβ LTMRs, touch sensory neurons. A subset of Aβ LTMRs expressing the Toll-like receptor 5 (TLR5) has been shown to drive mechanical itch transmission (Pan et al. 2019). Silencing or knocking out TLR5+ LTMRs abolishes scratching response toward mechanical itch in mice (Pan et al. 2019). Interestingly, sensory neurons have been shown not only to provide the excitatory drive but also to gate mechanical itch transmission. Feng *et al.* showed that the significant reduction of SAI-LTMR firing caused by the loss of touch-domes Merkel cells exacerbates both aging- and dry skin models of chronic itch. By contrast, enhanced SAI-LTMR firing by chemogenetic activation of Merkel cells suppresses alloknesis due to dry skin conditions (Hu et al. 2018). This Merkel cell associated LTMRs are thought to make connection with key spinal inhibitory interneurons that modulate mechanical itch transmission.

1.2.2 Spinal neural circuits of itch

1.2.2.1 Chemical itch pathways

The central projections of DRG pruriceptors terminate in the superficial dorsal horn of the spinal cord, where they synapse onto second-order neurons. In the spinal cord, glutamatergic (Glu+) excitatory neurons, differentiated from dI5/dIL$_B$ IN progenitor domains, are the key drivers for itch transmission (Xu et al. 2013). A number of neurotransmitters or neuromodulators, including glutamate, SP, GRP, Neuromedin B (NMB) etc., are released by these Glu+ neurons during acute and chronic itch conditions (Akiyama et al. 2014; Mishra et al. 2013; Wan et al. 2017). On the other hand, glycine and dynorphin released by GABAergic/Glycinergic inhibitory neurons in the cord modulate itch transmission by inhibiting sensory afferent, local interneuron or projection neuron activity (Kardon et al. 2014).

Glutamate is believed to be the key neurotransmitter for itch transmission, and antagonism of AMPA receptors, in the cord significantly reduces itch transmission (Akiyama et al. 2014). However, glutamate by itself may not suffice for itch processing. Genetic knockout of the vesicular glutamate transporter type 2 (VGLUT2) from nociceptors abolishes pain but strengthen itch sensitivity (Liu et al. 2010), suggesting that neurotransmitters (e.g. neuropeptides) other than glutamate may be required for itch transmission from the periphery to the spinal cord. Consistent with this idea, the natriuretic polypeptide b (Nppb) expressed primarily by NP3 sensory neurons was shown as a crucial neuropeptide for itch generation. Mishra *et al.* demonstrated that knockout of Nppb dramatically reduced itch sensitivity induced by histamine, chloroquine and a number of other pruritogens, while the intrathecal

application of the peptide could significantly induce scratching behavior in mice (Mishra et al. 2013).

Central projections of Nppb+ sensory neurons innervate lamina II of the spinal dorsal horn and synapse onto spinal interneurons (INs) that express Nppb receptor A (NPRA). Ablation of NPRA+ INs by intrathecal application of Nppb-conjugated saporin significantly reduces pruritogen-induced itch, showing NPRA+ INs in lamina II are major itch-relaying neurons that mediate itch information from the periphery (Mishra et al. 2013). Moreover, intrathecal application of Nppb peptide recovered itch sensitivity in Nppb knockout ($Nppb^{-/-}$) mice, and NPRA-ablation can suppress Nppb-induced itch (Mishra et al. 2013). Therefore, Nppb-NPRA signaling is indispensable for chemical itch transmission. More interestingly, most of NPRA-expressing neurons co-express the gastrin-releasing peptide (GRP). Pharmacological blockade of GRP signaling using a GRPR antagonist largely suppresses Nppb-induced itch (Mishra et al. 2013), suggesting that GRP signaling functions downstream of the Nppb-NPRA signaling.

NPRA/GRP neurons located in lamina II, synapse onto the excitatory GRPR+ neurons in laminae I/II. Ablation of GRPR+ neurons in the spinal cord using either GRP- or bombesin-conjugated saporin significantly reduces itch sensitivity induced by Nppb and a number of pruritogens, showing the importance of this neuron population as a downstream target of Nppb-NPRA/GRP pathway (Sun et al. 2007; Sun et al. 2009; Mishra et al. 2013). A model of the spinal chemical itch circuitry is that NPRA+/GRP+ INs in lamina II receive chemical itch inputs from the NP3 Nppb+ sensory afferents, and further relay this information to the downstream GRPR+ neurons by releasing GRP (Mishra et al. 2013). This Nppb/NPRA-GRP/GRPR neural circuitry forms a core spinal circuit for the coding of chemical itch. In

addition, spinal excitatory population marked by their expression of Tac1 and Sst have also been shown as important chemical itch transmission neurons (Fatima et al. 2019; Huang et al. 2019).

In the spinal cord, another group of neurons, rather than acting to drive itch, provide gate control or modulation for itch. These neurons are predominantly GABAergic and/or Glycinergic inhibitory INs that originate from dI4/dIL$_A$ (Ptf1α+/Pax2+) dorsal IN progenitor domains. Ablation of Ptf1α neuron causes a strongly deregulated scratch behavior (Escalante et al. 2020; Mona et al. 2020). Within this broad inhibitory population, a subset that express transcription factor Bhlhb5 has been shown to provide key modulation to chemical itch (Kardon et al. 2014). Bhlhb5+ inhibitory INs can inhibit GRPR+ or projection neurons when they are activated by counter-itch stimulus, like pain. Mice lacking Bhlhb5 develop significant itch sensitization to pruritogens (Kardon et al. 2014). However, Bhlhb5+ inhibitory INs are heterogeneous, which express a cohort of neurochemical markers including dynorphin, galanin, nNOS and NPY (Kardon et al. 2014). The majority of Bhlhb5+ neurons (~90%) co-express the somatostatin receptor 2a (Sst2a) (Kardon et al. 2014). Pharmacological blockade of Sst-Sst2a signaling reduces sensitivity to pruritogens (Fatima et al. 2019; Kardon et al. 2014), suggesting a key modulatory role for Sst-Sst2a signaling in chemical itch.

1.2.2.2 Mechanical itch pathways

Mechanical itch is an important protective mechanism against mosquito sting in healthy animals. Furthermore, in pathological conditions like alloknesis, light touch stimuli that to do not physiologically induce pruritus are experienced as itchy. Alloknesis is a feature

of chronic and acute pruritus, which is caused by several conditions, like dry skin, pruritogen sensitization (e.g. intradermal histamine injections) or dermatitis.

The first evidence for the existence of a dedicated spinal pathway for mechanical itch came from the discovery that an inhibitory spinal IN population, labeled using the neuropeptide Y (NPY) *Cre* line (NPY::Cre), gated mechanical itch transmission (Bourane et al. 2015). Manipulations of NPY::Cre+ INs was restricted to the cord using the $Lbx1^{FlpO}$ allele (NPY^{Lbx1}) and intersectional effector lines. Ablating and silencing of NPY^{Lbx1} neurons selectively promoted itch sensitization in response to low-threshold mechanical stimuli. In addition to inhibitory INs, multiple crucial spinal excitatory IN subsets that drive mechanical itch transmission have been discovered (Acton et al. 2019; Pan et al. 2019; Chen et al. 2020). Spinal excitatory neurons targeted by Neuropeptide Y1 receptor ($NPY1R^{Cre}$) and the $Lbx1^{FlpO}$ allele (hereafter referred as $Y1^{Lbx1}$ neurons) have been demonstrated as a key driver for mechanical itch (Acton et al. 2019). Activation of spinal $Y1^{Lbx1}$ neurons significantly elevated mechanical and spontaneous itch sensitivities, while their ablation or silencing dramatically suppressed mechanical itch (Acton et al. 2019). Patch recording onto the $Y1^{Lbx1}$ neurons showed they receive direct inhibitory inputs from the NPY^{Lbx1} neurons (Acton et al. 2019). Rabis retrograde tracing experiment demonstrated that $Y1^{Lbx1}$ neurons are preferentially innervated by Aβ LTMRs instead of C/Aδ pruriceptors (Acton et al. 2019). More interestingly, remarkable inhibition of mechanical itch can be achieved by simple application of exogenous NPY in mice (Acton et al. 2019). However, this NPY-inhibitory effect on mechanical itch diminishes in the mice lacking Y1 receptors in the spinal cord (Acton et al. 2019). Pharmacological blocking of Y1 receptors in the spinal cord by intrathecal application of Y1 antagonist BIBP3226 also sensitizes mechanical itch in mice (Acton et al. 2019). These

data demonstrated that NPY-Y1 signaling provides a key modulatory function for modulating mechanical itch transmission. Another crucial excitatory neuron population for mechanical itch is labeled by Urocortin 3 (*Ucn3*^{Cre}) and the *Lbx1*^{FlpO} allele (hereafter referred as Ucn3^{Lbx1} neurons). Ablation of Ucn3^{Lbx1} causes significant decrease of mechanical itch sensitivities in both acute and chronic conditions (Pan et al. 2019). Ucn3^{Lbx1} also receive direct GABA/Glycine-dependent modulatory inputs from NPY^{Lbx1} inhibitory neurons (Pan et al. 2019). Surprisingly, Ucn3^{Lbx1} population has been shown as substantially overlapping with Y1^{Lbx1} population (Pan et al. 2019), suggesting parallel spinal pathways may exist for mechanical itch transmission. Similarly, another excitatory neuron population expressing Tachykinin 2 (Tac2) also contributes to the mechanical itch transmission (Chen et al. 2020a).

In summary, the peripheral and spinal circuits processing chemical and mechanical itch function independently, and relay on a distinct set of sensory input. Retrograde rabies tracing shows that the neurons dedicated to sense mechanical itch preferentially receive LTMR sensory inputs whereas C-fiber nociceptors synapse onto chemical itch-related populations (Acton et al. 2019; Bourane et al. 2015; Chen et al. 2020; Pan et al. 2019; Kardon et al. 2014). And spinal INs processing chemical and mechanical itch are differentially localized in the dorsal spinal cord and have distinct molecular profiles (Acton et al. 2019; Bourane et al. 2015; Chen et al. 2020; Pan et al. 2019; Kardon et al. 2014; Mishra et al. 2013; Sun et al. 2009).

1.2.3 Supraspinal regions contributing to itch processing

The immediate scratch reflex can be elicited by a spinal reflex pathway, nevertheless the correct processing of itch sensation requires the involvement of supraspinal circuitries to

code the affective component and to sense the scratch-induced relief. Moreover, this ascending information is also used to suppress the itch-induced scratch before it becomes harmful, via descending inhibitory pathways (Akiyama et al. 2011; Koga et al. 2020; Samineni et al. 2019; Zhao et al. 2014).

1.2.3.1 Ascending circuitries of itch transmission

Itch, as a noxious sensory modality, is predominantly transmitted through the ALS to the supraspinal regions (Davidson et al. 2007, 2012, 2014; Mu et al. 2017). Electrophysiological recording in primate showed the spinothalamic tract (STT) neurons are tuned to respond to pruritogens including histamine or cowhage. However, none of the STT neurons respond to both pruritogens, indicating separate STT subpopulations may undertake the transmission of histaminergic and non-histaminergic itch (Davidson et al. 2007). The responses of STT neurons induced to pruritogens can be inhibited by counter-stimuli including scratching or capsaicin application (Davidson et al. 2009). In addition to the spinothalamic pathway, spinoparabrachial pathway, another key ascending tract within the ALS, has also been found important for itch transmission (Mu et al. 2017). Optogenetic inhibition of the spinoparabrachial projections in the PBN significantly reduces histamine- and chloroquine-induced itch (Mu et al. 2017).

The thalamus has been thought as a key relay station for multisensory modalities, including itch (Leknes et al. 2007; Mochizuki et al. 2003, 2013; Papoiu et al. 2013). The spinothalamic neurons, long-range projection neurons connecting the cord to the thalamus, respond to histaminergic and non-histaminergic itch (Davidson et al. 2007). These neurons deliver somatosensory signals (including itch) to contralaterally posterior and ventrobasal part

of the thalamus. fMRI imaging in human subjects showed activation in the contralateral thalamus in response to pruritogen-induced itch (Mochizuki et al. 2003, 2013). Previous imaging studies also demonstrated itch-induced activation in specific thalamic nuclei, including the medial dorsal nucleus (MD), the ventral posteromedial nucleus (VPM) and posterior thalamic nucleus (Po) (Leknes et al. 2007; Papoiu et al. 2013; Zhu et al. 2020). All these data established a clear argument that the thalamic nuclei are a major itch relay station in the supraspinal regions. Moreover, histaminergic and non-histaminergic itch seem processed by the thalamic nuclei by different mechanisms. Electrophysiological studies in primate showed that the STT neurons that relay both types of chemical itch into the ventral posterior lateral (VPL), ventral posterior inferior, and posterior nuclei, but only non-histaminergic itch seems relayed to the suprageniculate and medial geniculate nuclei (Davidson et al. 2012). Neuroimaging studies in human subjects confirmed this idea by showing a more extensive region of the thalamic nuclei was activated during cowhage-induced non-histaminergic itch (Leknes et al. 2007; Papoiu et al. 2012). However, the molecular identity of the itch relay neurons in the thalamus and how itch is relayed in the thalamic nuclei are still open questions.

The PBN has been recently found to be a crucial pontine nucleus for itch processing. In vivo fiber photometry imaging of mouse PBN neurons displayed a strong activation during histamine- and chloroquine-induced itch (Mu et al. 2017). This itch sensitization can be reversed when the PBN neurons are silenced (Mu et al. 2017). Glutamate release within the PBN circuitry has also been proved indispensable for itch transmission, since scratch was dramatically reduced in VGLUT2^{PBN}-deficit mice in response to both chemical and chronic itch (Mu et al. 2017). The role of PBN will be discussed in more details in the next chapter. It

is yet unknown if the PBN itch-relaying neurons are distinct from those relaying pain or other modalities, and how mechanical itch signals are processed in the PBN.

In addition to the PBN and the thalamus being involved in itch processing, several other brain regions have also been shown to respond to pruritogens-induced itch. Functional MRI in human subjects showed itch evoked activity was found in cortical regions, including the primary somatosensory cortex (S1) and the secondary somatosensory cortex (S2), the cingulate and prefrontal cortex, the amygdala, and the periaqueductal grey (PAG) (Ishiuji et al. 2009; Schneider et al. 2008). It has been shown that neurons in the PAG modulate both sensory and affective components of itch (Samineni et al. 2019b). As an well-known emotion center, the amygdala has been shown important for mediating affective itch (Sanders et al. 2019). Additionally, neurons in the central nucleus of the amygdala (CeA) is a key relay hub for itch modulation, potentially through their projections to the PAG, (Samineni et al. 2020).

1.2.3.2 Descending circuitries of itch modulation

Itch sensation can be alleviated by counter-itch stimuli such as scratching, pain or coldness. But neural mechanisms of itch modulation by those counter-itch stimuli remain largely unknown. In the spinal cord, GABAergic and Glycinergic inhibition have been proved as a key mechanism for itch modulation. Application of GABA or glycine antagonists suppresses scratch-evoked inhibition in superficial dorsal horn neurons (Akiyama et al. 2011). Also, descending inputs from the supraspinal region have been proved critical for itch modulation. Scratch-evoked itch inhibition in the lumbar cord was disrupted in the mice with upper cervical spinalization (Akiyama et al. 2011). Here, a few key brain regions providing descending modulation for itch will be discussed.

The periaqueductal grey (PAG), previous knowns as a pain modulation center, has also been reported as a critical brain nucleus for itch modulation (Gao et al. 2019; Samineni et al. 2019b). Chemogenetic activation of neurons in the ventrolateral PAG (vlPAG) significantly alleviates the chloroquine-induced itch, but silencing of them exacerbates the itch (Samineni et al. 2019a). Interestingly, glutamatergic and GABAergic neurons in the vlPAG modulate itch in an opposing manner, with the glutamatergic neurons promoting while GABAergic neurons suppressing itch (Samineni et al. 2019a). Recently, cell-type specific manipulation also identified Tac1+ glutamatergic neurons in the vl/lPAG facilitating itch-induced scratching (Gao et al. 2019). Ablation or silencing of Tac1+ neurons in the vl/lPAG significantly reduces itch-induced scratching, but not the nociceptive responses to thermal, mechanical or chemical stimuli (Gao et al. 2019), suggesting that itch and pain modulation may be governed by distinct populations in the PAG.

Multiple brainstem nuclei, including the rostral ventral medulla (RVM) and the locus coeruleus (LC), have also been reported to provide potential monosynaptic descending modulation for itch transmission (Koga et al. 2020; Zhao et al. 2014). The RVM is known for its modulatory roles for both pain and itch (Follansbee et al. 2018). Two key neuron populations, the ON and OFF cells, in the RVM have been found through electrophysiological recordings and show opposite responses to pruriceptive and nociceptive stimuli, with the ON cells excited while OFF cells inhibited by pruritogens or algogens (Follansbee et al. 2018). A number of the ON cells in the RVM has been found as neurokinin 1 receptor (NK1R)-expressing (Follansbee et al. 2021). Chemogenetic activation of NK1R+ ON cells in the RVM suppresses chemical and chronic itch (Follansbee et al. 2021), indicating ON cells may

downregulate the activity of spinal itch-related circuitries. One subnucleus of the RVM, the nucleus raphe magnus (NRM), has also been shown to provide descending modulation for itch by 5-hydoxytryptophan (5-HT, or serotonin) signaling (Zhao et al. 2014). Interestedly, serotonin released by descending projections of the NRM neurons in the cord coactivates HTR1A and GRPR receptors of spinal GRPR+ neurons and eventually enhances itch (Zhao et al. 2014). another study also demonstrates the locus coeruleus (LC) in the brainstem as a crucial descending modulation center for itch (Koga et al. 2020). The LC descending projection neurons achieve tonic inhibition to the spinal dorsal horn through noradrenergic signaling. Activation of spinal-projecting noradrenergic neurons in the LC inhibits both acute and chronic itch (Koga et al. 2020). Here, multiple studies demonstrated that monoamines, including serotonin and norepinephrine, are major neuromodulators that released by brainstem nuclei for modulating spinal neural circuitries that underlie pain and itch transmission. Also, spinal GRPR+ neurons that express receptors for both serotonin and norepinephrine are the key knots for integrating a cohort of descending inputs and modulating itch sensation (Koga et al. 2020; Zhao et al. 2014).

In summary, itch information is predominantly relayed by the anterolateral ascending pathways, including the spinothalamic and spinoparabrachial pathways, to the thalamic and parabrachial nuclei, which eventually facilitate the formation of affective components of itch and the modulation of itch. On the other side, multiple brain regions, including the PAG, RVM and LC, provide key descending modulation on itch transmission in the spinal cord. However, it is still mysterious that how the ascending inputs generate itch perception, and communicate to those itch modulation center and eventually provide sensory feedback for the descending modulation of itch.

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Chapter 2: The PBN is a key hub in the supraspinal processing of mechanical itch

2.1 Introduction

Mechanical itch is a distinct subtype of pruritus evoked by light touch stimuli detected by heavily myelinated LTMRs. In the spinal cord, mechanical itch is transmitted and gated by spinal interneuron circuits that are distinct from those that encode chemical itch. This there are unique molecular mechanisms and separate neural circuitries that are recruited for transmitting or modulating mechanical itch signals, as compared to those for itch induced by chemical pruritogens. Scratching responses to punctate light touch stimuli (touch-evoked itch) is the phenotypical hallmark of mechanical itch in physiological conditions. Chronic itch typically sensitizes animals to touch-evoked scratching, but it is often accompanied by repetitive scratching even in the absence of a tactile stimulus (spontaneous itch). Under chronic itch conditions, such as dry skin, atopic dermatitis and allergic contact dermatitis, light touch-evoked mechanical itch can induce aversive memories and emotional changes, which strongly suggests the involvement of supraspinal regions that elicit the affective components of itch related behaviors. How mechanical itch information is transmitted from the spinal cord to brain regions, or which brain centers encode mechanical itch, remain largely uninvestigated. In this study, I have combined mouse genetics and viral targeting with sensorimotor behavioral assays to define and characterize the projection neurons in the spinal cord and recipient structures in the brainstem that are responsible for mechanical itch transmission. The brain regions of particular interest are: 1) the dorsal column nuclei (DCN) that receive cutaneous mechanosensory information via the post-synaptic dorsal column (PSDC) pathway (Abraira et al. 2013; Paixão et al. 2019), and 2) the parabrachial nucleus

(PBN) that receive cutaneous nociceptive and pruriceptive information through the spinoparabrachial (SPB) pathway (Campos et al. 2018; Mu et al. 2017). I started by investigating the contribution of the parabrachial nucleus (PBN), a well-known pain and chemical itch relay station located in the dorsolateral pons of the brainstem (Campos et al. 2018; Mu et al. 2017).

2.2 Results

2.2.1 Chemogenetic silencing of the PBN neurons suppresses touch-evoked itch

It has been reported that the parabrachial nucleus (PBN) is an important relay center for chemical itch (Mu et al. 2017). To test whether the PBN modulates the behavioral responses to mechanical itch stimuli, I selectively silenced PBN neurons by injecting AAV-hSyn-HA-hM4D(Gi)-mCherry (hereafter referred as AAV-hM4D), an adeno-associated virus (AAV) carrying the G_i-DREADD hM4D, bilaterally into the PBN of wildtype mice, and examined mechanical itch sensitivity as indicated by hindlimb scratching movements (**Figure 2.7.1.A**). For these experiments, I had two sets of controls, one being the comparison of saline and CNO injection in the same mice, and the second being a control group of mice injected with AAV-CMV-EGFP (hereafter referred as AAV-EGFP), and similarly treated with CNO and Saline. This second control is very important given the side effects of CNO administration that can be converted to clozapine and cause neural activity changes in absence of the DREADD receptors (Gomez et al. 2017).

To measure the mechanical itch sensitivity in mice, innocuous mechanical stimulation (10 times) with von Frey hair (0.7mN) was delivered onto the nape of neck, and the scratching response rate of each mouse to the stimuli was assessed (**Figure 2.7.1.B**). Baseline sensitivity was assessed performing this assay in both groups treated with saline, and there was no difference between controls (AAV-EGFP injected) and mice injected with AAV-hM4D. Following CNO stimulation, the scratching responses were almost abolished in AAV-hM4D-injected mice while no changes in sensitivity were observed in the control group (**Figure 2.7.1.C**), suggesting the PBN is a critical hub in setting the sensitivity to mechanical itch stimuli.

2.2.2 Chemogenetic silencing of the PBN but not the DCN significantly reduces NPY ablation-induced touch-evoked and spontaneous itch

Previously it has been shown that NPY+ inhibitory neurons in the spinal cord modulate mechanical itch transmission (Bourane et al., 2015). Ablating or silencing the NPY+ neurons leads to a disinhibition of the mechanical itch circuitry, resulting in chronic spontaneous scratching and elevated scratch responses to mechanical stimuli (Bourane et al. 2015). Using this information, I then set out to test whether the PBN is required for these changes in mechanical itch sensitivity and chronic spontaneous scratching that are seen following the inactivation of these spinal NPY+ inhibitory neurons (**Figure 2.7.2.A**). To achieve a dual manipulation of the spinal NPY+ neurons and the PBN, *NPY::Cre; Lbx1^{FlpO}; Tau$^{ds\text{-}DTR}$* mice were used to selectively ablate spinal NPY+ neurons while delivering AAV-hM4D bilaterally to the PBN of these mice in order to broadly silence this nucleus (**Figure 2.7.2.B**). 7-10 days after DT application, a strong increase in mechanical and spontaneous itch

sensitivity was observed in NPY-ablated mice (**Figure 2.7.2.C and 2.7.2.D**). As a control, I tested response rate in NPY-ablated mice toward mechanical itch stimulation and spontaneous scratching following saline application. Upon CNO application, mice in which the NPY+ neurons had been ablated displayed a marked reduction in touch-evoked itch (**Figure 2.7.2.C**) as well as spontaneous itch (**Figure 2.7.2.D**). These results confirmed that the PBN is an important relay station for mechanical itch that strongly modulate itch-induced scratching, even in sensitized models where spinal NPY+ neuron-mediated inhibition of mechanical itch is reduced. I observed that mechanical itch sensitivity was not completely abolished after CNO-induced silencing of the PBN (**Figure 2.7.2.C**), suggested other brain nuclei may also be important for relaying NPY-modulated mechanical itch.

In view of the role that the DCN plays in transmitting light touch information and the residual mechanical itch sensitivity when the PBN was silenced (**Figure 2.7.2.C**), I set out to ask whether the DCN are important for relaying mechanical itch information through parallel ascending pathways. Indeed, touch-evoked itch might be relayed to the DCN as innocuous mechanical signal for further processing. To test this hypothesis, similarly as before, AAV-hM4D was delivered bilaterally into the DCN of *NPY::Cre; Lbx1*^{FlpO}*;Tau*$^{ds\text{-}DTR}$ mice (**Figure 2.7.2.E**). Somewhat surprisingly, touch-evoked and spontaneous itch sensitivity was unchanged following CNO-induced silencing of the DCN (**Figure 2.7.2.F and 2.7.2.G**), arguing that the DCN is not required for NPY-modulated touch-evoked and spontaneous itch. More importantly, this result also indicated that mechanical itch is not relayed as an "innocuous mechanical" signal to the DCN through the PSDC pathway, but rather as an "itch" signal to the PBN through the spinoparabrachial pathway. The remaining residue of

mechanical itch sensitivity after silencing the DCN might be due to the dose-dependent effect of CNO, or to the incomplete viral targeting of the PBN.

2.2.3 Chemogenetic silencing of the PBN suppresses BIBP3226-induced touch-evoked and spontaneous itch

The modulatory effects of spinal NPY+ neurons onto mechanical and spontaneous itch are executed by NPY-Y1R signaling. Blocking the NPY-Y1R signaling by application of Y1R antagonist, BIBP3226, results in a similar but shorter-lasting increase in touch-evoked and spontaneous itch sensitization in mice (Acton et al. 2019) (**Figure 2.7.3.A**), thus phenocopying the spinal NPY interneuron (IN) ablation phenotype. I therefore set out to test the function of the PBN in relaying this BIBP3226-induced sensitization to light touch-evoked itch and increase in episodes of spontaneous itch. AAV-hM4D was injected bilaterally to silence PBN neurons (AAV-EGFP for the control group) (**Figure 2.7.3.B**). Whereas intrathecal application of BIBP3226 resulted in a significant elevation of the response rate of touch-evoked itch and spontaneous scratching, both touch-evoked itch and spontaneous itch were almost abolished by CNO-induced PBN silencing (**Figure 2.7.3.C and 2.7.3.D**). This result demonstrated that the PBN is required for BIBP3226-induced mechanical and spontaneous itch, and further confirms that NPY-modulated mechanical itch information is dependent on PBN neural activity.

2.3 Conclusion and discussion

All the data shown in the Chapter 2 demonstrate a clear idea that the parabrachial nucleus (PBN) is a critical relay station for mechanical itch modulation (**Figure 2.7.1-2.7.3**).

Silencing the PBN neuronal activity substantially inhibits mechanical itch sensitivities under baseline (**Figure 2.7.1**), acute (**Figure 2.7.3**) and chronic (**Figure 2.7.2**) itch sensitization states. Combining the published results showing the PBN is a key relay center for pain (Han et al. 2015), chemical itch (Mu et al. 2017) and innocuous touch (Campos et al. 2018), our data further confirmed that the functional role of PBN as a multisensory integration and relay hub in the brainstem.

Itch usually provokes an unstoppable urge to scratch. Quick scratching towards the itchy sites is normally the first response for removing the harmful stimuli (like mosquito sting) and relieving the itch, which is thought to be a reflexive behavior that rely on spinal local neural networks. However, when itch becomes strong and long-lasting, the neural circuitries in the supraspinal structure start to be recruited and form affective components of itch (perception, aversion) and modulation towards scratching. On another aspect, mechanical itch, or touch-evoked itch, is one of the hallmark phenotypes under chronic itch conditions. The mechanical itch information relayed by the PBN neurons may be crucial for generating aversion and forming modulatory effects onto scratching by connecting to distinct postsynaptic regions in the brain.

However, a key question still remains unclear: how is the mechanical itch information relayed to the PBN? The spinoparabrachial neurons are the spinal projection neurons that directly innervate and deliver multisensory modalities, including pain, temperature, itch and touch, to the PBN (Barik et al. 2021; Mu et al. 2017; Choi et al. 2020). It is very likely that mechanical itch information is relayed from the spinal cord to the PBN by a subset of mechanosensitive spinoparabrachial neurons. In this sense, looking for spinoparabrachial

neuron population that underlie mechanical itch transmission becomes a crucial step for understanding the ascending transmission mechanism of mechanical itch.

2.4 Materials and methods

Key Resources Table

Reagent or Resource	**Source**	**Identifier**
Bacterial and Virus Strains		
AAV-hSyn-HA-hM4D(Gi)-mCherry	Salk Viral Core	
AAV-CMV-EGFP	Salk Viral Core	
Chemicals		
BIBP3226 trifluoroacetate	Tocris	Cat# C2707/1
Clozapine-N-oxide	Sigma	Cat# C0832
Experimental models: Organisms/Strains		
Mouse: *NPY::Cre*	Bourane et al. 2015a	
Mouse: $Lbx1^{FlpO}$	Bourane et al. 2015a	
Mouse: $Rosa26^{ds\text{-}tdTomato}$ $(Ai65^{ds\text{-}tdTomato})$	The Jackson Laboratory	JAX stock #021875; RRID:IMSR_JAX:021875
Mouse: $Tau^{ds\text{-}DTR}$	Britz et al. 2015	
Software and Algorithms		
Adobe Illustrator and Photoshop CS5	Adobe	
Prism 8	GraphPad	

ImageJ	Kurt de Vos, Univ. of Sheffiled, UK	

Experimental model and subject details

All protocols for animal experiments were approved by the IACUC of the Salk Institute for Biological Studies according to NIH guidelines for animal experimentation. Male and female mice were used in all studies. Animals were randomized to experimental groups and no sex differences were noted. The following mouse lines were used in this study: *NPY::Cre* (Bourane et al. 2015), *Lbx1*^{FlpO} *(Duan et al. 2014), Rosa26*$^{ds\text{-}tdTomato}$ *(as known as Ai65*$^{ds\text{-}tdTomato}$*) (Madisen et al. 2015), Tau*^{ds}-DTR (Britz et al. 2015).

Viral injection

10-14 weeks old *wildtype* or *NPY::Cre; Lbx1*^{FlpO}*; Tau*$^{ds\text{-}DTR}$ mice were placed on a stereotaxic frame (Kopf Instruments) and anaesthetized via inhalation of isoflurane (1.5–2.0%) using an isoflurane vaporizer during the surgery. Body temperature of the animal was maintained with a heating pad underneath the body during the surgery. Ophthalmic ointment was applied to maintain eye lubrication. Burr holes were made on the skull using high-speed stereotaxic drill (Kopf Instruments), and 500 nl of AAV-hSyn-HA-hM4D(Gi)-mCherry (or AAV-CMV-EGFP for controls) were injected into the PBN by a Nanoinjector (NANOJECT III, Drummond) at a rate of 2 nl/sec through glass pipettes (tip diameter 10-30μm) (Wiretrol II, Drummond). After each injection, the glass pipettes were left in place for 7-8 min before withdrawal. The animals were placed on a heating pad to recover from anesthesia before

returning to their home cage. Buprenex SR (0.1 g/kg) was injected intradermally in the back after surgery to provide analgesia.

The coordinates used for stereotaxic injections and implantation were as follows: PBN (-1.00 mm posterior to lambda, ± 1.50 mm from midline and -3.50 ventral from skull). The behavioral tests were performed 21-28 days after the viral injection.

Drug administration

Clozapine-N-oxide (Sigma) was dissolved in DMSO and then diluted with 0.9% sterile saline such that the concentration of DMSO did not exceed 1% of the injected solutions. For chemogenetic silencing of the PBN neurons by AAV injection and behavioral tests on touch-evoked and spontaneous itch in mice, all mice received intraperitoneal (i.p.) injections of saline on the first day and CNO (2 mg/kg) on the following day.

To test mechanical itch sensitivity under BIBP3226-induced acute itch sensitization, wildtype mice were anesthetized briefly under isoflurane and 10μl NPY 1 receptor antagonist, BIBP3226 (100nM) was injected intrathecally into the mice (Acton et al. 2019).

Behavioral testing

Littermate controls were used for behavioral tests, and the experimenter was blinded to genotype. Animals were habituated to the behavioral testing apparatus for 1 h on each of the two days prior to the testing day. 8-12 weeks old mice (both males and females) were used for touch-evoked and spontaneous itch testing upon chemogenetic silencing of the PBN neurons. On the testing day, animals were briefly habituated and injected intraperitoneally with CNO. Behavioral tests were performed 30-60 minutes after CNO injection.

Touch-evoked Itch

To quantify touch-evoked scratching behaviors induced by mechanical stimulation of the hairy skin, mice were placed in a plastic chamber and a 0.07 g (0.7mN) von Frey hair was applied to random spots of the nape of the neck for 3s (Acton et al. 2019). Hindlimb scratching responses over 10 trials were counted and reported as a percentage.

Spontaneous Itch

To quantify scratching induced in the absence of an experimental mechanical stimulus (spontaneous itch), mice were placed in a plastic chamber and video recorded for a period of 30 min; bouts of hindlimb scratching were counted (Acton et al. 2019).

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2.7 Figures

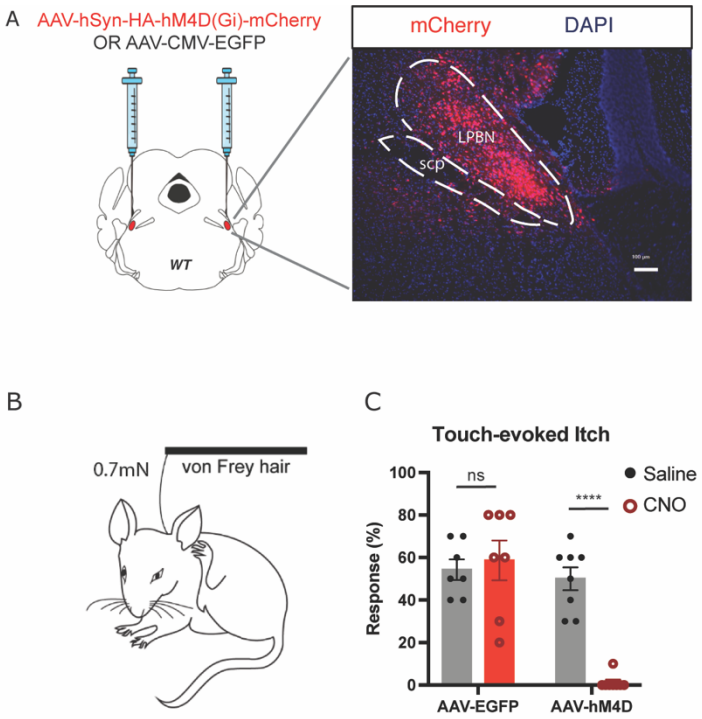

Figure 2.7.1. Chemogenetic silencing of the PBN neurons suppresses touch-evoked itch
(A) Schematic showing bilateral injection of AAV-hSyn-HA-hM4D(Gi)-mCherry or AAV-CMV-EGFP into the PBN of wildtype mice. Representative images of the PBN neurons infected by AAV-hSyn-HA-hM4D(Gi)-mCherry with DAPI staining. Scale bar, 100μm. (B) Schematic showing the touch-evoked itch test. (C) Effect of chemogenetic silencing of the PBN on the touch-evoked itch (AAV-EGFP, n = 7 mice; AAV-hM4D, n= 8 mice). Error bars represent SEM. Two-way ANOVA for multiple comparisons test for (C). ****p<0.0001, ns-no significant difference.

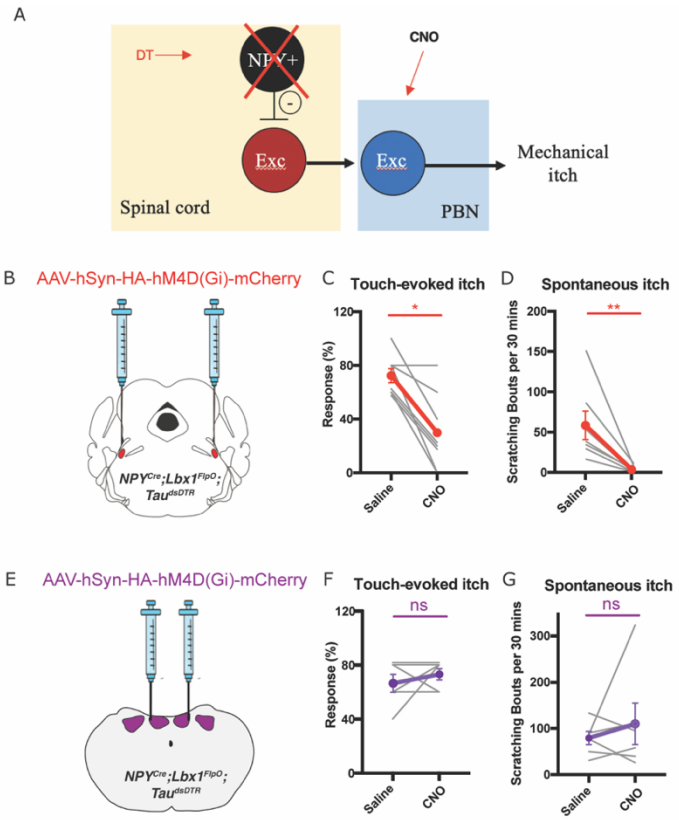

Figure 2.7.2. Chemogenetic silencing of the PBN neurons but not the DCN significantly reduces spinal NPY ablation-induced touch-evoked itch and spontaneous itch

(A) Schematic showing genetic ablation of spinal NPY+ inhibitory neurons induces chronic mechanical itch sensitization and the hypothesis that chemogenetic silencing of the PBN neurons might reverse the sensitization. (B) Bilateral injection of AAV-hSyn-HA-hM4D(Gi)-mCherry into the PBN of *NPY::Cre; Lbx1*^{FlpO}*; Rosa26*$^{ds\text{-}DTR}$ mice. (C-D) Effects of chemogenetic silencing of the PBN neurons on spinal NPY ablation-induced touch-evoked itch (n = 8 mice) and spontaneous itch (n = 8 mice). (E) Bilateral injection of AAV-hSyn-HA-hM4D(Gi)-mCherry into the DCN of *NPY::Cre; Lbx1*^{FlpO}*; Rosa26*$^{ds\text{-}DTR}$ mice. (F-G) Effects of chemogenetic silencing of the DCN neurons on spinal NPY ablation-induced touch-evoked itch (n = 6 mice) and spontaneous itch (n = 6 mice). Error bars represent SEM. Student's unpaired t test for (C), (D), (F) and (G); **p<0.01, ***p<0.001, ns-no significant difference.

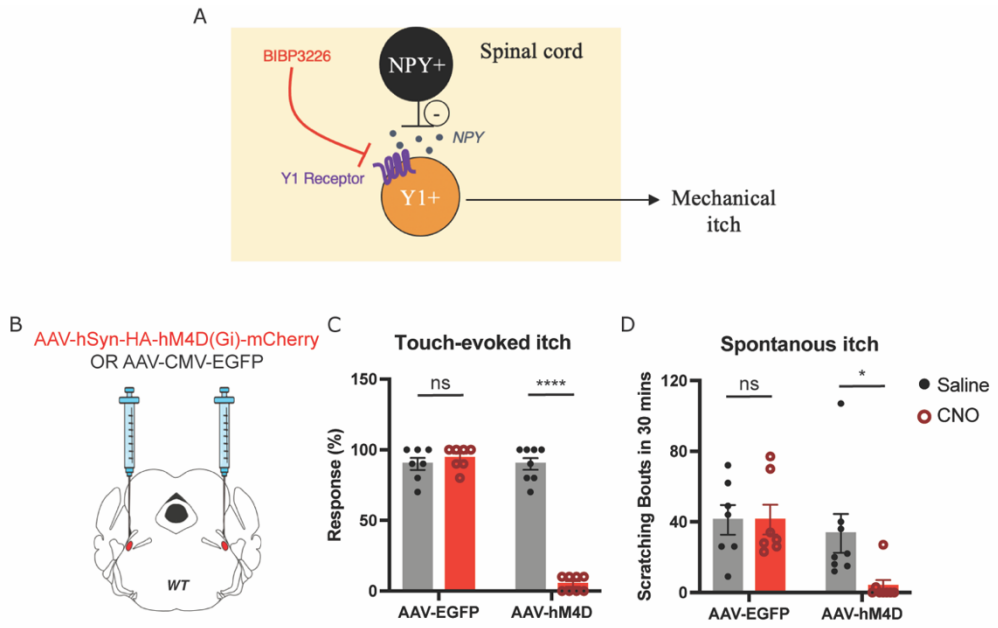

Figure 2.7.3. Chemogenetic silencing of the PBN neurons suppresses BIBP3226-induced touch-evoked and spontaneous itch

(A) Schematic showing blocking NPY-Y1 signaling pathway in the spinal cord by Y1 antagonist, BIBP3226, induces acute mechanical itch sensitization. (B) Bilateral injection of AAV-hSyn-HA-hM4D(Gi)-mCherry or AAV-CMV-EGFP into the PBN of *wildtype* mice. (C-D) Effects of chemogenetic silencing of the PBN neurons on BIBP3226-induced touch-evoked itch (AAV-EGFP, n = 7; AAV-hM4D, n = 8 mice) and spontaneous itch (AAV-EGFP, n = 7; AAV-hM4D, n = 8 mice). Error bars represent SEM. Two-way ANOVA for multiple comparisons test for (C) and (D). *p<0.05, ****p<0.0001.

Chapter 3: Calcrl, a novel marker for targeting spinoparabrachial projection neurons

3.1 Introduction

The spinoparabrachial (SPB) pathway is a crucial ascending sensory pathway that relays somatosensory information, including pain, touch and itch, from the spinal cord to the PBN (Barik et al. 2021; Choi et al. 2020; Mu et al. 2017, Roome et al., 2020). Silencing the axonal projections of the SPB neurons in the PBN suppresses itch induced by histamine and chloroquine (Mu et al. 2017). Mechanical itch, as a distinct itch subtype relayed to the PBN, is also likely to be transmitted by the SPB neurons. Recently, molecularly defined SPB neuron populations have been discovered for mediating distinct somatosensory modalities to the PBN (Barik et al. 2021; Choi et al. 2020). Tacr1+ (encodes Neurokinin 1 receptor) SPB neurons have been shown as a key a projection neuron population for mediating pain (Barik et al. 2021; Choi et al. 2020). Another SPB population that expresses Gpr83, which predominantly consists of Tacr1-negative neurons, is critical for affective touch (Choi et al. 2020). Additionally, the Tacr1+ neurons have been reported to mediate chemical but not mechanical itch (Acton et al., 2019a, Akiyama et al. 2015), which indicates these distinct itch subtypes are likely transmitted to the PBN by separate SPB populations. However, the molecular identity of the SPB population mediating mechanical itch has not been characterized yet.

3.2 Results

3.2.1 Characterization of ascending projection patterns of spinal neuron populations

In order to identify the SPB population that mediates mechanical itch, I started by screening a number of dorsal excitatory neuron populations to assess whether they encompassed SPB projection neurons. To this end, I crossed all the *Cre* lines that label the excitatory dorsal neuron populations with the *hCdx2::FlpO* and a double stop Td-Tomato (*Rosa26*$^{ds\text{-}Tomato}$) alleles, so as to restrict the labeling to only neuronal cell bodies in the spinal cord and their projections in the supraspinal areas (Huang et al. 2019) (**Figure 3.7.1.A**). As expected, the neuron populations predominantly involved in processing tactile feedback, like RORα+ neurons, projected almost exclusively to the DCN. Other broader populations projected to both DCN and PBN (**Figure 3.7.1.B**). We then screened Tacr1, which encodes Neurokinin 1 receptor (NK1R), and it has been found in a large population of SPB neurons (Cameron et al. 2015). Analysis of Tacr1^{Cdx2}-marked fibers showed projection into multiple brain regions including the lPBN (**Figure 3.7.2.A**), the lateral PAG (**Figure 3.7.2.B**), the VPL of the thalamus (**Figure 3.7.2.C**), as well as the DCN (**Figure 3.7.2.D**). To refine the targeting strategy, we assessed the projection pattern of a novel marker of dorsal horn neurons, Calcrl. Calcrl, or Calcitonin receptor-like, is a G protein-coupled receptor related to the calcitonin receptor that forms the functional CGRP receptor or adrenomedullin receptor together with RAMP proteins (Aldecoa et al. 2000; Kamitani et al. 1999; McLatchie et al. 1998). Surprisingly, Calcrl^{Cdx2}-marked fibers preferentially innervated the lPBN (**Figure 3.7.2.E**) without projections to the lateral PAG (**Figure 3.7.2.F**) and the thalamic nuclei (**Figure 3.7.2.G**), suggesting Calcrl-lineage neurons contain a SPB subpopulation with dedicated innervation to the PBN. Calcrl^{Cdx2}-marked fibers were also observed in the DCN (**Figure 3.7.2.H**). However, DCN-projecting neurons, or post-synaptic dorsal column neurons, are

thought to be potentially separated populations as they are differentially located in the dorsal horn and their axon runs in distinct regions, with SPB axons extending through the anterolateral funiculus and the PSDC axons at the tip of the dorsal funiculus.

3.2.2 Intersectional genetic labeling of Calcrl^{Lbx1} neurons targets a SPB population

The use of *hCdx2::FlpO* restricted Calcrl labeling to the spinal cord but also labeled glial cells, epithelial cells of blood vessel as well as other cell types. In order to selectively label Calcrl-lineage spinal neurons and better characterize their function, *Lbx1^{FlpO}*, a dorsal neuron-specific *FlpO* allele, was used here. We applied an intersectional genetic approach, by crossing the *Cre* mouse line with *Lbx1^{FlpO}* and *R26$^{ds\text{-}Tomato}$* mice, in order to label the dorsal spinal Calcrl neurons (hereafter referred as Calcrl^{Lbx1} neurons) (Duan et al. 2014; Bourane et al. 2015). (**Figure 3.7.3.A**). In the spinal dorsal horn, Calcrl^{Lbx1} neurons were predominantly located in the superficial (I-IIo) and deep (V) laminae, as well as in the lateral spinal nucleus (LSN) (**Figure 3.7.3.B**). To confirm these neurons were bona fide projection neurons, I injected CTB, a retrograde tracer, in the PBN of *Calcrl^{Lbx1}; Rosa26$^{ds\text{-}Tomato}$* mice to label ascending projecting neurons in the cord, and determine the percentage of those marked by Calcrl expression. CTB retrogradely traced neurons were found in multiple regions of the spinal dorsal horn, including laminae I, V and LSN (**Figure 3.7.3.C-3.7.3.E**). Most importantly, Calcrl^{Lbx1} neurons were found overlapping with CTB-backfilled cells in all these regions (**Figure 3.7.3.C-3.7.3.E**).

To further characterize spinal Calcrl^{Lbx1} neurons, I asked whether the Calcrl^{Lbx1} neurons represent a non-overlapping neuron populations as compared to the previously characterized Tacr1+ or Gpr83+ neuron populations, both of which include subsets of SPB

neurons (Barik et al. 2021; Choi et al. 2020). I observed a subpopulation of Calcrl^{Lbx1} neurons (44.7% ± 6.2%) that expresses Tacr1 mRNA (**Figure 3.7.4.A and 3.7.4.E**), suggesting these populations are partially overlapping. Most importantly, these data also show that the Calcrl^{Lbx1} population contains a Tacr1-negative subset (around 55%). I also observed a small proportion of Calcrl^{Lbx1} neurons (39.3% ± 1.3%) that express Gpr83 mRNA, showing that they also partially overlap (**Figure 3.7.4.B and 3.7.4.F**). Given the distinct spinal circuitry that has been described for chemical and mechanical itch, I then assessed the relationship between Calcrl^{Lbx1} neurons to known chemical itch neuronal populations, namely spinal neurons that express GRP and GRPR (Sun et al. 2007; Sun et al. 2009; Mishra et al. 2013). Very few Calcrl^{Lbx1} neurons were found to express either GRP (10.0% ± 1.6%) or GRPR (4.0% ± 2.6%) mRNA (**Figure 3.7.4.C, 3.7.4.D, 3.7.4.G and 3.7.4.H**), showing Calcrl^{Lbx1} neurons are a non-overlapping population with respect to both GRP+ and GRPR+ spinal neurons.

3.2.3 Calcrl^{Lbx1} neurons functionally contributes to the mechanical itch pathway

To assess the function of Calcrl^{Lbx1} neurons in transmitting mechanical itch sensation, we used the same intersectional strategy to manipulate the activity of these spinal interneurons. We started by crossing *Calcrl^{Cre}* and *Lbx1^{FlpO}* mice with the *Tau$^{ds\text{-}DTR}$* and *R26$^{ds\text{-}Tomato}$* effector alleles to express the diphtheria toxin receptor (DTR) for ablating and marking the neurons with tdTomato expression, respectively **(Figure 3.7.5.A**). We delivered the DT intrathecally to restrict ablation of Calcrl^{Lbx1} neurons to the spinal cord. As control for quantifying cell ablation efficiency, mice with same genotype (*Calcrl^{Cre}*; *Lbx1^{FlpO}*; *Tau$^{ds\text{-}DTR}$*; *R26$^{ds\text{-}Tomato}$*) were injected with saline. Our results showed a substantial portion of Calcrl^{Lbx1}

neurons in the spinal dorsal horn was ablated (from 26.2 ± 2.0 to 8.5 ± 0.9) following DT injection (**Figure 3.7.5.B and 3.7.5.C**), suggesting our ablation approach was successful. For the behavior tests, we used FlpO-negative littermate treated with DT as controls to maximize the number of mice available.

We tested the sensitivity to mechanical itch stimulation by applying a von Frey hair with low-threshold mechanical force (0.7mN) to the nape of the neck (area with low mechanical itch sensitivity) and to the ear (area with high mechanical itch sensitivity) in control and Calcrl^{Lbx1}-ablated mice (**Figure 3.7.6.A**). Scratching responses were recorded and response rate over 10 trials was quantified. In the control mice, mechanical itch stimulation induced a moderate scratching rate on the nape (29.0% ± 5.26%) and a slightly stronger on the ear (56.7% ± 9.5%) (**Figure 3.7.6.B and 3.7.6.C**). In the mice without Calcrl^{Lbx1} neurons, scratching responses to nape stimulation were totally abolished (1.7% ± 1.7%) compared to control mice (**Figure 3.7.6.B**). Likewise, scratching responses to ear stimulation were also substantially reduced (7.5% ± 5.2%) (**Figure 3.7.6.C**). The results demonstrated that Calcrl^{Lbx1} neurons are required for transmission of mechanical itch.

Neuronal ablation in the spinal cord can induce plasticity and thus changes in connectivity and synaptic strength. Here, in order to rule out potential rewiring of spinal circuitries following neuronal ablation, we used intersectional strategies to achieve acute silencing of Calcrl^{Lbx1} neurons. *Calcrl^{Cre}; Lbx1^{FlpO}* mice were crossed to a *Rosa26$^{ds\text{-}hM4D}$* effector line to selectively express the DREADD effector protein hM4D(G_i) in Calcrl^{Lbx1} neurons (**Figure 3.7.7.A and 3.7.7.B**). Thirty minutes following the CNO application, scratching responses evoked by nape mechanical stimulation were totally abolished (from 23.3% ± 4.2% to 0.0% ± 0.0%) (**Figure 3.7.7.C**), confirming the DT-ablation results.

To assess whether these neurons were not only necessary but also sufficient to induce mechanical itch-induced scratching, we crossed *Calcrl*^{Cre}*; Lbx1*^{FlpO} mice with a *Rosa26*$^{ds\text{-}hM3D}$ effector line to selectively express the DREADD effector protein hM3D(G_q) (**Figure 3.7.7.D and 3.7.7.E**). Mice in which Calcrl^{Lbx1} neurons were activated, following CNO stimulation, showed higher occurrence of spontaneous scratching (from 5.4 ± 2.1 to 28.0 ± 3.1) (**Figure 3.7.7.G**) and displayed higher levels of von Frey-induced scratching (from 22.8% ± 2.9% to 77.8% ± 6.4%) (**Figure 3.7.7.F**). In summary, I showed that Calcrl^{Lbx1} neurons are both necessary and sufficient for mechanical itch transmission.

3.2.4 Calcrl^{Lbx1} neurons mediate NPY-driven mechanical itch sensitization

Next, we assessed the function of *Calcrl*^{Lbx1} neurons in NPY-modulated mechanical itch transmission (Bourane et al. 2015; Acton et al. 2019). The NPY-dependent itch pathway can be sensitized either by NPY neuronal ablation, which induces chronic mechanical itch sensitization, or by acute blockade of the NPY-Y1R signaling (by application of Y1R antagonist, BIBP3326), which induces induce shorter-lasting mechanical itch sensitization. These manipulations were performed in mice where Calcrl^{Lbx1} neurons were ablated or in the relative controls.

I started by performing a genetic epistasis experiment to address whether the ablation of Calcrl^{Lbx1} neurons was sufficient to rescue mechanical itch sensitivity in NPY-ablated mice. The concomitant ablation of Calcrl^{Lbx1} neurons abolished the increased sensitivity to mechanical stimulation observed in NPY^{Lbx1} ablated mice (from 67.5% ± 7.5% to 0.0% ± 0.0%) (**Figure 3.7.8.A)**. Similarly, spontaneous scratching induced by NPY ablation was also

substantially reversed when Calcrl^{Lbx1} were co-ablated (from 96.1 ± 23.6 to 11.0 ± 4.6) (**Figure 3.7.8.B**).

Next, we tested the contribution that Calcrl^{Lbx1} neurons make to the acute Y1 antagonist-induced mechanical itch sensitization. Ablation of Calcrl^{Lbx1} neurons substantially reduced BIBP3226-induced mechanical itch (from 93.3% ± 3.3% to 6.7% ± 4.9%) (**Figure 3.7.9.A**). Additionally, spontaneous scratching induced by BIBP3226 (from 36.8 ± 9.9 to 4.3 ± 1.5) was also almost abolished in Calcrl^{Lbx1} ablated mice (**Figure 3.7.9.B**). These results showed that Calcrl^{Lbx1} neurons are required for NPY-modulated mechanical and spontaneous itch.

3.2.5 Calcrl^{Lbx1} neurons contribute to the occurrence of chronic itch

Mechanical itch sensitization is a hallmark of chronic itch (Akiyama et al., 2012; Hu et al., 2018; Pan et al. 2019). Thus, I proceeded to examine the role of Calcrl^{Lbx1} neurons in mechanical itch sensitization under chronic itch conditions. In order to induce robust and long-lasting itch sensitization, I applied three types of mouse chronic itch models including histamine alloknesis, dry skin and allergic contact dermatitis to mice with ablation of Calcrl^{Lbx1} neurons.

Histamine alloknesis is an acute mechanical alloknesis evoked by intradermal injection of histamine in the dermis, e.g. under the skin of the nape of the neck (Akiyama et al. 2012) (**Figure 3.7.10.A**). In control mice, nape stimulation with von Frey hair (0.7mN) around the histamine injection site induced robust scratching responses in the 30 to 60 minutes following the injection (Figure **3.7.10.B**). However, histamine-induced alloknesis was substantially reduced in Calcrl^{Lbx1}-ablated mice compared (Figure **3.7.10.B**). Next, I

assessed the function of Calcrl^{Lbx1} neurons in mechanical itch sensitization in two additional long-lasting chronic itch models: AEW-induced dry skin and SADBE-induced allergic contact dermatitis (Figure 12C and 12E). The AEW-induce dry skin was caused by chronic application of acetone, ether and water (AEW) twice a day for 7-10 days onto the nape of the neck (**Figure 3.7.10.C**). This treatment induced strong skin irritation and chronic mechanical itch sensitization (**Figure 3.7.10.D**). Allergic contact dermatitis in mice was induced by chronic application of squaric acid dibutylester (SADBE) onto the nape of the neck for 7-10 days (**Figure 3.7.10.E**), which resulted in a remarkable mechanical itch sensitization (**Figure 3.7.10.F**). Ablation of Calcrl^{Lbx1} neurons significantly reduced mechanical itch sensitization under both dry skin and allergic contact dermatitis conditions compared to controls (**Figure 3.7.10.D and 3.7.10.F**). Our results demonstrate that Calcrl^{Lbx1} neurons are critical for mechanical itch in both acute and chronic states. Nevertheless, we did observe that the mechanical itch sensitization in these three chronic itch models was not completely abolished, suggesting other spinal neuron populations might be involved in the process.

3.2.6 PBN neurons act as downstream effectors of Calcrl^{Lbx1} neuron projections in modulating mechanical itch

So far, our results have demonstrated three core findings: first, the PBN in the brainstem is a crucial nucleus for processing mechanical itch sensation (**Figures 2.7.1-2.7.3**); second, Calcrl neurons project to the PBN; and third, spinal Calcrl^{Lbx1} neurons are required for mechanical itch under both acute and chronic conditions (**Figures 3.7.6-3.7.10**). Thus, we hypothesized that the PBN may be the downstream target of spinal Calcrl^{Lbx1} neurons during mechanical itch transmission. First, in order confirm that the PBN is downstream of spinal

Calcrl^{Lbx1} neurons during mechanical itch, we combined intersectional genetic activation and viral silencing strategies for achieving dual manipulations of spinal Calcrl^{Lbx1} and the PBN neurons. hM3D(G_q) was introduced in spinal Calcrl^{Lbx1} neurons with the intersectional genetic approach and hM4D(G_i) was delivered to the PBN via bilateral AAV injection (**Figure 3.7.11.A**). CNO application activated spinal Calcrl^{Lbx1} neurons while simultaneously silenced the PBN neurons (**Figure 3.7.11.B**), allowing us to assess whether silencing the PBN is sufficient to rescue the mechanical itch sensitization induced by activation of Calcrl^{Lbx1} neurons. In the control group, with AAV-EGFP injected in the PBN, CNO application evoked robust response to mechanical itch stimuli due to the activation of Calcrl^{Lbx1} neurons (**Figure 3.7.11.C**). However, in the AAV-hM4D-injected mice, where the PBN was silenced by CNO, responses to mechanical itch stimuli were completely abolished (**Figure 3.7.11.C**). Similarly, the spontaneous itch observed following Calcrl^{Lbx1} activation was also reversed by silencing the PBN (**Figure 3.7.11.D**). These results show that PBN silencing completely reverses mechanical itch sensitization caused by Calcrl^{Lbx1} neuron activation, suggesting that the PBN is the downstream target of Calcrl^{Lbx1} neurons for relaying mechanical itch information.

Next, we wanted to analyze in detail whether the projections of spinal Calcrl^{Lbx1} neurons are responsible for the mechanical itch sensitization. To this end, we bilaterally implanted cannulae into the PBN of the *Calcrl^{Cre}; Lbx1^{FlpO}; Rosa26$^{ds\text{-}hM4D}$* mice. CNO infused through the cannula only diffuses locally into the PBN region and silences selectively the SPB projection terminals of Calcrl^{Lbx1} neurons (**Figure 3.7.12.A**). Littermate lacking the *FlpO* allele with the same implantation and treatment were used as controls. Following CNO infusion, touch-evoked mechanical itch responses were substantially reduced in the experimental mice compared to controls (**Figure 3.7.12.B**). Similarly, the acute mechanical

itch sensitization evoked by Y1 antagonist BIBP3226 was also significantly lowered in mice where the Calcrl projections were locally silenced in the PBN (**Figure 3.7.12.C**). Taken together, these results demonstrate that Calcrl^{Lbx1} ascending projections to the PBN mediate mechanical itch sensitization.

3.3 Conclusion and Discussion

All the data in the Chapter 3 characterizes a novel spinoparabrachial projection neuron population targeted by *Calcrl^{Cre}* and *Lbx1^{FlpO}* alleles (referred as Calcrl^{Lbx1} neurons) **(Figure 3.7.2 and 3.7.3)**. Calcrl^{Lbx1} neurons in the spinal cord consist of a key neuron population that underlie the transmission of mechanical itch under baseline **(Figure 3.7.6)**, acute **(Figure 3.7.9)** and chronic **(Figure 3.7.8 and 3.7.10)** itch sensitization states. However, the intersectional genetic targeting by *Calcrl^{Cre}* and *Lbx1^{FlpO}* alleles may target both interneurons and projection neurons in the spinal cord. Here, I implanted micro-cannula into the PBN for achieving a selective manipulation the spinoparabrachial projection neuron subset of Calcrl^{Lbx1} neurons, and demonstrated that Calcrl^{Lbx1} spinoparabrachial neurons is crucial for mechanical itch transmission **(Figure 3.7.12)**.

Spinal interneurons that targeted by Ucn3^{Cre} directly and preferentially receive Aβ LTMR inputs from periphery and are required for relaying mechanical itch (Pan et al. 2019). Our unpublished data revealed direct synaptic contact from axonal terminal of Ucn3+ interneurons to Calcrl^{Lbx1} spinoparabrachial neurons. This result established a core neural circuitry for mechanical itch transmission, which consists of Aβ LTMR sensory neurons, Ucn3+ spinal interneurons and Calcrl^{Lbx1} spinoparabrachial neurons.

Chemical itch transmission is also relied on the spinoparabrachial pathway (Mu et al. 2017). Our unpublished data also showed that ablation of Calcrl^{Lbx1} neurons reduces but not abolishes chemical itch sensation. Therefore, the open question is how the chemical and mechanical itch is relayed to the supraspinal regions. Are they relayed by separated or identical projection neuron populations in the spinal cord? Recently, functional roles of another two spinoparabrachial neuron populations marked by *Tacr1*^{Cre} and *Gpr83*^{Cre}, respectively, have been reported (Barik et al. 2021; Choi et al. 2020). Very interestingly, these two spinoparabrachial populations are substantially separate and constitute the majority of the spinoparabrachial neurons (around 90%) (Choi et al. 2020). For the spinal Tacr1+ population, it has been shown that ablation of them reduces chemical but not mechanical itch sensitivity (Acton et al. 2019), indicating that Tacr1+ neurons (including the spinoparabrachial subset) may relay chemical itch but not mechanical itch information. For the spinal Gpr83+ population, optogenetic activation of their PBN projections strengthens affective touch but not pain sensation (Choi et al. 2020), suggesting this spinoparabrachial population are low-threshold mechanosensitive and likely to be the mechanical itch-relay population. In the RNAscope data, I showed the Calcrl^{Lbx1} neurons partially overlap with Tacr1+ and Gpr83+ populations in the spinal dorsal horn, with around 45% of CalcrlLbx1 neurons expressing Tacr1 mRNA and 40% of them expressing Gpr83 mRNA **(Figure 3.7.4)**. To answer the questions, my working hypothesis is that the central nervous system recruits divergent ascending transmission circuitries, in which Calcrl+/Tacr1+/Gpr83- spinoparabrachial subgroup relaying chemical itch while Calcrl+/Gpr83+/Tacr1- or Calcrl+/Gpr83-/Tacr1- subgroup mediating mechanical itch information to the PBN. In the future, electrophysiology

with intersectional labeling will be needed to figure out the functional dissection of these spinoparabrachial subpopulations.

3.4 Materials and Methods

Key Resources Table

Reagent or Resource	**Source**	**Identifier**
Antibodies		
Rabbit α-DsRed (1:1000)	Clontech	Cat# 632496; RRID: AB_10013483
Goat α-CTB (1:4000)	List Laboratories	Cat# 703; RRID:AB_10013220
Oligonucleotides		
tdTomato RNAscope probe	ACDBio	Cat# 317041
Tacr1 RNAscope probe	ACDBio	Cat# 428781
Gpr83 RNAscope probe	ACDBio	Cat# 317431
Grp RNAscope probe	ACDBio	Cat# 317861
Grpr RNAscope probe	ACDBio	Cat# 317871
Bacterial and Virus Strains		
AAV-hSyn-HA-hM4D(Gi)-mCherry	Salk Viral Core	
AAV-CMV-EGFP	Salk Viral Core	
Chemicals		
BIBP3226 trifluoroacetate	Tocris	Cat# C2707/1

Cholera Toxin Subunit b (Recombinant), Alexa Fluor 647 Conjugate	Invitrogen	Cat# C34778
Diphtheria Toxin	List Laboratories	Cat# 150
Clozapine N-oxide	Sigma	Cat# C0832
Histamine	MilliporeSigma	Cat# H7250
SADBE	MilliporeSigma	Cat# 339792
Acetone	Sigma	Cat# 179124
Diethyl ether	Sigma	Cat# 309966
Experimental models: Organisms/Strains		
Mouse: *Calcrl*^{Cre}	Han et al. 2015	
Mouse: *NPY::Cre*	Bourane et al. 2015a	
Mouse: *hCdx2::FlpO*	Britz et al. 2015	
Mouse: *Lbx1*^{FlpO}	Bourane et al. 2015a	
Mouse: *Rosa26*$^{ds\text{-}tdTomato}$ *(Ai65*$^{ds\text{-}tdTomato}$*)*	The Jackson Laboratory	JAX stock #021875; RRID:IMSR_JAX:021875
Mouse: *Rosa26*$^{ds\text{-}hM3D}$	The Jackson Laboratory	JAX stock #026942; RRID:IMSR_JAX:026942
Mouse: *Rosa26*$^{ds\text{-}hM4D}$	Bourane et al. 2015a	
Mouse: *Tau*$^{ds\text{-}DTR}$	Britz et al. 2015	
Software and Algorithms		
Adobe Illustrator and Photoshop CS5	Adobe	

Prism 8	GraphPad	
ImageJ	Kurt de Vos, Univ. of Sheffiled, UK	

Experimental Model and subject details

All protocols for animal experiments were approved by the IACUC of the Salk Institute for Biological Studies according to NIH guidelines for animal experimentation. Male and female mice were used in all studies. Animals were randomized to experimental groups and no sex differences were noted. The *Calcrl*^{Cre} knockin mouse line was generated by Han et al. (Han et al. 2015).

The following mouse lines were also used in the study: *Lbx1*^{Cre} (Sieber et al. 2007), *Lmx1b*^{Cre} (Escalante et al. 2020), *Calbindin*^{Cre} (Gatto et al. 2021), *Calretinin*^{Cre} (Duan et al. 2014), *CCK*^{Cre} (Gatto et al. 2021), *Sst*^{Cre} (Duan et al. 2014), *ROR*α^{Cre} (Bourane et al. 2015b), *Sim1*^{Cre} (Zhang et al. 2008), *NPY::Cre* (Bourane et al. 2015a), *Lbx1*^{FlpO} (Bourane et al. 2015a), *hCdx2::FlpO* (Britz et al. 2015), *Rosa26*$^{ds\text{-}Tomato}$ (also known as *Ai65*$^{ds\text{-}Tomato}$) (Madisen et al. 2015), *Rosa26*$^{ds\text{-}hM3D}$ (Sciolino et al. 2016), *Rosa26*$^{ds\text{-}hM4D}$ (Bourane et al. 2015a), *Tau*$^{ds\text{-}DTR}$ (Britz et al. 2015).

Cholera Toxin-B Retrograde Tracing

6-10 weeks old *Calcrl*^{Cre}; *Lbx1*^{FlpO}; *Rosa26*$^{ds\text{-}tdTomato}$ mice were placed on a stereotaxic frame (Kopf Instruments) and anaesthetized via inhalation of isoflurane (1.5–2.0%) using an isoflurane vaporizer during the surgery. Burr holes were made on the skull using high-speed stereotaxic drill (Kopf Instruments), and 400 nl of Alexa Fluor–conjugated CTB 647 (Thermo

Fisher) were injected into the PBN through glass pipettes (Wiretrol II, Drummond) and a Nanoinjector (NANOJECT III, Drummond). The coordinates used for stereotaxic injections and implantation were as follows: PBN (-1.00 mm posterior to lambda, ± 1.50 mm from midline and -3.50 ventral from skull). The mice were perfused and dissected 7 days after the CTB injection. The spinal cords from 3 mice were cryosectioned and immunostained with goat-anti-CTB antibody for amplifying the signals.

Viral Injection

10-14 weeks old *Calcrl*^{Cre}; *Lbx1*^{FlpO}; *Rosa26*$^{ds\text{-}hM3D}$ mice were placed on a stereotaxic frame (Kopf Instruments) and anaesthetized via inhalation of isoflurane (1.5–2.0%) using an isoflurane vaporizer during the surgery. Body temperature of the animal was maintained with a heating pad underneath the body during the surgery. Ophthalmic ointment was applied to maintain eye lubrication. Burr holes were made on the skull using high-speed stereotaxic drill (Kopf Instruments), and 500 nl of AAV-hSyn-HA-hM4D(Gi)-mCherry (or AAV-CMV-EGFP for controls) were injected into the PBN by a Nanoinjector (NANOJECT III, Drummond) at a rate of 2 nl/sec through glass pipettes (tip diameter 10-30mm) (Wiretrol II, Drummond). After each injection, the glass pipettes were left in place for 7-8 min before withdrawal. The animals were placed on a heating pad to recover from anesthesia before returning to their home cage. Buprenex SR (0.1 g/kg) was injected intradermally in the back after surgery to provide analgesia.

The coordinates used for stereotaxic injections and implantation were as follows: PBN (-1.00 mm posterior to lambda, ± 1.50 mm from midline and -3.50 ventral from skull). The behavioral tests were performed 21-28 days after the viral injection.

Cannula Implantation

10–14 weeks old *Calcrl*^{Cre}; *Lbx1*^{FlpO}; *Rosa26*$^{ds\text{-}hM4D}$ mice were placed on a stereotaxic frame (Kopf Instruments) and anaesthetized via inhalation of isoflurane (1.5–2.0%) using an isoflurane vaporizer during the surgery. Burr holes were made on the skull using high-speed stereotaxic drill (Kopf Instruments). For cannula implantation, bilateral cannula (4mm in length, Gauge 33, P1 Technologies) were implanted into the bilateral PBN (-1.00 mm posterior to lambda, ± 1.50 mm from midline and -3.20 ventral from skull) and secured using a gel-type Super Glue (Loctite) with an accelerator application followed by application of dental cement (Metabond, Parkell). Behavioral experiments were performed one week after the cannula implantation.

Immunohistochemistry

P30-P40 *Calcrl*^{Cre}; *Lbx1*^{FlpO}; *Rosa26*$^{ds\text{-}tdTomato}$ mice were euthanized by a single intraperitoneal (i.p.) injection (10 ml/g body weight) of ketamine (10 mg/ml) and xylazine (1 mg/ml) immediately prior to perfusion with 20 mL ice-cold 4% paraformaldehyde in PBS. Spinal cords and brain were dissected and post-fixed for 1h at RT, then cryoprotected in 30% sucrose-PBS (w/v) overnight at 4 degree. Tissue was embedded in Tissue-Tek OCT Compound (Sakura Finetek) and sectioned by cryostat (Leica 3050S) at 40 mm. Sections were dried at RT and stored at -20 degree. In the day of immunostaining, sections were washed once with PBS (10 min), blocked with a solution of 10% donkey serum in PBST (PBS + 0.1% Triton X-100) for 1h at RT and then incubated overnight with primary antibodies in a solution of 1% donkey serum in PBT at 4 degree. Sections were then washed 3

times (10 min each) in PBST before being incubated for 1h at RT with fluorophore–conjugated secondary antibodies (1:1000; Jackson Laboratories) in PBT. Sections were again washed 3 times (10 min each) in PBST before being mounted with Aqua-Poly/Mount (Polysciences). A Zeiss LSM 700 confocal microscope was used to acquire images. 3-5 spinal cords were analyzed for each condition. ImageJ software was used to assess immunofluorescence, with thresholds set according to signal intensity (Jensen, 2013). The following primary antibodies were used in this study: goat α-CTB (1:4000; List Laboratories), rabbit α-DsRed (1:1000; Clontech).

RNA in situ hybridization.

RNA in situ hybridization was performed using the RNAscope Fluorescent Multiplex Assay using the probes and kits purchased from Advanced Cell Diagnostics (ACD, USA). P30-P40 *Calcrl*^{Cre}; *Lbx1*^{FlpO}; *Rosa26*$^{ds\text{-}tdTomato}$ mice were euthanized by a single intraperitoneal (i.p.) injection (10 ml/g body weight) of ketamine (10 mg/ml) and xylazine (1 mg/ml) immediately prior to perfusion with 20 mL ice-cold 4% paraformaldehyde in PBS. Spinal cords were dissected and post-fixed for 1h at RT, then cryoprotected in 30% sucrose-PBS (w/v) overnight at 4 degree. Tissue was embedded in Tissue-Tek OCT Compound (Sakura Finetek) and sectioned by cryostat (Leica 3050S) at 16 mm. Sections were dried at RT and stored at -80 degree. Sample preparation, pretreatment, and signal detection were performed according to the ACD protocols. Probes used are listed below: tdTomato (#317041), Tacr1 (#428781), Gpr83 (#317431), Grp (#317861), Grpr (#317871), Npy1r (#427021) and Calcrl (#452281). Two to three representative images lumbar spinal cord were selected from 3 mice, Quantification of the colocalization is done manually with ImageJ software according to the

ACDBio technical note. DAPI-stained nuclei were first identified, then the cell contour was defined with a 2μm radius surrounding the DAPI signals. Cells containing at least two puncta inside the imaginary boundary were labeled as positive.

Drug Administration

The preparation of CNO and Y1 antagonist, BIBP3226 was shown in the "Drug administration" of Chapter 2.3.

Cell Ablation

For ablation of neurons expressing Cre and FlpO drivers in addition to *Tau*$^{ds\text{-}DTR}$, mice were injected with diphtheria toxin (DT; 10ng in 10ul of 0.9% sterile saline, i.t.; List Biological Laboratories) at P42, P44 and P46 (Bourane et al., 2015). For the single -Cre ablation experiment, FlpO- littermates (*Calcrl*^{Cre}*; Tau*$^{ds\text{-}DTR}$) were used as controls and injected with DT in the same way. For the double-Cre ablation experiment, *NPY::Cre;Lbx1*^{FlpO}*; Tau*$^{ds\text{-}DTR}$ were used as positive controls that show robust mechanical itch sensitization. For i.t. injections, mice were anesthetized with 2.5% isoflurane in O2, delivered via a nose cone. The caudal paralumbar region was then secured between the thumb and index finger, and a 30-gauge needle was inserted into the fifth intervertebral space until it elicited a tail flick. To prevent outflow, the needle was held in place for 10 s and turned 90 degree prior to withdrawal.

Behavioral testing and assessment of ablation efficiency by immunohistochemistry were performed when all the behavioral tests were done.

For the single-Cre ablation experiment, behavioral tests were performed 14-21 days after the first injection of DT. For the double-Cre ablation experiment, behavioral tests were performed 7-10 days after the first injection of DT to assess spontaneous scratching induced by ablation of NPY::Cre neurons.

Chemogenetic Silencing or Activation

For silencing or activating neurons expressing Cre and FlpO drivers in addition to Rosa26$^{ds\text{-}hM4D}$ or Rosa26$^{ds\text{-}hM3D}$, respectively, mice were injected with Clozapine-N-oxide (CNO, 2g/kg, Sigma) intraperitoneally (i.p.) 30 mins before the behavioral tests. The preparation of CNO solution was done as shown in the "Drug Administration" of Chapter 2.3.

Behavioral Testing

Touch-evoked itch

The same as shown in Chapter 2.3.

Spontaneous itch

The same as shown in Chapter 2.3.

Histamine-induced alloknesis

The fur on the nape was shaved 5 days before behavioral tests. On the testing day, histamine (100 mg, MilliporeSigma, St. Louis, MO) in 20 mL saline was injected intradermally into the nape (Akiyama et al. 2012). After 30 mins, mice received three separate innocuous mechanical stimuli for 3 sec delivered using a von Frey hair (0.7 mN) at 3-5 s intervals at randomly selected sites that surround the injection site (1 cm away). Nape stimulation was given repetitively from 30-60 mins with a 10 mins interval. In total, 12 times

of nape stimulation were applied to each mouse within 30 mins. The overall alloknesis score was calculated as the total number of evoked scratching responses by hindlimb toward the stimulated site.

Chronic dry skin model

The fur on the nape and back was shaved 5 days before treatment. The skin of the nape and back of mice was treated topically with a mixture of acetone/ether (1:1, MilliporeSigma, St. Louis, MO) for 15 s followed by distilled water for 25 s (Acetone-Ether-Water, AEW). Topical application of AEW was performed twice a day for 10 consecutive days (day 1-10). Touch-evoked itch and spontaneous itch sensitivities of mice were measured at the end of day 7 and day 10, respectively.

To assess touch-evoked itch sensitivity, mice were habituated in the behavioral testing apparatus for 1h per day for two days before the testing day. On the testing day, mice were briefly habituated for 15 min and then received 10 separate innocuous mechanical stimuli for 1-second delivered using a von Frey filament (0.7 mN) at 10-second intervals at randomly selected sites on the nape surrounding the AWE-treated site (0.5-1 cm distance). The scratching responses evoked by nape stimulations were recorded and response rate in overall 10-time stimulation was calculated. To measure spontaneous itch sensitivity, mice were briefly habituated for 15 min in the behavioral testing apparatus. Then the mice were video recorded for 30 min. The spontaneous scratching bouts within 30 min were counted.

Allergic contact dermatitis model

The fur on the nape and abdominal skin was shaved 5 days before treatment. Mice were sensitized by the topical application of 25 ml of 1% squaric acid dibutylester (SADBE, MilliporeSigma, St. Louis, MO) in acetone to abdominal skin once a day for three consecutive

days (day 1-3) (Fu et al., 2014). Five days later (day 8), the nape skin close to the left ear was challenged with a topical application of 25 ml of 1% SADBE in acetone for three consecutive days (day 8-10). The touch-evoked and spontaneous itch sensitivities were tested on the day 10 and day 12, respectively. To measure touch-evoked itch sensitivity, mice were first habituated 1-hour per day for two days before the SADBE application. On the day 8, mice were briefly habituated for 15 min in the behavioral testing apparatus and then received 10 separate innocuous mechanical stimuli for 1-second delivered using a von Frey filament (0.7 mN) at 3-5 s intervals at randomly selected sites on the nape surrounding the treated ear (1 cm distance). The scratching response rate to overall 10 stimuli was calculated. To measure spontaneous itch sensitivity, on the day 10, mice were briefly habituated for 15 min in the behavioral testing apparatus and video record for 30 mins. The spontaneous scratching bouts were counted.

3.5 Acknowledgements

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3.7 Figures

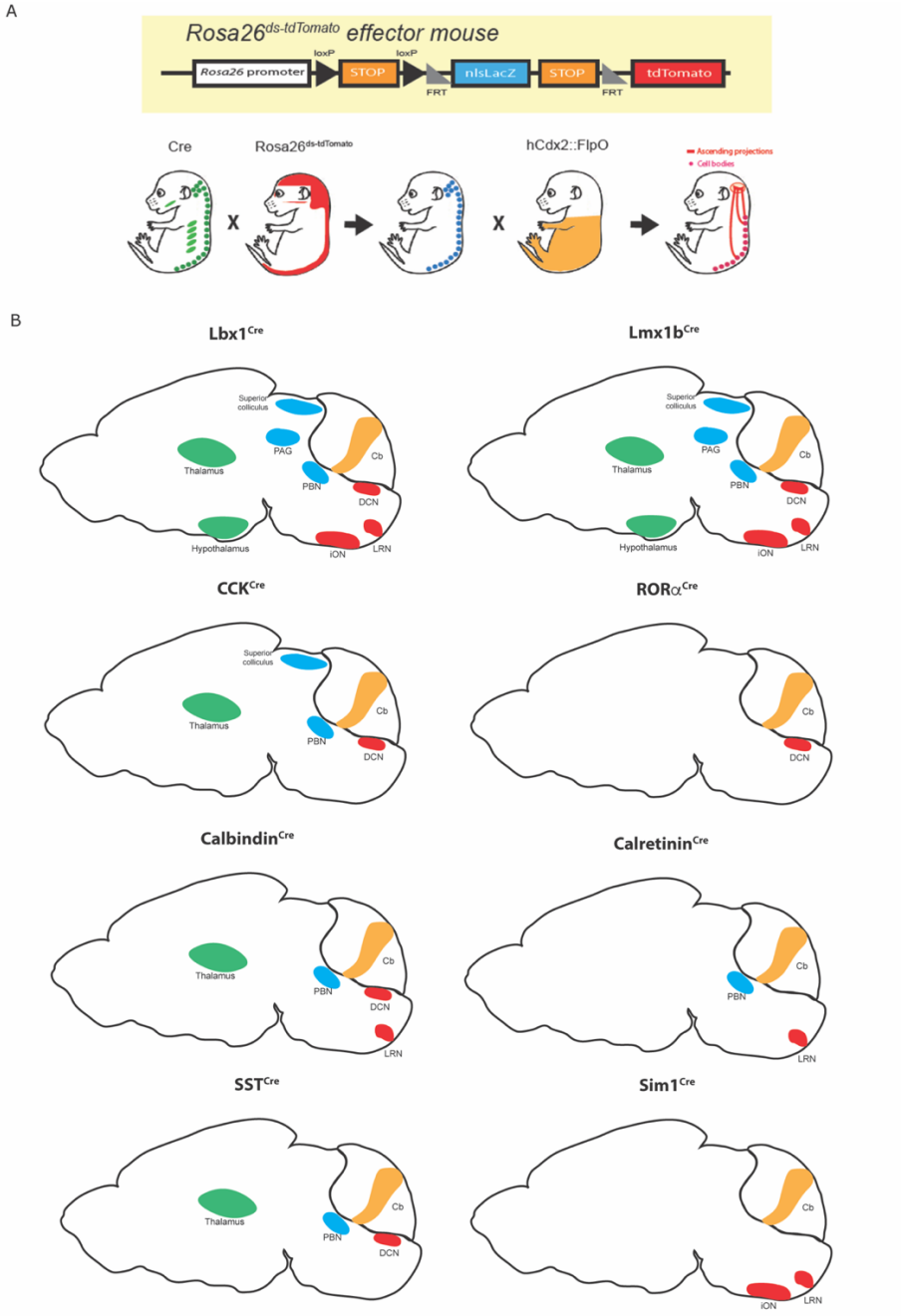

Figure 3.7.1. Characterization of the ascending projection patterns of spinal excitatory neuron populations in the supraspinal regions

(A) Schematic of intersectional genetic approach for targeting neuronal cell bodies in the spinal cord and axonal terminals in the supraspinal. (Adapted from Britz et al. 2014) (B) Summary of axonal projection pattern of spinal excitatory neuron populations in the supraspinal regions.

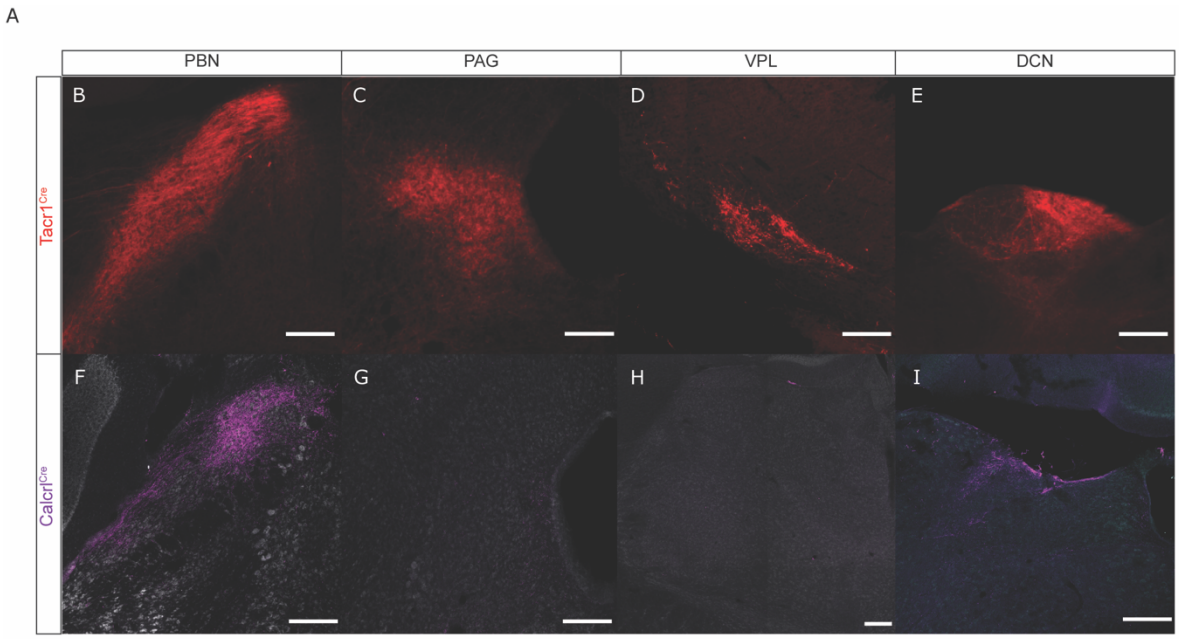

Figure 3.7.2. Characterization of the ascending projection patterns of spinal Tacr1- and Calcrl-lineage neurons in the supraspinal regions

(A-D) Representative images of ascending projections from spinal Tacr1-lineage neurons in the PBN (A), PAG (B), VPL of the thalamus (C), DCN (D). (E-H) Representative images of ascending projections from spinal Calcrl-lineage neurons in the PBN (E), PAG (F), VPL of the thalamus (G), DCN (H). Scale bar, 100μm.

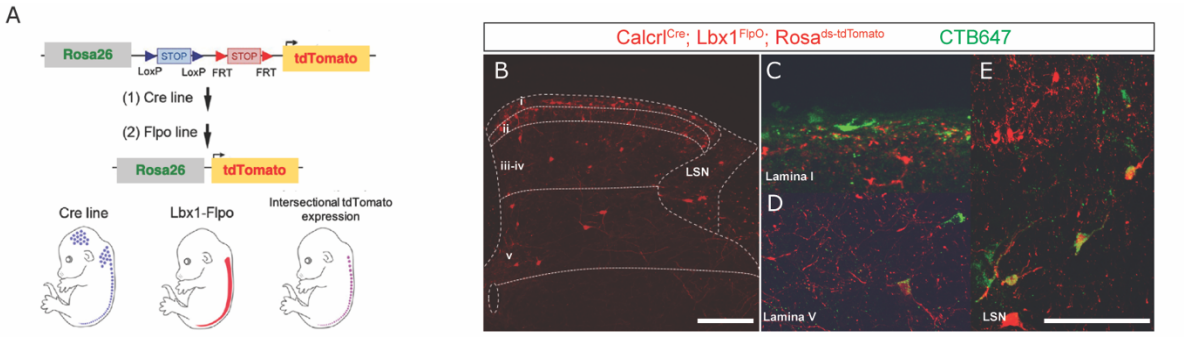

Figure 3.7.3. Intersectional genetic labeling of Calcrl^{Lbx1} neurons targets a SPB projection neuron population

(A) Schematic of intersectional genetic strategy for selectively labeling Calcrl^{Lbx1} neurons in the spinal cord by crossing *Calcrl^{Cre}* with *Lbx1^{FlpO}* and *Rosa26$^{ds\text{-}tdTomato}$* mouse lines. (B) Representative images showing expression of Calcrl^{Lbx1}-tdTomato neurons in the spinal dorsal horn. Scale bar, 100μm. (Adapted from Duan et al. 2014) (C-E) Representative images of Calcrl^{Lbx1}-tdTomato neurons overlapping with CTB backfilling cells (from the PBN) in the lamina I (C), lamina V (D) and LSN (E) in the lumbar spinal cord. Scale bar, 100μm.

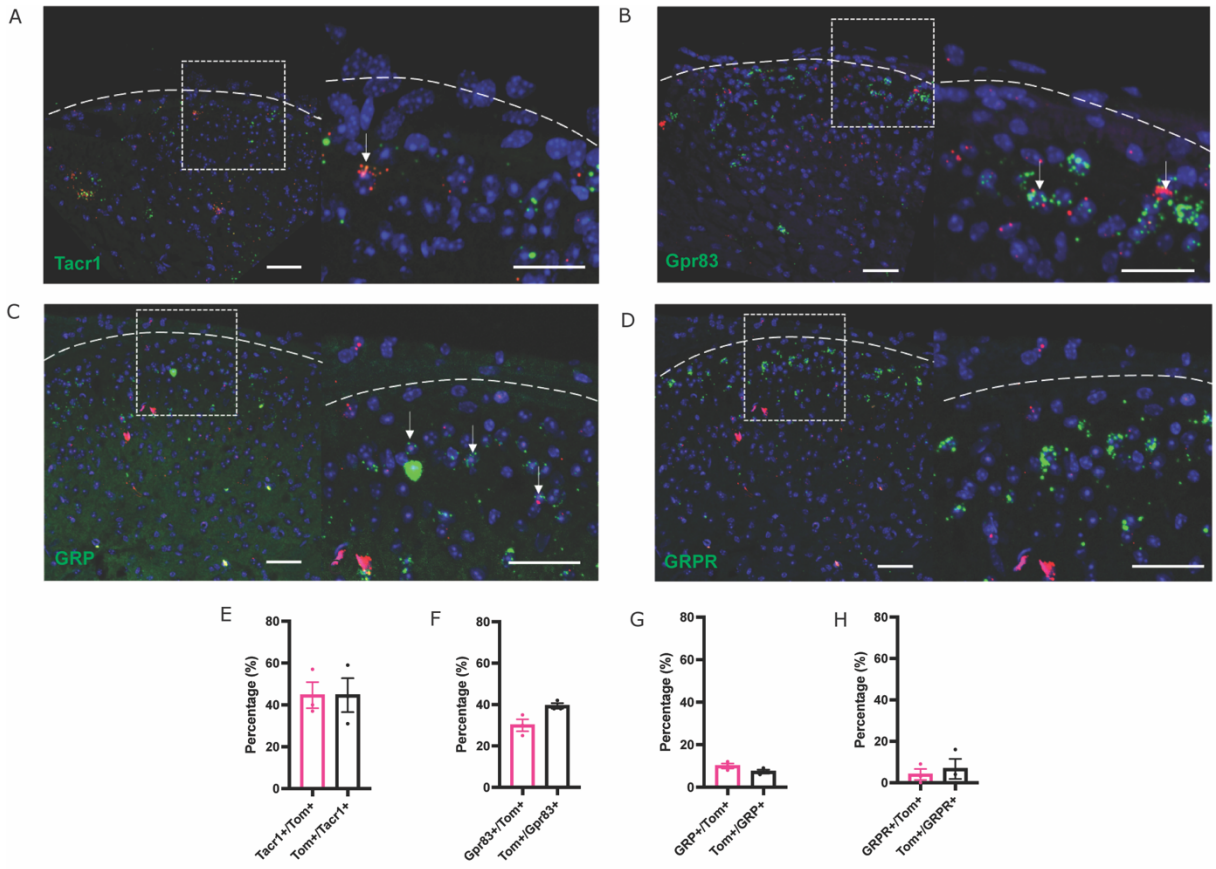

Figure 3.7.4. Characterization of the molecular profile of Calcrl^{Lbx1} neurons

(A-D) Representative RNAscope images of Tacr1(A), Gpr83(B), GRP(C), GRPR(D) in situ hybridization (green) in the spinal cord of Calcrl^{Lbx1}-tdTomato (red) mice. Scale bar, 100um. (E-H) Quantification of coexpression of Calcrl^{Lbx1}-tdTomato with Tacr1(E), Gpr83(F), GRP(G), GRPR(H) mRNA in P30 *Calcrl^{Cre}; Lbx1^{FlpO}; Rosa26$^{ds\text{-}tdTomato}$* mice.

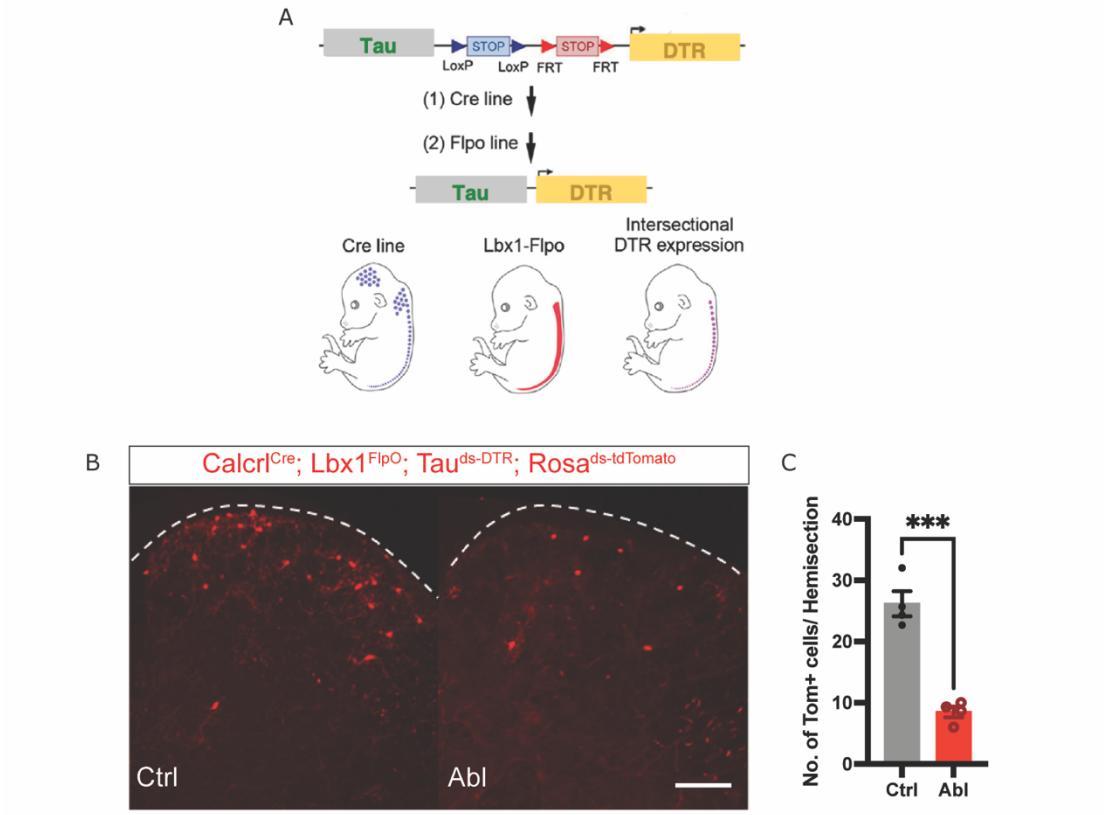

Figure 3.7.5. Intersectional genetic ablation of Calcrl^{Lbx1} neurons in the spinal cord
(A) Schematic of intersectional genetic ablation of Calcrl^{Lbx1} neurons in the spinal cord. (Adapted from Duan et al. 2014) (B) Representative images of spinal ablation of Calcrl^{Lbx1} neurons in control and ablated mice. Scale bar, 100um. (C) Quantification of Calcrl^{Lbx1}-tdTomato neurons in control (n = 4) and ablated (n = 4) mice. Error bars represent SEM. Student's unpaired t test; ***p<0.001.

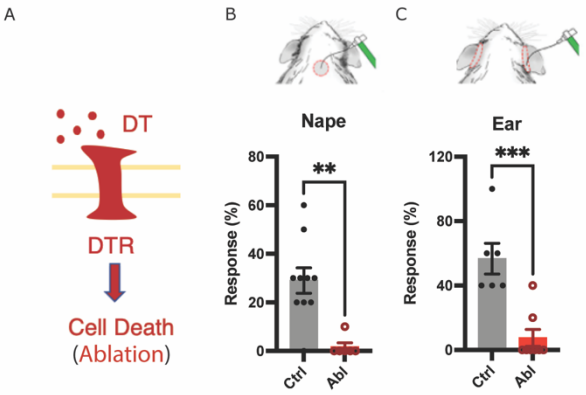

Figure 3.7.6. Ablation of spinal Calcrl^{Lbx1} neurons suppresses touch-evoked itch in different skin regions in mice

(A) Schematic showing neuronal ablation induced by diphtheria toxin (DT)-diphtheria toxin receptor (DTR) system. (B) Effects of Calcrl^{Lbx1} neuronal ablation on touch-evoked itch induced by nape stimulation in mice (Abl, n = 9; Ctrl, n = 8). (C) Effects of Calcrl^{Lbx1} neuron-ablation in the spina cord on touch-evoked itch induced by ear stimulation in mice (Abl, n = 8; Ctrl, n = 6) (Panel of stimulation sites adapted from Pan et al. 2019). Error bars represent SEM. Student's unpaired t test for (B) and (C); **p<0.01, ***p<0.001.

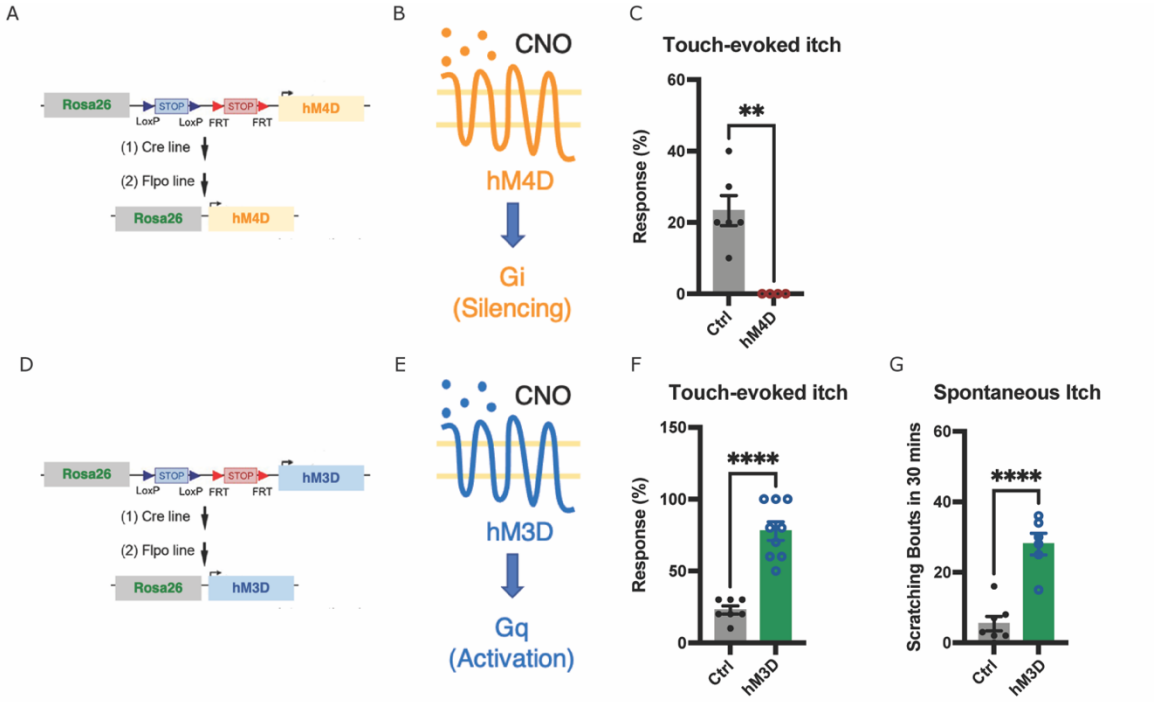

Figure 3.7.7. Chemogenetic silencing or activation suppresses or sensitizes mechanical itch, respectively

(A) Schematic of the tripartite genetic system used to selectively express hM4D(Gi) in Calcrl^{Lbx1} neurons. (B) Schematic of neuronal silencing of spinal Calcrl^{Lbx1} neurons by CNO-hM4D(Gi) system. (C) Effect of chemogenetic silencing of Calcrl^{Lbx1} neurons on touch-evoked itch induced by nape stimulation in mice (Ctrl, n = 6; hM4D, n = 4). (D) Schematic of the tripartite genetic system used to selectively express hM3D(Gq) in spinal Calcrl^{Lbx1} neurons. (E) Schematic of neuronal activation of Calcrl^{Lbx1} neurons by CNO-hM3D(Gq) system. (F-G) Effects of chemogenetic activation of Calcrl^{Lbx1} neurons on touch-evoked (F) and spontaneous (G) itch induced by nape stimulation in mice (Ctrl, n = 7; hM3D, n = 9). Error bars represent SEM. Student's unpaired t test for (C) (F) and (G); **p<0.01, ****p<0.0001.

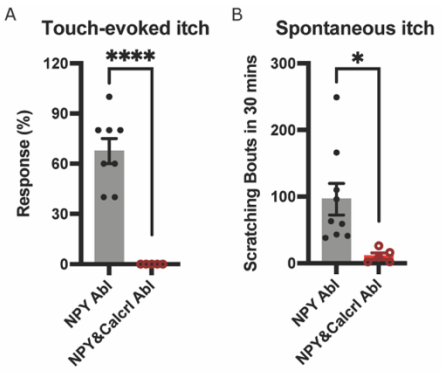

Figure 3.7.8. Ablation of Calcrl^{Lbx1} neurons suppresses spinal NPY ablation-induced touch-evoked and spontaneous itch

(A-B) Effects of Touch-evoked itch induced by nape stimulation (A) and spontaneous itch (B) on spinal NPY^{Lbx1} neuronal ablation-induced chronic mechanical itch sensitization in mice (NPY Abl, n = 8; NPY&Calcrl Abl, n = 5). Error bars represent SEM. Student's unpaired t test for (A) and (B); *p<0.05, ****p<0.0001.

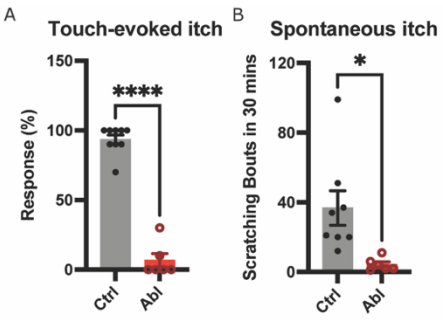

Figure 3.7.9. Ablation of Calcrl^{Lbx1} neurons suppresses BIBP3226-induced touch-evoked and spontaneous itch

(A-B) Effects of CalcrlLbx1 neuronal ablation on touch-evoked itch induced by nape stimulation (A) and spontaneous itch (B) under BIBP3226-induced acute mechanical itch sensitization in mice (Ctrl, n = 9; Abl, n = 6). Error bars represent SEM. Student's unpaired t test for (A) and (B); *p<0.05, ****p<0.0001.

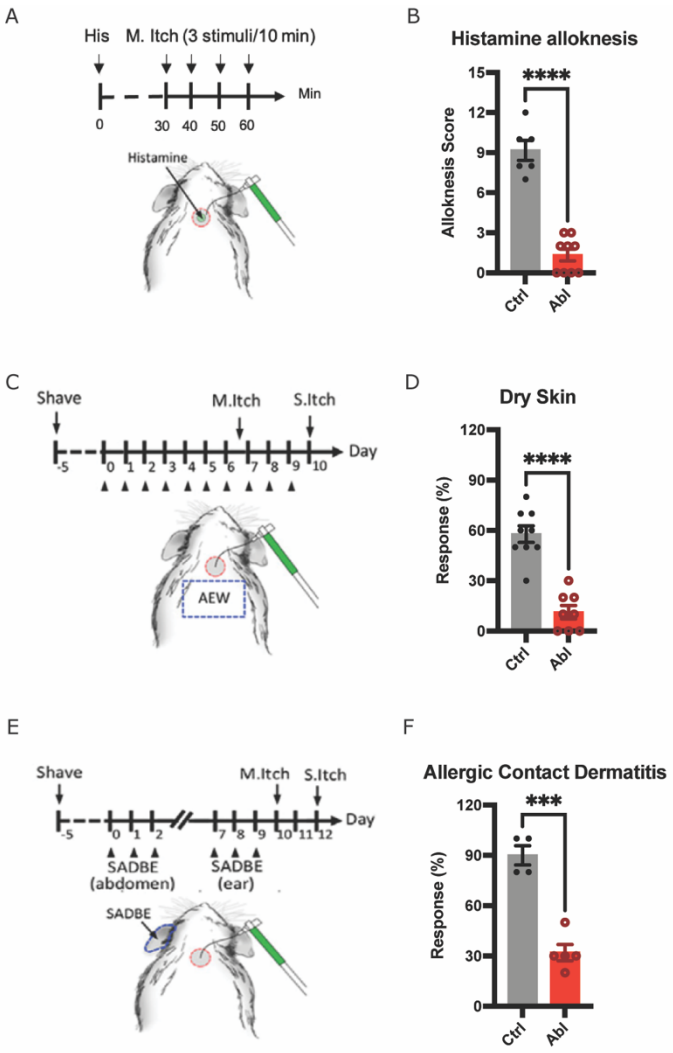

Figure 3.7.10. Ablation of Calcrl^{Lbx1} neurons suppresses touch-evoked itch under chronic itch conditions

(A) Schematic showing the histamine injection site on nape and surrounding sites for mechanical stimuli, and the procedure for behavioral tests. (B) Effect of Calcrl^{Lbx1} neuronal ablation on touch-evoked itch under histamine-induced acute alloknesis mice (Ctrl, n = 6; Abl, n = 9). (C) Schematic showing the procedure for AEW topical application and behavioral tests. (D) Effect of Calcrl^{Lbx1} neuronal ablation on touch-evoked itch under AEW-induced dry skin in mice (Ctrl, n = 9; Abl, n = 8). (E) Schematic showing the procedure for SADBE topical application and behavioral tests. (F) Effect of Calcrl^{Lbx1} neuronal ablation on touch-evoked itch under SADBE-induced allergic contact dermatitis in mice (Ctrl, n = 4; Abl, n = 5). Error bars represent SEM. Student's unpaired t test for (B) (D) and (F); ***p<0.001, ****p<0.0001. (A) (C) and (E) are adapted from Pan et al. 2019.

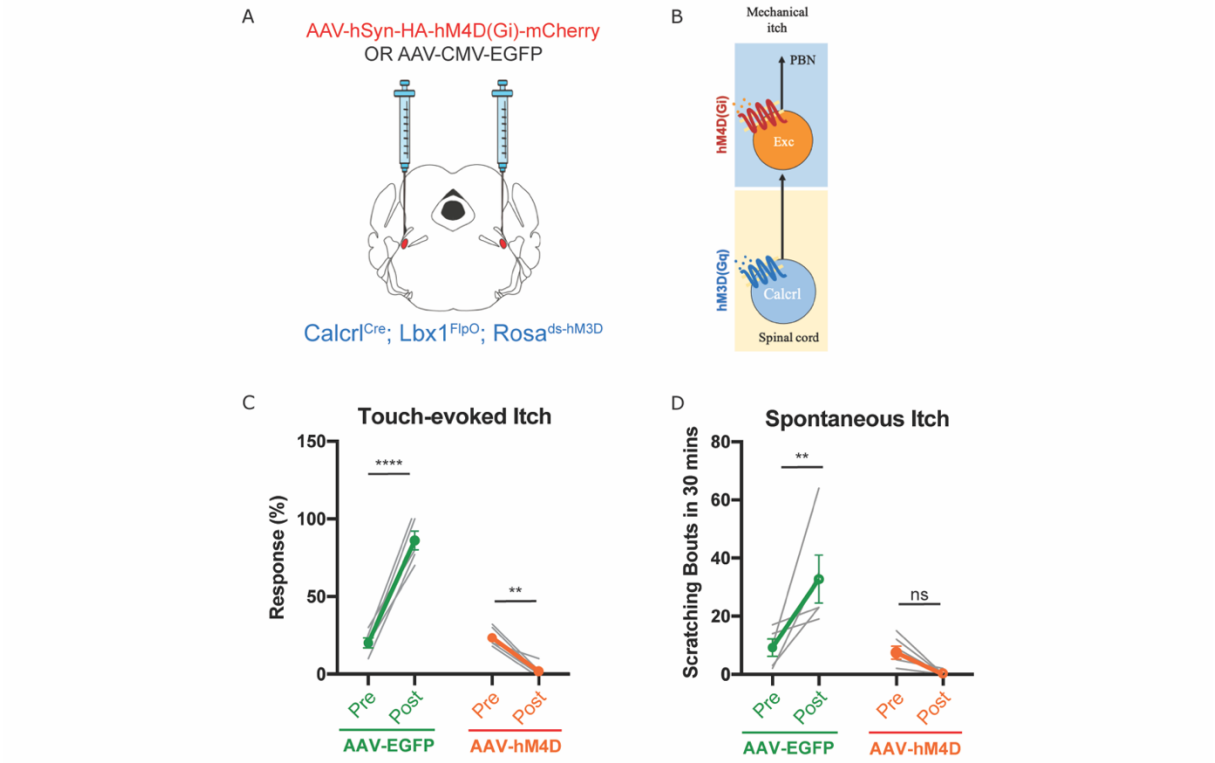

Figure 3.7.11. Silencing the PBN neurons reverses Calcrl^{Lbx1} neuron activation-induced mechanical itch sensitization

(A) Schematic showing bilateral injection of AAV-hSyn-HA-hM4D(Gi)-mCherry or AAV-CMV-EGFP into the PBN of *Calcrl^{Cre}; Lbx1^{FlpO}; Rosa26$^{ds\text{-}hM3D}$* mice. (B) Schematic of simultaneous manipulation of spinal Calcrl^{Lbx1} neurons (activation) and the PBN neurons (silencing) by CNO application in mice. (C-D) Effects of silencing the PBN neurons on touch-evoked itch induced by nape stimulation (C) and spontaneous itch (D) before and after CNO application in *Calcrl^{Cre}; Lbx1^{FlpO}; Rosa26$^{ds\text{-}hM3D}$* mice (AAV-EGFP, n = 5; AAV-hM4D, n = 6). Error bars represent SEM. Two-way ANOVA with multiple comparisons test for (C) and (D); **p<0.01, ****p<0.0001, ns-no significant difference.

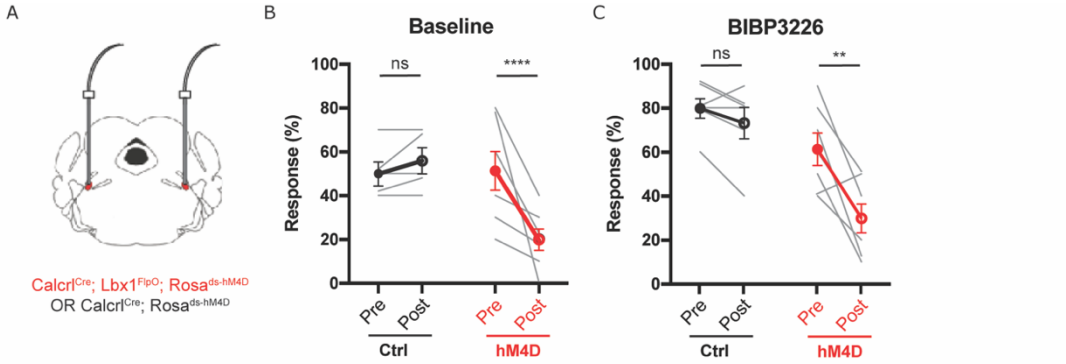

Figure 3.7.12. Silencing of Calcrl^{Lbx1} ascending projections in the PBN reduces touch-evoked itch

(A) Schematic showing bilateral cannula implantation into the PBN of *Calcrl^{Cre}; Lbx1^{FlpO}; Rosa26$^{ds\text{-}hM4D}$* or *Calcrl^{Cre}; Rosa26$^{ds\text{-}hM4D}$* mice. (B) Effect of silencing the Calcrl^{Lbx1} ascending projections in the PBN on touch-evoked itch induced by nape stimulation at baseline level before and after the CNO infusion into the bilateral PBN in mice (Ctrl, n = 5; hM4D, n = 6). (C) Effect of silencing the Calcrl^{Lbx1} ascending projections in the PBN on touch-evoked itch induced by nape stimulation under BIBP3226-induced acute itch sensitization before and after the CNO infusion into the bilateral PBN in mice (Ctrl, n = 5; hM4D, n = 6). Error bars represent SEM. Two-way ANOVA with multiple comparisons test for (B) and (C); **p<0.01, ****p<0.0001, ns-no significant difference.

Chapter 4: FoxP2^{PBN} neurons function the postsynaptic partners of spinal Calcrl neurons in the PBN for relaying mechanical itch

4.1 Introduction

We next asked which is the neuronal population within the PBN that receives and relays mechanical itch signals from spinal Calcrl neurons. Previous study identified CGRP+ neurons in the external lateral nucleus of the PBN (PBNel) as important relay population for chemical itch (Campos et al. 2018). However, considering the PBNel receive limited monosynaptic innervation from spinal neurons, CGRP+ neurons in the PBN are unlikely to be the postsynaptic neurons for the Calcrl neurons. In this case, I undertook a candidate approach to identify the post-synaptic neuron population to spinal Calcrl neurons. FoxP2 is expressed in the superior lateral, dorsal lateral and medial subnuclei as well as Kölliker-Fuse nuclei but not the external lateral subnucleus of the PBN (Geerling et al. 2016).

4.2 Results

4.2.1 FoxP2^{PBN} neurons are postsynaptic partner of spinal Calcrl-lineage neurons

To assess whether spinal Calcrl neurons form synaptic connections onto FoxP2^{PBN} neurons, we crossed *Calcrl^{Cre}; hCdx2::FlpO* mice with the *Rosa26$^{ds\text{-}Synaptophysin\text{-}EGFP}$* effector allele to label all axonal terminals of the spinal Calcrl-lineage neurons with Synaptophysin-EGFP (hereafter referred as Syn-EGFP) (**Figure 4.7.1.A**). In the PBN of adult mice, Syn-EGFP-labeled spinal Calcrl-lineage ascending projections were observed mainly in the dorsal part of the lPBN, including the superior lateral and central lateral subnuclei (**Figure 4.7.1.B**).

Very interestingly, we observed synaptic puncta labeled by synaptophysin-EGFP juxtaposed to FoxP2-immunoreactive cells in the dorsal lPBN (**Figure 4.7.1.B**), suggesting FoxP2^{PBN} neurons might be the post-synaptic partners of Calcrl-lineage neurons in the spinal cord.

4.2.2 FoxP2^{PBN} neurons are tuned to respond mechanical itch stimuli

Next, we examined the function of FoxP2^{PBN} neurons during mechanical itch transmission. We injected a Cre-dependent AAV bilaterally into the PBN of *FoxP2^{Cre}* mice to selectively express the Calcium indicator GCamP7s (or EGFP in control mice) in the FoxP2^{PBN} neurons and performed *in vivo* calcium imaging (fiber photometry) (**Figure 4.7.2.A**). Mice were stimulated 10 times on the nape of the neck using a von Frey hair with an interval of at least 10 seconds between stimulations. The calcium activity of FoxP2^{PBN} neurons were measured before and after the onset of scratching. Remarkably, a significant increase in FoxP2^{PBN} neuron activity was seen just before the onset of touch-evoked scratching (**Figure 4.7.2.B**), suggesting that FoxP2^{PBN} neurons were tuned to sense mechanical itch, even before the reflex response occurs.

A similar elevation of FoxP2^{PBN} neuron calcium activity was observed in mice with histamine-induced acute alloknesis, again just before the touch-evoked scratch response (**Figure 4.7.3.A**). Very interestingly, when we overlaid the two traces of calcium activity, the touch-evoked scratching in histamine alloknesis sensitization induced a significantly stronger neuronal activation of FoxP2^{PBN} neurons (**Figure 4.7.3.B**). This higher level of activity of FoxP2^{PBN} neurons during histamine-induced acute alloknesis implies a strong recruitment of PBN neurons in itch sensitized states.

4.2.3 Chemogenetic silencing of FoxP2^{PBN} neurons reduces mechanical itch sensitivities

In addition to monitoring the calcium activity of FoxP2^{PBN} neurons during mechanical itch, we also performed loss of function study in order to test the necessity of this neuron population during mechanical itch transmission. Cre-dependent AAV-hM4D (AAV-DIO-hM4D) was injected bilaterally into the PBN of *FoxP2^{Cre}* mice to selectively silence FoxP2^{PBN} neurons. Control mice as before were injected with a Cre-dependent AAV-EGFP (AAV-DIO-EGFP) (**Figure 4.7.4.A**). Touch-evoked scratching was substantially reduced after silencing the FoxP2^{PBN} neurons (**Figure 4.7.4.B**). We also observed reduction, albeit to a lesser extent, in the touch-evoked scratching in response to ear stimulation (**Figure 4.7.4.C**). These results show that FoxP2^{PBN} neurons are necessary for modulating the transmission of mechanical itch.

Next, we addressed whether the selective silencing of FoxP2^{PBN} neurons was also sufficient to suppress NPY-modulated mechanical itch sensitization (**Figure 4.7.5.A**). As before, we sensitized mice by injecting the Y1 antagonist, BIBP3226. Control mice showed increased scratch responses to touch-evoked itch and more frequent occurrence of spontaneous scratching. By contrast, FoxP2^{PBN} neuron silencing caused a strong reduction in the BIBP3226-induced mechanical and spontaneous itch (**Figure 4.7.5.A and 4.7.5.B**).

Finally, we assessed whether loss of FoxP2^{PBN} neuron was sufficient to suppress histamine-induced alloknesis. Intradermal injection of histamine in the nape of the neck caused a sensitization of the area and an elevated sensitivity to touch-evoked itch. This sensitization was substantially reduced after CNO application in the AAV-DIO-hM4D-injected compare to controls (AAV-DIO-EGFP injected) (**Figure 4.7.6.A**), demonstrating FoxP2^{PBN} neurons necessity for driving histamine-induced alloknesis.

In summary, we identified FoxP2^{PBN} neurons as a crucial postsynaptic target of spinal Calcrl^{Lbx1} neurons to mediate mechanical itch transmission in physiological and sensitized states. Spinal Calcrl^{Lbx1} and FoxP2^{PBN} neurons form a key relay pathway to transmit and modulate mechanical itch.

4.3 Conclusion and discussion

All the data in Chapter 4 shows that we found a functional postsynaptic partner of Calcrl^{Lbx1} neurons, which is marked by their expression of FoxP2, in the PBN during mechanical itch modulation **(Figure 4.7.1-4.7.6)**. FoxP2PBN neurons form direct synaptic contact with spinal Calcrl-lineage neurons **(Figure 4.7.1)**. FoxP2^{PBN} neurons are activated during mechanical itch-induced scratching under baseline **(Figure 4.7.2)** and histamine-induced acute alloknesis states **(Figure 4.7.3)**. Chemogenetic silencing of FoxP2^{PBN} neurons significantly reduces mechanical itch sensitivities under baseline **(Figure 4.7.4)**, acute mechanical itch sensitization states **(Figure 4.7.5 and .4.7.6)**.

My unpublished data (now shown) also showed that chemogenetic silencing of FoxP2^{PBN} neurons reduces chemical itch sensation. In this sense, both chemical and mechanical itch information from the spinoparabrachial tract converge into the FoxP2^{PBN} neurons and are further relayed to the downstream region as general itch information. Since information of these two itch subtypes are very like transmitted divergently through the spinoparabrachial tract, distinct relay mechanisms may be recruited by the spinal cord (divergence) and the PBN (convergence) to process chemical and mechanical itch.

The mechanical itch information relayed by FoxP2 neurons in the PBN for thought for generating affective components of mechanical itch and providing descending modulation for

mechanical itch-induced scratching. FoxP2^{PBN} neuron population are distributed primarily in the superior lateral (PBNsl), dorsal lateral (PBNdl), central lateral (PBNcl) as well as the medial nucleus, but not the external lateral nucleus (PBNel) of the PBN (Huang et al. 2020). Anterograde labeling of axonal terminals of the FoxP2^{PBN} neurons showed their projections in multiple brain regions, including the periaqueductal grey (PAG) (Huang et al. 2020). The PAG has been reported as a major descending control center for itch modulation: activation of the glutamatergic neurons promotes while activation of the GABAergic neurons inhibits itch (Gao et al. 2019; Samineni et al. 2019). Likely, parabrachial inputs to the PAG from FoxP2^{PBN} neurons may update the sensory feedback of mechanical itch from Calcrl^{Lbx1} spinoparabrachial pathway and drive the next-step itch modulation through the PAG and its downstream nuclei. On another side, CGRP+ neurons in PBNel are important for relaying affective components of somatosensation through their projections to the central amygdala (CeA), a well-known emotion center in the brain (Han et al. 2015). It is possible that FoxP2^{PBN} neurons in the dorsal part of the lateral PBN form mono- or poly-synaptic connection with CGRP^{PBN} neurons, which further relay the mechanical itch information to the CeA for aversion or motivation generation. In the future, manipulation of FoxP2^{PBN} axonal terminals will be needed to figure out the contribution of each of these projections on affection generation and itch modulation.

4.4 Material and methods

Key Resources Table

Reagent or Resource	**Source**	**Identifier**

Antibodies		
Rabbit α-FoxP2 (1:500)	MilliporeSigma	Cat# HPA000382
Chicken α-GFP (1:1000)	Aves	Cat # GFP-1020; RRID:AB_10000240
Bacterial and Virus Strains		
AAV-Ef1a-DIO-hM4D(Gi)-mCherry	Salk Viral Core	
AAV-Syn-DIO-jGCamP7s-WPRE	Salk Viral Core	
AAV-Syn1-DIO-EGFP	Salk Viral Core	
Chemicals		
BIBP3226 trifluoroacetate	Tocris	Cat# C2707/1
Clozapine N-oxide	Sigma	Cat# C0832
Histamine	MilliporeSigma	Cat# H7250
Experimental models: Organisms/Strains		
Mouse: *Calcrl*^{Cre}	Han et al. 2015	
Mouse: *hCdx2::FlpO*	Britz et al. 2015	
Mouse: *Rosa26*$^{ds\text{-}Synaptophysin\text{-}EGFP}$	Niederkofler et al. 2016	
Mouse: *FoxP2*^{Cre}	Palmiter 2018	
Software and Algorithms		
Adobe Illustrator and Photoshop CS5	Adobe	
Prism 8	GraphPad	

ImageJ	Kurt de Vos, Univ. of Sheffiled, UK	
Fiber Photometry System	pyPhotometry	
MatLab	MathWorks	

Experimental Model and subject details

All protocols for animal experiments were approved by the IACUC of the Salk Institute for Biological Studies according to NIH guidelines for animal experimentation. Male and female mice were used in all studies. Animals were randomized to experimental groups and no sex differences were noted. The *FoxP2*^{Cre} knockin mouse line was generated by the Palmiter lab at University of Washington (Palmiter 2018). The following mouse lines were used in this study: *hCdx2::FlpO* (Britz et al. 2015), *Rosa26*$^{ds\text{-}Synaptophysin\text{-}EGFP}$ (Niederkofler et al. 2016).

Immunohistochemistry

P21 *Calcrl*^{Cre}; *hCdx2::FlpO*; *Rosa26*$^{ds\text{-}Synaptophysin\text{-}EGFP}$ mice were euthanized by a single intraperitoneal (i.p.) injection (10 ml/g body weight) of ketamine (10 mg/ml) and xylazine (1 mg/ml) immediately prior to perfusion with 20 mL ice-cold 4% paraformaldehyde in PBS. The brain was dissected and post-fixed for 1h at RT, then cryoprotected in 30% sucrose-PBS (w/v) overnight at 4 degree. Tissue was embedded in Tissue-Tek OCT Compound (Sakura Finetek) and sectioned into coronal slices at 30 μm by cryostat (Leica 3050S). Sections were dried at RT and stored at -20 degree. In the day of immunostaining,

sections were washed once with PBS (10 min), blocked with a solution of 10% donkey serum in PBST (PBS + 0.5% Triton X-100) for 1h at RT and then incubated overnight with primary antibodies in a solution of 1% donkey serum in PBT at 4 degree. Sections were then washed 3 times (10 min each) in PBST before being incubated for 1h at RT with fluorophore–conjugated secondary antibodies (1:1000; Jackson Laboratories) in PBT. Sections were again washed 3 times (10 min each) in PBST before being mounted with Aqua-Poly/Mount (Polysciences). A Zeiss LSM 700 confocal microscope was used to acquire images, and 3-5 brain slices were analyzed. ImageJ software was used to assess immunofluorescence, with thresholds set according to signal intensity (Jensen 2013). The following primary antibodies were used in this study: chicken a-GFP (1:1000; Aves), rabbit a-FoxP2 (1:500; Sigma).

Drug Administration

The preparation of CNO and Y1 antagonist, BIBP3226 was shown in the "Drug administration" of Chapter 2.3.

Viral Injection

10-14 weeks old *FoxP2*^{Cre} mice were placed on a stereotaxic frame (Kopf Instruments) and anaesthetized via inhalation of isoflurane (1.5–2.0%) using an isoflurane vaporizer during the surgery. Body temperature of the animal was maintained with a heating pad underneath the body during the surgery. Ophthalmic ointment was applied to maintain eye lubrication. Burr holes were made on the skull using high-speed stereotaxic drill (Kopf Instruments). For chemogenetic silencing experiments, 500 nl of AAV-Ef1a-DIO-hM4D(Gi)-mCherry (or AAV-Syn1-DIO-EGFP for controls) were injected into the bilateral PBN of the

mice. For the fiber photometry experiments, AAV-Syn-DIO-jGCamP7s-WPRE (or AAV-Syn1-DIO-EGFP for controls) were injected into the bilateral PBN of the mice. The virus was injected by a Nanoinjector (NANOJECT III, Drummond) at a rate of 2 nl/sec through glass pipettes (tip diameter 10-30μm) (Wiretrol II, Drummond). After each injection, the glass pipettes were left in place for 7-8 min before withdrawal. The animals were placed on a heating pad to recover from anesthesia before returning to their home cage. Buprenex SR (0.1 g/kg) was injected intradermally in the back after surgery to provide analgesia.

The coordinates used for stereotaxic injections and implantation were as follows: PBN (-1.00 mm posterior to lambda, ± 1.50 mm from midline and -3.50 ventral from skull). The behavioral tests were performed 21-28 days after the viral injection.

Fiber Photometry

A fiber photometry system (405 and 465 nm Fiber Photometry System, pyPhotometry, USA) was used to record FoxP2^{PBN} neural activities. In the pyPhotometry system, GCaMP isosbestic fluorescence (405-nm excitation) and calcium-dependent fluorescence (465-nm excitation) were recorded with the 1-color time-division setting at 100 Hz, and data were analyzed with custom MATLAB scripts. F_0 was calculated by a least mean squares fitting of the 405-nm channel in reference to the 465-nm channel, and $\Delta F/F$ was calculated as (F_{465}-F_{405_fitted})/F_{405_fitted}.

To measure the neural activity of FoxP2^{PBN} neurons, the FoxP2^{Cre} mice were injected with AAV-DIO-GCamP7s (or AAV-DIO-EGFP for controls) in the PBN and implanted with optic fiber into the PBN. The fiber photometry recording was performed 2 weeks after the

viral injection and fiber implantation. The mice were handled for 3 days prior to the recording day. The mice were also habituated for 1h per day for two days before the recording day in the behavioral testing apparatus with the recording wire connecting to the optic ferrule on top of the mouse head. For recording the neural activity during touch-evoked itch under the baseline level, the mice were first habituated in the apparatus for 30 mins on the day of recording. Then each mouse was then video-recorded for 10 mins, during which 10-15 innocuous mechanical stimuli delivered by a von Frey hair (0.7mN) were applied to the nape of the mice with an interval of 30-40 seconds between two stimuli. For recording the neural activity during touch-evoked itch under histamine-induced acute alloknesis, the mice were first habituated in the apparatus for 30 mins and then injected with histamine (100 mg, MilliporeSigma, St. Louis, MO) in 20 mL saline intradermally into the nape. Each scratching response toward nape stimulation was captured and related neural activity was extracted and analyzed.

Behavioral Testing

Touch-evoked itch

The same as shown in Chapter 2.3.

Spontaneous itch

The same as shown in Chapter 2.3.

Histamine-induced alloknesis

The same as shown in Chapter 3.3.

4.5 Acknowledgements

Chapter 4 is a part of the material as it appears in Ren X*, David A*, Liu S*, Virlogeux A, Han S[#], Goulding M[#]. A Novel Spinoparabrachial Pathway Mediating Mechanical Itch. *In Preparation.* The dissertation author was the primary investigator and the first author of this material. The author would like to thank the Palmiter lab at University of Washington for sharing FoxP2-IRES-Cre mouse line; Shijia Liu for contributing on in vivo fiber photometry imaging experiment; Dr. David Acton for contributing on relevant tests with BIBP3226-induced acute mechanical itch sensitization model; Dr. Amandine Virlogeux for performing the FoxP2 immunostaining with the *Calcrl*^{Cre}*; hCdx2::FlpO; Rosa26*$^{ds\text{-}Synaptophysin\text{-}EGFP}$ mice; Dr. Graziana Gatto for technical assistance and insightful discussion; Dr. Sung Han for insightful discussion; Dr. Martyn Goulding for the experimental design and mentorship; and all the members of the Goulding lab at Salk Institute for comments and inputs.

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4.7 Figures

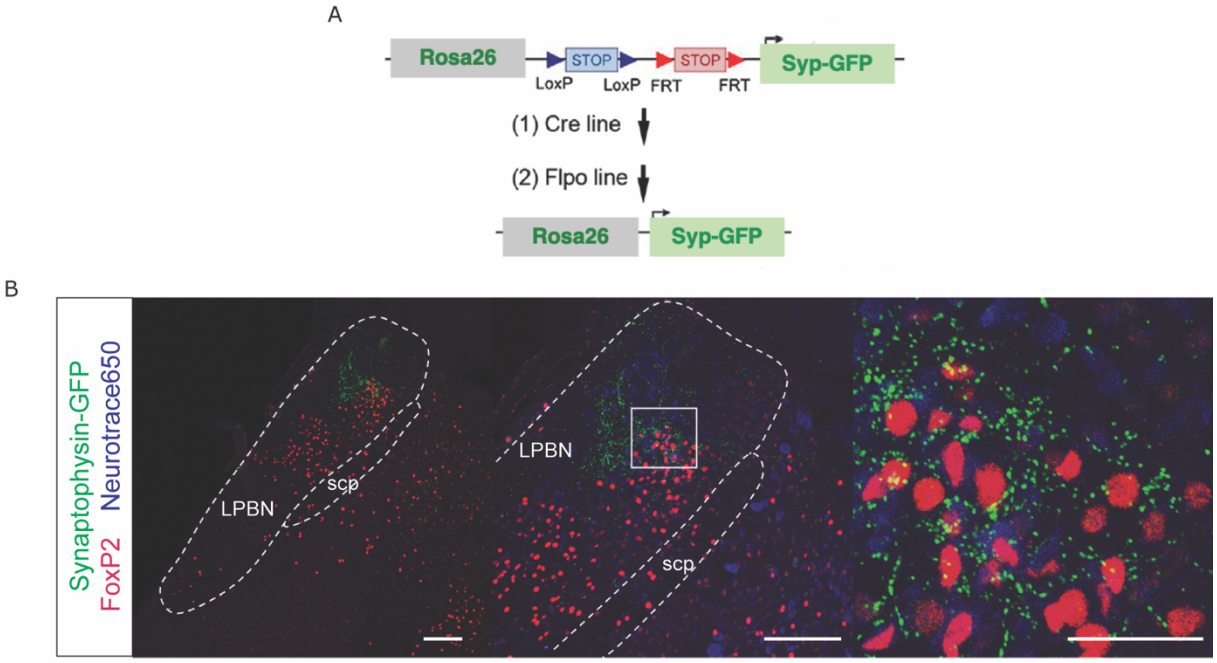

Figure 4.7.1. FoxP2^{PBN} neurons receive putative synaptic contacts from spinal Calcrl-lineage neurons.

(A) Schematic of intersectional genetic strategy for labeling axonal terminals of Calcrl^{Lbx1} neurons with synaptophysin-EGFP (Syp-GFP). (B) Representative IHC images of FoxP2 (red) and Neurotrace650 (blue) staining in the PBN of the brain slices of Calcrl^{Cdx2}-SynGFP (green) mice. Scale bar, 100um.

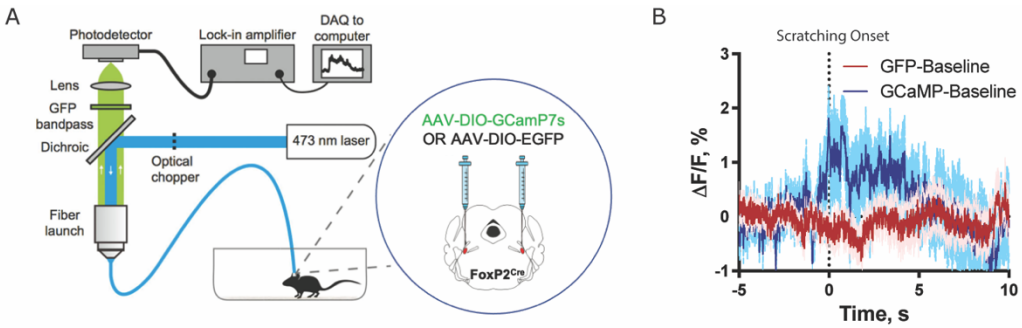

Figure 4.7.2. FoxP2^{PBN} neurons are activated during mechanical itch under baseline level.

(A) Schematic of in vivo fiber photometry calcium imaging using *FoxP2^{Cre}* mice injected with AAV-DIO-GCamP7s or AAV-DIO-EGFP. (B) Mean calcium fluorescence signal of FoxP2^{PBN} neurons in FoxP2^{Cre} mice injected with AAV-DIO-GCamP7s (n = 3, trials = 18) or AAV-DIO-EGFP (n = 2, trials = 10) during touch-evoked scratching. Shaded areas represent SEM.

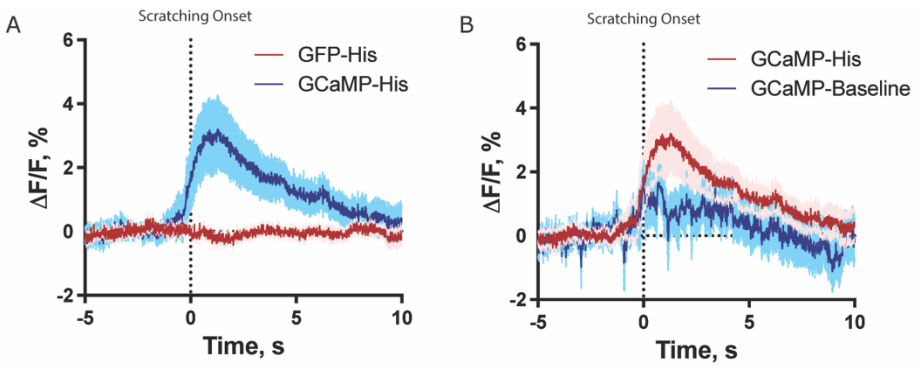

Figure 4.7.3. FoxP2^{PBN} neurons are activated during histamine-induced acute alloknesis
(A) Mean calcium fluorescence signal of FoxP2^{PBN} neurons in *FoxP2^{Cre}* mice injected with AAV-DIO-GCamP7s (n = 3, trials = 18) or AAV-DIO-EGFP (n = 2, trials = 10) during touch-evoked scratching under histamine-induced acute alloknesis. Shaded areas represent SEM. (B) Comparison of the mean calcium fluorescence of FoxP2^{PBN} neurons in *FoxP2^{Cre}* mice injected with AAV-DIO-GCamP7s (n = 3, trials = 18) under baseline and histamine-induced acute alloknesis. Shaded areas represent SEM.

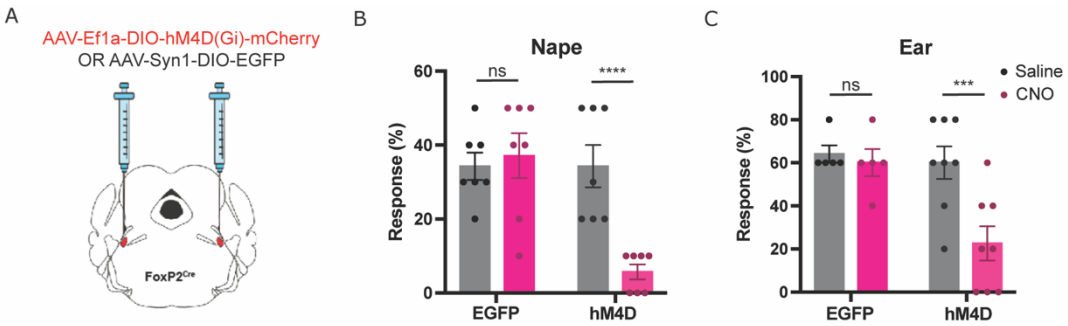

Figure 4.7.4. Chemogenetic silencing of FoxP2^{PBN} neurons reduces touch-evoked itch in different skin regions in mice

(A) Schematic showing bilateral injection of AAV-DIO-hM4D-mCherry or AAV-DIO-EGFP into the PBN of *FoxP2^{Cre}* mice. (B) Effect of chemogenetic silencing of the PBN on the touch-evoked itch induced by nape stimulation *FoxP2^{Cre}* mice (AAV-EGFP, n = 7 mice; AAV-hM4D, n= 7 mice). Error bars represent SEM. (C) Effect of chemogenetic silencing of the PBN on the touch-evoked itch induced by ear stimulation in *FoxP2^{Cre}* mice (AAV-EGFP, n = 5 mice; AAV-hM4D, n= 8 mice). Error bars represent SEM. Two-way ANOVA for multiple comparisons test for (B) and (C). ***p<0.001, ****p<0.0001, ns-no significant difference.

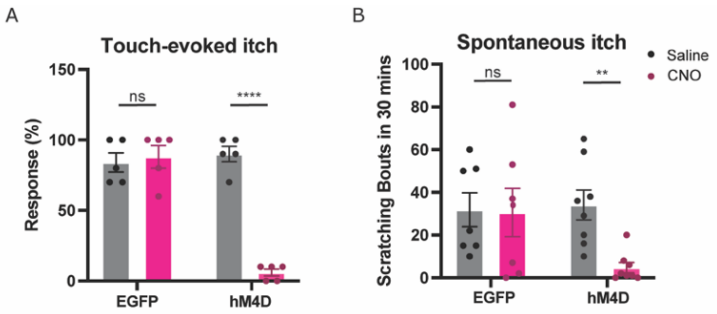

Figure 4.7.5. Chemogenetic silencing of FoxP2^{PBN} neurons suppresses BIBP3226-induced touch-evoked and spontaneous itch

(A) Effect of chemogenetic silencing of the PBN on the touch-evoked itch induced by nape stimulation under BIBP3226-induced acute mechanical itch sensitization in *FoxP2^{Cre}* mice (AAV-EGFP, n = 5 mice; AAV-hM4D, n= 5 mice). (B) Effect of chemogenetic silencing of the PBN on the spontaneous itch in *FoxP2^{Cre}* mice (AAV-EGFP, n = 7 mice; AAV-hM4D, n= 8 mice). Error bars represent SEM. Two-way ANOVA for multiple comparisons test for (A) and (B). **p<0.01, ****p<0.0001, ns-no significant difference.

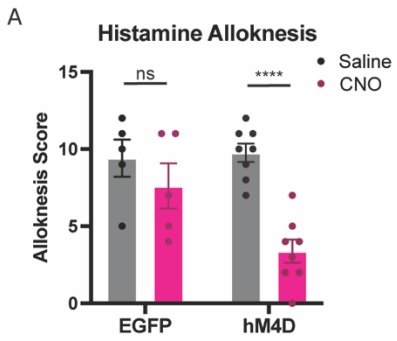

Figure 4.7.6. Chemogenetic silencing of FoxP2^{PBN} neurons reduces touch-evoked itch under histamine-induced acute alloknesis

(A) Effect of chemogenetic silencing of the PBN on the touch-evoked itch induced by nape stimulation *FoxP2^{Cre}* mice under histamine-induced acute alloknesis (AAV-EGFP, n = 5 mice; AAV-hM4D, n= 8 mice). Error bars represent SEM. Two-way ANOVA for multiple comparisons test for (A). ****p<0.0001, ns-no significant difference.