

Review

Parabrachial neurons promote nociplastic pain

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The parabrachial nucleus (PBN) in the dorsal pons responds to bodily threats and transmits alarm signals to the forebrain. Parabrachial neuron activity is enhanced during chronic pain, and inactivation of PBN neurons in mice prevents the establishment of neuropathic, chronic pain symptoms. Chemogenetic or optogenetic activation of all glutamatergic neurons in the PBN, or just the subpopulation that expresses the *Calca* gene, is sufficient to establish pain phenotypes, including long-lasting tactile allodynia, that scale with the extent of stimulation, thereby promoting nociplastic pain, defined as diffuse pain without tissue inflammation or nerve injury. This review focuses on the role(s) of molecularly defined PBN neurons and the downstream nodes in the brain that contribute to establishing nociplastic pain.

Nociplastic pain

Pain serves as an alarm and is a primary reason people seek medical attention. Acute pain due to burns, wounds, or trauma is a common event that elicits protective and coping behaviors. Nerve damage can elicit chronic, neuropathic pain that persists long after the original insult. A third category of pain – recently called nociplastic pain – occurs in the absence of identifiable injury or nerve damage [1–3]. Nociplastic pain presents itself as sensitivity to innocuous stimuli such as gentle touch (allodynia) and hypersensitivity to painful stimuli (hyperalgesia). Nociplastic pain is typically not localized to one specific region and is often associated with anxiety, depression, fatigue, abnormal sleep, and obesity. Twin studies suggest a strong genetic component, yet apart from rare mutations in sodium channels expressed in sensory neurons, most genes that have been associated with pain play a minor role [4]. Nociplastic pain may coexist with neuropathic pain [5,6] and it is generally unresponsive to commonly used pain medicines, making treatment difficult. These features make nociplastic pain difficult to study.

Nociplastic pain often encompasses what has been called ‘central sensitization’, a phenomenon that occurs when a restricted nerve is repeatedly stimulated and the area of hypersensitivity spreads to surrounding areas [7,8]. Central sensitization also occurs after repeated noxious stimulation (e.g., with capsaicin, or electrical) and promotes plasticity in the dorsal horn of the spinal cord resulting in hyperactivity of neurons that relay nociceptive signals to the brain [9–11]. A dominant hypothesis is that inhibitory neurons in the dorsal horn of the spinal cord – which normally provide a brake to prevent mechanical stimuli from reaching the nocifensive projection neurons – fail due to neuroplastic changes [12]. While tactile allodynia of the paws can be elicited by peripheral manipulations, it can also occur when trigeminal nerves in the head are traumatized, when specific neurons in the brain are artificially activated, or when animals are treated with compounds that activate the PBN as discussed below.

This review first summarizes data showing that the parabrachial neurons, and *Calca* neurons (expressing calcitonin gene-related peptide, CGRP) in particular, receive sensory input from the external environment and internal organs, including stimuli that are perceived as painful, and they project their axons to several forebrain regions that are implicated in pain processing.

Highlights

The parabrachial nucleus (PBN) is a gateway for establishment of chronic neuropathic pain caused by nerve injury.

In mouse models, neuropathic pain can be prevented, or reversed once established, by inhibition of PBN neurons or manipulation of other neuron populations in the brain.

Optogenetic or chemogenetic activation of all glutamatergic neurons in the PBN in mice, or just those that express the *Calca* gene, can induce long-lasting allodynia, a sign of nociplastic pain.

PBN neurons are activated by aversive events, and repeated exposure to them can promote allodynia.

PBN neurons are activated by painful stimuli, and they can develop heightened excitability in response.

The neural circuitry linking the PBN to pain phenotypes is unresolved; several potential nodes within the brain and spinal cord are considered.

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Thus, these neurons serve as a ‘general alarm’, alerting subjects to real or potential threats [13]. Herein, I outline the hypothesis that neural plasticity within the circuit from *Calca* neurons to the spinal cord promotes central sensitization, thereby promoting widespread hypersensitivity and allodynia, key features of nociplastic pain. Although nociplastic pain elicits sensory sensitivity, anhedonia, and lethargy, as well as conditions such as anxiety and depression in humans, this review focuses primarily on findings in mice showing long-lasting tactile allodynia in mice following activation of neurons in the brain.

Parabrachial neurons relay nocifensive signals to forebrain regions

During the mid-1980s, anatomically distinct regions of the PBN were described along with anatomical connections from neurons in the dorsal spinal cord and their trigeminal counterparts in the medulla; these observations led the authors to speculate on the role of the PBN in mediating pain [14–19]. These and many subsequent studies implicated the PBN in transmitting pain signals from the spinal cord to forebrain regions, including the central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST), periaqueductal gray (PAG), and several hypothalamic nuclei. A spinal–parabrachial–amygdala pain circuit was established by electrophysiological recording of PBN neurons that receive antidromic stimulation from the CeA and respond to thermal and/or mechanical stimulation of the skin [20]. The responsive neurons were primarily in the external lateral PBN where neurons expressing CGRP, substance P, and neurotensin are located [21–24]. Thus, these and other studies established that the external lateral PBN is a major target of the dorsal spinal cord and that PBN neurons respond to nocifensive stimulation and transmit aversive signals to the CeA. While this review focuses on the role of the PBN in mediating aversive signals, some PBN neurons relay pleasurable signals [25–29]; consequently, non-selective activation of PBN neurons could have mixed aversive and pleasurable effects. Figure 1 illustrates some of the inputs to the PBN, the subregions of the PBN, and some of the outputs to the forebrain via projection pathways that are described in this review.

Many spinal neurons that project their axons to the brain express neurokinin 1 receptor (NK1R, encoded by the *Tacr1* gene); they are a target of nociceptive, C-fiber afferents expressing substance P and CGRP [30]. Most of the *Tacr1* neurons send collaterals to both the PBN and the caudal ventrolateral medulla [31]. Another population of spinal projection neurons that express GPR83 was identified, a related G-protein-coupled receptor [32]. Spinal projection neurons expressing *Tacr1* [33] appear to be a subset of the *Tacr1* and *Gpr83* neurons [32]. *Tacr1* projection neurons receive inputs from nociceptive neurons in dorsal root ganglia, whereas *Gpr83* projection neurons receive inputs from both nociceptive and mechanoreceptor (touch) neurons in normal conditions. The distribution of axonal fibers within the PBN from GPR83 and NK1R neurons are distinct, with the GPR83 axonal fibers in closer proximity to *Calca* neurons, which is consistent with their preferential Fos activation of *Calca* neurons [32]. However, direct synaptic connections to *Calca* neurons from either *Tacr1* or *Gpr83* neurons have not been demonstrated, to my knowledge, suggesting that intra-PBN signaling is possible [34]. Recent evidence suggests even more diversity in spinal projection neurons; single-cell RNA sequencing of *Phox2a*-labeled nuclei revealed five distinct types of spinal projection neurons; three are in dorsal horn (layers 1–3) while two are in deeper layers, and they all include projections to the PBN [35,36]. Surprisingly, *Tacr1* and *Gpr83* were not defining molecular markers for any of the five *Phox2a* populations. Two spinoparabrachial pathways – a bilateral pathway that innervates both the ipsilateral and contralateral PBN, and a contralateral-only pathway that innervates the PBN and PAG – have been described [37]. Parabrachial neurons, including the *Calca* neurons, are also activated by itch [38,39], although they may be activated indirectly by other PBN neurons [40]. Trigeminal nerve ligation, inflammation, or application of capsaicin to the face activates the PBN neurons either directly or via the caudal spinal trigeminal nucleus, SP5C [29,41,42]. In summary, painful and

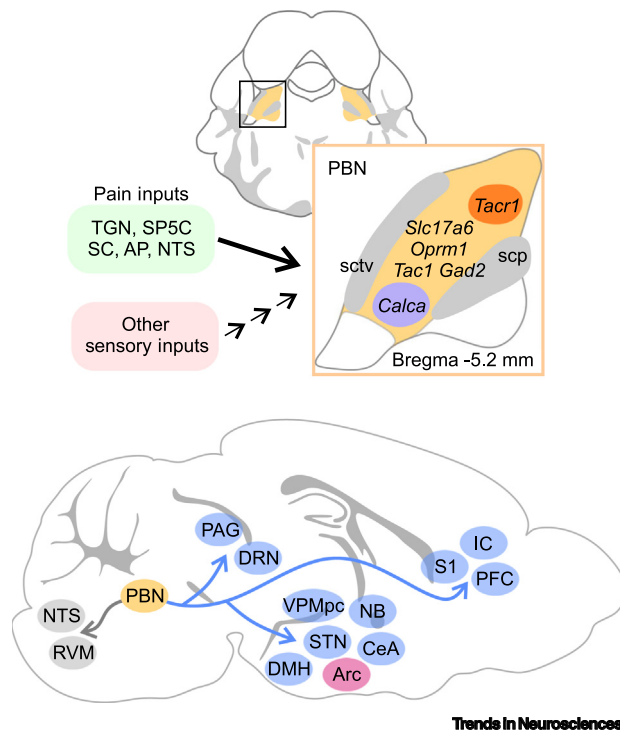


Figure 1. A diagram showing the neural inputs to the parabrachial nucleus (PBN) in mice and its outputs to the forebrain that may play a role in mediating pain. Almost every sensory modality can activate PBN neurons; direct activation of molecularly identified PBN neurons is only established (bold lines) for some inputs and unexplored for others (triple arrows). There are also inhibitory inputs to the PBN that affect pain; this includes inputs from the arcuate nucleus of hypothalamus (ARC) (pink) and the central nucleus of the amygdala (CeA) (not shown). The lateral PBN includes several subdivisions; the *Calca* neurons in the external lateral region and the *Tacr1* neurons superior lateral region have been shown to mediate pain phenotypes, along with neurons expressing *Slc17a6*, *Oprm1*, *Tac1*, and *Gad2* that are in several subdivisions (see main text). PBN neurons project their axons to the forebrain and the medulla via several pathways; only those targets mentioned in the review are shown. See [67] for more details. Abbreviations: AP, area postrema; DMH, dorsal medial hypothalamus;

DRN, dorsal raphe nucleus; IC, insular cortex; NB, nucleus basalis; NTS, nucleus tractus solitarius; PAG, periaqueductal gray; PFC, prefrontal cortex; RVM, rostral ventral medulla; S1, somatosensory cortex; SC, spinal cord; scp, superior cerebellar peduncle (fiber tract); sctv, ventral cerebellar tract; SP5C, spinal nucleus of the trigeminal, caudal part; TGN, trigeminal nucleus; VPMpc, parvocellular ventral posteromedial thalamus.

pruritic afflictions to the integumentary system reach the PBN via the spinal projection neurons or trigeminal neurons; however, the connectivity of these inputs to molecularly defined PBN neurons needs further research.

PBN *Calca* neurons also receive interoceptive inputs. The sensory vagus, with cell bodies in the nodose ganglia, innervates most internal organs. It responds to aversive events in internal organs (e.g., gastrointestinal malaise, bladder or colon distension, and inflammation) and transmits excitatory signals to the caudal region of nucleus of the solitary tract (NTS), which has prominent axonal projections to the PBN [43]. Neurons in the NTS that express dopamine β -hydroxylase (*Dbh*, noradrenergic neurons), cholecystokinin (*Cck*), and tachykinin 1 (*Tac1*) have been identified and shown to excite *Calca* neurons [44–46]. Neurons in the area postrema (AP), which lies above the NTS but outside the blood–brain barrier, are poised to respond to circulating molecules; lithium chloride (LiCl) (which induces visceral malaise), lipopolysaccharide (which induces inflammation), GDF15 (which is expressed in response to some cancers and other illnesses), and painful cancer-suppressing agents (e.g., cisplatin) activate molecularly identified neurons in the AP that project their axons to the PBN and induce *Fos* in *Calca* neurons [47]. Visceral organs are also innervated by spinal nerves. Thus, there are multiple pathways by which aversive signals in the soma can reach the PBN and *Calca* neurons.

Studies in mice using GCaMP-calcium imaging or *Fos* analyses demonstrated that in addition to aversive signals from the periphery, bitter tastes, loud or high-frequency noises, looming or

moving objects, predator odors, and motion sickness can activate *Calca* neurons [48,49]. The neural circuits that allow many of these sensory inputs to activate the *Calca* neurons are not fully established. Moreover, an innocuous cue (e.g., a tone, light, or taste) that normally does not activate *Calca* neurons will do so after being paired with aversive events like a foot shock or visceral malaise [38,42,50,51]. This phenomenon might reflect sensitization of sensory pathways that impinge on *Calca* neurons or reactivation of pain pathways.

An excitatory input to the PBN from the subthalamic nucleus (STN) can potentiate pain phenotypes [52]. The activity of the STN is normally suppressed by inhibitory inputs from the substantia nigra reticulata, but this inhibition is reduced after nerve injury [52]. Neurons in the PBN that project to the STN can promote pain [53], suggestive of a feed-forward mechanism.

The inputs to the PBN described are glutamatergic and they promote pain phenotypes. Many additional inputs to *Calca* neurons have been identified by rabies tracing studies, and they include both excitatory and inhibitory inputs [54,55]. Several inhibitory inputs have been identified that can suppress pain when they are activated. The CeA, one of the primary targets of neurons in the external lateral PBN, sends GABAergic signals back to the PBN. Acute, non-specific photoactivation of CeA projections to the PBN in naive mice suppress pain phenotypes due to formalin injection in the paw [41]. It also reduces acute pain induced by mechanical, thermal, or chemical means [56]. CeA neurons that express corticotrophin-releasing hormone (*Crh*), somatostatin (*Sst*), and/or dynorphin (*Pdyn*) and are among those that project to the PBN and suppress pain [41,57], but the molecular identity of the targets in the PBN have not been fully established.

Hunger can suppress pain at least in part by inhibiting neurons in the PBN. Neurons in the arcuate nucleus (ARC) that express agouti-related protein (AgRP), neuropeptide Y (NPY), and GABA are activated when mice are hungry [58]. Optogenetic activation of *Agrp*-neuron projections to the PBN inhibits inflammatory pain, which was blocked by an NPY1-receptor antagonist [59]. Other competing states (e.g., thirst and fear) can also signal to the PBN to suppress pain phenotypes [60].

Neuropathic and nociplastic pain are mediated by PBN neurons

Viral and genetic methods are used to monitor and manipulate the activity of molecularly defined neurons in mice to reveal their ability to establish pain-related phenotypes and assess their importance for mediating pain (see Box 1 for strategies used to manipulate neuron activity). This review

Box 1. Strategies for monitoring and manipulating the activity of molecularly defined neurons

Many of the experiments described in this review start with mice in which Cre recombinase was targeted to a gene of interest expressed within a particular brain region (e.g., the PBN). Such mice can then be transduced with an adeno-associated virus (AAV) that is stereotactically injected into the PBN. The virus can carry either an excitatory designer receptor: for example, a G-protein-coupled receptor (GPCR) that is selectively activated by a designer drug (DREADD) or an excitatory opsin that can be activated by light; in either case the expression of the receptor or the opsin depends on the action of Cre recombinase [119,120]. There are also inhibitory DREADDs and opsins that can suppress neuron activity. AAV carrying other effector genes can be used to express fluorescent proteins to measure the neurons' activity (e.g., the calcium indicator, GCaMP) or trace their axonal projections, toxins that inhibit synaptic signaling (e.g., tetanus toxin), or proteins that ablate the cells (e.g., caspase). Using these strategies one can achieve expression of proteins that are restricted to a particular brain region and cell type of interest, along with temporal control depending on when the virus is injected and the ligand or light is delivered. A commonly used GPCR for excitation is hM3Dq (Gq-coupled); for inhibition, hM4Di (Gi-coupled), which is activated by clozapine N-oxide (CNO) and its metabolite, clozapine. This chemogenetic strategy has the advantage that injection of the ligand leads to activation of the GPCR that lasts for several hours. The optogenetic strategy involves expressing an excitatory opsin, typically channelrhodopsin (ChR2) or its derivatives, or an inhibitory opsin that can be activated by placing a fiber-optic probe over the PBN and delivering light. An advantage of the optogenetic approach is that duration, frequency, and power of the stimulation can vary, and the fiber-optic probe can be placed over either the cell bodies in the PBN or the axon terminals in another brain region.

focuses on the enhanced sensitivity to mechanical (tactile) sensitivity that can last for several days or weeks after stimulation of molecularly defined neurons in the brain. Tactile sensitivity is typically measured by the von Frey method, which involves poking the foot pad of mice with synthetic filaments of graded stiffness and recording threshold (in grams) that reliably elicits paw withdrawal.

Chemogenetic activation of PBN glutamatergic neurons expressing the excitatory receptor, hM3Dq, with seven daily injections of its ligand (clozapine-N-oxide, CNO) results in robust tactile allodynia within a day that lasts several weeks after the last CNO injection [61]. This is a clear demonstration of nociplastic pain (chronic pain without nerve injury). In a separate study from our own group, we repeated this important observation, but also noted that the response to CNO is bi-phasic. If we measured tactile sensitivity 2 h after CNO delivery (when CNO effects are ongoing) there was robust analgesia, whereas when it was measured at 23 h, allodynia was observed [62]. Chemogenetic activation of PBN *Oprm1* neurons results in a similar analgesic effect at 2 h without allodynia later [62], suggesting that activating all glutamatergic neurons has a mixed effect; activating some neurons promotes short-term analgesia while activating others promotes longer-lasting tactile allodynia.

Because *Calca* neurons are activated by all threatening stimuli, they were a likely candidate to mediate the nociplastic phenotypes. Indeed, 1, 3, or 7 days of chemogenetic activation of *Calca* neurons with daily injections of CNO resulted in allodynia, whether measured at 2 or 23 h, that lasted for a few days after a single injection and for >2 weeks after seven daily injections [62]. Thus, there is a graded response to duration of *Calca* neuron activation. Two days of tactile allodynia was also produced by bilateral optogenetic activation of ChR2 in *Calca* neurons for just 5 min (20 Hz), while daily optogenetic stimulation for 5 days (5 min at 20 Hz each day) resulted in tactile allodynia lasting >2 weeks [62]. In addition to allodynia, chemogenetic activation of *Calca* neurons enhanced the sensitivity to radiant heat or a hot plate.

Partial sciatic nerve ligation (pSNL) is a well-established model of chronic neuropathic pain with long-lasting allodynia [63]. A pivotal study showed that inhibition of PBN glutamatergic neurons could prevent establishment of neuropathic pain using this model [61]. Selective inactivation of *Calca* neurons with tetanus toxin prior to pSNL completely prevented tactile allodynia, and inactivating *Calca* neurons after pSNL alleviated the allodynia, indicating that these neurons are necessary for the development and maintenance of neuropathic pain [62]. Considering that the neuropathic pSNL method results in long-lasting activation of *Calca* neurons and activation of *Calca* neurons is sufficient to elicit pain phenotypes in naive mice, it is likely that neuropathic pain develops into nociplastic pain as nerve injury heals.

Because direct activation of *Calca* neurons by either optogenetic or chemogenetic means induced persistent allodynia, and a wide variety of threats have been shown to activate these neurons (measured using either induction of Fos or GCaMP imaging) [13,48], we predicted that repeated exposure to many aversive conditions could induce allodynia. Indeed, exposure to 3 days of injections with LiCl (which induces nausea), nitroglycerin (which promotes vasodilation and migraine-like symptoms) [64], or cisplatin (a chemotherapy treatment often associated with pain) [65,66] induced allodynia that lasted for several days after the last injection [62]. Notably, allodynia did not develop if *Calca* neurons were inactivated prior to exposure to any of these threats. Even 3 days of mice being chased for 5 min/day by a robotic bug was sufficient to induce significant allodynia [62]. These results indicate that many threatening conditions, some of which are not considered painful, activate *Calca* neurons sufficiently to elicit nociplastic pain. The roles that *Calca* and other molecularly defined PBN neurons play in mediating pain are summarized in [Box 2](#).

Box 2. Roles of molecularly defined, murine PBN neurons in pain

***Slc17a6* (VGluT2, glutamatergic)**

Glutamatergic neurons represent about 85% of PBN neurons, and they have been grouped into about 12 molecularly defined clusters that have been spatially localized within the PBN [67]. Tactile allodynia is induced within 23 h of activation of hM3Dq with CNO in the *Slc17a6*-expressing neurons in the PBN, and repeated daily injections of CNO leads to long-lasting allodynia [61]. Brief photoactivation of all *Slc17a6* neurons promotes running and jumping as well as analgesia, depending on the brain region in which the fiber-optic probe is placed [34]. Chemogenetic inhibition of *Slc17a6* neurons or inactivation of a sodium leak channel (NALCN) in them alleviates inflammatory pain [116].

***Gad2* (GABAergic)**

Photoactivation of the GABAergic neurons in the PBN with ChR2 reduces capsaicin-induced mechanical hypersensitivity, while chemogenetic activation of hM3Dq in GABAergic neurons suppresses pain induced by complete Freund's adjuvant or dipping the tail in hot water [34]. Chemogenetic activation of GABAergic neurons prevents development of tactile allodynia induced by sciatic nerve ligation [61].

***Calca* (CGRP)**

Chemogenetic activation of *Calca* neurons promotes jumping on a hot plate [121] and long-lasting tactile allodynia as measured by the von Frey test [62]. Optogenetic activation of *Calca* neurons also promotes tactile allodynia that scales with the duration of activation [62]. CGRP itself is not necessary for pain phenotypes elicited by chemogenetic activation or partial sciatic nerve ligation (pSNL), inflammatory pain elicited by formalin injection into the toe pad, or acetic acid-induced pain [62,122]. However, CGRP plays a role in promoting pain in both arthritis and bladder models [123–125].

***Tac1* (substance P, neurokinin-1)**

Chemogenetic activation of hM3Dq targeted to *Tac1*-expressing neurons in the PBN enhances jumping on a hot plate [120,125], but spinal responses to heat or cold are unaffected. Ablation of the *Tac1* neurons reduces the number of paw licks in the formalin test [126].

***Tacr1* (substance P receptor)**

Tacr1 neurons reside in superior lateral PBN; they receive inputs from *Tac1*-expressing projection neurons in the spinal cord and project axons to the medial thalamus and hypothalamus [33,67,97,100]. These neurons are activated (based on GCaMP imaging) by tail pinch, exposure to a hot plate, hot water, or pungent mustard oil (wasabi). Chemogenetic activation of *Tacr1* neurons by hM3Dq and CNO makes mice skittish with escape-like behaviors, evokes robust licking, enhanced nocifensive responses to a clip on their tail, and enhances sensitivity to tactile stimulation. Their activation facilitates coping with pain [126,127]. Ablating or inhibiting *Tacr1* neurons attenuates nocifensive responses [97,100].

***Oprm1* (μ opioid receptor)**

There are several populations of *Oprm1*-expressing neurons in the lateral PBN, including those that express *Calca* [67,128]. The *Oprm1* neurons are activated by noxious thermal (hot plate), mechanical (pinch), and inflammatory (formalin in toe pad) stimuli, which results in nocifensive responses and increased breathing rate [55]. Despite being aversive, chemogenetic activation of *Oprm1* neurons results in analgesia [62], perhaps due to stress-induced analgesia [129]. Parabrachial *Oprm1* neurons normally suppress development of long-lasting mechanical allodynia [81].

How does activation of *Calca* neurons promote nociplastic pain?

To address this question, one would need to ascertain how parabrachial *Calca* neurons signal to postsynaptic cells and the neural circuits that are critical to induce nociplasticity. *Calca* neurons are glutamatergic and cholinergic and express several neuropeptides and other secreted proteins in addition to CGRP [67], which individually or in combination might be necessary to elicit nociplastic pain.

Dissecting the neural circuitry involved in the establishment of tactile allodynia after activating *Calca* neurons is a challenging problem. I begin with the premise that tactile allodynia is ultimately due to changes in the spinal cord (e.g., by suppression of an inhibitory gate), which allows A β and A δ low-threshold mechanoreceptor neurons to activate dorsal horn neurons that project to the brain [9,68,69]. The circuitry connecting the A β and A δ inputs to the spinal output is complicated, and plasticity in several nodes has been described [70]. For example, by using either genetic and/or

pharmacological techniques, manipulating signaling by spinal neurons expressing the neuropeptide Y receptor 1, protein kinase C γ , parvalbumin, retinoic acid receptor α , somatostatin, cholecystokinin, vesicular glutamate transporter 3, or norepinephrine has been shown to alleviate tactile allodynia induced by sciatic nerve injury [71–78]. While intrathecal infusion of NPY alleviates tactile allodynia caused by nerve injury, it did not mitigate the allodynia produced by activation of *Calca* neurons [62].

The spinal plasticity that occurs after activating *Calca* neurons could be modulated by descending projections from the brain – for example, from serotonergic neurons in the rostral ventral medulla (RVM) to the spinal cord [79,80] – and/or by *Pdyn* neurons in the dorsomedial hypothalamus [81]. Beginning with this perspective, the problem is reduced to understanding how the *Calca* neurons signal to the RVM, dorsomedial hypothalamus (DMH), or other descending spinal projection neurons [82], and characterizing the molecular identity of the neurons that initiate synaptic plasticity in the spinal cord. There are few axonal projections from *Calca* neurons to either the RVM or DMH; hence, a longer, indirect circuit is likely to be involved. Possible intermediate nodes in the signaling pathway from *Calca* neurons to the spinal cord are described in the following section.

Parabrachial neurons activate the nociceptive capsular region of the central amygdala

A circuit from parabrachial *Calca* neurons to the CeA that responds to pain was described in 1989 [83], and it has continued to receive considerable attention [84]. Indeed, the capsular region of the CeA is often called ‘nociceptive amygdala’ because of the rich projections from *Calca* neurons [85,86]. Targets of *Calca* neurons that project to the capsular CeA include neurons that express protein kinase C δ (PKC δ) encoded by the *Prkcd* gene and the CGRP receptor encoded by the *Calcrl* gene. There is a partial overlap of these two markers; neurons in the rostral CeA primarily express *Calcrl*, while those in the caudal CeA express both *Calcrl* and *Prkcd* [87,88]. The functions and projections of the rostral and caudal *Calcrl* neurons are distinct [87].

Chemogenetic activation of *Prkcd* neurons promoted tactile allodynia measured a few hours later, but it dissipated by the next day [89]. This transient tactile allodynia contrasts with allodynia lasting 3 days when *Calca* neurons are chemogenetically activated [62], suggesting that activation of *Prkcd* neurons by *Calca* neurons is insufficient to induce long-lasting allodynia. Chemogenetic activation of all PBN neurons that project to the CeA by injecting a retrograde adeno-associated virus (AAV) expressing Cre recombinase in the CeA and Cre-dependent hM3Dq in the PBN also produced transient allodynia [90]. This approach should include activation of *Calca* neurons; however, activating other PBN neurons may have opposing effects on the duration of allodynia. What might be missing? One possibility is that *Calca* neurons activate an ensemble of CeA neurons, not just the *Prkcd* neurons, and activating the ensemble may be necessary to establish longer-lasting allodynia. Another consideration is that chemogenetic activation of *Prkcd* neurons (or any other specific population of CeA neurons) may not mimic the activation of *Calca* neurons because *Calca* neurons may release neuromodulators that are necessary for the plasticity promoting long-lasting allodynia. For example, cerebellin 1 made by *Calca* neurons has been suggested to play a role regulating plasticity in the CeA via its binding to the GluD1 receptor [91]. Likewise, brain-derived neurotrophic factor (BDNF) released from *Calca* neurons would activate different signaling pathways than hM3Dq [92]. A third possibility is that *Calca* neurons need to activate neurons in multiple brain regions simultaneously to achieve longer-lasting allodynia. In line with the latter suggestion, photoactivation of individual *Calca* neuron projections to five major targets never recapitulated the behavioral effects of activating the *Calca* cell bodies in the PBN, while activating several targets simultaneously came closer to duplicating the effect of activating the cell bodies [93].

Activation of other brain regions can also promote long-lasting tactile allodynia

If activating a network of signaling pathways is necessary to establish chronic allodynia, then identifying other brain regions (and molecularly defined neurons within them) that can promote chronic tactile allodynia should provide a roadmap of the circuitry. Table 1 lists other brain regions along with defining genes for neurons that when activated (or in some cases inhibited) produce long-lasting tactile allodynia like that achieved by activating *Calca* or *Slc17a6* neurons in the PBN. These neurons reside in the prefrontal cortex, thalamus, dorsal raphe, hypothalamus, and rostroventral medulla. Some of the brain regions mentioned in the following section are not direct targets of *Calca* neurons [94]; thus, there are gaps in the roadmap that need to be filled to establish circuitry leading from *Calca* neurons to the spinal cord.

Dorsomedial prefrontal cortex (dmPFC)

Prolonged chemogenetic activation of an ensemble of neurons in the dmPFC can induce chronic pain-like behaviors in normal mice, and silencing these neurons can relieve both pain hypersensitivity and anxiety-like behaviors in mice with chronic inflammatory pain [95]. Chemogenetic activation of *Chat* neurons in the nucleus basalis promotes pain-related phenotypes via modulation of the dmPFC [96].

Anterior paraventricular nucleus of the thalamus (aPVT)

The aPVT is a post-synaptic target of aversive signals from the PBN, including *Tacr1*-expressing neurons [97–99] that are distinct from the projections of *Calca* neurons [100]. The PVT, in turn, has strong connections to the CeA, where it can modulate aversive events [101]. Direct optogenetic activation of aPVT neurons can give rise to long-lasting allodynia that depends on extracellular regulated kinase activity [102]. Neurons in the posterior PVT (pPVT) also promote pain-related phenotypes that can be chemogenetically suppressed [103].

Ventral posteromedial thalamus (parvocellular region) (VPMpc)

Another thalamic target of the PBN is the VPMpc, which is directly innervated by *Calca* neurons. The cellular targets of *Calca* neurons in the VPMpc express the *Cck* gene. Chemogenetic or optogenetic activation of the *Cck* neurons in the VPMpc promotes many aversive behaviors, including allodynia, and inactivation of these neurons has an analgesic effect [104].

Table 1. Neurons that can promote long-lasting nociplastic pain and prevent neuropathic pain by neural manipulation studies in mice

Location	Molecular marker	Method	Refs
Parabrachial Nucleus (PBN)	<i>Slc17a6</i> (<i>Vglut2</i>)	Stimulation: Chemogenetics (hM3Dq)	[61]
Parabrachial Nucleus (PBN)	<i>Calca</i>	Stimulation: Chemogenetics (hM3Dq) and optogenetics (ChR2)	[62]
Parabrachial Nucleus (PBN) (projection to DMH)	<i>Oprm1</i>	Inhibition: Chemogenetics (hM4Di) and ablation (taCasp3)	[81]
Prefrontal cortex (dmPFC)	Ensemble of trapped neurons	Stimulation: Chemogenetics (hM3Dq)	[95]
Paraventricular thalamus (aPVT)	Unknown	Stimulation: Optogenetics (ChR2)	[102]
Dorsal raphe nucleus (DRN) (projection to VTA)	<i>Slc17a8</i> (<i>Vglut3</i>)	Inhibition: Optogenetics (eNpHR) and Chemogenetics (hM4Di)	[105]
Dorsomedial Hypothalamus (DMH) (projection to sc)	<i>Pdyn</i>	Inhibition: Chemogenetics (hM4Di) and ablation (taCasp3)	[81]
Rostroventral Medulla (RVM)	<i>Tph2</i>	Stimulation: Optogenetics (ChR2)	[79]

Dorsal raphe nucleus (DRN)

The activity of glutamatergic neurons expressing VGluT3 (encoded by the *Slc17a8* gene) in the DRN that project axons to the ventral tegmental area (VTA) is inhibited in response to nerve injury. Optogenetic activation of these neurons can ameliorate not only tactile allodynia but also the anhedonia associated with nerve injury [105]. Activation of these DRN projections to the VTA stimulates dopaminergic action in the nucleus accumbens. Conversely, inhibiting the VGluT3 DRN neurons by either optogenetic or chemogenetic means elicits long-lasting allodynia and anhedonia in the absence of nerve injury. These results appear to be at odds with a report indicating that optogenetic activation of DRN glutamatergic neurons promotes pain-like phenotypes, including allodynia that lasts >24 h [106]. The latter study relied on viruses with the calmodulin kinase 2 (*Camk2a*) promoter to target glutamatergic neurons in the DRN; that promoter may drive expression in a different ensemble of neurons than the ones with Cre recombinase targeted to the *Slc17a8* locus.

Dorsomedial hypothalamus

Ablating or inhibiting lateral PBN *Oprm1* neurons that project to *Pdyn* neurons in the DMH induces long-lasting tactile allodynia [81]. Conversely, activating the *Pdyn* neurons in the DMH can suppress pain induced by ablating the *Oprm1* neurons in the PBN [81]. This observation suggests that there is ongoing suppression of nociplastic pain by *Oprm1*-expressing PBN neurons. The *Pdyn* gene encodes dynorphin, which is an agonist for the κ opioid receptor, an inhibitory GPCR expressed in the spinal cord. Inactivating the *Pdyn* gene in the DMH ameliorates allodynia, indicating that dynorphin signaling to κ receptors plays a significant role [81]. *Oprm1* is expressed in several molecularly distinct PBN neurons, including the *Calca* neurons that have sparse projections to the DMH [67]; thus, identifying a more specific marker for the PBN neurons that normally suppress pain is needed.

Ventrolateral periaqueductal gray (vlPAG)

The vlPAG-to-RVM circuit is an important part of the descending pain modulation pathway. Optogenetic and chemogenetic techniques have been used to document that activation of glutamatergic neurons in the vlPAG is antinociceptive, while activation of GABAergic vlPAG neurons is pronociceptive, consistent with extensive earlier observations [107]. The prevailing view is that the glutamatergic vlPAG neurons project to the RVM, and those neurons are regulated by vlPAG GABAergic interneurons. Consequently, activation of the GABA population in the PAG could promote nocifensive activity by inhibiting the antinociceptive projection neurons. However, there are caveats to this view. Activation of the somatostatin (*Sst*)-expressing population of glutamatergic neurons in the lateral PAG is pronociceptive, and inhibiting those neurons ameliorates pain [108]. Also, some GABAergic neurons in the PAG project directly to the RVM, but the targets of these GABAergic projections are not well defined. There are no reports, to my knowledge, showing that manipulation of defined populations of PAG neurons can give rise to long-lasting allodynia. An intriguing report demonstrated that neurons expressing calmodulin activated kinase II (*CamKII α*) in the CeA, PAG, RVM, and spinal cord are important for maintenance of tactile allodynia in a persistent pain model, and delivery of a *CamKII α* antagonist into the CeA reversed allodynia and the *CamKII α* activity in the PAG, RVM, and spinal cord [109].

Rostroventral medulla

Classical studies revealed that the RVM contains 'ON' cells that become active at the onset of nocifensive stimulation, and 'OFF' cells that become active after stimulation ceases [80,106,110]. Activation of GABAergic ON cells is predicted to promote nocifensive behaviors, including tactile allodynia. Inhibition or ablation of ON cells that express the μ opioid receptor in the RVM alleviates

tactile allodynia in a neuropathic pain model [111,112]. The PBN sends direct projections to the RVM that can control the activity ON and OFF cells, but the molecular identity of those PBN neurons has not been identified [113]. Of note, a study using optogenetic manipulation of serotonergic *Tph2*-expressing RVM neurons showed that activation of these neurons for just 5 min was sufficient to promote tactile allodynia that lasted several days, while daily 5-min photoactivation for 3 days promoted allodynia lasting >2 weeks [79]. This is among the first publications showing that artificial activation of a small, molecularly defined population of neurons in the brain can promote nociplastic pain. This result is confounded by the observation that there are both GABAergic and glutamatergic *Tph2*-expressing neurons in the RVM [82]. The major glutamatergic population of *Tph2* neurons is in the medial RVM, and activating this population is pronociceptive, whereas the lateral population is largely GABAergic and its activation is antinociceptive [79,82,114].

Anterior nucleus basalis (aNB) (substantia innominata)

Optogenetic inhibition of *Chat* neurons in the aNB during non-rapid eye movement (NREM) sleep at the onset of sciatic nerve injury prevented the development of tactile allodynia [115]. *Chat* neurons project to vasoactive intestinal peptide (*Vip*)-expressing neurons in the somatosensory cortex. Chemogenetic inhibition of *Vip* neurons in the S1 also prevented onset of allodynia in a nerve-injury model, and it was able to reverse allodynia that had already been established [115]. These authors went on to identify enhanced activity of the PBN as a major input to the aNB that is responsible for the NREM-dependent allodynia. The implication of these experiments is that a PBN → aNB → S1 circuit is necessary for development of allodynia after nerve injury; however, the authors did not examine whether activation of this circuit is sufficient to establish long-lasting allodynia. Most studies on chronic pain are conducted while mice are awake; consequently, the NREM sleep-dependence of the pain phenotypes described in this study is notable.

Pain alters neuroplasticity in PBN neurons

Chemogenetic activation of *Calca* neurons results in hypersensitivity 2 days later (measured as responses to graded current injections); hypersensitivity also developed after pSNL [62]. The increase in hypersensitivity might be due to downregulation of the NALCN sodium leak channel [116]. Capsaicin treatment elevates *Oprm1* neuron activity in the PBN that persists until the next day [81]. Evoked activity of PBN neurons to radiant heat or tactile stimulation increased and persisted long after intra-orbital nerve injury or inflammatory pain, but spontaneous activity was unchanged [42,117]. This enhanced activation of PBN neurons is due in part to decreased GABAergic input from the CeA [41]. The activity of individual *Calca* neurons measured by GCaMP-calcium imaging increased during nitroglycerin administration and lasted several days. The response to von Frey filaments was also enhanced by nitroglycerin treatment, and more *Calca* neurons were recruited without change in the magnitude or frequency of responses [62]. Thus, PBN neurons can maintain hyperexcitability for a few days after they are stimulated, but the duration of these effects and underlying mechanisms are not clearly established.

Concluding remarks and future directions

The PBN is a major target of spinal projection neurons, the sensory trigeminal nucleus, and the vagus; thus, it is a gateway to the brain for nociceptive signaling to many forebrain regions. Consistent with its proximal position in brain circuitry, activation of all excitatory neurons (*Slc17a6*) in the PBN (or just *Calca* neurons) can elicit long-lasting tactile allodynia that scales with degree of activation. The focus of this review is on *Calca* neurons, but it is likely that several populations of PBN neurons are either directly activated by nociceptive inputs or indirectly activated by intra-PBN signaling, in which case the possible efferent targets could increase, offering additional

Outstanding questions

Pain activates several molecularly distinct parabrachial neurons. Do they mediate selective aspects of neuropathic or nociplastic pain? Does intra-PBN connectivity play a role in mediating pain phenotypes?

There are several major output pathways from the PBN. Do the molecularly defined neurons that contribute to these pathways have distinct roles in mediating pain?

Based on studies in mice, most threatening stimuli can activate *Calca* neurons, but for many of these sensory inputs the neural circuitry leading to activation of *Calca* neurons is not established. What are the circuit pathways by which threatening stimuli activate *Calca* neurons and other molecularly defined PBN neurons?

Allodynia persists long after activation of the PBN, suggesting that it elicits synaptic plasticity either in PBN neurons or in downstream nodes. Which neurons undergo plasticity? What are the molecular mechanisms underlying synaptic plasticity in this context?

Activation of neurons in many brain regions can elicit long-lasting tactile allodynia and other pain-related phenotypes. A major challenge is to ascertain whether and how these brain regions are connected. Is there a common starting point (e.g., the PBN), with branching pathways to several nodes that then impinge on one (or more) descending pathways to the spinal cord?

circuit possibilities for signaling to the spinal cord. Examining these circuits is a major goal for future work (see [Outstanding questions](#)).

Activating neurons in several other brain regions beyond the PBN can also elicit long-lasting tactile allodynia, and future work may identify additional brain regions relevant in this context. A major challenge is to ascertain whether and how these brain regions are connected. Is there a common starting point (e.g., the PBN), with branching pathways to several nodes that then coalesce onto one (or more) descending pathways to the spinal cord? Are the different pathways redundant with respect to tactile allodynia: for example, would inhibiting one node prevent induction of allodynia by activating another node in naive mice? Do distinct pathways convey different emotional aspects of pain? Is there more than one descending pathway to the spinal cord that allows A β and A δ low-threshold mechanoreceptor neurons to activate spinal projection neurons resulting in allodynia? It is known that activating local GABAergic neurons in the PBN can prevent neuropathic pain [61], but it is not clear whether inhibitory neurons play an important regulatory role under physiological conditions. Likewise, the role of PBN glia remains largely unexplored, and accordingly has not been considered in the current review; however, scRNA sequencing revealed changes in gene expression in the PBN, including in glia, after nerve injury [118], suggesting interesting directions for future investigation.

The fact that allodynia persists long after activation of neurons ceases, in the PBN or elsewhere in the brain, suggests that activation elicits synaptic plasticity in the neurons that were directly activated and/or in downstream nodes. Assessing what neurons undergo plasticity and the molecular mechanisms underlying the synaptic plasticity that occurs is fundamental to better understanding nociplastic pain.

Acknowledgments

I appreciate input from students and colleagues that made this review possible, and I thank Dr Jane Chen for editorial help and preparation of the figure.

Declaration of interests

The author declares no conflicts of interest.

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