Uncovering a Monosynaptic Trigemino-parabrachial Circuit
Facilitating Heightened Craniofacial Pain Perception

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Neurobiology in the Graduate School of Duke University

2017
ABSTRACT
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Abstract

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

This dissertation is dedicated to my grandparents, Ana and Jose Rodriguez, Leticia Campos, my parents, Myriam and Alberto Rodriguez, my sister, Amanda Rodriguez, and my fiancée, Tyler Gibson.
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1. Introduction

Chapter 1 includes modifications on text and figures previously published in the F31-Diversity fellowship grant proposal awarded by NIDCR on February 2015.

1.1. Specific aims and hypothesis

Humans generally perceive craniofacial nociception as more severe than bodily nociception\(^1\)\(^2\). However, the mechanism behind this phenomenon is unclear. Gross anatomical studies have revealed that pain-related sensory information is delivered to the brain via two main processing streams. One pathway is from the dorsal horn or nucleus caudalis to the thalamus and then to the somatosensory cortex to process discriminative aspects of pain; the other is from a different subset of dorsal horn projection neurons to the lateral parabrachial nucleus (PB\(L\)), and then to various limbic regions (amygdala, hypothalamus, insular cortex, etc.), presumed to process the emotional and affective aspects of pain\(^3\)\(^5\).

PB\(L\) receives and processes somatic and visceral sensory inputs, as well as taste-related inputs\(^6\)\(^-\)\(^19\). Aside from anatomical tracing, evidence supporting the involvement of PB\(L\) in pain processing was largely derived from studies showing
the induction of the immediate early gene c-Fos in PBl upon painful stimuli. It is generally believed that activation of PBl neurons leads to the generation of “painful emotions”, as well as potentially eliciting descending pain inhibition to help cope with pain. Regardless of these apparent paradoxical functions from possibly distinct PBl neuronal populations, PBl could be mediating the differential processing of craniofacial and bodily nociception. Unfortunately, due to the lack of molecular tools, it has not been feasible to study the precise connectivity and causal functions of distinct PBl neurons activated by selected noxious stimuli.

Therefore, the objective of this investigation is to identify the input-output circuit of the noxious stimuli-activated PBl neurons and determine a potential mediator contributing to the differential processing of craniofacial and bodily nociception. This is accomplished using a highly innovative method, Capturing Activated Neuronal Ensembles (CANE), recently developed by our lab to investigate PBl neural circuits involved in processing noxious craniofacial stimuli in the mouse brain. We further uncovered and characterized the potential facilitator contributing to the differential processing of craniofacial and bodily nociception through slice electrophysiology and optogenetic activating/silencing behavioral paradigms. The rationale is that there may be
uncharacterized neuronal pathways related to affective pain processing that innervate the PB\textsubscript{L} that could be contributing to differential processing of craniofacial and bodily nociception, based on previous studies suggesting that primary sensory afferents are terminating in PB\textsubscript{L}\textsuperscript{33,34}. The long-term goal of this research is to refine the affective trigeminal pain circuit model and elucidate its role in heightened craniofacial pain perception, and possible role in chronic craniofacial pain disorders.

1.1.1. Test the hypothesis that PB\textsubscript{L} is more robustly activated by noxious craniofacial stimuli than by noxious bodily stimuli.

Humans perceive noxious craniofacial stimuli as more severe than noxious bodily stimuli\textsuperscript{2,35}. In parallel, fMRI studies reveal that there is robust activity in some of the limbic regions (i.e. CeA) as a result of application of noxious craniofacial stimuli in contrast to noxious bodily stimuli\textsuperscript{36}. This differential processing could be mediated by increased activity in the PB\textsubscript{L}, the gateway node of the affective pain pathway and a direct source of input to these various limbic regions\textsuperscript{37,38}. Therefore, determining the underlying presynaptic circuitry facilitating the activity changes in PB\textsubscript{L} can shed some light on whether the affective pain pathway is mediating this heightened qualitative pain processing. This aim analyzes the activity of the PB\textsubscript{L} in response to noxious
chemical stimuli injected in the whiskerpad or hindpaw of the mouse using immunohistochemistry (IHC) detection of c-Fos protein, an activity-dependent marker and surrogate for neuronal activity. This will provide insight into the differential activation of PB₄ neurons in the mouse and the PB₄’s role in valence detection. This will further characterize the possible diverse cell types of all noxious-stimuli activated PB₄ neurons.

_Hypothesis 1:_ Noxious chemical stimuli injected into the mouse whiskerpad will induce more neuronal activity in PB₄ as measured by c-Fos expression than comparable stimuli injected into the hindpaw, based on previous c-Fos IHC studies in the rat²⁰,²¹. Since the PB₄ is in the affective pathway and not discriminative pathway, c-Fos expression may be dependent on the intensity of the stimulus. Furthermore, based on _in situ_ hybridization results in the Allen Brain Atlas indicating that the majority of neurons in PB₄ are vGlut2+, noxious stimuli-activated neurons in PB₄ will consist of mostly glutamatergic neurons and a few GABAergic interneurons. Additionally, based on previous findings from Palmiter’s group on the role of CGRP⁺ neurons in PB₄ in affective pain perception and other studies on the role of FoxP2⁺ neurons in PB₄ in sodium deprivation, a percentage of neurons in PB₄ are CGRP⁺ and/or FoxP2⁺¹¹⁻¹³.
1.1.2. Examine where noxious stimuli-activate PB₇ neurons project to and what their presynaptic inputs are.

Levels of activity in any brain region can be modulated and driven by dynamic activity and number of presynaptic inputs. Therefore, determining the presynaptic inputs of noxious stimuli-activated PB₇ can reveal a difference in the level of connectivity from presynaptic inputs within the trigeminal pathway versus the spino-parabrachial pathway. This aim investigates the input-output circuit of selectivity tagged noxious stimuli-activated PB₇ neurons using CANE. This activity-dependent capturing system provides a more refined and selective outlook on the affective pain pathway stemming from the PB₇.

**Hypothesis 2:** In a recent study published in our lab, the CANE system efficiently and specifically captured activated neuronal ensembles in the ventrolateral medial hypothalamus (VMH)³². Therefore, the CANE system can reliably capture noxious stimuli-activated PB₇ neurons. This tool can facilitate in the visualization of axonal projections of noxious stimuli-activated PB₇ neurons. Similar to previous findings from anterograde studies of PB₇, CANE-captured noxious stimuli-activated PB₇ neurons will also project to PAG, BNST, CeA, hypothalamus, and insular cortex, but in a more restricted manner⁴,¹⁰. Previous studies have demonstrated that there are TrpV1⁺ afferents in PB₇, and have
hinted, but not demonstrated that these may be direct primary sensory inputs in PB\textsubscript{L}\textsuperscript{33,34}. Consequently, this leaves the possibility that the source of TrpV1\textsuperscript{+} afferents may be the primary sensory neurons located in the trigeminal ganglia. Therefore, transynaptic tracing of CANE captured noxious stimuli-activated PB\textsubscript{L} neurons and retrograde/anterograde tracing of TrpV1\textsuperscript{+} primary sensory neurons can demonstrate that a subset of PB\textsubscript{L} neurons that receives previously uncharacterized direct inputs from the trigeminal ganglion.

1.1.3. Investigate the physiological and behavioral function of the direct trigemino-parabrachial circuit in noxious craniofacial perception.

Previous studies have demonstrated that optogenetic/electrical activation of molecular/anatomical subsets of PB\textsubscript{L} neurons results in affective pain-like behavior, such as malaise, avoidance, and escape behavior\textsuperscript{11,26}. Additionally, optogenetic activation/silencing of Trpv1\textsuperscript{+} afferents innervating the skin results in changes thermal pain threshold\textsuperscript{39,40}. However, these findings do not definitely demonstrate whether the direct trigemino-parabrachial circuit plays a role in affective pain perception. This aim examines the synaptic connection between TrpV1\textsuperscript{+} afferents in PB\textsubscript{L} using optogenetic-assisted slice electrophysiology, elucidates the role of the direct trigemino-parabrachial circuit in affective pain perception using optogenetic activation/silencing during real time and
conditioned place preference tasks and determines whether this is specific to craniofacial pain using a von frey task while the animal experiences allodynia.

*Hypothesis 3:* Primary sensory TrpV1+ afferents in PBt can excite PBt neurons, and induced optogenetic stimulation of TrpV1+ afferents can result in an aversive behavior during a real-time place escape/avoidance task and aversive memory in a conditioned place avoidance assay. Optogenetic silencing of primary sensory TrpV1+ afferents in PBt can reduce craniofacial allodynia and induce preference in a real-time place preference task.

### 1.2. Background and Significance

Pain perception serves a crucial, protective role in preserving bodily homeostasis by reducing exposure to harmful stimuli and preventing further tissue damage, such as a burn, a bite, or excessive pressure. This is essential for an organism’s survival. Pain is a complex subjective experience that arises from nociception, the processing of noxious stimuli and a crucial aspect of the sensory nervous system. These noxious chemical, thermal, and mechanical stimuli are transduced, transmitted, and processed within the peripheral nervous system (PNS) and central nervous system (CNS) into acute pain perception and pain-related behavior⁴¹. In recent years, human psychometric studies have revealed that craniofacial nociception is qualitatively perceived as much more severe than
bodily nociception, despite similar pain intensities\textsuperscript{2,35}. These qualitative findings are further supported by fMRI studies demonstrating that craniofacial pain resulted in higher levels of amygdala activation compared to the same intensity stimulation applied to the hand\textsuperscript{36}. Despite these studies, the neurobiological underpinning for heightened craniofacial pain remained enigmatic.

Nociceptive signals are delivered via two main processing streams: discriminative vs affective/emotional. The less well understood affective/emotional pain pathway is thought to stem from the parabrainstem\textsuperscript{3-5}. Additionally, previous studies in the rat have shown that there is more robust c-Fos expression in PB\textsubscript{L} as a result of formalin injection into the whiskerpad in contrast to formalin injection into the hindpaw\textsuperscript{20,21}. Therefore, efficiently and selectively dissecting the PB\textsubscript{L} neural circuits involved in processing noxious craniofacial pain in mouse could provide greater insight into the neural mechanisms underlying heightened craniofacial pain. However, PB\textsubscript{L} contains an anatomically and molecularly diverse set of neurons that receive and regulate a multitude of visceral, affective, and emotional responses\textsuperscript{6-8,11,14,17,19}. Due to the lack of sufficiently precise molecular tools, it has not been possible to interrogate the entire population of PB\textsubscript{L} neurons activated by specific type of noxious stimuli. Hence, this will require the use of
more precise activity dependent capturing methods, such as the recently
generated highly innovative method, CANE, which allows expression of
fluorescent proteins (XFP), or opsins that can activate, or silence neuronal
activities in Fos+ neurons in any desired brain regions. This section discusses the
fundamental neural circuitry underlying the processing of nociception into
discriminative and affective pain perceptions, the differential circuit and activity
patterns of craniofacial versus bodily nociception, example of a chronic
craniofacial pain disorder and the current palliative methods of alleviating this
disorder, and how the use of the activity-dependent capturing method, CANE,
in combination with transgenic mouse models would facilitate in accurately
mapping the coarsely characterized affective craniofacial pain pathway.

1.2.1. Differential perception of craniofacial nociception versus bodily
nociception

It is thought that due to the location of organs serving vital functions such
as the brain, the sensory organs, organs for breathing, food intake and vocal
communications etc., noxious information in the head and face area may have
unique biological significance and therefore may be processed differently than
identical stimuli applied to other peripheral body parts. Human studies have
demonstrated that craniofacial nociception is qualitatively different from bodily
nociception. In previous psychometric studies, human subjects were presented with a similar intensity noxious thermal stimulus to the facial region versus the forearm region. These human subjects generally rank head and facial pain as much more severe and emotionally draining than body pain. Repeated application of the noxious thermal stimulus to the face induced sensitization, yet similar stimulation applied to the hand induced habituation\textsuperscript{2,35}. Fear induced by pain in human subjects was rated higher for face than for extremities, despite comparable ratings of the pain intensity\textsuperscript{2,35}. Furthermore, fMRI studies revealed that face pain resulted in higher levels of amygdala activation compared to the same intensity stimulation applied to the hand\textsuperscript{36}.

Nociceptive pain usually appropriately encodes noxious stimuli and normally acts as a warning for the organism that there is tissue damage. Nevertheless, pain can be neuropathic due to maladaptive plasticity occurring as a result from a lesion or disease affecting the somatosensory circuit. This type of pain generally serves no biological advantage for the organism and typically induces distress. When an organism experiences neuropathic pain, they undergo allodynia, the manifestation of sharp and burning painful sensations to non-painful stimuli and hyperalgesia, and also undergo the occurrence of increased pain sensation to painful stimuli. One example of a severe neuropathic
craniofacial disorder is trigeminal neuralgia. This disorder arises from the somatic sensory branches of the trigeminal nerve being compressed/lesioned, resulting in sharp, stabbing, or electro-shock pain lasting seconds to minutes. This chronic pain impacts the patient’s quality of life, which exacerbates the patient’s risk of developing affective comorbidities such as depression, anxiety, and sleep disorders. Although the mechanism of trigeminal neuralgia is not thoroughly understood, there are some pharmacological and surgical treatments\(^1\). A primary pharmacological treatment consists of antiepileptic medication targeting voltage-gated sodium channels. However, there is a percentage of pharmacological-resistant trigeminal neuralgia, in which surgical treatments, such as trigeminal tractotomy (TR) or doral root entry zone (DREZ), are typically considered. During these invasions procedures, the descending nociceptive trigeminal tractus innervating the medulla (Sp5C) is ablated, resulting in pain relief\(^{42-45}\). Although these procedures are effective in most patients initially, there is a recurrence of chronic pain in a percentage of patients, and repeating the procedure is usually less effective\(^{43-45}\).

Taking together the knowledge that humans perceive facial nociception as more severe than bodily nociception, and that trigeminal neuralgia is a terrible neuropathic facial pain disorder with an ambiguous neural mechanism, the
neurobiological underpinning for heightened craniofacial pain remains enigmatic.

**1.2.2. Initial signaling of a noxious stimulus**

Noxious chemical, thermal, and mechanical stimuli are detected and transduced by nociceptors which are highly specialized primary afferent sensory fibers whose cell bodies reside in the trigeminal ganglion in the facial region and the dorsal root ganglia in the torso region. These primary sensory neurons are pseudo-unipolar, in which their axons bifurcate and innervate both the peripheral tissues, for environmental detection and throughout the central nervous system transmitting noxious neural information. These nociceptors can be generally classified into two main groups: (1) thinly-myelinated medium-diameter “fast transmitting” Aδ-nociceptors which are activated by a specific noxious stimulus and (2) unmyelinated small-diameter “slow transmitting” C-nociceptors which are activated by various noxious stimuli. The C-nociceptors can be further divided into peptidergic or non-peptidergic C-nociceptors. Peptidergic C-nociceptors synthesize and release substance P and calcitonin-related gene peptide, express tropomyosin receptor kinase (TrKA) receptors and transient receptor potential cation channel member 1 (TRPV1). Generally these peptidergic C-nociceptors are activated by noxious heat stimuli (44°C),
capsaicin, or an acidic pH, resulting in a burning pain sensation. These nociceptors typically cause a long-term depolarization of neurons in the dorsal horn/Sp5C. Non-peptidergic C-nociceptors express purinergic receptor P2X3 or MRGPRD and are dependent on glia-derived neurotrophic factor (GDNF)\textsuperscript{47,48}. Little is known about their role, except that they transduce mechanical noxious stimuli\textsuperscript{47}.

These painful electrical signals are relayed through central axons terminating in a laminar fashion within the medullary/trigeminal or lamina I/IV of the spinal dorsal horn\textsuperscript{41}. Lamina I is innervated by both A\textsubscript{\delta}-nociceptors and peptidergic C-fibers, lamina II\textsubscript{o} is innervated by peptidergic C fibers, and lamina I\textsubscript{i} is innervated by non-peptidergic C-fiber\textsuperscript{49}. Lamina III and IV receive mostly A\textbeta-fibers that relay tactile information, and do not receive any nociceptive inputs. Lamina V is innervated by A\textbeta-fibers, A\textdelta-nociceptors, and some peptidergic C-fibers\textsuperscript{49}. Although the dorsal horn and Sp5C primarily consists of inhibitory and excitatory interneurons that modulate the local circuit, there are ascending projection neurons mainly located in lamina I and V that relay painful information to the brain\textsuperscript{50}. Although the general connectivity within the dorsal horn and Sp5C remains vague, it is certainly known that the nociceptive signals
emitted by the projection neurons are integrated and processed within the CNS into pain perception and pain-related behavior\(^3\).

1.2.3. Discriminative and affective pain pathways

Painful sensory information is delivered via two main processing streams: discriminative vs affective/emotional (Fig. 1.1). The discriminative aspects of pain comprise of the quality and location processing. In contrast, the affective/emotional aspect of pain comprises of the negative valance component of pain perception\(^3\)–\(^5\). The integration of the two dimensions of nociception contributes to the individual's overall pain experience.

The neural pathway necessary to process sensory discriminative aspects of pain has been extensively mapped. Second order neurons in the medullary/trigeminal or lamina I/IV of the spinal dorsal horn send axon projections to discrete subdivisions of the thalamus known as the ventral posterior lateral nucleus and the ventromedial nucleus in topographic fashion. Neurons in these thalamic nuclei then send projections to primary and secondary somatosensory cortices, which consequently result in the cognitive processing of the location and quality of pain\(^3\)–\(^5\).

However, the pathway presumed to process the emotional and affective aspects of pain is less well understood, and it is thought to involve a different
subset of dorsal horn projection neurons that project to the parabrachial nucleus (PB) located in the brainstem. Anatomical tracing studies have shown that PB commonly projects to various limbic structures that process emotions, such as the central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST), and lateral hypothalamis area (LHA). These in turn send projections to the insular cortex, cingulate cortex, and prefrontal areas. As the CeA and BNST are known to be involved in the acquisition and expression of emotion, arousal, and attention, the pathway through PB is thought to play central roles in the generation of unpleasant, affective responses to nociceptive inputs and motivation to escape/avoid a noxious stimulus.

Figure 1. 1. Schematic of Affective and Discriminative Pain Pathways.
1.2.4. The lateral parabrachial nucleus: a molecularly and physiologically diverse structure

Previous studies have suggested that PB receives and processes somatosensory, visceral, and taste-related inputs. These roles can be further assigned to different subdivisions of PB: lateral PB (PBₗ) and medial PB (PBₘ) divisions, as well as sub-nuclei within PBₘ and PBₗ (Fig. 1.2). PBₘ is thought to signal gustatory and taste information, whereas PBₗ is believed to signal aversive and appetitive taste, somatosensory, and visceral information. PBₗ, in turn, regulates autonomic processes, such as heart rate, digestion, respiratory rate, and perspiration⁶⁻⁸,¹¹⁻¹⁴,¹⁷⁻¹⁹.

Aside from anatomical tracing, evidence supporting the involvement of PBₗ in pain processing was largely derived from studies showing the induction of the immediate early gene c-Fos in PBₗ upon noxious stimuli²⁰⁻²³. Furthermore, Palmiter’s group recently further characterized a molecularly distinct subset of neurons in the external lateral portion of PBₗ that play a role in generating affective aversive and malaise behavior in mice¹¹,²⁶. Thus, it is generally assumed that activation of PBₗ neurons leads to the generation of “painful and unpleasant emotions”. However, some pharmacological inactivation studies also suggested PBₗ may elicit descending pain inhibition (anti-nociception). This modulation of
nociceptive information processing is based on the type of noxious stimulus and the individual’s behavioral state and is termed “Diffuse Noxious Inhibitory Controls” (DNIC)\textsuperscript{30,31,51,52}. These apparent paradoxical functions might have resulted from neurons in different divisions of PB\textsubscript{l} playing different anti- versus pro-nociception functions. Furthermore, PB\textsubscript{l} extends rostro-caudally from pons to midbrain, and it is possible that the caudal pontine region and rostral mesencephalic region of PB\textsubscript{l} have different roles in addition to the lateral-medial differences (Fig. 1.2). Unfortunately, due to the lack of molecular tools, it was not possible to study the precise connectivity and causal functions of different PB neurons activated by specific type of painful stimuli.

Figure 1.2. Schematic drawing of PB.

PB is divided into two regions: Medial PB (PB\textsubscript{m}) and Lateral PB (PB\textsubscript{l}). PB\textsubscript{l} is further subdivided into superior lateral (sl), caudal lateral (cl), dorsal later (dl), Ventral lateral (vl), external lateral (el), and Kölliker-Fuse (KF).
1.2.5. PB\textsubscript{L} receives thermal nociceptive inputs

Previous studies have shown the induction of cFos in PB upon noxious heat stimulation or capsaicin injection. Capsaicin, which is found in hot peppers, is known to activate TRPV1 expressed by thermal nociceptive neurons\textsuperscript{53}. Thus, capsaicin application has often been used to mimic noxious heat. Interestingly, a previous study using Trpv1::PLAP mice revealed that that fibers from a possible primary afferent source of Trpv1-lineage neurons were present in the PB\textsubscript{L}, especially in the PB-el, raising the possibility that thermo-noxious information from sensory neurons could directly activate PB\textsubscript{L} neurons instead of through relays in the spino/trigeminal dorsal horn\textsuperscript{33,34}. The authors speculated that the Trpv1\textsuperscript{+} fibers may have emerged from TG neurons which could provide an alternative circuit contributing to craniofacial pain experience. Furthermore, Panneton et al, observed labeled afferent fibers in regions near PB\textsubscript{L}, when neural tracer WGA-HRP was injected into the peripheral anterior ethmoidal nerve (AEN), which is originated from TG and innervate the nasal cavity\textsuperscript{54}. In a follow-up study, the authors showed that trigeminal rhizotomy resulted in loss of CGRP expressing fibers innervating the PB\textsubscript{L}\textsuperscript{55}. These and other studies have implied a possible direct TG→PB\textsubscript{L} pathway, but did not provide synaptic or behavior evidence to support this possibility.
1.2.6. (C)apturing (A)ctivated (N)euronal (E)nsembles

To investigate the PB\textsubscript{e} neural circuits involved in processing noxious thermal (orofacial-) pain in mouse, I will use a highly innovative method called “CANE” for capturing activated neuronal ensembles with mutant lentivirus (*LV), short named as CANE*LV (Fig 1.3). This was recently developed within the past seven years and published by our lab\textsuperscript{32}. CANE is designed to have two components that together allow tagging and manipulating strongly activated neurons that induce Fos-expression: (1) a knock-in mouse line where Fos expression triggers co-expression of a destabilized TVA receptor (Fos-dsTVA); and (2) engineered EnvA\textsuperscript{*} (explained below) coated lentivirus that only infect neurons expressing TVA (Fig. 1.3). In Fos-dsTVA line, TVA is an avian receptor for EnvA-coated viruses, and 2A-peptide enables co-translation of Fos and dsTVA. TVA receptor is fused with a “PEST” sequence that targets it for rapid degradation (dsTVA). Timed-injection of the EnvA\textsuperscript{*}-coated virus into the mouse’s brain allows us to selectively infect and even permanently tag neurons transiently activated in a physiological behavior. Since all neurons have none-zero basal level expression of Fos, to avoid background infection, we introduced 3 amino-acid mutations in the wildtype EnvA coat protein to generate EnvA\textsuperscript{*},
such that EnvA* binds TVA with low affinity and EnvA*-coated virus could only infect neurons with high-level Fos/dsTVA expression. Currently, we engineered EnvA*-coated lentiviruses (*LVs) expressing either fluorescent protein (XFP) or Cre-recombinase (*LV-Cre). Because LVs are non-toxic and enable stable transgene expression, they are ideal for introducing various transgenes into the captured ensembles.

**Figure 1.3. Schematic representation of the methodology for CANE.**

This exciting new technology will facilitate in testing the central hypothesis that: noxious stimuli-activated PBi neurons differentially process craniofacial nociception and bodily nociception and this may be mediated by a distinct trigeminal pathway. This hypothesis will be tested using a combination of the CANE*LV method, optogenetic tools, slice electrophysiology, and behavioral tasks.


2.2. Introduction

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

‘Suffering’ and ‘fear of pain’ are emotional aspects of pain that are not processed by the canonical discriminative pathway via the spino–thalamic–cortical somatosensory circuits. Instead, these feelings are relayed by the less-studied affective pain pathway, where nociceptive afferent information is routed from second-order neurons to PBₗ on to various limbic regions, such as the central amygdala (CeA), the bed nucleus stria terminalis (BNST), the lateral hypothalamus (LHA), the anterior cingulate and the insular cortices (also known as the spino–parabrachial circuit). Interestingly, it has been suggested that subregions of the PBₗ, a critical relay node in the affective pain circuit, might be differentially activated by noxious stimuli applied to the face versus the extremities in rats.
In this study, we show that painful stimuli applied to the face activate more PB\textsubscript{L} neurons, and do so more bilaterally, than those applied to the paw. We utilize our novel activity-dependent technology, called CANE\textsuperscript{32}, to identify PB\textsubscript{L}-nociceptive neurons and their connections with the affective pain system. We further discover the circuit mechanism underlying the more robust activation of PB\textsubscript{L} by noxious facial stimuli and show that activation of this circuit drives strong aversive behaviors, whereas its inhibition specifically reduces craniofacial nociception.

2.3. Materials and Methods

2.3.1. Animal Statement

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

2.3.2. Animals

Adult (P30-P60) male and female C57B/L6 mice (Jackson Laboratory) were used for immunohistochemistry and in situ hybridization. Male and female Fos\textsuperscript{TVA} mice\textsuperscript{32} (Jackson Laboratory, stock 027831) were used for capturing PB\textsubscript{L}-nociceptive neurons with the CANE technology, immunohistochemistry, electrophysiology, and input-output circuit mapping.
Male and female Ai65D\textsuperscript{57} mice expressing a Cre and Flp double-dependent STOP cassette in front of the tdTomato reporter (Jackson Laboratories, stock 024109) were used for Cre/FlpO based tracing of TrpV1Cre\textsuperscript{+} neurons that project to PB\textsubscript{l}. Male and female TrpV1-Cre mice\textsuperscript{53} were used for behavioral testing for both ChR2 or eArch experimental and GFP control groups, as well as electrophysiology experiments. Male and female Ai32 mice expressing a Cre-dependent ChR2 (Jackson Laboratories, stock 024109) were used for electrophysiology experiments. All mice were housed in a vivarium with normal light/dark cycles in cages with 1–5 mice. A day before experiments, we singly housed mice. We used two exclusion criteria for our subjects: (1) poor recovery or other health concerns following surgical intervention or (2) missed injection or implantation target, as determined by histological analysis. Animals were randomly selected from each litter. Random group allocation was maintained throughout the study, within constraints set by availability of in-house, purpose-bred lines. Experimenter blinding was sufficient to control for selection bias. Furthermore, behavioral analysis relied on objective, automatized measurements.
2.3.3. Viruses

CANE-LV-Cre (titer, $5 \times 10^8$ ifu/ml; pLenti-hSynapsin-Cre-WPRE [Addgene Plasmid #86641]; CANE-LV envelope [Addgene Plasmid #86666]) and CANE-RV-mCherry (titer, $5 \times 10^8$ ifu/ml) were produced as previously described\textsuperscript{32}. FuGB2-coated RG-LV-hSyn-FlpO and RG-LV-hSyn-DIO-FlpO were produced and concentrated as described previously\textsuperscript{58}. pAAV-SynP-DIO-TVA-EGFP-RG (pAAV-SynP-DIO-sTpEpB)\textsuperscript{59} was packaged in serotype AAV2/rh8 by the University of Pennsylvania Vector Core. AAV-CAG-flex-GFP, AAV-EF1$\alpha$-flex-ChR2(H134R)-eYFP\textsuperscript{60} and AAV-EF1$\alpha$-DIO-eARCH-eYFP\textsuperscript{61} were purchased from the University of Pennsylvania Vector Core.

2.3.4. Surgery

Animals were anesthetized with isoflurane in a stereotaxic frame (David Kopf Instruments) and small craniotomies were made over the target area. To target the PBt, mice were mounted in the stereotaxic frame at an angle such that lambda was $\sim 180 \mu m$ ventral to bregma (in practice, 140–240 $\mu m$). The stereotaxic coordinates of virus injection and custom-made optic fiber (200 $\mu m$ core diameter, Thorlabs) were AP $- 4.25 \pm 0.15$ mm, ML $1.45 \pm 0.15$ mm, and DV $-3.2 \pm 0.1$ mm. The thin glass capillary was slowly lowered to the target site to minimize the brain injury. Virus was delivered into the target site at a flow
rate of 100 nl per min using a pulled thin glass capillary (Warner Instruments) connected to an UltraMicroPump controlled by a SYS-Micro4 Controller 15 (World Precision Instruments).

For transsynaptic labeling experiment, CANE-LV-Cre and AAV-SynP-DIO-TVA-EGFP-RG were co-injected in animals subjected to 4% formalin injection; injected animals were singly housed for 2 weeks followed by CANE-RV-mCherry injection. For retrograde labeling experiment, RG-LV-hSyn-FlpO or RG-LV-hSyn-DIO-FlpO were injected in TrpV1Cre::Ai65D animals.

For neonatal intraperitoneal (IP) injections, postnatal day 1–2 pups were anesthetized with hyperthermia. 6 weeks after neonatal IP injection, mice were subjected to bilateral implantation of a custom-made optic fiber. After another 1–2 weeks of recovery, implanted animals were subjected to behavioral testing. The injected viruses and the waiting period for viral transgene expression for the different experiments are: for experiments in Fig. 2.4, CANE-LV-Cre (500 nl) together with AAV-CAG-flex-GFP (300 nl), waiting > 10 days or > 4 weeks; for experiments in Fig. 2.6a-o, CANE-LV-Cre (500 nl) together with AAV-SynP-DIO-TVA-EGFP-RG (200 nl), waiting 2 weeks, then CANE-RV-mCherry (1:200 dilution, 300 nl), waiting additional 10 days; for experiment in Fig. 2.9d-f, CANE-RV-mCherry (1:200 dilution, 300 nl), waiting 3 days. For
experiments in Figs. 2.6r-t and 2.9a-c,g-m, AAV9-CAG-flex-GFP (5 µL) or AAV9-EF1α-DIO-hChR2-eYFP (5 µL), waiting 4–6 weeks. For experiments in Fig. 5, AAV9-EF1α-DIO-eArch-eYFP (8 µL), waiting 4–6 weeks. For experiments in Supplementary Fig. 5, RG-LV-h-Syn-DIO-FlpO or RG-LV-hSyn-FlpO (800 nL), waiting 3 weeks.

2.3.5. Immunohistochemistry

All mice were deeply anaesthetized with isoflurane, and then transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4% PFA). Dissected brain samples were then post-fixed overnight in 4% PFA at 4 °C, cryoprotected in a 20% sucrose solution in PBS at 4 °C, frozen in Tissue-Tek O.C.T. Compound (Sakura) and stored at -80 °C until sectioning. Trigeminal and dorsal root ganglion samples were sliced at 20 µm using a cryostat (Leica Biosystems). All other coronal brain sections were sliced at 60-80 µm. The serial brain sections were collected in a 24 well plate and washed with PBS for 3 times. The sections were blocked with 2% bovine serum albumin (BSA) in PBS with 0.3% Triton X-100 (Blocking solution) at room temperature for 1 h. The sections were treated with 1st antibody in blocking solution at 4°C for overnight. The sections were washed 3 times followed by secondary antibody treatment at 4°C for 2 hours.
Sections were counter stained NeuroTrace fluorescent Nissl stain (fluorescent Nissl stain) (Invitrogen, N-21479) or 4’, 6-diamidino-2-phenylindole (DAPI) (Sigma, D9564). After this incubation, sections were washed, mounted and coverslipped. The primary antibodies used in this study are: goat anti-Fos (Santa Cruz Biotechnology, sc52-g, 1:300), rabbit anti-CGRP (Millipore, AB15360, 1:1000), sheep anti-FoxP2 (R&D Systems, AF5647, 1:5000), rabbit anti-NF200 (Sigma, N4142, 1:200), GS-IB4-Alexa 488–conjugated (Invitrogen, I21411, 1:1000), rabbit anti-VR1 (Abcam, ab31895, 1:1000), and rabbit anti-GFP (Abcam, ab290, 1:1000). The secondary antibodies are: Alexa Fluor 488 donkey anti-goat (Jackson immunoresearch, 705-545-147 1:1,000), Cy3 donkey anti-goat (Jackson immunoresearch, 705-165-147, 1:1,000), Alexa Fluor 488 donkey anti-rabbit (Jackson immunoresearch, 703-545-155, 1:1,000), Cy3 donkey anti-rabbit (Jackson immunoresearch, 711-165-152, 1:1,000), and Alexa Fluor 488 donkey anti-sheep (Abcam, ab150181, 1:1000)

2.3.6. Floating section in situ hybridization

For each mouse, six 60 µm sections containing the PBi were collected and in situ was performed as described previously. Gad1, Gad2, vGlut2 and Fos probes were created as previously described, and Gad1 and Gad2 probes were applied as a mixed probe. The probes were alternated across all sections to ensure
that one posterior section and one anterior section from each region was analyzed with each probe type.

2.3.7. Image Acquisition and Quantification

Samples were imaged using a Zeiss 700 laser scanning confocal microscope. In situ samples were imaged at 20× resolution at three z-positions. All z-positions for each slice were merged into a single image in Adobe Photoshop CS6 for quantification. All other samples were imaged at 10× resolution. The captured neurons and Fos expressing neurons in all immunohistochemistry and in situ hybridization experiments were manually counted, and percentages were calculated within each animal before averaging percentages across animals.

Axonal projections from captured PB-L-nociceptive neurons was quantified using a method previously described. The projection density for ROI’s was quantified across every other 80 μm coronal section. The data was normalized between animals by their own values in CeA (central amygdala). ROIs with densities in which the total pixel numbers of GFP-labeled axons divided by the area of the nuclei was less than 0.1 were excluded.

Again, using a method previously described, the number of transsynaptically labeled neurons from captured PB-L-nociceptive neurons was
quantified across every other 80 µm coronal section. Numbers of labeled cells in each ROI were manually counted. The data was normalized between animals by dividing with the number of starter neurons (GFP and mCherry double positive neurons in the PB\textsubscript{L}) in each animal.

### 2.3.8. Behavioral experiments for Fos immunostaining

Adult male and female C57B/L6 mice at ages more than 6 weeks were singly housed at least one day before noxious stimulation. Singly housed mice were directly perfused to stain for background Fos expression. For visualizing Fos expression induced by nociceptive stimuli, mice were lightly anesthetized with isoflurane, and unilaterally injected with 10 µL of saline, or 4% capsaicin, or 4% formalin into either the whisker pad or the hindpaw and returned to their home cage. 90 minutes later, the animals were perfused (as described in the method for immunostaining above).

### 2.3.9. Behavioral experiments for capturing PB\textsubscript{L}-nociceptive neurons with CANE virus

A brief description of CANE method: in Fos\textsuperscript{TVA} mice, activated neurons transiently express Fos which induces expression of a destabilized TVA (dsTVA) receptor. Lentivirus or deficient rabies virus pseudotyped with an engineered mutated envelope protein (CANE envelope) specifically binds cells
expressing high-level TVA receptor, which are strongly Fos+ neurons. In this way, CANE-viruses selectively infect Fos+ neurons and deliver desired transgenes to be expressed in Fos+ neurons.

Here, adult male and female FosTVA mice at ages more than 6 weeks were singly housed for at least one day, and then either handled without injection, or handled and subjected to noxious stimulation. Briefly, mice were taken out of their home cage, placed in the anesthesia chamber, lightly anesthetized with isoflurane, and injected unilaterally with 10 µl of saline or 4% capsaicin or 4% formalin into either the whisker pad or the hindpaw, and returned to their home cage. 60–90 min later, mice were anesthetized and underwent stereotaxic surgery for CANE-virus injection. Note that PB is a relatively large area and formalin/capsaicin activated neurons spread along both the dorsal-ventral as well as anterior-posterior axes; while we only injected CANE virus once using one stereotaxic coordinate, so we could only capture some of the neurons. Additionally, injections of formalin/capsaicin in whisker pad on different days could not hit the identical site, and this likely resulted in activation (Fos expression) of overlapping but non-identical populations of PB neurons.
2.3.10. Electrophysiological recording in acute brainstem slices

Four weeks after intraperitoneal injection of AAV9-EF1a-flex-ChR2-eYFP into TrpV1-Cre P1-2 mice, or 3 days after injection of CANE-RV-mCherry into the PBl of TrpV1-Cre::Ai32;FosTVA mice, mice were anesthetized with isofluorane, and transcardially perfused in ice-cold NMDG artificial cerebrospinal fluid (NMDG-ACSF; containing 92 mM NMDG, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 2 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 10 mM MgSO4, 0.5 mM CaCl2), and bubbled with 5% CO2 / 95% O2. The brain was then extracted and sectioned into 250 µm thick sagittal slices using a vibratome (VT-1000S, Leica Microsystems) containing ice-cold oxygenated NMDG-ACSF. Sagittal sections including the PBl were then bubbled in same solution at 37 °C for 8 min, and transferred to bubbled, modified-HEPES ACSF at room temperature (20-25 °C; 92 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 2 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 2 mM MgSO4, 2 mM CaCl2) for at least 1 h before recording. Recordings were performed in a submerged chamber, superfused with continuously bubbled ACSF (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 20 mM glucose, 2 mM CaCl2, 1.3 mM MgSO4) at near-physiological temperature (34 ± 1°C). Cells expressing GFP were
visualized by infrared differential interference contrast and fluorescence video microscopy (Examiner.D1, Zeiss). Whole-cell current clamp recordings were amplified with Multiclamp 700B (filtered at 2 kHz), digitized with Digidata 1440A (5 kHz), and recorded using pClamp 10 software (Axon). Both unlabeled and mCherry+ PB cells surrounded by axon terminals expressing a virally encoded fluorescent marker (ChR2-EYFP) were visualized by infrared differential interference contrast and fluorescence video microscopy (Examiner.D1, Zeiss). Whole-cell voltage-clamp recordings were amplified with Multiclamp 700B (filtered at 2 kHz), digitized with Digidata 1440A (5 kHz), and recorded using pClamp 10 software (Axon). The patch-clamp electrode (4–6 MΩ) was filled with an intracellular solution containing 130 mM D-gluconic acid, 130 mM CsOH, 5 mM NaCl, 10 mM HEPES, 12 mM phosphocreatine, 3 mM MgATP, 0.2 mM Na2GTP, 1 mM EGTA. Photostimulation was performed using a 473 nm LED (CoolLED, pE4000) controlled by pClamp 10 software (Axon). Light intensity was set to be 100% for generation of spikes in the axon terminals of projecting TrpV1Cre::ChR2+ neurons with a pulse length of 10 ms. To confirm whether postsynaptic currents were monosynaptic, tetrodotoxin (TTX; 1 µM) was initially bath applied, followed by a combination of TTX and 4-aminopyridine (4-AP; 100 µM).
All electrophysiology data were analyzed off-line using Neuromatic package (Think Random) in Igor Pro software (WaveMetrics). Off-line analysis was performed by averaging five traces. Light-evoked EPSC and IPSC peak amplitude, half-width, onset latency, time to peak, rise time, and decay time were analyzed. The onset latency of the light-evoked EPSCs and IPSCs was defined as the time from the onset of the stimulus to the first measurable deflection of the potential from the baseline. Similarly, time to peak was defined as the time from the onset of the stimulus to the peak of the potential. Rise time and decay time were defined as the time between 10% and 90% of the rise or decay of the potential, respectively.

2.3.11. Optogenetic activation of TrpV1Cre::ChR2⁺ sensory afferent terminals in PB₇ in a real-time place escape/avoidance (PEA) test and in circular chamber for audio recording

Channelrhodopsin (ChR2) or control GFP was expressed in TrpV1-Cre⁺ primary sensory neurons by neonatal IP injection of either AAV9-EF1α-DIO-hChR2-eYFP or AAV9-CAG-Flex-GFP in TrpV1-Cre pups (as described above). Six weeks later, virus injected mice were implanted with custom-made optic fibers which were placed above PB-el on both sides and fixed on the skull with dental cement (Parkell). One week later, the animals were subjected to a
2-chamber real-time PEA test in light cycle, using a modified method described previously\textsuperscript{66,67}. The size of custom-made behavior chamber is $50.1 \times 27.7 \times 31.2$ cm, made with clear acrylic Plexiglas that had distinct stripe patterns from one another. For optogenetic stimulation, laser is delivered through patch cables attached to the implanted optic fiber as described previously\textsuperscript{32}. The mouse is placed in the center of the box and allowed to explore both chambers without light stimulation (pre-stimulation) for 10 min. Generally, after exploration, the mouse shows a small preference for one of the two chambers. Subsequently, blue light stimulation (10 Hz, 20 ms pulse-width, $\sim 3.5$ mW) is delivered whenever the mouse enters or stays in the preferred chamber, and light is turned OFF when the mouse moves to the other chamber (stimulation phase, total 10 min). Finally, the mouse can freely explore both chambers without blue light stimulation (post-stimulation) for 10 min. We recorded behavioral data via a webcam (Logitech web-camera, PN 960-000764) interfaced with Bonsai software\textsuperscript{68}. Real-time laser stimulation was controlled by Bonsai software through Arduino with a custom-made Arduino sketch (Arduino UNO, A00073). After 1 week, the same group of mice were subjected to another behavioral test, where the mouse was placed in a circular field in a sound proof chamber. The mouse’s movements and audible vocalizations were
recorded from the top of field using the webcam with audio control at a frame rate 30 fps. The experimental mouse was placed in the center of the circular field and allowed to explore freely. Blue light was delivered as described above. The duration of each light stimulation was 30 s and the interval between light stimuli was > 2 min. The number of light stimulation for each mouse in each behavioral test was 4. The number of pips was calculated for each interval and averaged offline.

After all behavior tests were completed, the mice were given a train of strong light stimulations (15 s on and 15 s off, 50 ms pulses, 10 Hz, ~3.5 mW, repeated 3 times) to elicit ChR2- or photostimulation-dependent Fos expression in their home cage. Subsequently, animals were perfused at 90 min after the final stimulation and processed for Fos immunostaining.

2.3.12. Optogenetic activation of TrpV1Cre::ChR2⁺ sensory afferent terminals in PB in a classical conditioned place aversion (CPA) test

Channelrhodopsin (ChR2) or control GFP was expressed in TrpV1-Cre⁺ primary sensory by neonatal IP injection of either AAV9-EF1α-DIO-hChR2-eYFP or AAV9-CAG-Flex-GFP in TrpV1-Cre pups (as described above). Six weeks later, virus injected mice were implanted with custom-made optic fibers which were placed above PB-el on both sides and fixed on the skull with dental cement.
(Parkell). One week later, the animals were subjected to a 2-chamber classic conditioned place aversion (CPA) test in same behavior chamber used for PEA. The mouse is first habituated to the chamber on day 1. On day 2, the mouse is placed in the center of the box and allowed to explore both chambers without light stimulation (pre-stimulation) for 10 min. Generally, after exploration, the mouse shows a small preference for one of the two chambers. In the following two days (day 3 and day 4), the mouse is closed off in the non-preferred chamber with no stimulation for 15 min in the morning, and then closed off in the preferred chamber with blue light stimulation (10 Hz, 20 ms pulse-width, ~3.5 mW) for 15 min in the afternoon. On the final day (day 5), the mouse can explore both chambers without blue light stimulation (post-stimulation) for 10 min, and their behaviors are recorded and analyzed.

2.3.13. Optogenetic silencing of TrpV1Cre::eArch⁺ sensory afferent terminals in PBŁ in von Frey tests and real-time place preference (RTPP) test

Enhanced archaerhodospin (eArch) or control GFP was expressed in TrpV1-Cre⁺ primary sensory by neonatal IP injection of either AAV9-EF1α-DIO-eARCH-eYFP or AAV9-CAG-Flex-GFP in TrpV1-Cre pups (as described above). Six weeks later, virus injected mice were implanted with custom-made optic
fibers which were placed above PB-el on both sides and fixed on the skull with
dental cement (Parkell). More than one week later, the animals were subjected to
von Frey tests. All mice were first habituated to handling and testing equipment
at least 30 min before experiments. Behavioral responses to mechanical stimuli
applied to face or hindpaw at baseline (without capsaicin injection) were
examined first and both in the absence and in the presence of photo illumination.
Subsequently, capsaicin (Sigma-Aldrich, 1µg/10µl, dissolved in normal saline
with 4% ethanol and 4% tween-80) was subcutaneously injected into either right
hindpaw or right whisker pad. Between 10 and 20 min after capsaicin injection,
behavioral responses to mechanical stimuli were tested either in the absence or
the presence of photo-silencing/illuminating of TrpV1-Cre+ axons in PB-L. The
mice were tested for hindpaw and face responses on different days with a
randomized order (i.e. some were tested for face first, others were tested for paw
responses first). There was at least one week interval separating the paw versus
face (or vice versa) tests. For the hindpaw test, mice were individually placed on
an elevated metallic wire mesh floor in polyethylene cages (4x4x5.5 inch,
Comerio-VA, Italy). A graded series of von Frey filaments (0.04-2g, Stoelting)
was inserted through the mesh floor and applied to the plantar surface of the
hindpaw. For face test, mice were individually placed in a custom-made box
(3x3x4 inch) with the top, bottom and four walls made of silver wire mesh and
allowed for free movement. Again, a graded series of von Frey filaments (0.02-1g) was inserted through the mesh wells from the lateral side and applied to the
skin of the vibrissa pad within the infraorbital nerve territory. A brisk
withdrawal of the paw or head was considered a positive response. Mice were
tested 3 times with at least 2 withdrawal behaviors out of 3 trials indicated a
positive result. Mechanical threshold was defined as the minimum force
necessary to elicit a response. For optogenetic silencing during von Frey tests,
a continuous green light (561nm) stimulation (~12 mW) was delivered during
both the hindpaw and face tests (with and without capsaicin injections). Again,
mice were tested 3 times with at least 2 withdrawal behaviors out of 3 trials
indicated a positive result. Mechanical threshold was defined as the minimum
force necessary to elicit a response.

TrpV1Cre::eArch and TrpV1Cre::GFP mice were also subjected to a real-
time place preference test (RTPP). Individual mouse was placed in the center of
the box and allowed to explore both chambers without light stimulation
(baseline) for 10 min. Generally, after exploration, the mouse shows a small
preference for one of the two chambers. After recording the baseline behavior,
individual mouse was injected with 5μl 4% capsaicin into the left whisker pad
and placed in the chamber again to freely explore both chambers without light stimulation (no stimulation) for 10 min again. Subsequently, a continuous green light stimulation (561 nm, ~12 mW) was delivered through the optic fiber to silence the TrpV1Cre::eArch fibers (or illuminate the control GFP fibers) in PB whenever the mouse entered or stayed in the non-preferred chamber, and light was turned OFF when the mouse moved to the other chamber (total 10 min of real-time stimulation). We recorded behavioral data via a webcam (Logitech web-camera, PN 960-000764) interfaced with Bonsai software. Real-time laser stimulation was controlled by Bonsai software through Arduino with a custom-made Arduino sketch (Arduino UNO, A00073). Matlab codes for controlling laser and triggering stimulation are available upon request. Subsequently, animals were perfused for post-hoc analysis.

2.3.14. Statistics

No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications. Values in text are reported as mean ± standard error of mean. All data (with the exception of behavioral data for the real-time PEA test) were analyzed using two-tailed paired and unpaired Student’s t test between 2 groups (experimental or control), or in the case of multiple groups, one-way or two-way ANOVA followed by
Tukey’s test. The criterion for statistical significance was $P < 0.05$. Regarding the assumption of normality for large/medium data sets, D’Agostino and Pearson normality test was used. When the sample size was less than four, Shapiro-Wilk normality test was used. We provided mean values with associated standard error of the mean values. To determine whether the variance was similar between the groups that are being statistically compared, F test was used for t tests, and Brown-Forsythe was used for one-way ANOVA. The results showed that the variance was similar.

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

2.4. Results and Discussion
2.4.1. Noxious facial stimuli activate the PB\textsubscript{L} more robustly and bilaterally compared to noxious bodily stimuli.

We injected 4% formalin (a noxious chemical) either unilaterally into the whisker pad, or unilaterally into one hindpaw, and immunostained for the immediate early gene Fos as a marker for activated neurons in the PB\textsubscript{L} (Fig. 2.1a). Whisker pad formalin injection activated the PB\textsubscript{L} with significantly more Fos\textsuperscript{+} neurons than paw injection of an equivalent amount of formalin (Fig. 2.1c; Whisker: 952 ± 100.7; Paw: 616 ± 75.1 total Fos\textsuperscript{+} neurons; P = 0.04; n = 7), especially in the external lateral sub-nucleus of the PB\textsubscript{L} (PB-el) (Fig. 2.1b). Furthermore, unilateral formalin whisker pad injection induced Fos\textsuperscript{+} neurons in PB-el bilaterally with a trend of more Fos\textsuperscript{+} cell on the ipsilateral side (Fig. 2.1b, d; Contra: 213.8 ± 32.8; Ipsi: 281.5 ± 22.3 Fos\textsuperscript{+} neurons; P = 0.053; n = 4). By contrast, unilateral paw formalin injection preferentially activated the contralateral PB-el with significantly more Fos\textsuperscript{+} neurons on the contralateral than on the ipsilateral side (Fig. 2.1b, d; Contra: 253.3 ± 24.1; Ipsi: 129.7 ± 14.3 Fos\textsuperscript{+} neurons; P < 0.01; n = 3), which is consistent with the fact that spino-parabrachial projection neurons in dorsal spinal cord are known to predominantly send axons to the contralateral side\textsuperscript{5,21}. Additionally, consistent with the fact that the affective pain circuit does not discriminate the types of pain\textsuperscript{3}, we found that capsaicin, formalin, and even the
minor pain associated with control injection of saline unilaterally into the whisker pad all activated the PBv neurons (including neurons in PB-el) compared to no-injection controls, with formalin the most potent stimulus to evoke Fos+ neurons (Fig. 2.2 a, b; Home Cage: 73 ± 26; Saline: 421 ± 94; Capsaicin: 673 ± 72; Formalin: 952 ± 101 Fos+ neurons, n = 3,3,3,4). In the same animals, we also observed Fos+ neurons in spinal trigeminal nucleus caudalis (Sp5C), which was expected since Sp5C is a main relay in the trigeminal-thalamic-cortical pain pathway (Fig. 2.2c, n = 3)10,15,20.
Figure 2. 1. Lateral parabrachial nucleus (PB\textsubscript{L}) is differentially activated by the same noxious stimulus applied to the face versus hindpaw.

a, Schematic illustration of Fos induction protocol. Ninety minutes after 10 µL 4% formalin was injected, brainstem slices containing PB\textsubscript{L} were stained for Fos expression. TG, trigeminal ganglion; Sp5C, trigeminal nucleus, caudalis; DRG, dorsal root ganglion; S.C., spinal cord. b, Representative images of Fos\textsuperscript{+} neurons in PB\textsubscript{L} after formalin injection into right whisker pad (top) and right hindpaw (bottom). Large white dashed circle (left) indicates the entire structure of PB\textsubscript{L}, whereas small white dashed circle (right) indicates ventral region of PB\textsubscript{L} including PB-el. Blue, DAPI stain. Scale bars, 200 µm. c, Total numbers of Fos\textsuperscript{+} neurons in PB\textsubscript{L} on both sides combined ($n = 4, 3$; two-tailed unpaired Student’s $t$ test; *$P = 0.0445$; $t_{4.962} = 2.674$). d, Numbers of Fos\textsuperscript{+} neurons in ipsilateral (magenta) and contralateral (teal) PB-el in mice unilaterally injected with formalin into one whisker pad ($n = 4$) or one hindpaw ($n = 3$ mice; two-way ANOVA; whisker: $P = 0.0533$; hindpaw: **$P = 0.0090$; $F_{1,5} = 32.75$). n.s., nonsignificant. Data are mean ± s.e.m.
Figure 2.2. Fos expression patterns in the PBl after different types of noxious injections into the right whisker pad.

(a) Anti-Fos staining was performed 90 minutes after each injection. Blue; DAPI stain. Scale bar, 100 µm. (b) Quantification of Fos+ neurons in the PBl. (n = 3, 4, 4, 4; one-way ANOVA; Home Cage vs. Saline: *P = 0.0437; Home Cage vs. Capsaicin: **P = 0.0014; Home Cage vs. Formalin: ***P = 0.0001; F_{3, 11} = 18.25) Data are mean ± SEM. (c) Anti-Fos staining was performed on Sp5C 90 minutes after formalin injection. Blue; DAPI stain. Scale bar, 100 µm.
2.4.2. PB\textsubscript{L} neurons activated by noxious facial stimuli are molecularly heterogeneous.

Two-color fluorescence in situ hybridization further showed that most Fos\textsuperscript{+} PB\textsubscript{L}-nociceptive neurons were slc17a6\textsuperscript{+} (i.e. vGlut2\textsuperscript{+}) (Fig. 2.3 a, b; glutamatergic; 80 ± 1\%, n = 3), while only a minority of Fos\textsuperscript{+} cells were gad1/2\textsuperscript{+} (Fig. 2.3 a, b; GABAergic; 7 ± 2\%, n = 3). A recent study showed that the gene calca encoding calcitonin gene-related peptide (CGRP) is expressed in PB-el. These CGRP\textsuperscript{+} PB-el neurons were activated by intense foot shock, and transmitted affective pain signals to the CeA\textsuperscript{15}. We therefore decided to focus on CGRP expression and found a subset of Fos\textsuperscript{+} PB\textsubscript{L}-nociceptive neurons in the ventral region indeed expressed CGRP (Fig. 2.3 c-d; 56 ± 5\% of ventral; 2 ± 1\% of dorsal; 34 ± 3\% of total Fos\textsuperscript{+} PB\textsubscript{L}-nociceptive pain neurons were CGPR\textsuperscript{+}; n = 3). Another marker, the Forkhead box protein P2 (FoxP2), implicated in circuits related to vocal communication and sodium intake, has also been found to be expressed in the PB\textsubscript{L}\textsuperscript{12,13}. We found that again only a subset of Fos\textsuperscript{+} PB\textsubscript{L} neurons in the dorsal region expressed FoxP2 (Fig. 2.3 c-d; 9 ± 4\% of ventral; 46 ± 10\% of dorsal; 21 ± 5\% of total Fos\textsuperscript{+} PB\textsubscript{L}-nociceptive neurons were FoxP2\textsuperscript{+}; n = 3).
Figure 2.3. Molecular characterization of PB-L-nociceptive neurons

(a) Two-color fluorescent in situ hybridization showing formalin-activated (Fos+, green) and Vglut2-expressing neurons (right) and Gad1/2-expressing neurons (left) (both magenta) in the PB-L. Scale bar, 20 µm. (b) Quantification of co-expression of Fos+ neurons and Vglut2 and Gad1/2-expressing neurons in the PB-L. (n = 3; two-tailed paired Student’s t test; **P = 0.001; t = 29.73). Data are mean ± SEM. (c) Left panel, staining of formalin-activated neurons (anti-Fos, green) and CGRP+ neurons (magenta). Right panel, staining of formalin activated neurons (anti-Fos, green) and FoxP2+ neurons (magenta). Scale bar, 100 µm. (d) Quantification of co-expression of Fos+ neurons and FoxP2+ and CGRP+ neurons in PB-L-dorsal, PB-L-ventral, and total PB-L. (n = 4; two-tailed paired Student’s t test; P = 0.0544, *P = 0.0496, P = 0.1503; t = 4.109, t = 4.32, t = 2.279). Data are mean ± SEM.

2.4.3. CANE is efficient and selective in activity-dependent capturing of facial nociceptive relay PB-L neurons.

How might noxious facial stimuli activate more neurons in the PB-L, particularly in the PB-el, compared to noxious bodily stimuli, especially on the
ipsilateral side? To answer this question, we needed to identify neurons that provide presynaptic inputs to face-nociception-activated PB\(_l\) neurons. Previous studies using anterograde and retrograde tracer dyes labeled the general afferents to the entire PB\(_l\) region\(^5,9,10,14,15\). However, the PB\(_l\) contains diverse populations of neurons in addition to neurons responsive to noxious stimuli, such as cells activated by innocuous warm and cool temperatures, as well as cells responsive to various taste stimuli\(^6-8\). The PB\(_l\) is also known for its significant role in regulating instinctive behavior, namely thirst for water, sodium appetite and hunger for food\(^17-19\). Thus, tracer based studies lack the resolution to identify specific inputs to the PB\(_l\)-nociceptive neurons. Since CGRP and FoxP2 only label subsets of PB\(_l\)-nociceptive neurons (Fig. 2.3 c-d), we reasoned that transsynaptic tracing of inputs to either CGRP\(^+\) or FoxP2\(^+\) neurons may miss certain types of inputs that innervate the non-CGRP\(^+\), or non-FoxP2\(^+\) PB\(_l\)-nociceptive neurons. We therefore turned to our newly developed technology called CANE for viral-genetic tagging of transiently-activated neurons to capture noxious stimuli-activated PB\(_l\) neurons. CANE uses a pseudotyped lentivirus or rabies virus to selectively infect Fos\(^+\) neurons genetically engineered to transiently express the receptor for the pseudotyped viruses (Fos\(^{TVA}\) mice), and consequently, the viruses mediate expression of desired transgenes in activated cells\(^32\).
We first validated that CANE could indeed selectively label PB\textsubscript{i}-nociceptive neurons. In a two-bout experimental paradigm, CANE was used to capture PB\textsubscript{i} neurons activated by a noxious stimulus (capsaicin or formalin injection) by co-injecting CANE-LV-Cre and AAV-flex-GFP into the PB\textsubscript{i}. Three weeks later, the same animal was given a second painful stimulus to induce Fos expression and sacrificed for immunostaining (Fig. 2.4a). In both capsaicin-capsaicin, and formalin-formalin paradigms, 55 ± 3% (n=9) and 55 ± 2% (n=9) of CANE-captured PB\textsubscript{i} neurons were Fos\textsuperscript{+}, respectively (Fig. 2.4 d, f, h, i). This indicated that the second noxious injection reactivated many (~55%) of the same cells excited by the first stimulus. By contrast, without noxious stimulation, there was only a small number of background captured neurons (due to Fos-expression in PB\textsubscript{i} induced by handling/restraining the animals but without application of noxious stimuli), which had significantly less overlap with Fos\textsuperscript{+} neurons induced by noxious stimuli (Fig. 2.4 b, e, h, i; 27± 3% CANE\textsuperscript{+} cells were Fos\textsuperscript{+} in no stimulus-formalin paradigm (n=5; P < 0.0001); 31 ± 5% CANE\textsuperscript{+} cells were Fos\textsuperscript{+} in the no stimulus-capsaicin paradigm (n=4; P < 0.0001)). In the capsaicin-saline paradigm, 36 ± 3% CANE-captured cells were Fos\textsuperscript{+} activated by saline injection (Fig. 2.4 e, h, n=4; P = 0.0005), consistent with the fact that saline injection only caused moderate PB\textsubscript{i} activation. Previous electrophysiological studies reveal that the same PB\textsubscript{i}
neurons could be activated by different noxious modalities\textsuperscript{25}, prompting us to ask whether CANE-captured capsaicin-activated PB\textsubscript{l} neurons overlapped with formalin-activated neurons and vice versa. Indeed, we observed a similar percentage of CANE\textsuperscript{+} neurons that were Fos\textsuperscript{+} regardless of whether the capsaicin-formalin paradigm was used (51 ± 2\%; n = 7) or vice-versa (55 ± 2\%; n = 6) (Fig. 2.4 c, h, i). We also examined the overlap between CANE-captured face-activated PB\textsubscript{l} nociceptive neurons and Fos\textsuperscript{+} cells induced by contralateral hindpaw nociception and vice versa. About 30\% of CANE\textsuperscript{+} neurons were Fos\textsuperscript{+} in both whisker-hindpaw and hindpaw-whisker nociception paradigms (Fig. 2.4 g, j; W:H, 26 ± 3\%; H:W, 33 ± 4\%, n = 6). Our observations are consistent with the current concept that the PB\textsubscript{l} mediated affective pain circuit plays a limited role in discriminating the types and locations of injury\textsuperscript{25,75}. As an additional control for the specificity of CANE, we co-injected CANE-LV-Cre, AAV-flex-GFP (CANE::GFP), and AAV-tdTomato into the PB\textsubscript{l} after formalin injection into the whisker pad, and compared the labeling resulted from the two viral methods. CANE::GFP labeled a specific subset of PB\textsubscript{l} neurons, whereas AAV-tdTomato labeled a majority of neurons at the injection site (Fig. 2.5, n = 4), thus further confirming the specificity of our method.
Figure 2. 4. Capturing and mapping the axonal projection targets of PB\textsubscript{L}-nociceptive neurons.

\textbf{a}, Schematic illustration of strategy to express GFP in nociceptive relay PB\textsubscript{L} neurons in Fos\textsuperscript{TVA} mice using CANE. \textbf{b}–\textbf{g}, Examination of CANE-captured neurons activated by the first stimulus (magenta) versus Fos\textsuperscript{+} neurons activated by the second stimulus (green) in the PB\textsubscript{L}. In all six conditions, CANE method was used to capture neurons activated by stimulus/no stimulus, and 2 weeks later, Fos was induced by the second stimulus. Blue, DAPI. Scale bars, 10 µm. \textbf{h}–\textbf{j}, The percentages of Fos\textsuperscript{+} neurons among CANE\textsuperscript{+} neurons in the different conditions. Data are mean ± s.e.m. (from left to right: \textbf{h}, n = 4, 9, 7, 4; one-way ANOVA; ****P ≤ 0.0001, **P = 0.0005, P = 0.3952, P = 0.3223; **P = 0.0005, *P = 0.0047; F\textsubscript{3,20} = 12.49; \textbf{i}, n = 5, 5, 9; one-way ANOVA; ****P ≤ 0.0001, ****P ≤ 0.0001, P = 0.6876; F\textsubscript{2,17} = 52.17; \textbf{j}, n = 3, 3; two-tailed unpaired Student’s \textit{t} test; P = 0.2759; \textit{t}\textsubscript{3.505} = 1.289). \textbf{k}–\textbf{p}, Representative images of axonal projections from captured formalin-activated PB\textsubscript{L} (magenta) in several brain nuclei expressing Fos (green) induced by formalin. Insets, schematics of coronal view of location (in red box) in brain. * in \textbf{k} denotes very large terminal boutons from labeled PB\textsubscript{L} axons in BNST; some of boutons surround the Fos\textsuperscript{+} BNST neuron cell.
bodies. q. Quantification of normalized density of innervations (total pixels divided by the area of each nucleus; \(n = 3\)). All data shown are mean ± s.e.m. r. Schematic summary for output targets of PBl-nociceptive neurons. BNStov, oval nucleus of the bed nucleus of the stria terminalis; PVH, paraventricular hypothalamic nucleus; PVT, paraventricular nucleus of the thalamus; CeAc, central amygdalar nucleus, capsular part; SNpc, substantia nigra pars compacta; PAGvl, ventrolateral periaqueductal gray; NST, nucleus of the solitary tract; IRt, intermediate reticular tract. Scale bars: k,o, 20 \(\mu\)m; l–n,p, 50 \(\mu\)m (\(n = 3\)). Data are mean ± s.e.m.

Figure 2.5. Additional evidence for the specificity of CANE captured PBL-nociceptive neurons

Representative image of CANE captured PBl-nociceptive neurons (Green) and generally labeled mCherry+ PBl neurons (magenta) after co-injection of CANE-LV-Cre; AAV-flex-GFP; AAV-tdTomato. (\(n = 4\) hemispheres in 2 mice). Scale bar, 50 \(\mu\)m (both low and high mag).

2.4.4. PBl-nociceptive neurons project axons to multiple emotion- and instinct-related centers in the brain.

We next traced the axonal projections of CANE::GFP-captured PBl-nociceptive neurons. The targets of PBl-nociceptive neurons include: the bed nucleus of the stria terminalis (BNST, where PBl axons form large axonal boutons surrounding BNST neuron cell bodies), the paraventricular thalamic nucleus (PVT), the paraventricular nucleus of the hypothalamus (PVH), CeAc (the
capsular division), the ventral tegmental area (VTA), the ventrolateral periaqueductal grey (PAG\textsubscript{vl}), the nucleus of the solitary tract (NST) and the intermediate reticular nucleus in the hindbrain (IRt) (Fig. 2.4 k-r). Quantitative measurements of the densities of innervation (n = 3) using a previously described method\textsuperscript{32,76} showed that the majority of projections were ipsilateral with small numbers of axons innervating the contralateral side (Fig. 2.4 q). A schematic summary of the projections is shown (Fig. 2.4 r). Notably, all the targets of PB\_L-nociceptive neurons contained Fos\textsuperscript{+} neurons induced by noxious facial stimulation (Fig. 2.4 k-p, green signals).

2.4.5. PB\_L-nociceptive neurons receive reciprocal inputs from emotion-related limbic regions and bilateral inputs from various reticular brainstem regions.

Having validated that CANE selectively captured PB\_L-nociceptive neurons that relay signal to emotion- and instinct-related centers, we mapped the presynaptic inputs to these neurons using a CANE-based transsynaptic tracing method\textsuperscript{32}. Briefly, CANE-LV-Cre and the helper virus AAV-SynP-DIO-TVA-EGFP-RG\textsuperscript{59} were co-injected into the ipsilateral PB\_L to express the TVA receptor, rabies glycoprotein G, and GFP selectively in the PB\_L neurons which were activated by formalin injection into the whisker pad. Two weeks later, CANE-RV-
mCherry was injected into the same location in PB\textsubscript{l}. The GFP/mCherry double-positive neurons are the starter PB\textsubscript{l}-nociceptive neurons, while mCherry\textsuperscript{+} neurons outside of the PB\textsubscript{l} are presynaptic neurons (Fig. 2.6 a, b). We observed mCherry\textsuperscript{+} neurons in BNST, CeAm (medial division), and several hypothalamic nuclei including the PVH, substantia nigra pars compacta (SNpc), PAG\textsubscript{vl}, brainstem reticular regions, NST, spinal trigeminal nucleus caudalis (Sp5C), and the dorsal horn of the spinal cord (Fig. 2.6 c-j; m; quantification represents numbers of labeled presynaptic neurons/number of starter neurons; n = 6). Note that the labeled neurons in the reticular regions, NST, and Sp5C neurons were distributed bilaterally with an ipsilateral dominance (Fig. 2.6 m; number of transsynaptically labeled cells/number of starter cell: Ipsi. [IRt: 6.3 ± 1.3; PCRt: 6.4 ± 1.4; MRn: 1.3 ± 0.4; GRn: 3.1 ± 0.7; NST: 1.9 ± 0.7; Sp5C: 5.3 ± 1.6]; Contra. [IRt: 0.9 ± 0.2; PCRt: 1.4 ± 0.5; MRn: 3.8 ± 0.8; GRn: 2.1 ± 0.5; NST: 0.8 ± 0.2; Sp5C: 0.5 ± 0.2]), consistent with previous dye tracing studies\textsuperscript{5,9,10,14,15}. Additionally, there were a few labeled cells in the contralateral PB\textsubscript{l} (Fig. 2.6 m; 0.7 ± 0.2). A schematic summary of the projections is shown (Fig. 3o).
2.4.6. CANE-captured PB_L-nociceptive neurons receive direct input from primary sensory neurons in the ipsilateral trigeminal ganglion.

Interestingly, transsynaptically labeled mCherry+ neurons were also observed in the ipsilateral TG, but not in any of the DRG on either side (n = 6, Fig. 2.6 k, l), suggesting that TG sensory neurons innervating head and face provide direct monosynaptic inputs to ipsilateral PB_L-nociceptive neurons. A few previous anatomical studies hinted at the possibility of a direct TG-PB connection33,34,54,55. Interestingly, transsynaptic tracing of inputs to hindpaw formalin-activated PB_L-nociceptive neurons also revealed labeled neurons in TG but not in any DRG (n = 4, Fig. 2.6 n), suggesting craniofacial but not body primary sensory neurons provide direct, monosynaptic inputs onto PB_L-nociceptive neurons. The result is also consistent with the idea that some PB_L-nociceptive neurons receive convergent inputs from both face and body. We examined the expression of IB4 (a marker for non-peptidergic c fibers), CGRP, TrpV1 (the receptor for capsaicin and a marker for a subset of c fibers and a small subset of Aδ-fibers), and NF200 (a marker for both Aδ- and Aβ- fibers) among the transsynaptically labeled TG neurons. The TG neurons directly presynaptic to the PB_L included NF200+ (45 ± 4%), TrpV1+ (38.5 ± 4%), CGRP+ (26.2 ± 7%), and IB4+ cells (12 ± 4%; n = 8; Fig. 2.6 p, q). Taken together, the transsynaptic tracing studies suggest that there are two
separate pathways transmitting craniofacial nociception from TG to the PB\textsubscript{l}: (1) the previously known indirect TG\textarrowright\text{Sp5C\textarrowright PB\textsubscript{l}} and (2) the newly revealed direct TG\textarrowright PB\textsubscript{l} projection. By contrast, there is only one indirect pathway transmitting somatosensory body nociception from DRG to the PB\textsubscript{l}: DRG\textarrowright\text{spinal dorsal horn\textarrowright PB\textsubscript{l}}.

Notably, a previous study using TrpV1::PLAP mice observed that fibers from a possible primary afferent source of TrpV1-lineage neurons were present in the PB\textsubscript{l}, especially in the PB-el\textsuperscript{33,34}. The authors speculated that the TrpV1\textsuperscript{+} fibers may have emerged from TG neurons which could provide an alternative circuit contributing to craniofacial pain experience\textsuperscript{33,34}. These previous findings, in addition to our finding that \textasciitilde40\% of transsynaptically labeled TG neurons are TrpV1\textsuperscript{+}, led us to postulate that TrpV1\textsuperscript{+} fibers may be a major source of noxious TG inputs to PB-el. Therefore, we performed neonatal intraperitoneal (IP) injection of AAV to selectively label periphery-derived TrpV1-Cre\textsuperscript{+} axons\textsuperscript{53,77,78}. Briefly, Cre-dependent AAV9-flex-GFP was injected into TrpV1-Cre mouse pups at postnatal day 1-2\textsuperscript{53}. The IP injection resulted in selective labeling of TrpV1-Cre\textsuperscript{+} primary sensory neurons with GFP without labeling of TrpV1-Cre\textsuperscript{+} CNS neurons (Fig. 2.6 r, s and Fig. 2.7, n = 3). Furthermore, axonal terminals from labeled TrpV1\textsuperscript{+} primary sensory neurons were observed near nociceptive Fos\textsuperscript{+} neurons in PB-el and in
Sp5C (Fig. 2.6; Fos was induced by capsaicin injection into the ipsilateral whisker pad).

We further designed a TrpV1-Cre and retrograde-FlpO intersectional strategy (Fig. 2.8a) to determine whether PB\textsubscript{l} projecting TG neurons also project to Sp5C. Briefly, retrograde-lentivirus expressing either FlpO (RG-LV-hSyn-FlpO, n=4) or Cre-dependent FlpO (RG-LV-hSyn-DIO-FlpO, n=6) was injected into PB in TrpV1-Cre; Ai65 mice (Fig. 2.8a). Retrograde-lentivirus infects axons and is transported back to cell bodies\textsuperscript{58,65}. Ai65 is a Cre and Flp co-dependent tomato reporter\textsuperscript{57}. In this strategy, only TrpV1-Cre expressing neurons that project axons into PB will express both Cre and FlpO, and therefore only these neurons will express tomato, allowing us to visualize their cell bodies and axon projections. The Cre-dependent RG-LV-hSyn-DIO-FlpO gave sparser labeling results than the RG-LV-hSyn-FlpO. We observed tdTomato\textsuperscript{+} neurons in ipsilateral TG (Fig.2.8 c, f) but not in any DRG (data not shown). Interestingly, tdTomato\textsuperscript{+} axons can be seen in both PB\textsubscript{l} and in Sp5C (Fig. 2.8 b, d, e, g), indicating that at least some of the labeled TG neurons project bifurcated axons to innervate both PB\textsubscript{l} and Sp5C. The peripheral axons of labeled TrpV1-Cre\textsuperscript{+} TG→PB\textsubscript{l} neurons form either free-nerve endings or circular endings around hair follicles (Fig. 2.8h).
Figure 2.6. Trans-synaptic labeling of presynaptic neurons for PB₁-nociceptive neurons reveals the direct TG→PB₁ pathway.

**a.** Schematic illustration for trans-synaptic tracing of presynaptic inputs to PB₁-nociceptive neurons. **b.** Representative image of CANE-RV-mCherry-infected PB₁-nociceptive neurons. Green, PB₁-nociceptive neurons expressing TVA and RG; red, RV-mCherry⁺; yellow, starter cells. Scale bar, 10 µm. **c–I.** Representative images of trans-synaptically labeled neurons in several brain regions. Scale bars: c–h,k,l, 50 µm; i, 100 µm; j, 20 µm. **m,n.** Quantification of trans-synaptically labeled neurons in each brain area contralateral (teal) and ipsilateral (magenta) to injected site (m) after whisker-pad formalin injection and (n) after hindpaw formalin injection. The
value is normalized against the number of starter neurons and averaged across animals. Data are mean ± s.e.m. (n = 6; n = 3).

**o.** Schematic summary for input sources for PB1-nociceptive neurons. BNST, bed nucleus of the stria terminalis; PVH, paraventricular hypothalamic nucleus; LHA, lateral hypothalamus; CeAm, central amygdaloid nucleus, medial; SNpc, substantia nigra pars compacta; PAG, periaqueductal gray; DRn, dorsal raphe nucleus; DRnvl, ventrolateral DRn; NST, nucleus of the solitary tract; Sp5C, trigeminal nucleus, caudalis; Pr5, principal sensory trigeminal nucleus; TG, trigeminal ganglion; DRG, dorsal root ganglion; S.C., spinal cord (dorsal horn). Reticular (ret.) nuclei: PRn, pontine reticular nuclei; IRt, intermediate reticular tract; PCRt, parvicellular reticular tract; MRn, medullary reticular nuclei; GRn, gigantocellular reticular nuclei.

**p.** Molecular characterization of trans-synaptically labeled TG neurons. Green, bottom to top: IB4+, CGRP+, NF200+, TrpV1+. Left, colocalized trans-synaptically labeled TG neurons. Right, non-colocalized labeled TG neurons. Scale bars, 20 µm.

**q.** Percentage of trans-synaptically labeled trigeminal ganglion neurons expressing IB4, CGRP, NF200, or TrpV1 (n = 8; one-way ANOVA; *P = 0.0135, **P = 0.0008, *P = 0.0468, ****P ≤ 0.0001, ****P ≤ 0.0001, P = 0.4653; F = 3.27 = 22.7). Data are mean ± s.e.m.

**r.** Schematic illustration and timeline of intraperitoneal injection in 1–2-day-old TrpV1-Cre pup with AAV9-CAG-flex-GFP. Four weeks after injection, TrpV1Cre::GFP mouse was injected with capsaicin in the whisker pad and sections stained for Fos (n = 4 mice).

**s.** Representative image of trigeminal ganglion with TrpV1Cre::GFP+ neurons. Scale bar, 200 µm.

**t.** Representative image of PB1 with TrpV1Cre::GFP+ axon terminals (green) and capsaicin-induced Fos+ neurons (magenta). Scale bar, 50 µm (high magnification).

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**Figure 2. 7.** Labeling of TrpV1-Cre+ primary sensory neurons but not CNS neurons

(a) Schematic illustration and timeline of intraperitoneal injection in 1–2-day old TrpV1-Cre pup with AAV-CAG-flex-GFP. Four weeks after injection, TrpV1Cre::GFP mouse was injected with capsaicin in the whisker pad and stained for Fos (n = 3 mice).

(b-d) Representative images of a cortical section (b), Sp5C (c), and DRG (d) from a TrpV1-Cre mouse intraperitoneally injected with AAV-CAG-flex-GFP and stained for Fos (magenta). Note that there is no GFP expressing neuronal cell bodies in CNS.

Scale bar, (b, c) 500 µm and (d) 50 µm.
Figure 2. Selective labeling of PBL projecting TrpV1-Cre+ neurons using a retrograde-FlpO and TrpV1-Cre intersectional strategy revealed that these neurons project to both PBL and Sp5C.

(a) Schematic illustration of the intersectional strategy (retrograde FlpO together with TrpV1-Cre) to selectively label TrpV1-Cre+ neurons projecting to PBL. (b-d) Representative images of sparse labeling results using RG-LV-hSyn-DIO-FlpO in combination with TrpV1-Cre in Ai65 reporter (n=6): (b), labeled axon terminals in PBL. (c), few labeled TG neuron cell bodies. (d), labeled axon terminals in Sp5C. Scale bars, 50, 20, 50µm. (e-h) Representative images of dense labeling results using RG-LV-hSyn-FlpO in combination with TrpV1-Cre in Ai65 reporter (n = 4): (e), labeled axon terminals in PBL. (f), labeled TG neurons. (g), labeled axon terminals in Sp5C. (h), labeled peripheral axon terminals in lower lip and whisker pad. Scale bars, 50µm (e), 50µm (f), 100µm (g), 50µm (h).
3.4.1. \( \text{TrpV1-Cre}^+ \) primary trigeminal sensory neurons provide functional monosynaptic excitatory input onto PB\(_L\)-pain neurons.

To directly examine whether TG→PB\(_L\) axons form functional synaptic connections in PB\(_L\), we injected Cre-dependent AAV9-flex-ChR2-YFP into TrpV1-Cre pups intraperitoneally to express channelrhodospin-YFP (ChR2-YFP) in peripheral TrpV1-Cre\(^+\) neurons (TrpV1Cre::ChR2), and performed whole-cell patch clamp recording of PB\(_L\) neurons in slices from these animals (Fig. 2.9a). Photoactivation of TrpV1Cre::ChR2\(^+\) terminals elicited excitatory post-synaptic currents (EPSCs) in 15 out of 54 neurons (Fig. 2.9b, c and Fig. 2.10). Furthermore, the EPSCs persisted in the presence of action potential blockade using 1 \(\mu\)M TTX and 100 \(\mu\)M 4-AP (Fig. 2.9b). In a complementary set of experiments, we captured PB\(_L\)-pain neurons using CANE-RV-mCherry in TrpV1Cre::ChR2 animals (Fig. 2.9d). In 6 CANE-captured mCherry\(^+\) PB\(_L\)-pain neurons, photoactivation of TrpV1\(^+\) terminals elicited EPSCs that were not blocked by TTX (Fig. 2.9e, f). These results corroborate and extend the circuit tracing findings that the inputs from TG TrpV1-Cre\(^+\) fibers to PB\(_L\)-nociceptive neurons are monosynaptic and excitatory.
Figure 2.9. Optogenetic activation of TrpV1-Cre+ sensory axons activates PBi-nociceptive neurons and elicits aversive behavior and stress calls in a real-time place escape/avoidance task.

a, Schematic illustration of intraperitoneal injection of a 1–2-day-old TrpV1-Cre pup (n = 3), followed by optogenetic-assisted whole-cell patch-clamp recording from a PBi neuron in acute brain slices. b, Representative traces from a cell showing no light-evoked IPSC at a holding potential of 10 mV, but observed to have light-evoked EPSC at a holding of −65 mV. Cell, held at −65 mV, was bath applied 1 µM TTX, followed by 100 µM 4-AP and 1 µM TTX, and showed a light-evoked monosynaptic EPSC. c, Averaged current amplitude. Data are mean ± s.e.m. (closed circles represent individual cells, n = 15). d, Representative high-magnification image of TrpV1Cre::ChR2+ axon terminals and CANE-RV-mCherry captured PBi-pain neurons (n = 3 mice; scale bar, 50 µm). e, Representative example of an mCherry+ PBi-pain neuron recorded to have light-evoked EPSC at a holding of −65 mV. Cell was bath applied 1 µM TTX, followed by 100 µM 4-AP and 1 µM TTX, and showed a light-evoked monosynaptic EPSC. f, Averaged current amplitude. Data are mean ± s.e.m. (closed circles represent individual cells, n = 6). g, Schematic illustration of real-time place escape/avoidance (PEA) test. h,i, Representative spatial tracking maps showing the location of (h) an experimental mouse before, during, and after optogenetic stimulation of TrpV1Cre::ChR2+ axon terminals and (h) a control mouse before,
during, and after illumination of TrpV1Cre::GFP+ axon terminals in the PB. In the preferred chamber. j, Percentage of preference (per 30 s) the experimental and control groups had before, during, and after optogenetic stimulation (n = 8, 3) shown across time (min). Data are mean ± s.e.m. k, Quantification of time the TrpV1Cre::ChR2 group spent in preferred chamber before, during, and after optogenetic stimulation (n = 8 one-way repeated measures ANOVA; ****P ≤ 0.0001, *P = 0.0128, ****P ≤ 0.0001; F 2,14 = 49.41). Data are mean ± s.e.m. l, Quantification of time the TrpV1Cre::GFP group spent in preferred chamber before, during, and after light illumination (n = 3; one-way repeated measures ANOVA; P = 0.8867, P = 0.6377, P = 0.8886; F 2,6 = 0.4412). Data are mean ± s.e.m. m, Schematic illustration of vocalization recording chamber. n, Quantification of frequency of pips induced by optogenetic stimulation of TrpV1Cre::ChR2 (experimental) or TrpV1Cre::GFP (control) axon terminals in the PB. Data are mean ± s.e.m. (ChR2, n = 8; GFP, n = 3; two-tailed unpaired Student’s t test; **P = < 0.0001; t 7 = 10.13).

![Figure 2. 10. EPSC characterization of PBL neurons receiving direct TrpV1Cre::ChR2+ TG afferent inputs](image)

**a-c**, Quantification of the rise time, half-width, and decay time of the photo-stimulating TrpV1Cre::ChR2 axons evoked EPSCs in recorded PB neurons (n = 15 cells). Data are mean ± SEM. **d**, Correlation of the TrpV1Cre::ChR2 evoked EPSC amplitude over the onset latency of the EPSC. (Nonlinear regression; n = 15 cells).
3.4.2. Activation of TrpV1-Cre\(^+\) axon terminals in PB\(_L\) induces robust aversive behavior and audible vocalization.

To address the behavioral impact of the direct TG→PB\(_L\) monosynaptic projection in awake behaving animals, we asked whether its activation would be sufficient to elicit aversive responses in a modified real-time place escape/avoidance (PEA) assay, which has been used in recent studies to assay affective components of pain\(^{79-81}\). Optic fibers were implanted bilaterally above PB-el in either TrpV1Cre::ChR2 mice (n = 8) or control mice TrpV1Cre::GFP (n = 3) mice (Fig. 2.9g). Mice were habituated and placed in a two-chamber arena. Their behaviors were recorded under three conditions: (i) freely exploring with no stimulation for 10 min (baseline), followed by (ii) 10 min of conditioned photoactivation when the mouse is in its preferred chamber (stimulation), and followed again by (iii) 10 min without stimulation (post-stimulation). Upon photo stimulation of TrpV1Cre\(^+\) axons in PB-el, TrpV1Cre::ChR2 mice immediately fled to the opposite chamber (Fig. 2.9h), and subsequently they moved less and spent significantly more time on the un-stimulated side (Fig. 2.9h, j-k, P < 0.0001). In the post-stimulation period, some but not all mice still showed avoidance of the chamber in which they received photostimulation (Fig. 2.9j-k). Light illumination
had no effect on movement and behavior of the control TrpV1Cre::GFP mice (Fig. 2.9i, j, l; P = 0.66). These results suggest that the optogenetic stimulation of the TG→PB monosynaptic projection caused a drastic aversive effect likely due to activation of the downstream affective pain pathway.

We further wanted to determine whether optogenetic activation would be sufficient to induce an aversive affective memory using the conventional conditioned place aversion (CPA) assay (Fig. 2.11a). Mice were habituated first by placing them in the two-chamber arena and allowing free exploration. Subsequently, they were subjected to two days of conditioning: mice were paired with photostimulation in the preferred chamber for 15 min and four hours later, they were placed in the non-preferred chamber with no stimulation for 15 min. On the fourth day, they explored the arena freely with no light stimulation for 10 min (post-stimulation). All TrpV1Cre::ChR2 mice (n = 7) spent less time in the chamber where they were stimulated previously (Fig. 2.11b, c; P = 0.008). Light illumination had no effect on the movement and behavior of the control TrpV1Cre::GFP mice (n = 5; Fig. 2.11d, e; P = 0.258). These results suggest that repeated optogenetic activation of the TG→PBL.
Figure 2.11. Optogenetic activation of ChR2-expressing TG afferents in the PBL induces place avoidance in a conditioned place aversion (CPA) assay.

(a) Schematic illustration of the conventional conditioned place aversion (CPA) test. (b) Representative spatial tracking map showing the location of an experimental mouse before and after optogenetic stimulation of TrpV1Cre::ChR2+ axon terminals in the PBFL in the preferred chamber. (c) Quantification of time the experimental group spent in preferred chamber before and after optogenetic stimulation (n = 7 two-tailed paired Student’s t test; **P = 0.0080; t₆ = 3.899). Data are mean ± SEM. (d) Representative spatial tracking map showing the location of an experimental mouse before and after light illumination of TrpV1Cre::GFP+ axon terminals in the PBFL in the preferred chamber. (e) Quantification of time the control group spent in preferred chamber before and after light illumination (n = 6, two-tailed paired Student’s t test; P = 0.2576; t₄ = 1.319). Data are mean ± SEM.

Monosynaptic projection induces an aversive memory. We further recorded audios of mice placed in a circular arena (Fig. 2.9m). Optogenetic activation of TrpV1-Cre+ afferents in PB-el induced audible vocalizations in TrpV1Cre::ChR2 (n = 8) resembling distress calls, but not in control TrpV1Cre::GFP mice (n = 3)
(Fig. 2.9n, Fig. 2.12, on average 66 ± 7 pips with 2 ± 0.2 pips/second were elicited; P < 0.0001). Distress vocalization stopped when laser light was turned off.

![Image](image1.png)

**Figure 2.12. Optogenetic activation of ChR2-expressing TG afferents in the PBL induces vocalization**

Representative spectrograms of induced audible vocalizations of an adult mouse during photo activation (20ms pulses at 10Hz) of TrpV1Cre::ChR2+ axon terminals within the PBl. Vocalization stops when laser light turns off (n = 8 mice).

Post-hoc immunostaining conducted after photo-stimulation of the TrpV1Cre::ChR2 axon terminals in the PBl showed marked Fos expression in this region photo-stimulation of the TrpV1Cre::ChR2 axon terminals in the PBl showed marked Fos expression in this region, whereas only background Fos expression was observed in Sp5C (Fig. 2.13a, b), indicating that there was little back propagation of activities from PBl axon-terminal photo-stimulation to the axon branches of TG sensory neurons in Sp5C. Post-hoc immunostaining after photo-stimulating TrpV1Cre::GFP axon terminals only showed background level Fos expression (Fig. 2.13c, n = 5). Taken together, these data demonstrate that
activating the direct axonal projection from TrpV1-Cre⁺ terminals in PB-el is sufficient to induce robust escape/avoidance behavior, aversive memory, and audible distress vocalizations, which are surrogates of pain behavior and pain associated negative affect.

**Figure 2.** Post-Hoc analysis after optogenetic stimulation of TrpV1Cre::ChR2⁺ axons in PBL

(a) Representative image from a TrpV1-Cre mouse (n = 5 mice) intraperitoneally injected with AAV-flex-ChR2-EYFP which underwent photo stimulation. Numerous Fos⁺ (magenta) neurons in PBₐ were observed after photo stimulation of TrpV1Cre::ChR2⁺ axon terminals (green). Scale bar, 100 µm. (b) Relatively few Sp5C neurons expressed Fos (magenta) after photo stimulation of TrpV1Cre::ChR2⁺ axon terminals in the PBₐ. Scale bar, 100 µm. (c) Representative image from a TrpV1-Cre mouse intraperitoneally injected with AAV-flex-GFP which underwent photo illumination (n = 3 mice). Few PBₐ neurons expressed Fos (magenta, background expression) after photo stimulation of TrpV1Cre::GFP⁺ axon terminals (green). Scale bar, 100 µm.

### 3.4.3. Silencing TrpV1-Cre⁺ axon terminals in PBₐ partially reduces mechanical allodynia in face but not in hindpaw after capsaicin injection

We next asked whether silencing the direct TG→PBₐ monosynaptic projection would affect pain-related behaviors. Previous studies showed that the optogenetic silencer archaerhodopsin (Arch) could effectively silence nociceptors
including TrpV1+ neurons\textsuperscript{39,40}. We therefore used the neonatal IP injection strategy to express eArch\textsuperscript{61} or GFP in TrpV1-Cre\textsuperscript{+} sensory neurons. Optic fibers were implanted bilaterally above PB-el in TrpV1Cre::eArch mice (n = 9) or TrpV1Cre::GFP (n = 8) mice (Fig. 2.14 a, b). A von Frey test was used to assess the mechanical threshold of face or paw withdrawal responses before and after capsaicin injections into the whisker pad or hindpaw, and with or without photo-silencing of TrpV1-Cre\textsuperscript{+} axons in PBel (Fig. 2.14a). After capsaicin injection into either the face or the paw, both TrpV1Cre::eArch and TrpV1Cre::GFP mice drastically lowered the withdrawal threshold in responses to von Frey application to face or paw, respectively (Fig. 2.14 c, d). Hence, capsaicin-injection induced mechanical allodynia in both face and hindpaw as expected (Fig. 2.14 c, d). Importantly, eArch-mediated photo-silencing of TrpV1-Cre\textsuperscript{+} axons in PB-el partially alleviated the capsaicin induced alldynia in the face but had no effect on the mechanical hypersensitivity of the hindpaw (Fig. 2.14 c, d; face P = 0.0046, paw P = >0.9999). Light illumination had no effect on TrpV1Cre::GFP mice (Fig. 2.14 c, d; P = >0.9999). These results confirmed that the TG→PBel direct pathway indeed specifically contributes to face nociception.

We further tested whether photo-silencing of TrpV1-Cre\textsuperscript{+} axons in PBel after facial capsaicin injection would elicit conditioned place preference for the light
illuminated chamber. The effect of capsaicin only lasts about 20 min, we therefore performed a real-time place preference (RTPP) assay (Fig. 2.14e, 10 min no light and 10 min with light illumination in the non-preferred chamber). After capsaicin injection into the whisker pad, TrpV1Cre::eArch mice spent significantly more time in the chamber with photo-silencing of the TrpV1-Cre$^+$ terminals in PBl (Fig. 2.14f, n = 6, P = 0.029). By contrast, control TrpV1Cre::GFP mice show no preference (Fig. 2.14g, n = 7, P = 0.6). Taken together, these data demonstrated that when mice are subjected to noxious facial stimulation, silencing the neural activity of the direct TG→PBl pathway reduces facial allodynia and induces place preference, indicating that this pathway contributes significantly to the manifestation of facial-pain equivalents.
Figure 2.14. Optogenetic silencing of TrpV1-Cre$^+$ axon terminals in PB selectively reduces face allodynia after capsaicin injection.

a, Schematic illustration of intraperitoneal injection of a 1–2-day-old TrpV1-Cre pup followed by face and hindpaw von Frey tests in the same individual mice in TrpV1Cre::eArch ($n=9$) and TrpV1Cre::GFP groups ($n=8$). The order of face versus hindpaw tests was randomized. Each mouse was tested before and after the injection of 10 µL 4% capsaicin into either face or hindpaw. b, Representative post-hoc image of TrpV1Cre::eArch$^+$ axon terminals in PB and labeled TrpV1Cre::eArch$^+$ cell bodies in TG ($n=9$ mice; scale bars, 50 µm). c, Quantification of mechanical thresholds of face withdrawal responses in von Frey tests. Measurements were taken before and after capsaicin injection into right whisker pad, as well as without and with optogenetic
silencing, in TrpV1Cre::eArch \((n = 9)\) or in control TrpV1Cre::GFP groups \((n = 8,\) two-way repeated measures ANOVA; \(eArch vs. GFP\) \(P \geq 0.9999,\) \(P \geq 0.9999,\) \(P \geq 0.9999,\) 
\(*P = 0.0440;\) \(\) (no light vs. light) \(eArch: P \geq 0.9999,\) \(\) **\(P = 0.0046,\) 
GFP: \(P \geq 0.9999,\) \(P \geq 0.9999;\) \(F_{3,45} = 2.671).\) Data are mean ± s.e.m. d, Quantification of mechanical thresholds of hindpaw withdrawal responses in von Frey tests. Measurements were taken before and after capsaicin injection into right hindpaw, as well as without and with optogenetic silencing in TrpV1Cre::eArch \((n = 9)\) or in TrpV1Cre::GFP groups \((n = 8,\) two-way repeated measures ANOVA; \(Arch vs. GFP\) \(P \geq 0.9999,\) \(P \geq 0.9999,\) \(P \geq 0.9999,\) \(P \geq 0.9999;\) \(\) (no light vs light) 
Arch: \(P \geq 0.9999,\) \(P \geq 0.9999,\) GFP: \(P \geq 0.9999,\) \(P \geq 0.9999;\) \(F_{3,45} = 0.03048).\) Data are mean ± s.e.m. e, Schematic illustration of real-time place preference (RTPP) test of mouse injected with capsaicin into left whisker pad. f, Quantification of time the experimental group spent in non-preferred chamber before capsaicin, after capsaicin, and without or with optogenetic silencing \((n = 6\) one-way repeated-measures ANOVA; \(P = 0.5356,\) \(*P = 0.0174,\) \(\) **\(P = 0.0031;\) \(F_{2,10} = 10.92).\) Data are mean ± s.e.m. g, Quantification of time the control group spent in non-preferred chamber before capsaicin, after capsaicin, and without or with optogenetic silencing \((n = 7;\) one-way repeated measures ANOVA; \(P = 0.7320,\) \(P = 0.2086,\) \(P = 0.5537;\) \(F_{2,10} = 1.695).\) Data are mean ± s.e.m.

2.5. Conclusion

In this study, we discovered that nociceptive trigeminal afferents transmit painful signal to the affective pathway through both the direct monosynaptic TG→PB\(_{ll}\) and the indirect disynaptic TG→Sp5C→PB\(_{ll}\) projections. Previously Panneton et al injected neural tracer WGA-HRP into the peripheral anterior ethmoidal nerve (AEN), which is originated from TG and innervate the nasal cavity, and observed labeled afferent fibers in regions near PB\(_{ll}\)\(^{54}.\) In a follow-up study, the authors showed that trigeminal rhizotomy resulted in loss of CGRP expressing fibers innervating the PB\(_{ll}\)\(^{55}.\) These and other studies have implied a possible direct TG→PB\(_{ll}\) pathway\(^{33,34,54,55},\) but did not provide synaptic or behavior
evidence to support this possibility. Here we used a combination of activity-dependent tagging, monosynaptic transsynaptic tracing, intersectional genetic labeling, optogenetic-assisted slice electrophysiology, and in vivo optogenetic activation and silencing experiments to definitely establish the monosynaptic connection between TG and PBl-nociceptive neurons, and revealed the important functions of this pathway in craniofacial pain-related aversive behaviors.

Our findings have several important implications. First, the dual and bilateral pain-transmitting pathways compared to the single indirect DRG→dorsal horn→PBl pathway could explain why similar intensity stimuli applied to face activate more PBl neurons than when applied to limbs. This could in turn lead to heightened and bilateral activations of the affective pain responses, such as a higher level and more persistent activation of CeA, BNST, hypothalamus, and insular cortex through the axonal projections from PBl-nociceptive neurons (Fig. 2.4k-r). This projection pattern can provide a circuit basis for the perception of trigeminally-mediated pain as more severe, fear-inducing and emotionally-draining than other body pain. The monosynaptic TG→PBl connection also provides a mechanism for rapid, short-latency direct connections of nociceptive inputs from the head and face to brain centers involved in homeostatic regulation and emotional processing\textsuperscript{8,11,18,35,36,82}. Second, current palliative neurosurgical
procedures aimed at alleviating refractory trigeminal pain target the descending spinal trigeminal tract (Fig. 2.15) including making thermal lesions, referred to as “dorsal root entry zone coagulation” (DREZ), to lesion pain-transmitting pathways in Sp5C, a contemporary adaptation of the classic trigeminal tractotomy\textsuperscript{44,45}. Based on our study, DREZ coagulation will only lesion the TG→Sp5C connection, while leaving the TG→PB\textsubscript{L} connection intact (Fig. 2.15). This may explain the lack of therapeutic response or post-operative pain relapse in some patients subjected to trigeminal DREZ surgery\textsuperscript{43-45}. Future surgical procedures should consider severing both TG→Sp5C and the TG→PB\textsubscript{L} connection for providing invasive palliation of chronic, refractory orofacial pain, e.g. for trigeminal neuralgia. Notably, our discovery presented here critically relied on the CANE methodology, although CANE does have qualifiers, namely the 60–90min waiting interval between the stimulus application and the surgery (in order for Fos/TVA protein to reach peak levels) inevitably resulted in some background labeling. Nevertheless, CANE is still the most well-validated tool to selectively label and transsynaptically trace the presynaptic inputs to transiently activated neurons as shown here and in our previous studies\textsuperscript{32}. Our input-output circuit mapping of PB\textsubscript{L}-nociceptive neurons revealed many limbic centers that are reciprocally connected with PB\textsubscript{L}, providing a circuit basis for understanding
closely associated and clinically highly-relevant comorbidities with pathologic trigeminal pain, namely anxiety, depression, disturbance of circadian rhythm and altered intake behavior\textsuperscript{42-44,56,83,84}. Future studies on mechanisms underlying chronic craniofacial pain disorders can now take advantage of this circuit diagram including the newly unveiled monosynaptic TG→PB\textsubscript{i} pathway to identify specific maladaptive.

![Diagram of trigeminal sensory pathways](image)

**Figure 2. 15. Schematic illustration of trigeminal sensory pathways**

Schematic summary for output targets of tactile (green) and nociceptive (red) trigeminal ganglion (TG) sensory neurons, including the newly discovered TG→PB\textsubscript{i} projection. Schematic illustration demonstrates location of where DREZ (dorsal root entry zone coagulation) is performed to lesion the TG→Sp5C pathway to treat refractory craniofacial pain. Note that DREZ lesion will not affect the TG→PB\textsubscript{i} projection. Pr5, principal sensory trigeminal nucleus; Sp5O, trigeminal nucleus, oral; Sp5I, trigeminal nucleus, interpolaris; Sp5C, trigeminal nucleus, caudalis; DREZ, dorsal root entry zone coagulation.
2.6. Chapter Acknowledgements

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3. Conclusion
3.1. Dissertation summary

This research was motivated by the need to find alternative approaches to alleviate chronic pain, in particular craniofacial pain. Overall, chronic pain is a major health problem in the US and the economic burden approaches $600 billion per year in lost productivity and medical interventions. Pathological craniofacial chronic pain, in particular, is not only a physical ordeal, but also an emotional drain, and is refractory to currently available treatments. Knowledge gained from this work should significantly further our understanding of central emotional/affective pain processing circuit, and provide the basis for understanding the abnormal processing in chronic pathological craniofacial pain. As discussed in chapter 1, the neurobiological mechanism underlying heightened affective craniofacial pain perception has been poorly understood. Furthermore, conventional molecular tools targeting genetically specific neuronal populations have been limited in their ability to study the precise connectivity and causal functions of a molecularly diverse set of PBt neurons activated by specific type of noxious stimulus. Therefore, the hypotheses tested were as follows: 1) Noxious chemical stimuli injected into the mouse whiskerpad activates more neurons in PBt than noxious chemical stimuli injected into the hindpaw. 2) The CANE
system can specifically and efficiently capture noxious stimuli-activated PB neurons, and facilitate in the visualization of all axonal projection targets and presynaptic inputs of noxious stimuli-activated PB neurons. 3) Activation of primary sensory TrpV1+ afferents in PB can excite PB neurons, resulting in aversive behavior. Whereas, silencing primary sensory TrpV1+ afferents in PB can reduce craniofacial pain perception. The aims of this research were to address each of the above hypotheses by 1) evaluating whether PB is more robustly activated by noxious craniofacial stimuli than by noxious bodily stimuli; 2) identifying what the presynaptic inputs of noxious stimuli-activate PB; and 3) investigating the physiological and behavioral function of the direct trigemino-parabrachial circuit in noxious craniofacial perception.

Chapter 2 described the discovery that nociceptive trigeminal afferents transmit painful signal to the affective pathway through both the direct monosynaptic TG→PB and the indirect disynaptic TG→Sp5C→PB projections. A combination of activity-dependent tagging, monosynaptic transsynaptic tracing, intersectional genetic labeling, optogenetic-assisted slice electrophysiology, and in vivo optogenetic activation and silencing experiments was used to definitely establish the monosynaptic connection between TG and PB-nociceptive neurons,
and revealed the important functions of this pathway in craniofacial pain-related aversive behaviors.

Taken as a whole, these results are indicative of the pain pathways required for heightened perception of craniofacial pain. Specifically, the data showed that formalin injection in the whiskerpad induces robust, bilateral expression in a molecularly diverse set of neurons in PB\textsubscript{L}, these noxious stimuli-activated PB\textsubscript{L} neurons receive direct excitatory input from molecularly diverse set of primary sensory trigeminal ganglion neurons, and this trigemino-parabrachial pathway is required for craniofacial pain-related aversive behavior.

3.2. Future Work

The results from Chapter 2 show that unlike body sensory neurons, craniofacial nociceptive neurons directly synapse with noxious stimulus-activated PB\textsubscript{L} neurons which in turn project to multiple limbic centers processing emotions and affects. Furthermore, these findings are the first to definitely prove this monosynaptic pathway is both sufficient and necessary for craniofacial-pain-activated aversive behaviors. Despite these incredible findings, they fall short of conclusively determining the synaptic mechanism in which this direct TG→PB\textsubscript{L} interacts with the Sp5C→PB\textsubscript{L} circuit and how this
differs from SC → PBl. For example, do these two pathways converge on the same PBl neuron or do they synapse onto two distinct PBl populations. Do TG synapses facilitate in amplifying the signal sent from Sp5C synapses terminating in PBl? To further address this question, future work could include anterograde tracing from Sp5C and TG to uncover the spatial distribution pattern of axonal terminals in PBl. Other work could include determining the synaptic connectivity of TG and Sp5C afferents on PBl neurons and assessing whether activation of these two circuits induce synaptic potentiation. Additionally, one can compare whether activation of Sp5C afferents can induce larger post-synaptic currents in comparison to activation of SC afferents using slice or in-vivo electrophysiology.

Additionally, the findings lacked evidence of whether SC or Sp5C signaling induces similar aversive pain-like behavior responses in mice. It is not yet certain whether activation of SC afferents would induce a less escape-like behavior and little/no audible vocalization, or if silencing would reduce thermal allodynia. To further address this question, future work could include optogenetic activation or silencing of Sp5C or SC axon terminals in PBl during various behavioral assays, such as place preference tests or pain reflexive tests.
Furthermore, it is unclear whether this pathway contributes to the maladaptive plasticity that results in chronic facial pain disorders. It would be interesting to see if in future studies, chronic pain models, such as nerve ligation would induce synaptic changes in the TG → PB l and Sp5C→PB l pathways. Additionally, other future work could include ligating the facial nerve and silencing the TG → PB l and Sp5C→PB l pathways to see if this completing reverses allodynia and hyperalgesia.

Moreover, little is known about the local circuitry within the functionally diverse PB l and how this would modulate the nociceptive signals relaying through PB l. It would be interesting to determine whether neuronal ensembles activated by appetitive taste/gustatory/thermal signals attenuates/dampens the excitability of neuronal ensembles activated by various aversive signals, and vice versa. Future studies could include using a combination of activity-dependent capturing methods to label appetitive and aversive neuronal ensembles (one with channelrhodopsin and another with gfp/tdtomato) and performing optogenetic-assisted slice electrophysiology to assess whether activation/silencing of one set of neurons changes the threshold to fire of the opposing set of neurons and vice versa. This work could begin to unravel how PB l regulates which
valence signal to convey to the rest of the limbic system resulting in either an organism’s avoidance or attraction to any particular stimuli.

Appendix A: Identification of distinct ChAT+ neurons and activity-dependent control of postnatal SVZ neurogenesis


4.1. Abstract

Postnatal and adult subventricular zone (SVZ) neurogenesis is believed to be primarily controlled by neural stem cell (NSC)-intrinsic mechanisms, interacting with extracellular and niche-driven cues. Although behavioral experiments and disease states have suggested possibilities for higher level inputs, it is unknown whether neural activity patterns from discrete circuits can directly regulate SVZ neurogenesis. We identified a previously unknown population of choline acetyltransferase (ChAT)+ neurons residing in the rodent
SVZ neurogenic niche. These neurons showed morphological and functional differences from neighboring striatal counterparts and released acetylcholine locally in an activity-dependent fashion. Optogenetic inhibition and stimulation of subependymal ChAT+ neurons in vivo indicated that they were necessary and sufficient to control neurogenic proliferation. Furthermore, whole-cell recordings and biochemical experiments revealed direct SVZ NSC responses to local acetylcholine release, synergizing with fibroblast growth factor receptor activation to increase neuroblast production. These results reveal an unknown gateway connecting SVZ neurogenesis to neuronal activity-dependent control and suggest possibilities for modulating neuroregenerative capacities in health and disease.

4.2. Introduction

Robust generation of adult-born neurons, from the rodent subventricular and subependymal zone (SVZ/SEZ) neurogenic niche, is a useful experimental system for studying regenerative capacities in the mammalian brain. SVZ neurogenesis provides tractable assays to tackle molecular and cellular-level mechanisms regulating addition of new neurons into established neural circuits. It also serves as a wonderful model for understanding how tissue stem cells and their progeny respond to injury and
The consensus view currently is that postnatal and adult SVZ neurogenesis is mediated by subependymal glial fibrillary acidic protein (GFAP)+ B-type astrocytes functioning as NSCs, producing transiently amplifying Mash1+ progenitors that differentiate into doublecortin (DCX)+ neuroblasts, which then migrate to the olfactory bulb through the rostral migratory stream. Although the term SVZ neurogenesis is widely used to describe this process, neuroblasts are born in the subependymal space around the lateral brain ventricles.

As in other tissue stem cell niches, self-renewal of SVZ NSCs and production of differentiating progeny are controlled by well-conserved cell-intrinsic molecular pathways. In addition, extracellular factors and cell-cell interactions in the NSC microenvironment are also critical. For example, blood vessels in the SVZ niche have been shown to regulate NSC function by acting as sources for neurogenic signals. In addition, ependymal cells lining the ventricular surface can provide instructive cues to sustain new neuron production, as well as redirecting NSC responses to local tissue damage. Together, the SVZ niche provides a rich environment for trophic factors, coordinating NSC homeostasis.
Other than classical stem cell niche factors, neurotransmitters, common currencies for neural circuit activity and modulation, have also been shown to be important during adult SVZ neurogenesis\textsuperscript{98,99}. Excitatory neurotransmitter glutamate can influence proliferation and differentiation of neural progenitors through mGluR activation\textsuperscript{100,101}. Glutamate also enhances survival of DCX\(^+\) neuroblasts and newborn neurons through activating NMDA receptors\textsuperscript{101,102}. Inhibitory neurotransmitter GABA is believed to control progenitor proliferation through GABA\(_A\) receptor\textsuperscript{103,104}, activating voltage-gated calcium channels in SVZ astrocytes\textsuperscript{105}. Modulatory neurotransmitter dopamine has also been shown to stimulate SVZ proliferation\textsuperscript{106} through increased epidermal growth factor (EGF) secretion\textsuperscript{107}. Serotonin\textsuperscript{108,109}, as well as cholinergic activation\textsuperscript{110,111}, are believed to have similarly positive effects on SVZ cellular proliferation. Despite this knowledge, it remains unclear whether neuronal activity can directly regulate postnatal and adult SVZ neurogenesis, as the exact neurons capable of performing such functions have not been identified. It is currently unknown whether the SVZ niche contains resident neurons that provide local innervation.

We performed a direct comparison of neurotransmitters \textit{in vitro} for their neurogenic properties. We found that acetylcholine (ACh) markedly increased
DCX⁺ neuroblast production. As we searched for potential ACh sources in vivo, we uncovered a previously unknown population of ChAT⁺ neurons residing in and innervating the SVZ niche, with distinct morphological/functional properties from cholinergic neurons in the neighboring striatum. After identifying these subependymal ChAT⁺ neurons, we sought to determine their function and found an important gateway connecting postnatal and adult SVZ neurogenesis to neuronal activity-dependent modulation.

4.3. Materials and Methods

4.3.1. Animals

All mouse experiments were performed according to an approved protocol by the Institutional Animal Care and Use Committee at Duke University. The following mouse lines were purchased from JAX: Chat<sup>res</sup>-<i>cre</i>+/+ (#006410); Chat<sup>loxP</sup>-+/+ (#016920); Nkx2.1-Cre (#008661); R26R-ChR2EYFP (#012569); R26R-ArchGFP (#012735); Chat-ChR2EYFP(#014545); GFAP-GFP (#003257). Drd2-Cre<sup>112</sup> and Chat-EGFP<sup>113</sup> mice were purchased from MMRRC. Generation of Ank3 mutant allele was as described<sup>114</sup>. 

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4.3.2. Cell culture, imaging, and analyses

Adherent SVZ NSC culture and differentiation were performed as described\textsuperscript{115}. ACh, dopamine, muscimol, serotonin, glutamate, mecamylamine, atropine (Tocris), bFGF and EGF (Invitrogen) and α-FGF neutralizing Ig\textsuperscript{116} (#05-117, Millipore) were used at concentrations as described for each experiment, and added fresh daily during \textit{in vitro} differentiation. For FGF ELISA assay, SVZ culture was differentiated in 2 ml of media, and 100 μl was collected for analysis at times indicated following manufacturer's protocol (FGF mouse ELISA kit, Abcam). \textit{In vivo} tamoxifen induction was performed as described\textsuperscript{95}. All IHC staining images were acquired on Leica TCS SP5 confocal microscope, with control and experimental samples imaged under identical instrument settings. Imaris software was used for three-dimensional image projections, signal colocalization and quantifications as described\textsuperscript{96}. Counting of SVZ proliferating cells was performed as described\textsuperscript{117}.

4.3.3. IHC staining and electron microscopy

Preparation of brain tissue for IHC staining was as described\textsuperscript{95,118}. We used primary antibodies to GFP (#GFP-1020, 1:500, Aves Labs), RFP (#600-401-379, 1:1,000, Rockland), ChAT (#AB144P, 1:100, Millipore), VACHT (#G4481, 1:100, Promega), DCX (#AB2253, 1:200, Millipore), Phospho-S6 Ribosomal
Protein (#2215, 1:200, Cell Signaling), Ki67 (#ab15580, 1:800, Abcam), Mash1 (#556604, 1:100, BD Pharmigen), Nestin (#Rat-401, 1:50, Developmental Studies Hybridoma Bank), S100β (#SAB1402349, 1:200, Sigma), Iba1(#ab15690, 1:200, Abcam), CD11b (#MCA711G, 1:200, AbD Serotec), NG2 (#MAB6689, 1:200, R&D Systems), Caspase 3 (#9664, 1:200, Cell Signaling), Thbs4 (#AF2390, 1:200, R&D Systems), α3-NicotinicR (#AB5590, 1:500, Millipore), MuscarinicR (#10-217, 1:500, Argene), GFAP (#G3893, 1:1,000, Sigma), phospho-FGFR (#3476, 1:500, Cell Signaling). All antibodies used were validated in our previous publications or by publications available on vendor website specific to each antibody. For phospho-FGFR staining, samples were blocked and stained in PBST (0.1% Triton X-100 (vol/vol), in PBS), washed in phosphate-buffered saline (PBS). For immunogold electron microscopy, animals were perfused with 1% glutaraldehyde (wt/vol) and 4% paraformaldehyde (wt/vol, Polysciences) in 0.1 M PBS according to an online protocol (http://synapses.clm.utexas.edu/lab/howto/protocols/Perfusion_Harris_v20090413.pdf). 50-µm vibratome sections were incubated for 30 min at 23–25 °C in 1% sodium borohydride (wt/vol, EMS), followed by 1 h at 23–25 °C blocking in 1% BSA (wt/vol), and incubation in primary Ig to RFP ((#600-401-379, 1:1,000,
Rockland) for 48 h at 4 °C. Ultra Small Immunogold (0.8 nm)-conjugated secondary Ig incubation (rabbit, 1:50, EMS) was performed in 0.8% BSA and 0.1% fish gelatin (wt/vol, CWFS gelatin, Aurion) buffer, 3 h at 23–25 °C. Gold particles were enhanced using Silver IntenSE kit (Amersham GE) for 12 min, followed by incubation in 2% osmium (wt/vol) and dehydrated in a graded percentage of acetone solutions, all at 23–25 °C, followed by epoxy mounting. Thin sections were stained using uranyl acetate (EMS) and Reynold's lead citrate (EMS).

**4.3.4. Cell-attached and whole-cell electrophysiology**

Animals were anesthetized with isofluorane, transcardially perfused and then ventricular wall and striatal sections were dissected as whole mounts in ice-cold NMDG artificial cerebrospinal fluid (ACSF; containing 92 mM NMDG, 2.5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 30 mM NaHCO$_3$, 20 mM HEPES, 2 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 10 mM MgSO$_4$, 0.5 mM CaCl$_2$), and bubbled with 5% CO$_2$/95% O$_2$. Tissues were then bubbled in same solution at 37 °C for 8 min, transferred to bubbled, modified-HEPES ACSF at 23–25 °C (92 mM NaCl, 2.5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 30 mM NaHCO$_3$, 20 mM HEPES, 2 mM glucose, 5 mM sodium
ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 2 mM MgSO₄, 2 mM CaCl₂) for at least 1 h before recording. Recordings were performed in submerged chamber, superfused with continuously bubbled ACSF (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 20 mM glucose, 2 mM CaCl₂, 1.3 mM MgCl₂) at 2–5 ml min⁻¹ at 23–25 °C. Cell-attached recordings were performed using ACSF-filled micropipettes (4–6 MΩ). Signals were amplified with Multiclamp 700B (filtered at 2 kHz), digitized with Digidata 1440A (5 kHz), recorded using pClamp 10 software (Axon). Internal solutions for whole-cell recordings contained 130 mM potassium gluconate, 2 mM NaCl, 4 mM MgCl₂, 20 mM HEPES, 4 mM Na₂ATP, 0.4 mM NaGTP, 0.5 mM EGTA and Alexa 488 dye (Invitrogen) to visualize patched cells. Signals were amplified with Multiclamp 700B (filtered at 10 kHz), digitized with Digidata 1440A (20 kHz), recorded using pClamp 10. Pipette puff application was performed via transistor-transistor logic (TTL) control of Picospritzer III (Parker). Light-activation of channelrhodopsin was delivered by TTL control of 473-nm laser (IkeCool). Tracings were analyzed using Neuromatic package (Think Random) in Igor Pro software (WaveMetrics). Voltage threshold for action potential generation was identified as point of most rapid change in membrane potential, determined by analyzing first and second derivatives in current-
clamped whole-cell recordings. Average spike shape was generated via aligning traces to peak of first action potential after current injection. Time zero = mean time at current pulse initiation.

4.3.5. Dil and DiO-labeling

Dil-labeling was performed according to previously described methods\textsuperscript{119} with the following modifications: following dissection, samples were fixed for 1 h at 23–25 °C with 4% paraformaldehyde (wt/vol) in PBS, then washed 3 × 20 min in PBS. DiI (Invitrogen, 40 \(\mu\)M CM-DiI in ethanol) was loaded into cells using micropipettes and +4-nA current injection for 10 min, followed by 10 min without current for equilibration. Samples were then returned to 4% paraformaldehyde in PBS at 37 °C for 24 h to allow diffusion through neuronal processes. For ventricular views, SVZ niche and striatum were dissected in whole-mount preparation\textsuperscript{120}. For coronal views, brain samples were sliced into 300-\(\mu\)m sections with VT1000S vibratome (Leica). Neuronal processes were traced and analyzed using three-dimensional filament tracer in Imaris software (Bitplane). Dendritic fields were enveloped with three-dimensional hull surface to provide ellipsoid axis length measurements, with 'a' designated as the long axis in either ventricular or
coronal views. Imaris filament statistics provided dendritic branch point numbers and three-dimensional Sholl analysis was run to determine filament crossings through concentric spheres centered on soma. Vybrant DiO (Invitrogen, 40 μM) was dissolved in dichloromethane (Sigma).

4.3.6. M1-CNiFER cell imaging and analyses

Excitation and imaging of CNiFER cells expressing Ca\(^{2+}\) indicator protein TN-XXL\(^{121}\) was performed at 920 nm with 40× (0.8 NA, Nikon) water-dipping objective on multiphoton microscope (Prairie Technologies). Emission light was directed through filter-cube containing T505LPXR dichroic beamsplitter, HQ470/40-2p emission filter, and ET535/30-2p emission filter (Chroma Technologies). Regions of interest were drawn around individual M1-CNiFER cells with average fluorescence for each time point analyzed using Igor Pro software (WaveMetrics). Fluorescence and FRET ratio changes were analyzed as described previously\(^{122}\). For SVZ transplantation experiments, cultured M1-CNiFER cells were triturated from dishes by pipetting without trypsin, re-suspended in ACSF, followed by injection into acute brain slices with pulled (inner diameter = 40 μm) capillary tube using Picospritzer III (Parker). Donepezil (100 μM) was added to ACSF for acute slice experiments.
Light-activation of ChR2EYFP was delivered by TTL control of 473-nm laser (IkeCool).

### 4.3.7. Optogenetic stimulation and analyses

Placement of cannula under isofluorane anesthesia to target the lateral ventricle was performed as described\textsuperscript{123}, using implantable mono fiber-optic fiber (200 µm, 0.22 NA, Doric). Protruding ferrule end of cannula was then connected via fiber cord to rotary coupling joint (Doric), allowing free animal movement. Light-stimulation of ChR2EYFP was delivered by TTL control (Master 8, AMPI) of 473-nm laser (IkeCool), 5-ms pulse duration bursts at 5 Hz, lasting 10 s, given once every 2 min as described\textsuperscript{124}. Light inhibition with ArchGFP was performed by TLL-controlled 556-nm laser (IkeCool), on for duration of experiment. For SVZ p-rpS6, Ki67, Mash1, DCX, Nestin, Iba1, CD11b and NG2 analyses, 50-µm brain coronal sections were cut and collected serially on Leica VT1000S vibratome. Position of optical fiber tract entering the lateral ventricle was first verified, and five coronal sections surrounding the fiber tract were selected for analyses, comparing P30 littermates with light versus no-light stimulation.
4.3.8. SDS-PAGE and immunoblotting

Protein extracts were prepared as described\textsuperscript{125}, and resolved by electrophoresis through SDS-PAGE and transferred onto nitrocellulose membranes. For FACS-sorting of ChAT\textsuperscript{+} neurons, brain tissues were first dissociated with neural tissue dissociating kit (Miltenyi) according to manufacturer’s protocol, followed by sorting on BD FACS DiVa sorter via genetically labeled tdTomato fluorescence. Antibodies were diluted in PBS containing 0.2\% Triton X-100 (vol/vol) and 4\% non-fat dry milk (wt/vol), followed by overnight incubation at 4 °C. Detection was accomplished through secondary antibodies conjugated to horseradish peroxidase (#111-036-003, 115-036-003, 1:5,000, Jackson ImmunoResearch) and treated with enhanced chemiluminescence (Thermoscientific). We used primary antibodies to Ank3 (rabbit, 1:1,000, V. Bennett), DCX (#4604, 1:600, Cell Signaling), pFGFR1 (#06-1433, 1:2,500, Millipore), pEGFR (#2238, 1:800, Cell Signaling) and actin (#ab3280, 1:2,000, Abcam). All antibodies used were validated in our previous publications\textsuperscript{90,96} or by publications available on vendor website specific to each antibody.
4.3.9. Statistical analysis

No statistical methods were used to pre-determine samples sizes, but they are similar to those reported elsewhere\textsuperscript{90,95,96}. IHC staining cell counting from animal experiments was performed blind to the experimental condition; other data collection and analyses were not performed blind to the conditions. Blocking of experimental design was assigned by animal genotype and was not randomized. Data sets were tested for normality with Igor Pro (WaveMetrics) using serial randomness test. Depending on sample size, Student’s $t$ test (≥10) or Wilcoxon two-sample test (≥5) were used for statistical comparisons between two datasets. One-way ANOVA was performed for multivariate comparisons. \textit{In vivo} optogenetic experiments were performed on sets of three littermates and compared via one-way ANOVA for correlated samples. Cell recording data was acquired first in control followed by pharmacological conditions, and compared by one-way ANOVA for correlated samples. Throughout, Tukey box-and-whisker plots were generated using BoxPlotR (http://boxplot.tyerslab.com/) to depict mean (+), median (line), low and high quartiles (boxes), range (whiskers), and outliers (o).

4.4. Result
4.4.1. Neurotransmitter effect on neuroblast production in vitro

We reasoned that defining neurotransmitters with potent neurogenic capabilities may reveal the exact neurons that directly control SVZ neurogenesis. Starting with the SVZ NSC adherent culture assay, we compared the abilities of several key neurotransmitters to enhance DCX⁺ neuroblast production. We differentiated passage two SVZ adherent cultures in the presence of select concentrations of neurotransmitters, focusing on glutamate, GABA, serotonin, dopamine and acetylcholine (Fig. A.1a). We scored the effects of these neurotransmitters on neuroblast production after 5 d of in vitro differentiation by making protein lysates from individually treated culture plate wells and performed western blotting analyses on DCX protein levels (Fig. A.1b). This revealed a potent neurogenic effect of the modulatory neurotransmitter ACh on the production of DCX⁺ neuroblasts from differentiating SVZ NSC cultures (Fig. A.1a,b), which was sensitive to nicotinic or muscarinic inhibition (Fig. A.1c).
Figure A.1. Neurotransmitter effects on DCX+ neuroblast production in vitro.

(a) Representative DCX staining of differentiated SVZ adherent cultures treated pharmacologically. (b) Western blot analyses of protein lysates from individually treated culture plate wells, showing titratable increase in DCX protein levels following acetylcholine (ACh) treatment. Ser: serotonin; Dopa: dopamine; Glut: glutamate; Muscimol: GABA agonist. (c) Western blot analyses of control or ACh-treated (10 µM) cultures in the presence of muscarinic antagonist atropine (Atrop) or nicotinic antagonist mecamylamine (Mec). Western blot analyses for each condition were repeated 5 times with consistent results. Scale bar: 50 µm.
4.4.2. Genetic disruption of cholinergic circuit activity

Given the overall physiological importance of ACh, genetic deletions of ChAT\textsuperscript{126} or the vesicular acetylcholine transporter (VAcT)\textsuperscript{127} result in similar lethal phenotypes shortly after birth. It has been shown that disruption of Ankyrin 3 (Ank3 or AnkyrinG), a large adaptor protein that is necessary for proper axonal initial segment assembly and function, in cerebellar Purkinje neurons results in substantial defects in their abilities to initiate action potentials\textsuperscript{128}. To adopt a similar strategy, we generated a conditional \textit{loxP}-flanked allele for the \textit{Ank3} locus (Fig. A.2a). We crossed \textit{Ank3}\textit{loxP}+/+ mice with \textit{Actb-cre} driver mice to generate \textit{Ank3}−/+ mice, which were then used to generate \textit{Ank3}−/− mutants. These mice died shortly after birth. Protein extracts made from whole-brain lysates confirmed a protein-null mutation for the 480- and 270-kDa isoforms of Ank3 (Fig. A.2b), expressed by wild-type ChAT\textsuperscript{+} neurons (Fig. A.2b), which are known to localize to axon initial segments\textsuperscript{129}.

To conditionally remove Ank3 from cholinergic neurons, we crossed \textit{Chat}\textit{IRES-cre}+/+; \textit{Ank3}\textit{loxP}/+ mice to \textit{Ank3}\textit{loxP}/+; \textit{R26R-tdTomato}\textit{loxP}/loxP mice to generate \textit{Chat}\textit{IRES-cre}+/+, \textit{Ank3}\textit{loxP}/+, \textit{R26R-tdTomato}\textit{loxP}/+ (control), \textit{Chat}\textit{IRES-cre}+/+; \textit{Ank3}\textit{loxP}/+, \textit{R26R-tdTomato}\textit{loxP}/+ (Het), and \textit{Chat}\textit{IRES-cre}+/+; \textit{Ank3}\textit{loxP}/loxP, \textit{R26R-tdTomato}\textit{loxP}/+ (cKO).
mice. The presence of Cre-dependent tdTomato reporter allowed us to visualize ChAT+ neurons for functional studies. Ank3-cKO mice were born in a Mendelian ratio and showed no evidence of perinatal lethality (32 of 232 animals genotyped at postnatal day 7 (P7) were cKO mutants, 13.8% compared to expected 12.5%). We observed occasional shaking in the movements of cKO mice, but otherwise did not detect obvious phenotypic defects.

Immunohistochemical (IHC) staining of tdTomato+ cholinergic neurons from cKO mice showed the lack of Ank3 signal in their proximal axonal segments (Fig. A.2c). Similar to what had been observed in Purkinje neurons, cholinergic neurons from the striatum of cKO mice showed noticeable defects in action potential generation to stimuli, with increasing defects to stronger inputs (Fig. A.2d). Notably, we also detected defects in Ank3-Het ChAT+ neurons, and this was likely a result of differences in Ank3 expression, as Ank3−/+ heterozygous mice showed lower overall Ank3 protein levels in the brain than wild-type littermates (Fig. A.2b). These functional defects are consistent with the notion that cholinergic neurons in cKO mice are unable to effectively relay inputs into action potentials and subsequent release of ACh.

When we examined DCX+ neuroblast production in these Ank3-cKO animals, we saw a marked reduction in neuroblast chains along the ventricular
wall (Fig. A.3a). This defect in SVZ neurogenesis was noticeable at P14, becoming severe at P30 (DCX+ IHC staining coverage/ventricular area: control = 11.71 ± 0.98%, cKO = 4.09 ± 0.83% (mean ± s.e.m.), n = 5; P< 0.01, z = 2.611, Wilcoxon two-sample test; Fig. A.3b,c). Ki67 IHC staining showed a corresponding decrease in SVZ cell proliferation in these animals (Fig. A.3d,e), whereas caspase 3 staining showed no obvious increase in cellular apoptosis (data not shown). Consistent with this decrease in SVZ neuroblasts, we observed diminished Mash1+transiently amplifying progenitors in the SVZ niche of P30 Ank3-cKO mice as compared with controls (Fig. A.3d,e).

ACh exerts its function locally, as it is rapidly degraded by extracellular acetylcholinesterases. Given that striatal cholinergic neurons are anatomically adjacent to the SVZ niche, to understand whether their ACh release could be an important contributor to sustaining robustness of SVZ DCX+ neuroblast production, we analyzed a genetic mouse model in which ACh release from striatal cholinergic neurons is largely eliminated\textsuperscript{130}. We performed IHC staining for DCX, Ki67 and Mash1 and found no obvious SVZ neurogenesis defects in P30 Nkx2.1-cre; Chat\textsuperscript{loxP/loxP} mutant mice versus Nkx2.1-cre; Chat\textsuperscript{loxP/loxP} littermate controls (Fig. A.4).
Figure A. 2. Generation of Ank3-mutant ChAT+ neurons.

(a) Genomic map of ank3 floxed-allele showing insertion of LoxP sites around exons 22 and 23, within Spectrin binding domain of Ank3. (b) Western blot analysis of whole brain lysates from P0 animals, showing successful deletion of higher molecular weight isoforms of Ank3 in ank3KO/KO (KO) mutants (left panel). Western blot analysis of FACS-sorted ChAT+ neurons from ChATires-Cre/+; R26R-tdTomato mice, showing expression of higher molecular weight isoforms of Ank3 protein (right panel). (c) Ank3 IHC staining in tdTomato-labeled ChAT+ neurons in control (Ctrl) and Ank3-cKO (cKO) mice, showing proximal axonal segments (arrows). Imaris software was used to create 3D image reconstructions (lower panels). (d) Representative whole-cell, current-clamp recordings from striatal ChAT+ neurons in response to 100 ms of 250 or 500 pA current pulses in Ctrl, Het, and Ank3-cKO animals. (Right) Quantification of spike numbers to 100 ms of current pulse, 250 or 500 pA. * P < 0.002, t28 = 3.661, unpaired Student’s t test, n = 15 in each group (5 animals). Box plots show mean, median, quartiles, range. Scale bar: 2 µm (c).
Figure A. 3. Ank3 deletion in ChAT+ neurons results in postnatal SVZ neurogenesis defects.

(a) Representative whole-mount DCX staining of SVZ neuroblast chains showing neurogenesis defects in P30 Ank3-cKO mice. DCX fluorescence signal is inverted to black on white for clarity. R, rostral; C, caudal; D, dorsal; V, ventral; Ctrl, control. (b,c) Close-up views of SVZ DCX neuroblast defects in P14 and P30 whole-mount preparation (b) or P30 coronal sections (c) from control and Ank3-cKO mice. (d) Representative IHC staining of Ki67 and Mash1 expression in P30 SVZ niche showing decreased Ki67+ and Mash1+ cell numbers in Ank3-cKO mice. (e) Quantifications of SVZ Ki67 and Mash1 IHC staining data from P30 control and Ank3-cKO animals. *P < 0.008, Wilcoxon two-sample test, n = 5, z = 2.611. Box plots show mean (+), median (−), quartiles (boxes) and range (whiskers). Scale bars represent 100 µm (a) and 50 µm (b–d).
Figure A. 4. SVZ neurogenesis in Nkx2.1-Cre; ChAT^floxy^floxy^mice.

(a) Representative views of ventricular whole-mount DCX staining from P30 Nkx2.1-Cre; ChAT^floxy/+ (Ctrl) and Nkx2.1-Cre; ChAT^floxy^floxy (cKO) mice. (b) Representative Ki67 and Mash1 IHC staining of SVZ niche from P30 Nkx2.1-Cre; ChAT^floxy/+ (Ctrl) and Nkx2.1-Cre; ChAT^floxy^floxy (cKO) mice. Fluorescence signals inverted to black on white for clarity in both (a) and (b). (c) Quantifications of SVZ Ki67^+, Mash1^+, DCX^+ cell numbers. n = 5. Box plots show mean, median, quartiles, range. Scale bars: 50 µm (a,b).

4.4.3. Identification of subependymal ChAT^+ neurons

We next set out to investigate potential sources for ACh in the SVZ neurogenic niche. To determine whether the SVZ received direct cholinergic innervation, we used IHC staining of SVZ whole mounts with antibody to ChAT and found extensive ChAT^+ processes in the niche (Fig. A.5a). Three-dimensional reconstruction of these ChAT^+ processes showed that they reside
in close proximity to the subependymal space (Fig. A.5a). We did not detect ChAT expression in SVZ ependymal niche cells or B-type astrocytes and their progeny in the niche (data not shown). To determine whether these ChAT⁺ SVZ processes might be from ChAT⁺ cholinergic neurons, we performed IHC antibody staining on SVZ whole mounts from ChatIRES-cre; Rosa26R-tdTomato (R26R-tdTomato) transgenic mice, in which Cre is expressed in ChAT⁺ neurons and induces tdTomato expression. We found that tdTomato⁺ processes in the SVZ colabeled with ChAT, as well as with VACHT (Fig. A.6a).

We noticed large ChAT⁺ neuronal cell bodies residing in the SVZ niche in the subependymal space (Fig. A.6a,b). DiI-filling of these subependymal ChAT⁺ (subep-ChAT⁺) neurons, visualized via Chat-EGFP mice, showed that they have complex arborization of neuronal processes (Fig. A.6c). Using Ank3 IHC co-staining of axon initial segments to distinguish axons from dendrites, we observed that these subep-ChAT⁺ neurons projected their axonal processes locally in the subependymal space (Fig. A.5c,d and data not shown). Compared with similarly DiI-filled striatal ChAT⁺ neurons, a noticeable morphological difference was that these subep-ChAT⁺ neurons appeared to be mostly planar, parallel to the ependymal surface above, as compared with the
three-dimensional space-filling profile seen in striatal ChAT$^+$ neurons (Fig. A.5d,e, Fig. A.6b-d and data not shown). In Ank3-cKO mice, as expected, we found defective action potential generation in subep-ChAT$^+$ neurons (Fig. A.7a-c). When we performed IHC staining against tdTomato in Nkx2.1-cre; R26R-tdTomato mice, we found that, unlike their striatal counterparts$^{130}$, subep-ChAT$^+$ neurons were mainly tdTomato$^-$ (Fig. A.7d), and therefore not targeted in Nkx2.1-cre; Chat$^{loxP/loxP}$ mice. The Gsx2-cre driver also did not label subep-ChAT$^+$ neurons (Fig. A.7e). It has been reported that Drd2-Cre can target cholinergic neurons in the striatum$^{131}$. We found tdTomato$^+$ subep-ChAT$^+$ neurons in Drd2-cre; R26R-tdTomato mice (Fig A.7e), and Drd2-cre; Chat$^{loxP/loxP}$ mice showed SVZ neurogenesis defects compared with littermate controls (Fig. A.7f-h).
Figure A. 5. Identification of subependymal ChAT+ neurons.

(a) Imaris three-dimensional projections of IHC staining from SVZ niche whole mounts from a P30 Foxj1-GFP mouse. Red, ChAT+ processes; green, ependymal cells (Ep) visualized by Foxj1-GFP transgene. Note that the ChAT+ processes were subependymal. Dashed box indicates neuronal cell body. LV, lateral ventricle. (b) P30 SVZ niche coronal sections from Chat^{IRES-cre+}, R26R-tdTomato mice stained with tdTomato antibody. Note the presence of ChAT+ neurons (arrows) beneath ependymal cells (dashed lines). (c) Representative DiI-filling of subep-ChAT+ neuron. Top, en-face ventricular view shows dendritic and axonal processes. Bottom, side view of above neuron in three-dimensional reconstruction demonstrates planar arrangement paralleling ependymal surface above (dotted line between a and b indicates orientation of side view). (d) Traces from ventricular and coronal section views of representative DiI-filled ChAT+ neurons. Note the planer versus non-planar morphologies of subependymal versus striatal ChAT+ neurons in coronal view. Blue lines indicate dendrites and gray areas represent axonal fields. (e) Quantifications of neuronal morphology from ventricular and coronal views for subependymal (Subep) and striatal (Str) ChAT+ neurons. Traced neurons were fit with an ellipse, and ellipse axis lengths were measured as the long (a) and short axis (b), followed by calculation of the axis ratio (a/b). Note the significantly increased axis ratio for subep-ChAT+ neurons in coronal view. *P < 0.002, t_{18} = 4.469 unpaired Student’s t test, n = 10. Box plots show mean, median, quartiles and range. Scale bars represent 30 µm (a), 20 µm (b) and 50 µm (c,d).
Figure A. 6. Anatomical characteristics of subependymal ChAT$^+$ neurons.

(a) IHC staining for tdTomato, ChAT, VACHT in ChAT$^{IRES-Cre/+}$; R26R-tdTomato transgenic mice, showing co-localization in subep-ChAT$^+$ neurons. (b) Additional example traces for subependymal (Subep) or striatal ChAT$^+$ neurons in ventricular and coronal views. (c) Quantifications of subependymal and striatal ChAT$^+$ neuron dendritic branch point numbers. Box plots show mean, median, quartiles, range, $n = 10$. (d) 3D Sholl analyses of subependymal (Subep) and striatal (Str) ChAT$^+$ neuron dendritic morphology. Box plots show mean, median, quartiles, range, $n = 10$. Scale bars: 20 $\mu$m (a), 50 $\mu$m (b).
Figure A. 7. Defects in subependymal ChAT+ neuron action potential generation and SVZ neurogenesis.

(a) Representative current-clamp recordings from subep-ChAT+ neurons in response to 100-ms current pulses (250 pA = green, 500 pA = black trace) in Ank3 control (top), heterozygous (Het, center) and cKO (bottom) mice. Square trace indicates the duration of the current pulse. (b) Quantifications of spike numbers to 100-ms current pulses at 250 or 500 pA, spike threshold, and input resistance in subep-ChAT+ neurons from control, heterozygous and cKO animals. *P < 0.0001, t_{34} = 4.283 (250 pA), t_{34} = 7.532 (500 pA), **P < 0.0006, t_{34} = 3.281, unpaired Student’s t test, n = 18 in all groups (6 mice). Box plots show mean, median, quartiles and range. (c) Mean action potential traces from Ank3 control, heterozygous and cKO subep-ChAT+ neurons to 500-pA current injections, showing delayed spiking in cKO neurons. (d) ChAT and tdTomato IHC staining of coronal sections from P30 Nkx2.1-cre; R26R-tdTomato mice showing colocalization in striatal (*), but not subep-ChAT+ (arrows) neurons. (e) ChAT and tdTomato IHC staining in subep-ChAT+ neurons from P30 Gsx2-cre; R26R-tdTomato or Drd2-cre; R26R-tdTomato mice showing colocalization with Drd2-cre driver, but not Gsx2-cre driver (arrows). (f) Representative views of ventricular whole-mount DCX staining from P30 Drd2-cre; Chat^{loxP/+} (Ctrl) and Drd2-cre; Chat^{loxP/loxP} (cKO) mice. (g) Representative Ki67, Mash1 and DCX IHC staining of SVZ niche from P30 Drd2-cre; Chat^{loxP/+}(Ctrl) and Drd2-cre; Chat^{loxP/loxP} (cKO) mice. Fluorescence signals are inverted to black on white for clarity. (h) Quantifications of SVZ Ki67+, Mash1+, DCX+ IHC staining data from g. *P < 0.008, z = 2.611, Wilcoxon two-sample test, n = 5. Box plots show mean, median, quartiles and range. Scale bars represent 15 µm (d,e) and 50 µm (f,g).
4.4.4. Functional properties of subependymal ChAT\(^+\) neurons

The morphological and anatomical location differences between subependymal and striatal ChAT\(^+\) neurons raise the question of whether these neurons exhibit functional variations. Whole-cell recordings of ChAT\(^+\) neurons from P28–35 \(\text{Chat}^{\text{IRES-cre}^+/}\; R26R-\text{tdTomato}\) mice revealed that the subependymal population and their striatal counterparts had similar firing rates in response to current injection (Fig. A.8a). Both neuronal populations showed \(I_h\) sag current, but exhibited some differences in membrane electrophysiological properties (Fig. A.8b,c). In cell-attached recording configuration from similarly prepared acute brain slices, striatal ChAT\(^+\) neurons showed characteristic patterns of spontaneous activity\(^{132}\) (spontaneous firing frequency = 1.786 ± 0.266 Hz (mean ± s.e.m.), \(n = 21\); Fig A.9a,b). Notably, cell-attached recordings of subep-ChAT\(^+\) neurons showed little spontaneous activity (spontaneous firing frequency = 0.042 ± 0.013 Hz (mean ± s.e.m.), \(n = 53\), \(P < 0.0001\), \(t_{72} = 5.089\), Student’s \(t\) test; Fig A.9c). However, they could be induced to fire action potentials after local release of glutamate via puff pipette (Fig A.9d,e).

To further confirm these differences in evoked versus spontaneous activities between subependymal and striatal ChAT\(^+\) neurons, we crossed the \(\text{Chat}^{\text{IRES-cre}^+}\) driver line to \(\text{Rosa26R-ChR2EYFP}\ (R26R-\text{ChR2EYFP})\) mice to
express channelrhodopsin in ChAT+ neurons. Repeating the same cell-attached recording experiments, we observed a blue light–activated transient pause in spontaneous activity in striatal ChAT+ neurons (Fig A.9f), similar to previous observations133. Again, subep-ChAT+ neurons showed little spontaneous activity, but responded robustly to blue light activation by continuously firing action potentials during the duration of stimulation (Fig A.9g). These results indicate that, not only do subependymal and striatal ChAT+ neurons differ morphologically, they also exhibit different activity states.
Figure A. 8. Comparisons of striatal and subependymal ChAT+ neuron electrophysiological properties.

(a) Representative whole-cell, current-clamp recordings of striatal and subep-ChAT+ neurons in response to 100 (red), 250 (green), or 500 pA (black) current injections for 100 ms. (Right) Number of evoked spikes during 100 ms current pulses of 100, 250, or 500 pA. Box plots show mean, median, quartiles, range, n = 15 in each group (5 animals). (b) Representative whole-cell current-clamp recordings of striatal and subep-ChAT+ neurons in response to 500 ms negative current injection (following 100 ms 20 pA positive current pulse). Dotted-lines indicate amplitude of sag potential. (c) Analyses of striatal and subep-ChAT+ neuron resting membrane potential, spike initiation threshold, and depolarization to spike, acquired by increasing depolarizing current pulses (100 ms duration) until spike initialization. Box plots show mean, median, quartiles, range, n = 15 (5 animals).
Figure A. 9. Electrophysiological properties of subependymal ChAT+ neurons.

(a) tdTomato IHC antibody staining of P30 brain coronal section from ChatIRES-cre/+; R26R-tdTomato mice showing spatial relationships between subependymal and striatal ChAT+ neurons. (b,c) Representative traces of cell attached recordings from striatal (b) or subependymal (c) ChAT+ neurons. (d,e) Representative traces of cell-attached recordings from striatal (d) and subependymal (e) ChAT+ neurons in response to 1 s of local application of 100 µM glutamate. Bar indicates duration of puffed drug. Peristimulus-time histogram and raster plots for 15 consecutive sweeps, as well as corresponding average spikes per second (mean ± s.e.m.) are shown below, demonstrating baseline spontaneous and glutamate-evoked frequencies. Note the robust spike frequency of subep-ChAT+ neuron during stimulation. Red dashed lines indicate start of drug application across trials. (f,g) Responses of striatal (f) or subependymal (g) ChAT+ neurons, expressing ChR2EYFP to 100 ms (top left), 10 s (top right) or 10 ms (bottom) pulses of 473-nm light. Blue bars indicate duration of light pulse. For 10-ms light pulses, peristimulus-time histogram and raster plots for 15 consecutive sweeps are shown below representative traces. Scale bar represents 20 µm (a).
4.4.5. Activity-dependent ACh release in the SVZ niche

ChAT-expressing neurons can synthesize and release ACh. To detect ACh release from subep-ChAT+ neurons, we used an ACh sensor: the M1 muscarinic receptor cell-based neurotransmitter fluorescent engineered reporter (M1-CNiFER)\textsuperscript{122}. These modified HEK293 cells express the Ca\textsuperscript{2+} indicator protein TN-XXL and the M1 muscarinic receptor. ACh binding to M1 receptor results in enhanced fluorescence resonance energy transfer (FRET) between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) domains of TN-XXL indicator\textsuperscript{122} (Fig A.10a). We used 920-nm multiphoton laser excitation and simultaneously recorded M1-CNiFER fluorescence emissions at 475 nm (CFP) and 530 nm (YFP). ACh application to adherent M1-CNiFER cells resulted in consistent and opposing responses in CFP versus YFP fluorescence (Fig A.11a), and corresponding fractional changes in the FRET ratio (Fig A.11b), consistent with previously published results\textsuperscript{122}. To determine whether M1-CNiFER cells can detect ACh release in the SVZ niche, we transplanted them into the subependymal space of SVZ niche in acute brain slice preparations from P30 wild-type mice (Fig A.10b). Using the same imaging protocol described above on transplanted M1-CNiFER
cells, we detected consistent $\Delta F/F$ fluorescence and fractional changes in FRET ratio following ACh application (Fig A.11c).

We next repeated this SVZ transplantation experiment, using acute slices prepared from P30 $\text{Chat}^{\text{IRES-cre}^{\text{+}}}; R26R-\text{ChR2EYFP}$ mice. Instead of direct ACh application, we used 473-nm light pulses to locally excite subep-
ChAT$^+$ neurons and measured M1-CNiFER cell responses (Fig A.10c). This resulted in consistent $\Delta F/F$ fluorescence and fractional FRET changes in M1-
CNiFER cells (Fig A.11d), consistent with activity-dependent release of ACh from subep-ChAT$^+$ neurons. Focal 473-nm light stimulation of ChR2EYFP-
expressing striatal ChAT$^+$ neurons, either directly adjacent to the SVZ or deeper into the striatum, did not result in measurable SVZ M1-CNiFER cell responses (Fig A.11e). As controls, 473-nm light pulses on M1-CNiFER cells in adherent cultures or after transplantation into the SVZ of brain slices prepared from non-transgenic mice showed no $\Delta F/F$ fluorescence and FRET changes (data not shown).
Figure A. 10. M1-CNiFER experimental design.

Schematic representations of (a) M1-CNiFER cell detection of ACh; (b) M1-CNiFER cells transplanted into SVZ niche in acute slice preparation; (c) M1-CNiFER cells transplanted into SVZ niche in acute slice preparation from ChATIRES-Cre/+; R26R-ChR2EYFP mice.

Figure A. 11. Detecting activity-dependent release of ACh in the SVZ niche.

(a) Representative changes in M1-CNiFER fluorescent responses at 475- and 530-nm emission wavelengths, with and without ACh application. Excitation source, 920-nm laser. (b–e) Average traces of M1-CNiFER cell baseline FRET ratios and ACh- or light-induced changes in FRET ratios ($\Delta R/R$). (b) ACh applied to adherent M1-CNiFER cells in culture. Images sampled once every 2.6 s ($n = 10$). Data are presented as mean ± s.e.m. (c) ACh applied to M1-CNiFER cells transplanted into SVZ in acute brain slice preparation. Images sampled once every 5.4 s ($n = 16$). Data are presented as mean ± s.e.m. (d) M1-CNiFER cells transplanted into SVZ niche in acute slice preparation from ChATIRES-cre/+; R26RChR2EYFP mice, followed by 473-nm light stimulation to activate subepi-ChAT+ neurons (5 Å~ 250-ms light pulses, 2 Hz). Images sampled once every 1.6 s ($n = 14$). Data are presented as mean ± s.e.m. (e) The focal light-stimulation protocol and slice preparation to image SVZ transplanted M1-CNiFER cells shown in d was used to activate striatal ChAT+ neurons adjacent to the SVZ (top trace). Activating striatal ChAT+ neuron at various distances from SVZ: averages of maximum $\Delta R/R$ from multiple slice imaging experiments ($n = 15$). Data are presented as mean ± s.e.m. Scale bar represents 10 µm (a).
4.4.6. SVZ NSCs respond to local ChAT\(^+\) neuron activity

Detection of activity-dependent ACh release led us to look for anatomical relationships between cholinergic processes and the SVZ niche. We performed transmission electron microscopy (TEM) on RFP antibody–stained, immunogold-labeled SVZ samples from \(\text{Chat}^{\text{IRES-cre/+}}; \ R26R-tdTomato\) animals. NSCs in the SVZ niche are identified on TEM through contacts with the brain ventricular surface and their characteristic invaginated nuclei\(^3\). We detected immunogold-labeled axonal projections from ChAT\(^+\) neurons, showing intracellular vesicles adjacent to SVZ NSCs (Fig. A.12a). Antibody staining against nicotinic and muscarinic receptors revealed their localizations in subependymal GFAP\(^+\) astrocytes near ChAT\(^+\) neuronal processes (Fig. A.13).

To determine whether SVZ NSCs can directly respond to local ACh release, we prepared acute brain slices from P30 \(\text{nestin-Cre}ER^{\text{tm}4}; \ R26R-tdTomato; \ \text{Chat-ChR2EYFP}\) mice. P7 tamoxifen injection in these mice results in tdTomato expression in SVZ NSCs\(^9\). We performed whole-cell patch recording on subependymal tdTomato\(^+\) NSCs (Fig. A.14a,b) while focally activating subep-ChAT\(^+\) neurons via 473-nm laser, using 10-ms pulses at 15, 30 or 50 Hz for 1 s (Fig. A.12b). This resulted in consistent inward currents in SVZ NSCs, which could be blocked by cholinergic receptor antagonists (Fig. A.12b-d).
Similar results were obtained from subependymal GFP$^+$ type B astrocytes in *Gfap-GFP; Chat$^{IRES-cre/}$; R26R-Chr2EYFP* mice (data not shown). Although glutamatergic inhibition did not block this light-induced response in tdTomato$^+$ SVZ NSC, it was tetrodoxin (TTX) sensitive (Fig. A.12b-d). Identical 473-nm light-stimulation experiments performed using brain slices from *nestin-CreER$^{tm4}$; R26R-tdTomato* mice (without Chat-Chr2EYFP) did not induce measurable currents in tdTomato$^+$ SVZ NSCs (Fig. A.12b). We did not detect measurable light-induced currents in *nestin-CreER$^{tm4}$; R26R-tdTomato* lineage-traced S100β$^+$ SVZ ependymal niche cells or Mash1$^+$ transiently amplifying progenitors, but we did observe light-induced cholinergic currents in DCX$^+$ neuroblasts in the SVZ (Fig. A.14c,d).
Figure A. 12. SVZ NSCs respond directly to local ACh release

(a) TEM analysis of P30 immunogold-labeled (indicated by black dots) ChAT+ neuronal terminals in the SVZ niche. Close-up view showing synaptic vesicles in axon from ChAT+ neuron adjacent to SVZ B-type astrocytic stem cells (B1). Asterisk indicates axon containing synaptic vesicles. (b) Representative voltage-clamp recordings from tdTomato+ SVZ NSCs showing evoked inward currents following 10-ms, 473-nm light pulses at 15, 30 or 50 Hz for 1 s (top traces, red trace is baseline without light). Optogenetically stimulated currents were sensitive to the cholinergic blockers mecamylamine (Mec, 40 µM) and atropine (Atrop, 5 µM), and were unaffected by glutamatergic blockers CNQX (10 µM) + AP5 (100 µM) (black trace), but were abolished by blockade of action potentials (TTX 2 µM, red trace). No light-evoked response was found in tdTomato+ SVZ NSCs using identical experimental conditions from P30 nestin-CreERtm4; R26R-tdTomato mice (without Chat-ChR2EYFP). Blue bars represent the duration of light-stimulation train (10-ms pulses, 30 Hz unless otherwise noted). (c) Quantifications of current responses to different light-stimulation frequencies shown in b (n = 8). Box plots show mean, median, quartiles and range. (d) Quantification of light-evoked current responses under pharmacological conditions shown in b. *P < 0.0006, F3,21 = 8.81 (Mec/Atrop), F2,13 = 14.02 (TTX), one-way ANOVA, n ≥ 5 in all groups. Box plots show mean, median, quartiles and range. Scale bars represent 250 nm (a).
Figure A. 13. Nicotinic and muscarinic receptor expression in subependymal GFAP+ cells.

IHC staining of P30 SVZ coronal sections, with antibodies against α3-nicotinic (α3-nAChR), α4-nicotinic (α4-nAChR), or pan-muscarinic (mAChR) receptors (red); GFAP (blue); GFP (from ChAT-GFP transgene, green); and DAPI (red, in left panels). GFAP+ SVZ NSCs (white dashed-lines) touching the lateral ventricle (LV) are positive for nicotinic and muscarinic receptors (Imaris 3D). They are adjacent to GFP+ processes from ChAT+ neurons (arrows). Antibodies against nicotinic and muscarinic receptors that showed IHC specificity were made in rabbit, and were stained individually. Scale bar: 10 μm.
Figure A.14. Detecting activity-dependent electrical currents in SVZ cell types.

(a) Schematic representation of experimental setup in P30 SVZ acute slices from nestin-CreERtm4 (N4); R26R-tdTomato; ChAT-ChR2EYFP mice. (b) Representative images of tdTomato+ subependymal NSCs in P30 brain slices, lineage-traced via nestin-CreERtm4; R26R-tdTomato following tamoxifen injection, filled with DiO (green) through glass micropipette. (c) Representative images showing DiO injections and subsequent IHC staining with Nestin, S100β, Mash1, or DCX antibodies. (d) Representative voltage-clamp recordings from tdTomato+ ependymal niche cells, Mash1+ “C” cells, DCX+ neuroblasts, following 10 ms 473 nm light pulses @ 30 Hz for 1 second (red traces are baselines without light). Inward current in DCX+ neuroblast is sensitive to cholinergic blockers mecamylamine (Mec., 40 μM), atropine (Atrop., 5 μM). Blue bar = duration of light-stimulation train. (e) Quantifications of light-evoked current responses in (d). * P < 0.0002, F2,8 = 34.49, one-way ANOVA, n = 5. Box plots show mean, median, quartiles, range. Scale bars: 5 μm (b,c).
4.4.7. Optogenetic modulation of SVZ cellular proliferation

Given that our *in vitro* data suggests that ACh has substantial neurogenic effects (Fig A.1), we sought to examine whether activation of subep-ChAT\(^+\) neurons can result in increased cellular proliferation *in vivo* by implanting optical fibers targeting the lateral ventricle of P30 *Chat\(^{IRES-cre/+}\); R26R-ChR2EYFP* mice (Fig A.15 & Fig A.16a). Phosphorylated ribosomal protein S6 (p-rpS6) is an activity-dependent marker for cholinergic neurons *in vivo*.\(^{135,136}\)

IHC staining using antibody to p-rpS6 showed that subep-ChAT\(^+\) neurons have robust p-rpS6 expression under physiological conditions (Fig A.15d).

Following a light-train protocol that is effective for optogenetic activation of cholinergic neurons\(^{124}\) (Fig A.15a,b), p-rpS6 expression in subep-ChAT\(^+\) neurons became enhanced after 48 h of *in vivo* 473-nm light stimulation (Fig A.15d). Concurrently, we observed consistent increases in the numbers of Ki67\(^+\) proliferating cells in the SVZ niche as compared with fiber-implanted controls without light (Fig A.15e & Fig A.16b). Expression of Mash1 and DCX and the numbers of Nestin\(^+\)Ki67\(^+\) SVZ NSCs increased following 48 h of light stimulation versus no-light controls (Fig A.15e & Fig A.16b). Identical 48 h of light stimulation, in control mice lacking ChR2 expression in ChAT\(^+\) neurons, resulted in no noticeable changes to p-rpS6 expression in subep-
ChAT+ neurons (data not shown) or SVZ Ki67+, Mash1+, DCX+ or Nestin’Ki67+ cell numbers (Fig A.15e).

Conversely, in P30 ChatIRES-cre; R26R-ArchaerhodopsinGFP (R26R-ArchGFP) mice, we performed the same optogenetic experiment, using a 556-nm laser to silence subep-ChAT+ neurons (Fig A.15c & Fig A.16c). This resulted in reduced p-rpS6 expression in subep-ChAT+ neurons (Fig A.15f) and corresponding decreases in the numbers of Ki67+, Mash1+, DCX+ and Nestin’Ki67+ cells in the SVZ niche (Fig A.15g and Fig A.16d). Although p-rpS6 expression changes were robust in subep-ChAT+ neurons following light stimulation (Fig A.15d,f), we did not observe concurrent p-rpS6 changes in striatal ChAT+ neurons either adjacent to the SVZ or deeper into the striatum (Fig A.16e,f). Similar optogenetic experiments using ChatIRES-cre+; R26R-ChR2EYFP or ChatIRES-cre+; R26R-ArchGFP mice, but with light fibers targeting the striatum instead of SVZ, resulted in no obvious changes to SVZ neurogenesis (data not shown). Implantation of optical fiber to target the lateral ventricle represents a form of injury. To understand whether glial proliferation contributed to the observed increases in SVZ Ki67+ cells following ChR2 stimulation, we performed IHC staining for Iba1, CD11b or NG2, 48 h post fiber implantation, and found no significant differences in their cell
numbers or proliferation comparing light stimulation versus no-light controls (Fig A.17a,b). Consistent with our previous findings⁹⁰, we detected a delayed onset of Thbs4 protein induction in the SVZ niche 3+ d after fiber placement (Fig A.17c). Given that the cascade of injury responses in vivo is complex, we limited our optogenetic analyses to the first 48 h after fiber placement, prior to substantial Thbs4 protein upregulation.
Figure A. 15. Optogenetic modulation of SVZ niche cellular proliferation and neurogenesis.

(a) Images of ChR2EYFP-expressing subep-ChAT+ neuron (*) and cell-attached configuration. (b) Representative trace of cell attached recordings from subep-ChAT+ neurons from P30 ChatIRES-cre/+; R26R-Chr2EYFP mice responding to pulses of 473-nm light stimulation. Blue bars indicate duration of light induction. (c) Optogenetic silencing of subep-ChAT+ neurons during whole-cell recording from ChatIRES-cre/+; R26R-ArchGFP mice. Neuronal spiking was induced via 15-s, 100-pA depolarizing current (indicated by lower bar). A 5-s pulse of 556-nm light (green bar) abolished spiking. (d) Representative p-rpS6 IHC staining of subep-ChAT+ neurons following 48 h of 473-nm light stimulation, comparing induced ipsilateral (ipsil.) to uninduced contralateral (contra.) SVZ, imaged at identical settings from the same section. Note the increase in p-rpS6 expression in subep-ChAT+ neuron in the light-induced condition (arrow). (e) Quantifications of SVZ Ki67+, Mash1+, DCX+ and Nestin+Ki67+ IHC staining data from ChatIRES-cre/+; R26R-Chr2EYFP littermates without light stimulation (control, +ChR2, −light), with light-stimulation (+ChR2, +473 nm) and from R26R-Chr2EYFP (no ChatIRES-cre+/) littermates with light stimulation (−ChR2, +473 nm). *P < 0.003, F2,15 = 26.08 (Ki67), F2,15 = 8.91 (Mash1), F2,12 = 14.11 (DCX), F2,12 = 173.7 (Nestin/Ki67), one-way ANOVA, n ≥ 5 in all groups. Box plots show mean, median, quartiles and range. (f) Representative p-rpS6 IHC staining of subep-ChAT+
neurons following 48 h of 556-nm light stimulation, comparing induced ipsilateral to uninduced contralateral SVZ, imaged at identical settings from the same section. Note the decrease in p-rpS6 expression in subep-ChAT+ neuron in light-induced condition (arrowhead). Cy5 channel used for p-rpS6 secondary antibody staining, for clarity represented in green channel for colocalization with tdTomato. (g) Quantifications of SVZ Ki67+, Mash1+, DCX+ and Nestin+Ki67+ IHC staining data from ChatIRES-cre/++; R26R-ArchGFP littermates without light stimulation (control, +Arch, −light), with light stimulation (+Arch, +556 nm) and from R26R-ArchGFP (no ChatIRES-cre/+) littermates with light stimulation (−Arch, +556 nm). *P < 0.005, F2,15 = 16.85 (Ki67), F2,15 = 13.21 (Mash1), F2,12 = 26.3 (DCX), F2,12 = 28.28 (Nestin/Ki67), one-way ANOVA, n ≥ 5 in all groups. Box plots show mean, median, quartiles and range. Scale bars represent 10 µm (a) and 5 µm (d,f).
Figure A. 16. Optogenetic modulation of subependymal ChAT+ neuron activity in vivo.

(a,c) Schematic representations of in vivo light-stimulation. LV = lateral ventricle, Ctx = cortex. (b,d) Representative Ki67, Mash1, DCX, Nestin/Ki67 IHC staining of SVZ niche from P30 ChATlRES-Cre/+; R26R-ChR2EYFP (b) or ChATlRES-Cre/+; R26R-ArchGFP (d) mice, with (+) or without (–) 48 hrs of light-stimulation in vivo (473nm for ChR2 or 556 nm for Arch). Fluorescence signals inverted to black on white for clarity. Representative subependymal Nestin+ cells (*) co-localizing with Ki67 (arrows) in right panels. (e,f) Representative p-rpS6 IHC staining of striatal ChAT+ neurons (arrows) adjacent to the SVZ, from P30 ChATlRES-Cre/+; R26R-ChR2EYFP mice following 48 hrs of 473 nm light-stimulation (e), or from P30 ChATlRES-Cre/+; R26R-ArchGFP mice following 48 hrs of 556 nm light-stimulation (f), comparing ipsilateral to uninduced contralateral striatum after ventricular stimulation, imaged at identical settings from same section. Cy5 channel used for p-rpS6 secondary antibody staining, for clarity represented in green channel for co-localization with tdTomato. Scale bars: 20 µm (b,d), 10 µm (e,f).
Figure A. 17. Assessing in vivo injury responses from light-fiber implant.

(a) Representative Iba1, CD11b, NG2 IHC staining of SVZ niche from P30 ChATIRESCre/+; R26RChR2EYFP mice, with (+) or without (−) 48 hrs of 473 nm light-stimulation in vivo. Dashed-lines = proliferating SVZ regions. (b) Quantifications of SVZ Iba1, CD11b, NG2 IHC staining data from (a). P = 0.169, F2,12 = 2.07 (NG2); P = 0.175, F2,12 = 2.02 (NG2/Ki67). One-way ANOVA, n = 5. Box plots show mean, median, quartiles, range. (c) Representative Thbs4 IHC staining on SVZ sections, 48 and 84 hrs post cortical optical fiber implantation to target the lateral ventricle (LV). Note the delayed Thbs4 upregulation in SVZ niche ipsilateral (ipsil) to fiber implantation (arrows). contra = contralateral hemisphere. Scale bars: 20 µm (a), 50 µm (c).
4.4.8. ACh enhances SVZ neurogenesis through the FGFR pathway

It had been reported previously that embryonic cortical progenitors proliferate to ACh stimulation through fibroblast growth factor receptor (FGFR) signaling\textsuperscript{137}. On FGF ligand binding, FGFR is known to activate via receptor phosphorylation on tyrosine 653/654\textsuperscript{138}. Following removal of growth factors from primary SVZ NSC culturing media, we detected similar effects on FGFR tyrosine 653/654 phosphorylation in SVZ cultures after re-introduction of FGF (Fig A.18a). Addition of ACh without FGF resulted in a similar increase in FGFR phosphorylation (Fig A.18a). To determine whether this increase in ACh-mediated FGFR phosphorylation is a direct effect downstream of ACh signal transduction or an indirect pathway perhaps through increased FGF ligand production, we performed similar experiments as above with shorter incubation periods. Although we were able to detect FGF-mediated FGFR activation and phosphorylation 90 min after FGF addition to media, we did not observe noticeable increase in FGFR phosphorylation after ACh addition in the same time course (Fig A.18b), making it less likely that ACh-mediated intracellular signals can directly activate FGFR.

To test whether this delayed increase in ACh-mediated FGFR phosphorylation may, in part, be secondary to upregulation of FGF
production, we used an antibody to FGF to block FGF ligand binding to its receptor (Fig A.18a). Although addition of ACh alone resulted in increased FGFR phosphorylation (Fig A.18a), concurrent incubation with antibody to FGF blunted this increase (Fig A.18a). ELISA assay to detect the presence of FGF in the culture media during *in vitro* differentiation revealed corresponding FGF increases in the ACh-treated conditions compared to controls (Fig A.18c). In similar experiments, we did not detect EGF receptor (EGFR) activation following ACh addition to SVZ NSC cultures (Fig A.18d). Furthermore, co-incubation with antibody to FGF blunted ACh-induced DCX⁺ neuroblast production in culture (Fig A.18e), consistent with the notion that local release of ACh in the SVZ niche in an activity-dependent manner induces NSC production of new neuroblasts. This idea was further supported by our observation of decreased levels of phospho-FGFR staining in subependymal GFAP⁺ NSCs in P30 Ank3-cKO mice compared with littermate controls (Fig A.18f).
Figure A. 18. ACh and FGFR activation.

(a) Western blot analyses on effects of Ach or FGF (12 ng/ml) in SVZ NSC FGFR phosphorylation. Anti-FGF (α-FGF) was used at 13 µg/ml, except for “low” condition in lane 8 (6.5 µg/ml). (b) Western blot analysis of FGFR phosphorylation following application of either 100 µM ACh, or 12 ng/ml FGF to SVZ NSC adherent cultures. Note the lack of ACh-mediated increase in FGFR phosphorylation 90 minutes after treatment.

(c) ELISA analyses of FGF concentrations in culture media during SVZ NSC differentiation in vitro, with ACh added (100 µM once at time zero) compared to control (no ACh). * P < 0.008, z = 2.739 (24 hrs), z = 2.611 (48 hrs), Wilcoxon two-sample test, n = 5. Box plots show mean, median, quartiles, range. (d) Western blot analysis of EGFR phosphorylation in SVZ NSC adherent cultures after treatment with 100 µM ACh, 20 ng/ml EGF (EGF Cond1), or 40 ng/ml EGF (EGF Cond2). (e) Western blot analyses on the effects of ACh and FGF on DCX+ neuroblast production in SVZ NSC adherent culture.

All Western analyses (a,b,d,e) were repeated 5 times with consistent results. (f) Representative IHC staining and quantifications of phosphorylated FGFR (pFGFR) co-localization with GFAP+ SVZ NSCs in P30 niche using Imaris, showing decreased expression in Ank3-cKO animals compared to littermate controls. pFGFR fluorescence signal inverted to black on white for clarity. * P < 0.008, z = 2.611 Wilcoxon two-sample test, n = 5. Box plots show mean, median, quartiles, range. Scale bar: 10 µm (f).
4.5. Discussion

4.5.1. Summary

Starting with an *in vitro* assay to identify neurogenic neurotransmitters, we found that ACh has a potent effect on DCX⁺ neuroblast production from postnatal SVZ NSCs. In search of potential sources for ACh in the SVZ niche, we uncovered direct cholinergic inputs from local subep-ChAT⁺ neurons. This previously undescribed subpopulation of cholinergic neurons showed morphological and functional differences from their neighboring striatal counterpart and could release ACh into the niche in activity-dependent fashion. *In vivo* optogenetic manipulation of subep-ChAT⁺ neurons revealed that their activity was both necessary and sufficient to modulate SVZ neurogenic proliferation. Lastly, we found that SVZ NSCs can respond to ACh release, and then act through FGFR signaling pathway to increase neuroblast production. These results will have important implications for understanding circuit-level control of postnatal and adult SVZ neurogenesis in health and disease.
4.5.2. Cholinergic circuit control of SVZ neurogenesis

The systemic importance of ACh is perhaps best demonstrated by the early postnatal lethality of animals lacking either ChAT\textsuperscript{126} or VACHT\textsuperscript{127}. ACh can be released via both bulk (non-vesicular) and vesicular (neuronal activity dependent) mechanisms. Together they modulate wide-ranging cellular and neural circuit-level functions such as neuromuscular control and striatal gating of cortical versus thalamic inputs\textsuperscript{133}. Cholinergic signaling has also been reported to influence rodent SVZ neurogenesis: \textit{in vivo} infusion of nicotinic agonist can result in increased SVZ cellular proliferation, as measured by BrdU incorporation\textsuperscript{111}. Given that the endogenous sources for the ACh mediating these effects were not yet identified, it was possible that indirect actions of cholinergic pharmacology may be responsible for observed phenotype.

To genetically test the importance of cholinergic circuitry on SVZ neurogenesis and to overcome early lethality associated with ChAT and VACHT deletions, we took an approach to blunt cholinergic neurons' ability to properly scale action potential generation to the strengths of stimuli. We found that cholinergic neurons' ability to fire precise action potentials is important to maintain the robustness of adult SVZ neurogenesis. As a neurotransmitter, ACh exerts its function locally as a result of rapid degradation by extracellular
acetylcholinesterases. Given that striatal ChAT\(^+\) neurons are anatomically adjacent to the SVZ niche, we analyzed a genetic mouse model in which ACh from striatal ChAT\(^+\) neurons is largely eliminated\(^{130}\), but detected no obvious SVZ neurogenesis defects.

Our discovery that the SVZ niche is directly innervated by local subep-ChAT\(^+\) neurons points to exciting future directions for understanding circuit-level control of new neuron production. Subep-ChAT\(^+\) neurons highly expressed the neuronal activity-dependent marker p-rpS6\(^{136}\), indicating that these neurons are normally active \textit{in vivo}. However, they did not spontaneously fire action potentials in acute slice preparations. Given that spontaneous firing of striatal cholinergic neurons is generated by intrinsic membrane properties instead of synaptic drive\(^{132}\), it is possible that membrane property differences contribute to the lack of spontaneous activity in subep-ChAT\(^+\) neurons. It is also possible that the functional connectivity for subep-ChAT\(^+\) neurons differs from striatal counterparts, although potential sources for excitatory and inhibitory inputs are currently unknown and will require circuit-tracing strategies to identify. Functional experiments have suggested that the rates of adult SVZ neurogenesis can be influenced by pregnancy\(^{139,140}\), male pheromone preference during mating\(^{141}\) and paternal recognition of
offspring\textsuperscript{142}. Although it is currently unclear whether subep-ChAT\textsuperscript{+} neuron activity patterns can be influenced by these behavioral procedures, known neural circuits involved in mediating these behaviors may serve as entry points into understanding the connectivity of subep-ChAT\textsuperscript{+} neurons.

4.5.3. NSC proliferation and neuroblast production

Throughout embryonic and postnatal development, NSCs self-renew and generate progeny through cell-intrinsic mechanisms that interact with microenvironmental cues. Recent results from adult hippocampal neurogenesis have shown that local neural circuits can be important for NSC proliferation and differentiation\textsuperscript{143-145}. This emerging view on connections between neural circuits and stem cell biology is exciting, as it can elegantly tie together external inputs, circuit-level coding and NSC fate choices to make lasting structural changes via new neuron production. SVZ NSCs require an array of growth factors to sustain self-renewal and balance proliferation and differentiation: EGF is perhaps one of the best studied and is a key ingredient for successful culturing of NSCs \textit{ex vivo}, as it promotes NSC proliferation. Another important growth factor for SVZ NSCs is FGF. We found that ACh preferentially synergizes with FGFR, but not EGFR, activation, which begin to
suggest cellular mechanisms for how neural modulation may feed into canonical stem-cell regulatory loops.

The inhibitory neurotransmitter GABA has been shown to enhance the maturation of newborn SVZ neuroblasts\textsuperscript{146}. Although GABA's important function on neuronal inhibition is not lost in this system, as its increased level feedback onto NSCs to dampen their proliferation\textsuperscript{103,104}. This elegant parallel usage of neurotransmitter for neural circuit and NSC control may ensure efficient integration of these two biological processes in the brain. Future experiments aimed to address intersections between neurotransmitters and known pathways controlling NSC proliferation and differentiation should shed further light on our understanding of circuit-level control of neurogenesis.

### 4.6. Chapter acknowledgments

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Biography

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