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Genetic evidence of the function of Phox2a-expressing anterolateral system neurons in the transmission of chronic pain

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Abstract

The development of the chronic neuropathic pain state often originates at the level of peripheral sensory neurons, whose abnormal function elicits central sensitization and maladaptive plasticity in the nociceptive circuits of the spinal dorsal horn. These changes eventually reach supraspinal areas bringing about cognitive and affective co-morbidities of chronic pain such as anxiety and depression. This transmission presumably relies on the function of spinal projection neurons at the origin of the anterolateral system (AS). However, the identity of these neurons and the extent of their functional contribution remain unknown. Here, we asked these questions in the context of the mouse AS neurons that require the transcription factor Phox2a for their normal target connectivity and function in transmitting acute nociceptive information to the brain. To this end, we examined the effects of a spinal cord-specific loss of Phox2a (Phox2a^{cKO}) on the development of central sensitization evoked by the spared nerve injury (SNI) model of chronic pain. We found that SNI-treated Phox2a^{cKO} mice developed normal reflexive spinal responses such as mechanical allodynia evidenced by a decreased withdrawal threshold to von Frey filament stimulation and dynamic brush. On the other hand, Phox2a^{cKO} attenuated the development of cold but not mechanical hyperalgesia, in behavioral paradigms that require the relay of nociceptive information to the brain. Furthermore, Phox2a^{cKO} attenuated anxio-depressive-like behaviors evoked by SNI, measured by performance in the open field test and tail suspension test. Thus, Phox2a AS neurons play a critical role in the generation and maintenance of chronic neuropathic pain.

Keywords

Chronic pain model, Phox2a, anterolateral system, spinal projection neurons, nocifensive behaviour, allodynia, hypersensitivity, anxiodepressive- like behaviours, spinal cord, spared nerve injury

Introduction

The sensation of acute pain is required for avoidance of harmful stimuli. Various conditions such as peripheral or central injury hijack nociceptive circuits through activity-driven plastic changes, resulting in the development of chronic neuropathic pain (CNP). This maladaptive state is characterised by the perception of innocuous stimuli as noxious (allodynia) or enhanced sensitivity to noxious stimuli (hyperalgesia), and is eventually coupled with comorbid anxiety and depression, in humans and model animals.[1–5] Many central sensitization mechanisms have been proposed.[6,7] At the spinal cord level, loss of a local inhibitory gate and central sprouting of sensory afferents drive allodynia and hyperalgesia.[8,9] At supraspinal levels, long-term

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potentiation induces functional plasticity-related alterations in brain regions linked to anxiety and depression, such as the medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), and nucleus accumbens (NAc).[10,11] Malfunctions of descending inhibitory and facilitatory controls may further contribute to central sensitization at the spinal level.[12,13]

Central sensitization at the supraspinal level follows its appearance in the spinal cord suggesting that projection neurons linking the spinal cord to the brain may be involved in this transmission. In acute nociception, anterolateral system (AS) neurons integrate spinal interneuron and sensory afferent information and relay it through glutamatergic projections to nociceptive brain regions such as the periaqueductal gray, the parabrachial nucleus, and the medial, posterior and lateral thalamus. These areas are gateways to the mPFC, central amygdala, ACC, and somatosensory cortex, where discriminatory and motivational/affective components of pain are decoded.[14–16] The evidence linking human AS neurons to CNP is the relief of persistent pain by lesioning their axons.[17,18] Animal models of CNP provide evidence that AS neurons are subject to central sensitization via synaptic plasticity: their activity increases as does the probability of responding to innocuous stimuli.[19–21] Ablation of AS neurons via their neurokinin-1 receptor (NK-1R) expression also imply their contribution to central sensitization, however, this conclusion is confounded by the fact that such manipulations also ablated many NK-1R-expressing interneurons.[22,23] Our recent identification of the developmentally-expressed transcription factor Phox2a as a selective handle of a large population of AS neurons allows their manipulation exclusive of interneurons. The spinal cord-specific loss of Phox2a (Phox2a^{cKO}) in mice results in impaired AS brain target innervation, and decreased supraspinal responses to noxious stimuli but normal spinal-level nocifensive reflexes.[24] These findings imply that Phox2a AS neurons may relay persistent pain information between the spinal cord and the brain, and their maladaptive recruitment in CNP may evoke plastic changes in supraspinal regions linked to anxiety or depression comorbidities.

Materials and methods

Mouse lines and the generation of Phox2a^{cKO} *mutants and controls*

Only adult male mice (6–17 weeks of age), maintained on a mixed background of 129/Sv and C57Bl/6 were used in this study. Mice were kept on a 12-h light:12-h dark cycle (light on from 6h00–18h00) with food and water provided *ad libitum*. All procedures were approved by the IRCM Animal Care Committee, using regulations and guidelines provided by the Canadian Council for Animal Care (CCAC). *Phox2a*^{cKO} mice were generated as previously[24] by breeding parents carrying two *Phox2a*^{flox} (*Phox2a*^{f}) alleles with *Hoxb8*^{Cre}*, Phox2a*$^{f/+}$ or *Hoxb8*^{Cre}*, Phox2a*$^{f/f}$. Phox2a^{cKO} mice carried *Hoxb8*^{Cre}*; Phox2a*$^{f/f}$. Control mice were littermates that bear either *Hoxb8*^{Cre}*, Phox2a*$^{+/+}$ or *Phox2a*$^{f/f}$, as close as possible to the ratio of 1:1. *Phox2a*^{f} (RRID:IMSR_EM:04,758) and *Hoxb8*^{Cre} genotyping were as described previously.[24]

Spared nerve injury model

Adult mice (6–8 weeks of age), under anesthesia with isoflurane (Isoflurane USP CP0406V2, 4% for induction and 2% for maintenance) with a mixture of 70% N_2O and 30% O_2 underwent spared nerve injury (SNI) or sham surgery. The detailed procedure of SNI surgery has been previously described.[25,26] Briefly, under anesthesia, the tibial and common peroneal nerves on the left side were tightly ligated with 6-0 Sofsilk^{TM} sutures (Coviien, S1172), and 2–3 mm of the nerves were severed between ligations, while the sural nerve was left intact. Muscle and skin were closed in two layers using the suture. For sham groups, mice received surgery as described except that tibial and common peroneal nerves were shortly exposed without manipulation. Mice received 1 mg/kg buprenorphine for analgesia prior to surgery and recovered for at least 15 min post-surgery in a heated chamber before being returned to their home cage.

Mouse behavioral assays

All behavioral assays were conducted largely as in Roome et al., 2020. X. Z. performed all behavioral assays except for the dynamic brush, tail suspension and place avoidance task that were conducted by M. M. Experimenters were always blinded to experimental groups in all behavioral tests. Mice from control and *Phox2a*^{cKO} groups were littermates, no mouse was housed alone, and only male mice were used. Prior to every test, mice were habituated in a dedicated behavior test room for at least 30 min. For the von Frey test, acetone test, and dynamic light brush test, mice were habituated in a small plexiglass chamber (4 × 2.2 × 2.5 cm) on a mesh floor. For all the other assays, mice were habituated in their home cages.

Algometric behavioral assays

For the paw withdrawal threshold (von Frey) test, each mouse was placed in the plexiglass chamber on a mesh floor. The von Frey filaments (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4 g; North Coast Medical, Cat#: NC12775-01 - NC12775-10) were used to stimulate the hindpaw plantar surface of the sural nerve territory. Mice were tested using an "up-down" method of Dixon, as described previously.[24,27,28]

For the Dynamic Brush (DB, dynamic tactile hypersensitivity) test, animals were habituated to the testing room in their home cage for 60 min and then were placed in individual 5 × 5 × 10 cm cubicles on a wire mesh platform for an additional 20 min before testing. Using short-handled

paintbrushes with synthetic gold taklon bristles (Round tip, size: #1), each animal was stimulated 10 times on each foot of the sural nerve territory, in an alternating fashion with a 30-s interval. A stimulation consisted of a slow and fluid movement of the brush tip, gliding lightly from heel to toe. For each stimulation, the intensity of reaction was recorded as follows: 0—No reaction, 1—Normal withdraw (brief repositioning of the foot withdraw), 2—Withdraw +1 or 2 mild flinching, 3—Withdraw + intense flinching (>1 s), 4—Withdraw + licking. Averaged intensity of reaction (average of the reaction intensity for each withdrawal) was calculated for each foot and each animal.

For the acetone test, each mouse was placed in a plexiglass chamber (4 × 2.2 × 2.5 cm) on a mesh floor. A drop of acetone extruded from the blunt end of a 5 mL syringe and carefully applied to the plantar surface of the hind paw, without touching the skin. Mice were recorded for 1 min following the application. Such procedure was repeated 5 times, with an inter-trial interval of 5 min. The total licking time as a sum of 5 trials was reported.[29]

The Place Escape/Avoidance Paradigm assay (PEAP)[30] was conducted as adapted for mice.[31] The apparatus consisted of a light/dark box apparatus (20 × 20 × 40 cm) positioned on top of a mesh screen. The chamber was divided into 2 equal compartments (one with black walls and one with white ones), separated by a wall with a 7 × 7 cm opening. Animals were placed into the white chamber and allowed to freely move throughout the apparatus for the duration of a 30-min test period. A 9.804 mN (filament 4.08, 1 g, Stoelting Co, Catalog# 58,011) von Frey monofilament was applied to the plantar surface of a hindpaw every 15 s. The left (ipsilateral injured or sham) paw was stimulated in the black chamber, and the right (contralateral undisturbed) paw in the white chamber. This paradigm creates a conflict between the aversive light compartment and aversive noxious mechanical stimulation in the dark compartment. The percentage of time mice spent in the light chamber was calculated for every 5 min of testing (for details, see LaBuda and Fuchs, 2000) and was expressed as the animal's preference. As the innate preference of a mouse is for the dark environment, the time spent in the light compartment is a measure of the aversion to mechanical stimulation of the injured paw relative to aversion to the light compartment.[31]

Measurement of anxio-depression-like behaviors

As in a standard Open Field test,[32] an individual mouse was placed in the center of an acrylic chamber (matt white, no odors, 40 × 40 × 30 cm) with an overhead dim light. Mice were recorded for 10 min with a camera (iPhone X, rear camera) placed overhead. The chamber was cleaned between each test. Videos were analyzed using autotyping[33] for time spent in the center region (20 × 20 cm) and movement trace in MATLAB R2018a. Data was exported and sorted in Microsoft Excel.

For the Tail Suspension assay (TS),[34] mice were individually suspended by the tail underneath a platform. Adhesive tape was used to attach the tail at two points (0.5–1 cm from the base and the tip of the tail) to the platform for 8 min. The animal behavior was videorecorded and analyzed using digital software (Anymaze®) to report the duration of time spent in immobility (not moving but stretched out). Videos were analyzed by minute for the entire test period by an observer blinded to experimental condition. The duration of immobility in the final 3 min (minute 5–8) was reported as depression-like behavior.

Tissue fixation, freezing and sectioning

Mice were anesthetized with an intraperitoneal (i.p.) injection of a Ketamine/Xylazine solution (10 mg/mL Ketamine, 1 mg/mL Xylazine, in 0.9% saline), 10 mL/kg of body weight. Transcardial perfusion was conducted at least 30 min post injection, with the verification that mice reached surgical plane of anesthesia by confirming loss of pedal withdrawal (toe pinch) reflex. Mice were perfused with 15 mL of ice-cold 0.9% NaCl solution followed by 20 mL of ice cold 4% paraformaldehyde (PFA) in 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4.; Fisher) using 25 mL syringes. Brains and spinal cords were collected and post-fixed in 4% PFA in 1x PBS on a gentle tilt shaker at 4°C overnight, followed by overnight washing in 1x PBS, and then transferred to 30% sucrose for at least 2 days until sink. Tissue was stored at −80°C until cryosectioning. Before sectioning, tissue was embedded in Tissue-Tek O.C.T. Compound (Sakura, 4583) and cryosectioned at a temperature between −20 and −23°C. Brain tissue was sectioned at a thickness of 50 µm and spinal cord tissue at a thickness of 30 µm for all experiments.

Immunohistochemistry

Tissue sections were stored at −80°C. Slides were first warmed to room temperature for at least 15 min and washed 3 times with 1x PBS, 10 min each, and blocked in a blocking solution (5% heat-inactivated horse serum (HIHS) and 0.1% Triton X-100 in 1x PBS (0.1% tPBS)) for 30 min. Following blocking, sections were incubated with primary antibody solution overnight at 4°C. Primary antibody solutions were prepared with Rabbit anti-Fos (Cell Signaling Technology, Cat#: 2250S), Rabbit anti-Iba1 (Abcam, Cat#: ab178846), Mouse anti-NeuN (Millipore, Cat#: MAB377), diluted at 1:1000 in 1% HIHS, 0.1% tPBS. For spinal cord section, Isolectin GS-IB4 From *Griffonia simplicifolia*, Alexa Fluor™ 488 Conjugate (Thermo Fisher Scientific, Cat#: I21411) was incubated with primary antibodies, diluted at 1:500 in 1% HIHS, 0.1% tPBS. On the following day, sections were washed 3 times with 1x PBS, 10 min each, and were incubated with secondary antibody solution at room temperature for 2h. Secondary antibodies were prepared with Alexa 488 Donkey anti-Mouse (Cat#: 715-545-150), Cy3 Donkey

anti-Mouse (Cat#: 715-165-150), Cy5 Donkey anti-Rabbit (Cat#: 711-175-152), Cy5 Donkey anti-Guinea Pig (Cat#: 706-175-148), all from Jackson Immunoresearch Laboratories, diluted at 1:500 in 1% HIHS, 0.1% tPBS. Following the incubation, sections were washed 3 times with 1x PBS, 10 min each, and coverslipped using a Mowiol solution (10% Mowiol, 25% glycerol; Sigma). Slides were allowed to dry and stored in the dark at 4°C prior to imaging.

Quantification and statistical analysis

Fos and Iba1 quantification. For baseline analysis, animals were allowed to explore their home cages for 90 min, followed by anesthesia with Ketamine/Xylazine (100 mg Ketamine and 10 mg Xylazine in 10 mL saline, 0.1 mL per 10 g of body weight), followed by perfusion after 30 min. For ice-water induced analysis, animals were first anesthetized with Ketamine/Xylazine solution for 5 min. Subsequently the hindpaw ipsilateral to the surgery site was immersed in 5 mL of ice-water mixture for 1 min. The immersion was repeated 5 times with 1-min intertrial interval, and the animals were perfused as above. Following immunohistochemical staining (see above), micrographs of tissue sections were obtained with confocal microscope (Leica SP8) with the same optical and laser settings for all sections. Cell counts were performed using the cell counter plugin in ImageJ software (author: Kurt De Vos). Laminae of the spinal dorsal horn were defined with reference to the IB4 staining (above being Lamina I) and were adapted from Rexed,[35] 1952 with reference to Allen mouse spinal cord P56 Adult Annotated Atlas. The subnuclei of the parabrachial nucleus were delineated by cell and neuropile morphologies as per the Allen Brain Mouse P56 Coronal 2011 reference atlas and the more recent molecular oncology study.[36] For Fos quantification, images were thresholded at the same level as indicated in the legends. For Iba1 quantification, only star-shaped cells (activated form) were counted. Data were exported and sorted in Microsoft Excel. The sum of total cell numbers from 5 sections for spinal cord and 3 sections for the brain per animal was reported.

Statistical analyses. All statistical analyses were performed using Prism 9 (Graphpad Software). Statistical tests of non-parametric and parametric unpaired t-test, paired t-test, and Ordinary Two-way ANOVA followed by Tukey's multiple comparisons were used as specified in figure legends. Significance is represented as *n*. s.: non-significant ($p > 0.05$), *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, or ****: $p < 0.0001$.

Results

Fos and Iba1 expression in the spinal cord of SNI-treated Phox2a^{cKO} mice

Damage to peripheral nerves induces maladaptive changes in spinal and supraspinal nociceptive circuits. To determine the role of Phox2a AS neurons in these effects, we combined the Phox2a^{cKO} genetic model of their loss of function with the spared nerve injury (SNI) mouse model of neuropathic pain.[25] SNI induces a wide range of neuropathic effects that include spinal allodynia, mechanical hyperalgesia, and recapitulates the anxio-depression comorbidities observed in human patients.[25,37] We thus performed SNI or sham surgery on adult Phox2a^{cKO} (*Hoxb8^{Cre}; Phox2a$^{flox/flox}$*) and control (*Phox2a$^{flox/flox}$* or *Hoxb8^{Cre}*) mice to ask if the loss of Phox2a AS neurons impacted the emergence of molecular and behavioral maladaptive changes caused by SNI. All experiments were performed with male animals since females do not consistently model the full range of SNI-associated behavioral phenotypes found in males.[38,39]

We first verified whether Phox2a^{cKO} mice exhibit molecular changes normally associated with SNI; in this model of neuropathic pain, the dorsal horn of the spinal cord exhibits increased expression of Fos, a protein associated with increased neuronal activity.[40,41] Another consequence of SNI is increased microglial activity in the dorsal horn, evidenced by the increased number of microglia expressing the activation marker Iba1 with star shape morphology.[42] We thus asked whether similar effects were evident in Phox2a^{cKO} mice treated with SNI 4 weeks following SNI induction, on the side ipsilateral (ipsi) and contralateral (contra) to the surgery. Thus, we quantified Fos and Iba1 expression in Lamina I and Lamina V/lateral spinal nucleus (V/LSN), the locations of many Phox2a AS neurons, and the total number of Fos + or Iba1 cells in these regions (Lamina$^{Sup\ +\ Deep}$). First, we examined the number of Iba1 expressing (Iba1+) cells with star-like morphology (Figure 1(a) and (b)). In line with previous reports, examination of Iba1 + cells in control mice (ctrl) revealed their significant increase on the ipsilateral side of SNI animals, when compared to the contralateral side (paired parametric t-test, ctrl SNI ipsi vs. contra, Lamina I $p = 0.0173$, Lamina V/LSN $p = 0.0223$, Lamina$^{Sup\ +\ Deep}$ $p = 0.0106$). Further analysis revealed elevated numbers of Iba + cells on the ipsilateral side following SNI treatment, when compared to sham treated control mice (unpaired parametric t-test, ipsi ctrl sham vs. SNI, Lamina I $p = 0.0138$, Lamina V/LSN $p = 0.0057$, Lamina$^{Sup\ +\ Deep}$ $p = 0.0012$). While this effect was preserved in Phox2a^{cKO} mice treated with SNI compared to sham as total Iba + numbers (unpaired parametric t-test, ipsi Phox2a^{cKO} sham vs. SNI, Lamina I $p = 0.0973$, Lamina V/LSN $p = 0.2075$, Lamina$^{Sup\ +\ Deep}$ $p = 0.0177$), there is no apparent effect when comparing ipsilateral with contralateral in SNI-treated Phox2a^{cKO} mice (paired parametric t-test, Phox2a^{cKO} SNI ipsi vs. contra, Lamina I $p = 0.2123$, Lamina V/LSN $p = 0.2593$, Lamina$^{Sup\ +\ Deep}$ $p = 0.2134$).

Next, in the same animals, we quantified Fos-expressing (Fos+) neurons defined by NeuN expression (Figure 1(c) and (d)). In line with previous observations, SNI treatment increased the number of Fos + neurons in the ipsilateral Lamina V/LSN and in total number of Lamina$^{Sup\ +\ Deep}$, when

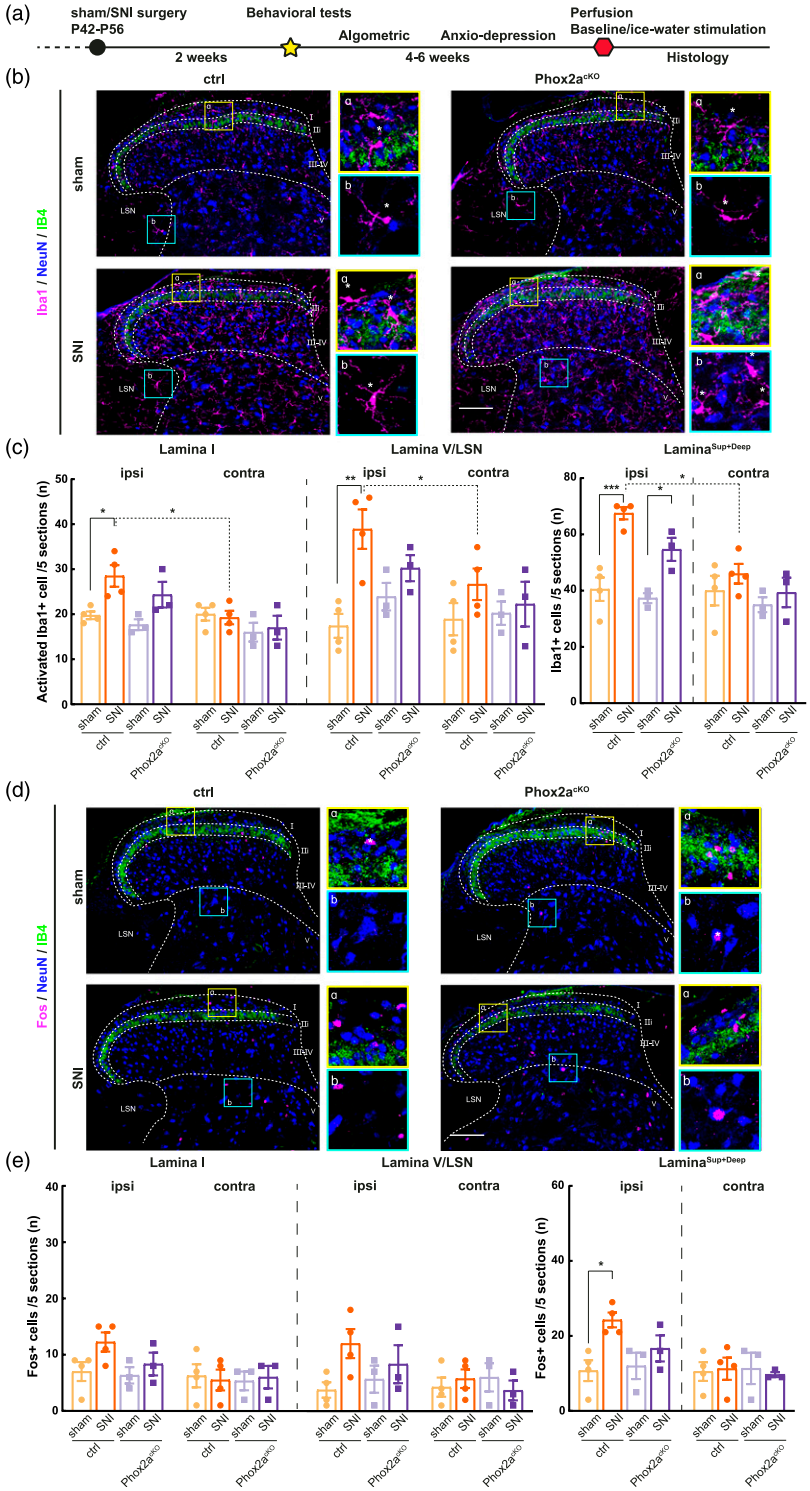

Figure 1. Attenuation of SNI-induced Fos and Iba1 expression in the spinal cord in Phox2a^{cKO} mice. (a), Representative spinal cord dorsal horn transverse sections of sham- or SNI53operated control (ctrl) or Phox2a^{cKO} animals. Star-shaped Iba1+ (magenta) cells not co-labelled with NeuN (blue) were quantified as Iba1-expressing (Iba1+, white asterisk in zoomed view (a, b). Lamina I is defined as dorsal to the IB4 band (green) in Lamina IIi. Lam V/LSN were defined as per Rexed, 1952. (b)**,** Quantification of Iba1 + cells in Lamina I, Lamina V/LSN, or as a total (LaminaSup + Deep) on the ipsilateral (ipsi) side or contralateral (contra) side to the operated side. Summation of Iba + cells of 5 sections with 30 μm thickness is reported. (c)**,** Representative spinal cord dorsal horn transverse sections. Fos (magenta) colocalizing with NeuN (blue) were quantified as Fos-expressing neurons (Fos+, white asterisk). Laminae defined as in (a) Scale bar same for all images, 100 μm. (d)**,** Quantification and statistical analysis of Fos + cells the same as in (b). In both experiments, n = 4 for control groups. = 3 for Phox2a^{cKO} groups. Bars represent mean ± SEM with individual values as dots. Unpaired parametric t-test (solid line) for sham versus SNI within ctrl or Phox2a^{cKO} group. Paired parametric t-test (dotted line) for ipsi versus contra within SNI-treated ctrl or Phox2a^{cKO} group. Absence of comparison bars indicates a non-significant difference, *: $p < 0.05$, **: $p < 0.01$ or ***: $p < 0.001$. See Results for numerical data and statistical results. Scale bar same for all images is 100 μm.

compared to sham controls (unpaired parametric t-test, ipsi ctrl sham vs. SNI, Lamina I $p = 0.0707$, Lamina V/LSN $p = 0.0304$, Lamina$^{Sup + Deep}$ $p = 0.0075$,[40,41]). We did not detect a significant increase in Fos + cells in either location or in total number in Phox2a^{cKO} mice with SNI treatment compared to sham animals (unpaired parametric t-test, ipsi Phox2a^{cKO} sham vs. SNI, Lamina I $p = 0.2943$, Lamina V/LSN $p = 0.5555$, Lamina$^{Sup + Deep}$ $p = 0.3987$). Together, these results suggest that Phox2a AS loss of function impairs the induction of Fos and Iba1 expression in the spinal dorsal horn of SNI-treated mice.

One of the features of SNI-associated cold allodynia is that the increased neuronal activity in the spinal dorsal horn can be further augmented by exposure to cold stimuli.[43] We asked whether this effect can also be observed in Phox2a^{cKO} mice using the cold-water immersion paradigm in which the sham or SNI-subjected hindlimb was immersed in ice-water mixture (0°C) for 1 min with 5 repeats, while under anesthesia (Figure 2). This treatment resulted in increased number of Fos + neurons on the side ipsilateral to the stimulus when compared to the contralateral side of SNI treated control animals (paired parametric t-test, ctrl SNI ipsi vs. contra, Lamina I $p = 0.0669$, Lamina V/LSN $p = 0.0500$, Lamina$^{Sup + Deep}$ $p = 0.0183$). A similar increase was also observed in Phox2a^{cKO} mice (paired parametric t-test, Phox2a^{cKO} SNI ipsi vs. contra, Lamina I $p = 0.0162$, Lamina V/LSN $p = 0.0572$, Lamina$^{Sup + Deep}$ $p = 0.0078$). In addition, cold exposure resulted in more Fos + neurons in ipsilateral Lamina I, V/LSN, and Lamina$^{Sup + Deep}$ combined when compared to controls (unpaired parametric t-test, ipsi ctrl sham vs. SNI, Lamina I $p = 0.0194$, Lamina V/LSN $p = 0.0353$, Lamina$^{Sup + Deep}$ $p = 0.0404$). However, in cold-exposed Phox2a^{cKO} mice, when compared to sham treated animals, SNI treated mice had an increased number of Fos neurons in Lamina I, but not in V/LSN or when expressed as Lamina$^{Sup + Deep}$ (unpaired parametric t-test, ipsi Phox2a^{cKO} sham vs. SNI, Lamina I $p = 0.0462$, Lamina V/LSN $p = 0.1870$, Lamina$^{Sup + Deep}$ $p = 0.0564$). Together, our results demonstrate that Phox2a^{cKO} attenuated the induction of Fos and Iba1 expression changes normally induced by SNI, with and without cold stimulation.

SNI-induction of Fos expression in the parabrachial nucleus of Phox2a^{cKO} mice

SNI elevates Fos expression in multiple brain regions such as the parabrachial nucleus (PBN), periaqueductal gray and anterior cingulate cortex.[40,41] We therefore asked whether the loss of AS neuron function caused by Phox2a^{cKO} may attenuate these effects in the PBN since we previously demonstrated that Phox2a^{cKO} markedly reduces the number of spinofugal afferent axons in the PBN. In particular, we investigated two subregions of the PBN – dorsal lateral PB (PBdl) being innervated primarily by the Lamina I PNs, and the internal lateral PB (PBil) innervated by the Lamina V/LSN PNs whose connections are reduced in Phox2a^{cKO} mutants.[24,44,45] We first examined the number of Fos + neurons in the PBN of mice exposed only to their home cage environment (Figure 3(a) and (b)). In the PBdl, compared to sham operated mice, SNI treatment induced Fos expression in both control and Phox2a^{cKO} groups, predominantly on the contralateral side (unpaired parametric t-test, PBdl, ctrl sham vs. SNI, ipsi $p = 0.0721$, contra $p = 0.0011$, total $p = 0.0061$; Phox2a^{cKO} sham vs. SNI, ipsi $p = 0.0664$, contra $p = 0.0482$, total $p = 0.0438$). Similarly, in the PBil, SNI elevated the total number of Fos-expressing neurons compared to sham treated mice, in control and Phox2a^{cKO} groups (unpaired parametric t-test, PBil, ctrl sham vs. SNI, total $p = 0.0156$; Phox2a^{cKO} sham vs. SNI, ipsi total $p = 0.0254$). On the other hand, although the number of contralateral Fos + cells in SNI-treated mice was significantly elevated compared to sham operated animals in control mice, this effect did not occur in Phox2a^{cKO} mice (unpaired parametric t-test, PBil, ctrl sham vs. SNI, contra $p = 0.0152$; Phox2a^{cKO} sham vs. SNI, contra $p = 0.2943$).

We also examined Fos expression in the PBN of control and Phox2a^{cKO} mutants with SNI or sham, exposed to a cold stimulus, as above (Figure 3(c) and (d)). SNI-operated groups again showed significantly more Fos + neurons in the PBdl, both in the control groups and the Phox2a^{cKO} groups (unpaired parametric t-test, PBdl, ctrl sham vs. SNI, ipsi $p = 0.0158$, contra $p = 0.01{,}016$, total $p = 0.0149$; Phox2a^{cKO} sham vs. SNI, ipsi $p = 0.0808$, contra $p = 0.0257$, total $p = 0.0417$). However, we failed to observe any significant differences in the PBil between sham and SNI-operated groups either in control or Phox2a^{cKO} mice (unpaired parametric t-test, PBil, ctrl sham vs. SNI, ipsi $p = 0.8933$, contra $p = 0.1575$, total $p = 0.3285$; Phox2a^{cKO} sham vs. SNI, ipsi $p = 0.4918$, contra $p = 0.2794$, total $p = 0.1242$). Together, these analyses imply that the SNI-evoked Fos expression activation in the PBN is largely unaffected in Phox2a^{cKO} mutants.

Spared nerve injury-induced mechanical allodynia in Phox2a^{cKO} mice

We then turned to a behavioral analysis of Phox2a^{cKO} mice with SNI by first examining the development of allodynia manifested by nocifensive paw withdrawal in response to application of von Frey (vF) filaments.[26] These are mostly reflexive responses elicited by spinal cord nocifensive circuits; however, because of the limited impact of Phox2a^{cKO} on spinal circuits function, we hypothesized that Phox2a^{cKO} would not affect the development of SNI-associated allodynia. Indeed, both SNI-operated Phox2a^{cKO} and control mice showed decreased withdraw threshold compared to sham-operated controls in the vF assays (Figure 4(a) left, Ordinary Two-way ANOVA, Tukey's multiple comparison, and same for the subsequent statistics in Figure 4, ctrl sham vs. SNI and Phox2a^{cKO} sham vs. SNI $p < 0.0001$). There was no difference between the SNI-operated groups or between the sham-operated control and Phox2a^{cKO} groups (sham ctrl vs. Phox2a^{cKO} $p = 0.9977$; SNI ctrl vs. Phox2a^{cKO} $p = 0.9934$). Another allodynic effect of SNI is revealed by the

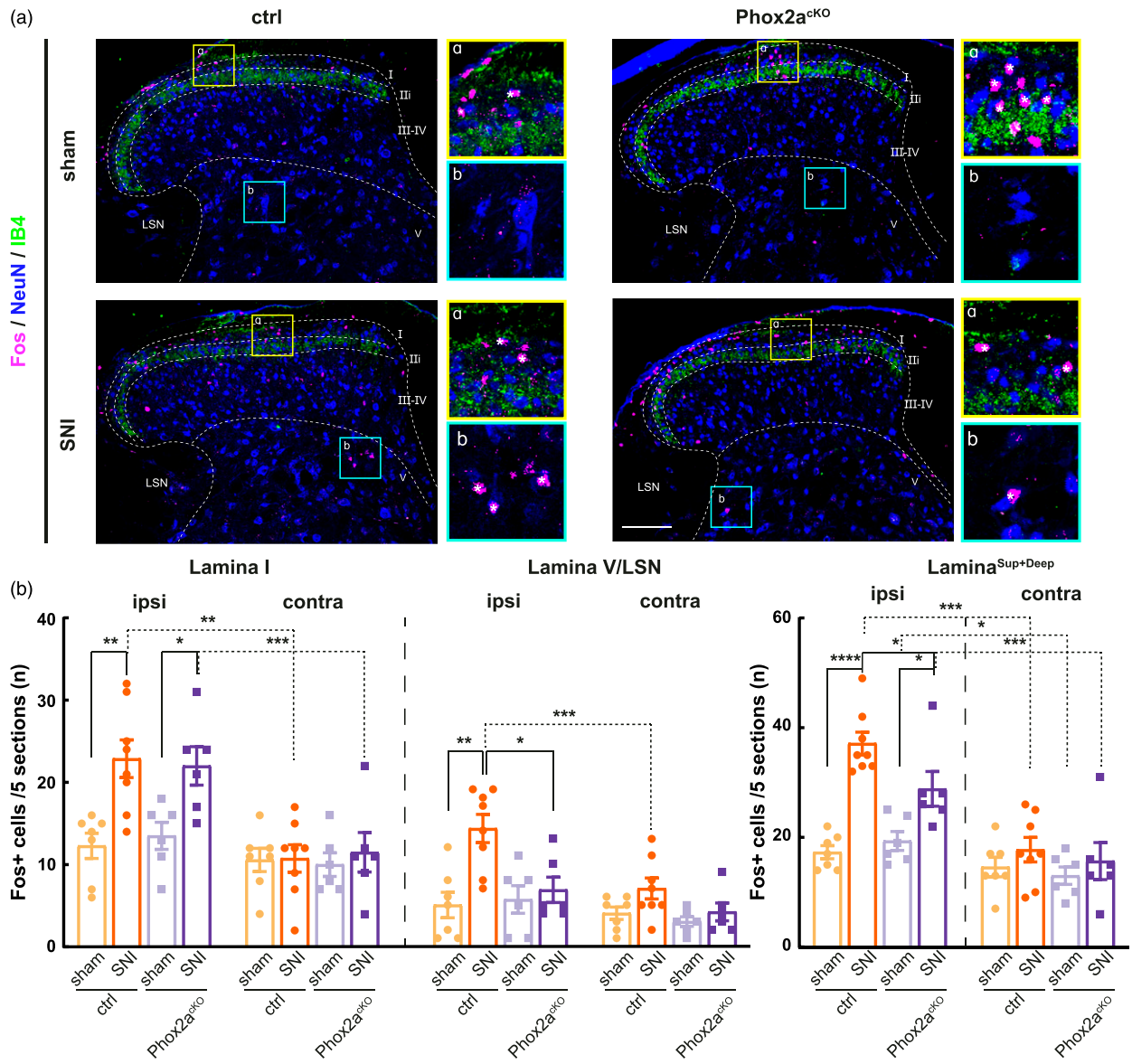

Figure 2. Attenuated ice-water induced Fos expression in SNI-treated Phox2a^{cKO} mice. (a), Representative spinal cord dorsal horn images of sham- or SNI-operated control (ctrl) or Phox2a^{cKO} animals 2 h post ice-water stimulation on the ipsilateral to the operation side. Fos (magenta) colocalizing with NeuN (blue) were quantified as Fos-expressing (Fos+). (b), Quantification and statistical analysis of Fos + cells. Summation of Fos + cells of 5 sections with 30 μm thickness is reported. Bars represent mean ± SEM with individual values as dots. Unpaired parametric t-test (solid line) for sham versus SNI within ctrl or Phox2a^{cKO} group. Paired parametric t-test (dotted line) for ipsi versus contra within SNI-treated ctrl or Phox2a^{cKO} group. Absence of comparison bars indicates a non-significant difference, *: $p < 0.05$, **: $p < 0.01$ or ***: $p < 0.001$. $n = 3$ for all groups. See Results for numerical data and statistical results. Scale bar same for all images is 100 μm.

dynamic brush (DB) test in which the hindpaw on the operated side is stimulated by repeated gentle brushing.[46] Compared to sham-operated animals, SNI mice exhibited a significantly lower withdrawal threshold (Figure 4(a) right, ctrl sham vs. SNI $p = 0.0078$). SNI also induced allodynia in Phox2a^{cKO} mice, when compared to sham-operated mice (Phox2a^{cKO} sham vs. SNI $p = 0.0048$). And similar to the vF results, there was no difference between the control and Phox2a^{cKO} groups received either treatment (sham ctrl vs. Phox2a^{cKO} $p = 0.7015$; SNI ctrl vs. Phox2a^{cKO} $p = 0.7265$). Together, these results demonstrate that Phox2a^{cKO} model of Phox2a AS neuron loss of function does not impair the induction of spinal-level allodynia by SNI.

Reduced SNI-induced cold but not mechanical hyperalgesia in Phox2a^{cKO} mice

One of the hallmarks of the SNI model of neuropathic pain is the development of hypersensitivity to cold stimuli, manifested by increased licking evoked by acetone or menthol

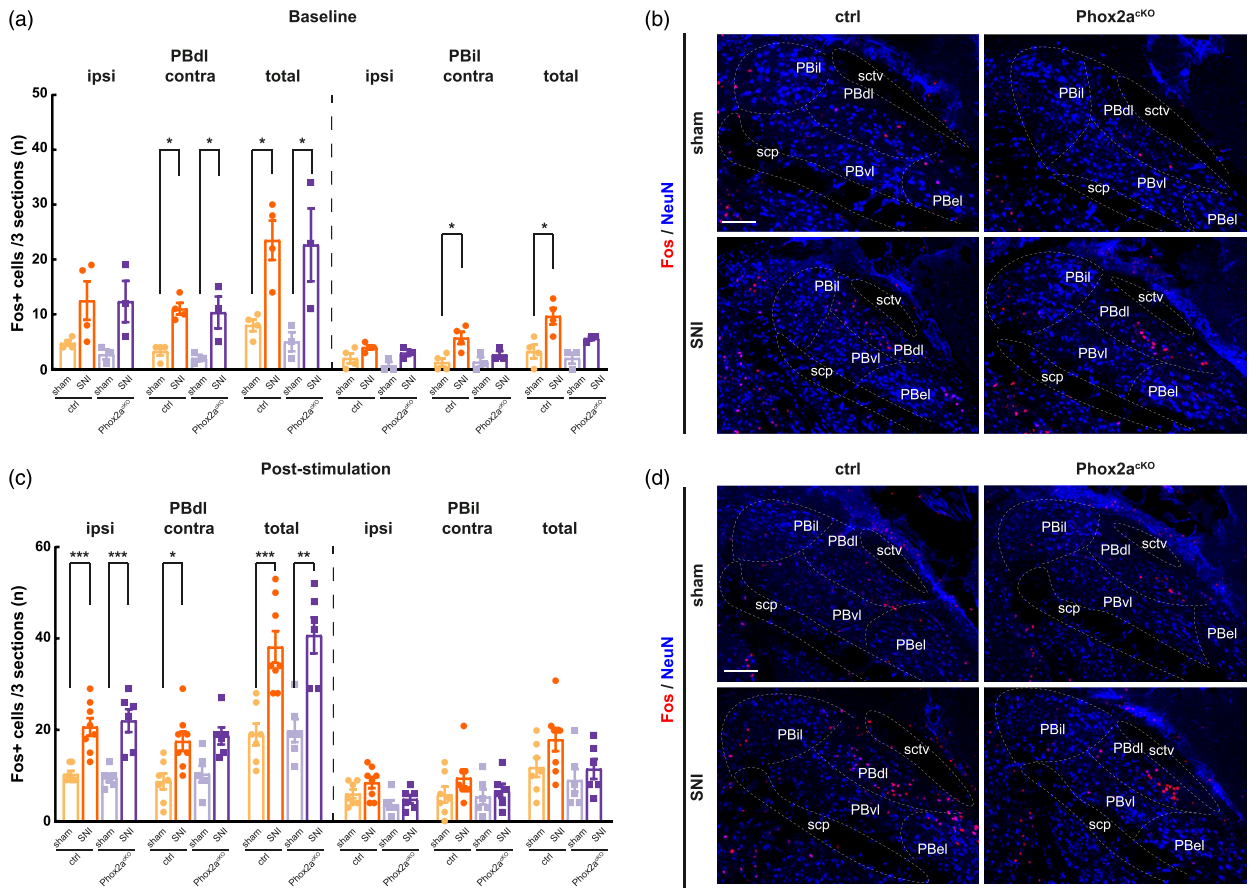

Figure 3. SNI-induction of Fos expression in the PBil of Phox2a^{cKO} and control mice. (a), Quantification and statistical analysis of Fos + cells in the PBN dorsal lateral (PBdl) and internal lateral (PBil) subnucleus, on the ipsilateral and contralateral side to the surgery, or as combined (total). Summary of 3 sections of 50 μm thickness is reported. $n = 4$ for control groups, and $n = 3$ for Phox2a^{cKO} groups. Unpaired parametric t-test for sham versus SNI within ctrl or Phox2a^{cKO} group. (b), Representative coronal section of the PBN of sham- or SNI-operated control (ctrl) or Phox2a^{cKO} animals. Fos (red) signal co-localizing with NeuN (blue) was quantified as Fos-expressing neurons (Fos+). (c,d), Same as (a,b) except that mice received ice-water stimulus to induce Fos expression. Bars represent mean ± SEM with individual values as dots. Unpaired parametric t-test (black line) for sham versus SNI within control or Phox2a^{cKO} groups. Paired 572 parametric t-test (brown line) for ipsi versus contra within SNI-treated control or Phox2a^{cKO} group. $n = 3$ for all groups. Absence of comparison bars indicates a non-significant difference, *: $p < 0.05$, **: $p < 0.01$ or ***: $p < 0.001$. See Results for numerical data and statistical results. Scale bar same for all images is 100 μm.

applied to the limb.[25,37] Since this behavior is a nocifensive response requiring the relay of sensory information from the spinal cord to supraspinal structures, we reasoned that the reduced spinofugal connectivity in Phox2a^{cKO} mice might limit the magnitude of SNI-induced allodynic response. We thus applied acetone to the ipsilateral hindlimb of SNI or sham surgery adult Phox2a^{cKO} and control mice and scored the total time spent licking the application site of 5 trials (Figure 4(b) left). Control SNI-operated mice showed significantly more paw licking following the application of acetone compared to sham-operated mice (ctrl sham vs. SNI $p = 0.0024$). In line with previously reported acetone test deficiency in Phox2a^{cKO} mice, we observed reduced licking time in sham-operated Phox2a^{cKO} mice compared to sham-operated controls, (sham ctrl vs. Phox2a^{cKO} $p = 0.0024$;).[24] However, in the Phox2a^{cKO} SNI and sham mice, no significant licking time difference was observed between sham- and SNI-operated groups (Phox2a^{cKO} sham vs. SNI $p = 0.4167$; SNI ctrl vs. Phox2a^{cKO} $p = 0.0467$, genotype effect $p = 0.0116$), suggesting that the allodynic response to cold was attenuated by the loss of Phox2a AS function.

Next, we asked whether the hyperalgesia to a mechanical stimulus commonly observed in SNI mice would also be impaired by a loss of Phox2a AS neuron function (Figure 4(b) right). To test this, we used the place escape/avoidance paradigm (PEAP) that assesses the extent to which noxious mechanical stimuli activate neuronal circuits concerned with affect and motivation.[30] This paradigm takes advantage of the innate preference of mice for a dark chamber over a bright one. Mice that perceive the mechanical stimulus of 9.804 mN (vF filament 4.08, 1 g) as noxious in a black

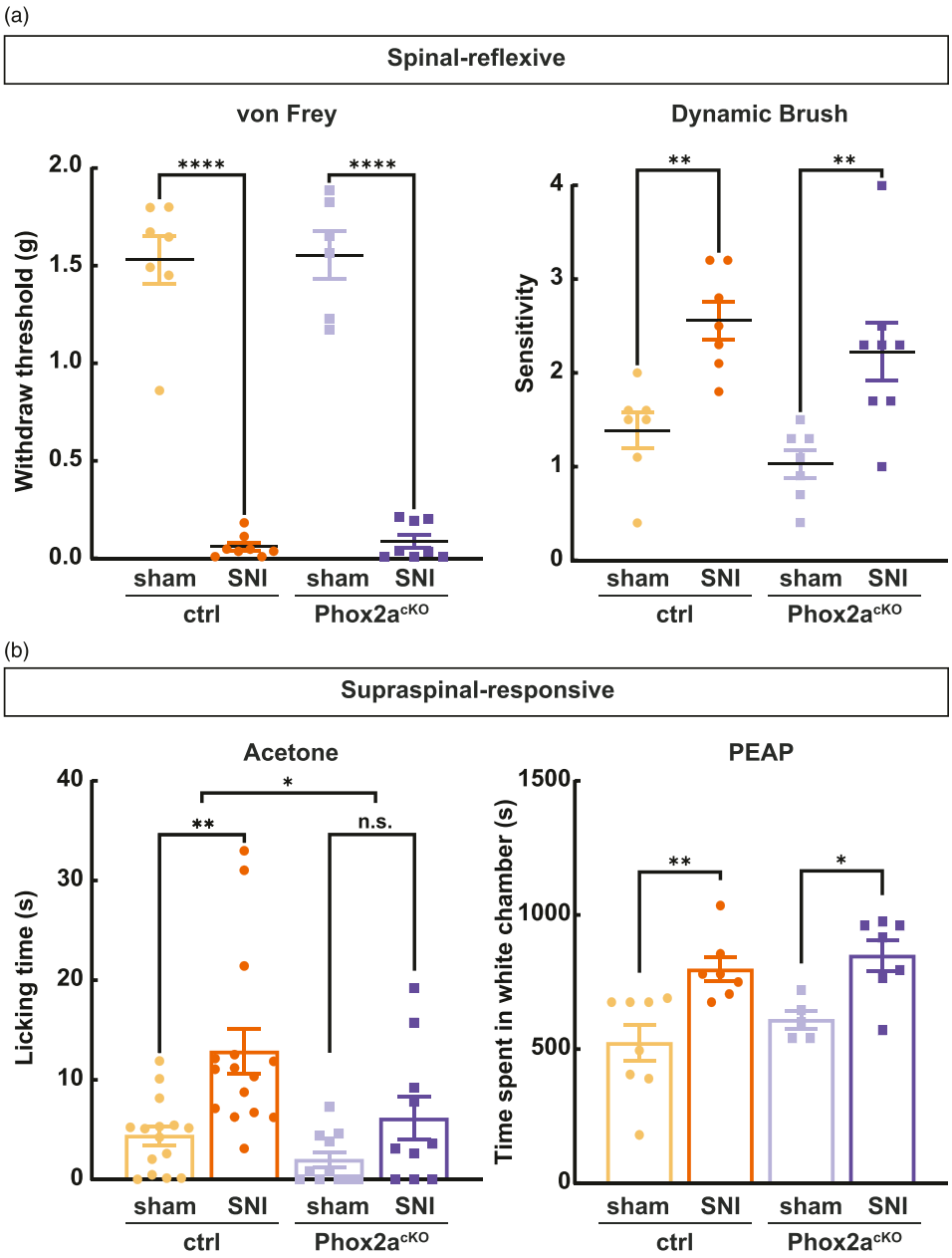

Figure 4. Phox2a^{cKO} mice develop spinal-level mechanical allodynia but their supraspinal licking response to cold allodynia is reduced. (a), Spinal-reflexive mechanical allodynic. 579 behavioral tests in control and Phox2a^{cKO} mice with sham or SNI treatment. Von Frey (vF) showing withdrawal thresholds to von Frey hairs in sham- (n = 7 ctrl; n = 6 Phox2a^{cKO}) and SNI-operated (n = 8 ctrl and Phox2a^{cKO}) mice, average of 3 trials on the hindpaw ipsilateral to the operated side. Dynamic brush (DB) showing response sensitivity to a paintbrush in sham- (n = 7 ctrl and Phox2a^{cKO}) and SNI-operated (n = 7 ctrl; n = 8 Phox2a^{cKO}) mice, average of 10 repeats on the hindpaw ipsilateral to the operated side. Lines represent mean ± SEM with individual values as dots. (b), Supraspinal responses to cold allodynia (acetone) and mechanical allodynia (PEAP). Acetone test showing the summation of licking duration after acetone application in 5 trials in sham- (n = 15 ctrl; n = 11 Phox2a^{cKO}) and SNI-operated (n = 15 ctrl; n = 10 Phox2a^{cKO}) mice. PEAP test showing sham- (n = 8 ctrl; n = 5 Phox2a^{cKO}) and SNI-operated (n = 7 ctrl and Phox2a^{cKO}) mice spending in the white chamber of the total 30-min test. Bars represent mean ± SEM with individual values as dots. Ordinary Two-way ANOVA with Tukey's multiple comparisons. n. s.: non-significant, *: $p < 0.05$, **: $p < 0.01$ or ***: $p < 0.001$, or ****: $p < 0.0001$. See Results for numerical data and statistical results.

chamber and no stimulus in a white chamber, spend more time in the white chamber indicating an avoidance of a noxious stimulus. This paradigm has been adapted to take advantage of the SNI-induced allodynia in which a stimulation with a normally innocuous thin vF filament is perceived as noxious (Figure 4(a)). In line with previous studies,[30,31] we observed that SNI-operated control mice preferentially chose the white chamber (ctrl sham vs.

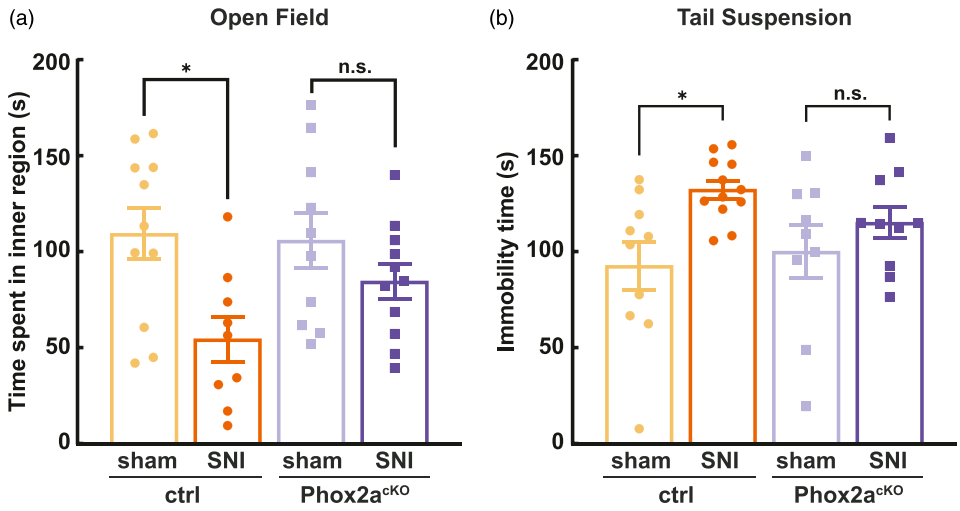

Figure 5. Impaired Phox2a AS neuron function attenuates SNI-induced anxio-depressive like behavior. (a), Open field test (OFT) showing time spent in the center region of the open chamber in sham- (n = 11 ctrl; n = 10 Phox2a^{cKO}) and SNI-operated (n = 9 ctrl; n = 11 Phox2a^{cKO}) mice during a 15-min test. (b), Tail suspension (TS) showing the total time mice were immobile during last 3 min (minute 5 to minute 8) of an 8-min test. Bars represent mean ± SEM with individual values as dots. Ordinary Two-way ANOVA with Tukey's multiple comparisons. *n.* s.: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, or ****: $p < 0.0001$. See Results for numerical data and statistical results.

SNI $p = 0.0071$). Similarly, the difference in time spent in white chamber SNI-operated Phox2a^{cKO} mice to sham-operated ones was statistically significant (Phox2a^{cKO} sham vs. SNI $p = 0.0461$; SNI ctrl vs. Phox2a^{cKO} $p = 0.9111$). Thus, Phox2a^{cKO}-mediated loss of Phox2a AS function impairs the supraspinal nocifensive responses to SNI-induced cold allodynia, but the PEAP response to mechanical allodynia. This implies that supraspinal projections of Phox2a AS neurons are important for the transfer of some of the SNI-induced maladaptive changes from the spinal cord to supraspinal nociceptive centers.

Attenuation of SNI-associated anxio-depressive-like behaviors by Phox2a AS loss of function

Modeling the depression and anxiety comorbidities that are common in chronic pain patients, SNI increases the frequency of anxio-depressive-like behaviors in male rodents, 2 weeks following the development of spinal-level allodynia.[5,38] The implied transfer of maladaptive plasticity from the spinal cord to the brain is presumably mediated by spinal projection neurons.[47] Thus, we asked whether the normal function of Phox2a AS neurons was required for the increased occurrence of anxio-depressive-like behaviors caused by SNI in rodents. One of these is the increased avoidance of the center of the open field test (OFT) arena and more time spent near the walls surrounding the arena, which is interpreted as anxiety-like increased aversion to brightly lit open spaces[4,39] Consistent with these studies, we observed that control SNI-operated mice spent significantly less time exploring the center of the OFT arena compared to sham-operated control mice (Figure 5(a), Ordinary Two-way ANOVA, Tukey's multiple comparison, and same for the subsequent statistics in Figure 5, ctrl sham vs. SNI $p = 0.0176$). In contrast, the time spent in the center of the OFT arena by SNI-operated Phox2a^{cKO} and sham-operated Phox2a^{cKO} mice did not differ (Phox2a^{cKO} sham vs. SNI $p = 0.6039$), although not significantly lower from the SNI-operated control group (SNI ctrl vs. Phox2a^{cKO} $p = 0.3326$). Moreover, the time spent in the center of the OFT arena by sham-treated control and Phox2a^{cKO} mice was not significantly different (sham ctrl vs. Phox2a^{cKO} $p = 0.9969$), suggesting that Phox2a^{cKO} does not result in an increased propensity to explore or decreased baseline anxiety-like behaviors.

To address the impact of Phox2a^{cKO} on the development of depressive-like behaviors caused by SNI, we used the tail suspension (TS) task (Figure 5(b)) in which mice are suspended by their tail and the time that they remain immobile is an indication of their loss of drive to right themselves.[34] SNI-treated male rodents exhibit significantly more TS task immobile time compared to sham treated animals, which has been interpreted as decreased motivation associated with a depression-like state.[39] Thus, in control groups, SNI-operated mice showed increased immobility compared to sham-operated males (ctrl sham vs. SNI $p = 0.0269$). On the contrary, in the Phox2a^{cKO} groups, SNI-operated mice do not show significant increase in immobility time in comparison to the sham operated group (Phox2a^{cKO} sham vs. SNI $p = 0.7247$), although not significantly lower than the SNI-operated control group (SNI ctrl vs. Phox2a^{cKO} $p = 0.5804$). Together, these behavioral experiments suggest that impaired Phox2a AS neuron function attenuates the development of SNI-induced anxio-depressive-like comorbidities.

Discussion

Previously, we demonstrated that the developmental expression of Phox2a identifies a major subpopulation of the AS, and its loss of function in Phox2a^{cKO} mice impairs acute nocifensive responses requiring the relay of nociceptive information to the brain.[24] Here we demonstrate that Phox2a AS neurons also participate in the development of maladaptive plastic changes associated with the SNI neuropathic pain model. Their normal function is required for supraspinal nocifensive responses evoked by allodynic and hyperalgesic stimuli, and the development of anxio-depression-related behaviors. Here, we discuss the extent to which Phox2a AS neurons may participate in central sensitization, at the level of spinal circuits or through supraspinal transmission of maladaptive plasticity, and the possibility that cold and mechanical allodynia recruit divergent ascending pathways.

Phox2a AS neurons function in the development of spinal maladaptive plastic changes

Previous AS neuron loss-of-function studies in the context of CNP took advantage of their NK1R expression. However, since NK1R is widely expressed by many spinal interneurons, the resulting manipulation disrupts the function of local spinal circuits that underlie reflexive withdraw responses, as well as the function of AS neurons in the transmission of nociceptive information from the spinal cord to the brain.[22,23] Here we used the Phox2a^{cKO} model, where the loss of Phox2a impairs the molecular specification of Phox2a AS neurons in Lamina I, V and the LSN, without any evidence of perturbed development of spinal interneurons. Moreover, while the spinoparabrachial connections of Lamina I AS neurons are relatively spared in Phox2a^{cKO} mice, those of Lamina V and the LSN AS neurons are disrupted. This is commensurate with the normal reflexive withdrawal in response to mechanical and thermal stimuli but disrupted supraspinal-driven behaviors in Phox2a^{cKO} mice.[24] The present data show that in Phox2a^{cKO} mice local spinal circuit function is also preserved under neuropathic pain conditions and argue against a broad impact of a developmental loss of Phox2a beyond AS neurons themselves. An alternative interpretation is that if Phox2a AS neurons participate extensively in the development of spinal-level maladaptive responses to SNI, for example by regulating interneuron activity, this effect does not require Phox2a expression during development and would imply a very focussed function of the Phox2a transcription factor. Phox2a^{cKO} mice have a normal induction of mechanical allodynia by SNI, suggesting that the normal function of Phox2a AS neurons is not required for the development of maladaptive changes within spinal circuits. We interpret the Fos expression in baseline and cold treatment experiments in the context of the behavioral observations: some metrics reveal that the loss of Phox2a attenuates the number of spinal neurons increasing their Fos expression in response to SNI. Although we cannot assess the number of Phox2a AS neurons activating Fos expression since Phox2a is no longer expressed in the adult spinal cord, previous work suggests that few AS activate Fos expression in response to noxious stimuli.[48] Thus, the decreased number of Fos-expressing neurons following SNI likely results from the impairment of Phox2a AS neuron functional integration into local neuronal networks, which would impede the SNI-evoked recruitment of spinal interneurons pre- or post-synaptic to AS neurons. However, in the context of SNI-induced activation of microglia, Phox2a^{cKO} did not markedly attenuate their induction of Iba1 compared to sham treatment. This coincides with the previous report that Phox2a AS neurons are not surrounded by perineural nets that interact with microglia to promote the development of plastic changes in chronic pain.[49]

Phox2a AS participate in the transmission of chronic pain effects from spinal cord to brain and the development of anxio-depressive-like behaviors

Phox2a AS neurons relay acute nociceptive information from the spinal cord to the brain.[24] Our data suggest that they are also required for the analogous transmission in a chronic pain model. The evidence for this is twofold: (1) behaviorally maladaptive sensitization and allodynia develop normally at the level of the spinal cord in Phox2a^{cKO} mice post-SNI induction, (2) increased licking in response to acetone and anxiety- and depressive-like behaviors induced by the SNI do not develop to their normal extent in mice with impaired Phox2a AS neuron function. Our behavioral analyses do not assess spontaneous pain induced by SNI by measuring weight bearing, paw grooming or analgesic consumption. However, our analyses do reveal that spinal-level hypersensitivity and allodynia develop normally in Phox2a^{cKO} mice, but their supraspinal relay is impaired. The idea that central sensitization at the level of spinal networks eventually leads to the central sensitization of its supraspinal targets is not new and studies in different models of CNP have demonstrated that second-order nociceptive spinal neurons, including interneuron and AS neurons with direct or indirect connections to brain regions, are subject to central sensitization.[47] The present experiments are the first manipulation restricted to spinal projection neurons that allows to tease away the contribution to central sensitization of AS neurons from that of interneurons. Our experiments argue that a molecularly-defined population of AS neurons, namely that expressing Phox2a in the developing spinal cord, plays an important role in the transmission of central sensitization, and imply that Phox2a AS neurons themselves are subject to maladaptive plasticity under neuropathic pain conditions. Our data also raise the question of the function in the transmission of chronic pain of other AS neuron populations such as those expressing *Gpr83* or *Tacr1* mRNA.[50,51] Given the estimates that Phox2a neurons comprise half of all AS neurons in Lamina I[52] and a third of those in Lamina V/LSN,[24] the

observed behavioral effects suggest that Phox2a AS neurons make a significant contribution to supraspinal transmission of pain chronicisation.

There are at least two complementary concepts related to the transmission of central sensitization by AS neurons: one is that it favors a particular ascending pathway to a supraspinal "hub", connected to different centers mediating CNP effects on emotion and motivation. Alternatively, there are several parallel pathways to these brain regions obviating the requirement for a hub.[53] Which model is consistent with the effects that we observe in Phox2a^{cKO} mice? Concomitant with the attenuated anxiety- and depressive-like behaviors in Phox2a^{cKO} mice was an SNI-associated Fos induction in the parabrachial nucleus, one of the principal supraspinal targets of Phox2a AS neurons. The magnitude of this change was similar between controls and Phox2a^{cKO} mice and is somewhat surprising given that Phox2a^{cKO} mice exhibit a strong decrease of Lamina V/LSN spinoparabrachial connections. Thus, the Phox2a^{cKO} model uncouples Fos induction in the PB from the development of anxio-depressive behaviors. It would argue against the idea that SNI is predominantly sensitizing a PBN hub, which then promotes anxio-depressive-like behaviors through other brain regions connected to it.

Cold and mechanical allodynia may take divergent pathways in CNP

Interestingly, we observed a possible discrepancy between mechanical and cold sensitization induced by SNI. Although Phox2a^{cKO} mice had reduced supraspinal licking responses in the acetone test in SNI-treated mice, the response to mechanical stimulus of SNI-treated Phox2a^{cKO} mice in the PEAP test was not significantly attenuated in Phox2a^{cKO} mice. This could be explained by two possibilities. The first one is that thermal hypersensitivity and mechanical allodynia induction by SNI occur through distinct spinofugal pathways, with the former being disrupted by Phox2a^{cKO} while the latter being minimally affected by Phox2a^{cKO}.[54,55] This raises the question of whether molecularly-distinct AS neuron populations are devoted to specific pain modalities or whether neuronal population coding supersedes molecular differences.[51] Mechanical allodynia may involve the Aβ - low threshold mechanoreceptor - dorsal column nuclei pathway, but the role of this pathway in cold allodynia has not been demonstrated.[56,57]

Alternatively, the differential effect of Phox2a^{cKO} on thermal versus mechanical sensation could also reflect a diversity of brain regions that evoke the different coping behaviors. Although both cold-evoked licking and avoidance of a chamber with a mechanical stimulus are driven by the affective-motivational dimension of nociception, the former may primarily rely on the recruitment of motor control and limb-mouth coordination circuits, while PEAP avoidance could engage prefrontal cortex decision making circuits.[58]

Summing up, our genetic manipulation strongly argues that Phox2a AS neurons play an important role in transmitting chronic pain-associated maladaptive plasticity from the spinal cord to the brain, which drives the development of anxio-depressive comorbidities. Certainly, our study bears the standard caveats associated with developmental loss of function, but it is the first approach to specifically test the role of AS neurons in chronic pain, without directly interfering with spinal interneuron function. Given that a disruption of AS axons provides at least temporary relief from intractable chronic pain in humans, a reversible and molecularly-precise inactivation of human Phox2a AS neurons could be a more refined therapeutic avenue.

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Author contribution

All authors have contributed to conceptualisation of the experiments and manuscript writing.

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Data availability

All data and reagents are available upon request to lead contact.

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References

1. Costigan M, Scholz J, Woolf CJ. Neuropathic Pain: A Maladaptive Response of the Nervous System to Damage. *Annu Rev Neurosci* 2009; 32: 1–32. DOI: 10.1146/annurev.neuro.051508.135531

2. Finnerup NB, Kuner R, Jensen TS. Neuropathic Pain: From Mechanisms to Treatment. *Physiol Rev* 2021; 101: 259–301. DOI: 10.1152/physrev.00045.2019
3. Kuner R, Kuner T. Cellular Circuits in the Brain and Their Modulation in Acute and Chronic Pain. *Physiol Rev* 2021; 101: 213–258. DOI: 10.1152/physrev.00040.2019
4. De Gregorio D, McLaughlin RJ, Posa L, Ochoa-Sanchez R, Enns J, Lopez-Canul M, Aboud M, Maione S, Comai S, Gobbi G. Cannabidiol modulates serotonergic transmission and reverses both allodynia and anxiety-like behavior in a model of neuropathic pain. *Pain* 2019; 160: 136–150. DOI: 10.1097/j.pain.0000000000001386
5. McWilliams LA, Goodwin RD, Cox BJ. Depression and anxiety associated with three pain conditions: results from a nationally representative sample. *Pain* 2004; 111: 77–83. DOI: 10.1016/j.pain.2004.06.002
6. Woolf CJ. Pain amplification—A perspective on the how, why, when, and where of central sensitization. *J Appl Biobehav Res* 2018; 23: e12124. DOI: 10.1111/jabr.12124
7. Woolf CJ, Salter MW. Neuronal Plasticity: Increasing the Gain in Pain. *Science* 2000; 288: 1765–1769. DOI: 10.1126/science.288.5472.1765
8. Boyle KA, Gradwell MA, Yasaka T, Dickie AC, Polgár E, Ganley RP, Orr DPH, Watanabe M, Abraira VE, Kuehn ED, Zimmerman AL, Ginty DD, Callister RJ, Graham BA, Hughes DI. Defining a Spinal Microcircuit that Gates Myelinated Afferent Input: Implications for Tactile Allodynia. *Cell Rep* 2019; 28: 526–540.e6. DOI: 10.1016/j.celrep.2019.06.040
9. Woolf CJ, Shortland P, Coggeshall RE. Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 1992; 355: 75–78. DOI: 10.1038/355075a0
10. Yalcin I, Barthas F, Barrot M. Emotional consequences of neuropathic pain: Insight from preclinical studies. *Neurosci Biobehav Rev* 2014; 47: 154–164. DOI: 10.1016/j.neubiorev.2014.08.002
11. Lançon K, Qu C, Navratilova E, Séguéla P. Decreased dopaminergic inhibition of pyramidal neurons in anterior cingulate cortex maintains chronic neuropathic pain. *Cell Rep* 2021; 37: 109933. DOI: 10.1016/j.celrep.2021.109933
12. Huang J, Gadotti VM, Chen L, Souza IA, Huang S, Wang D, Ramakrishnan C, Deisseroth K, Zhang Z, Zamponi GW. A neuronal circuit for activating descending modulation of neuropathic pain. *Nat Neurosci* 2019; 22: 1659–1668. DOI: 10.1038/s41593-019-0481-5
13. Liu Y, Latremoliere A, Li X, Zhang Z, Chen M, Wang X, Fang C, Zhu J, Alexandre C, Gao Z, Chen B, Ding X, Zhou J-Y, Zhang Y, Chen C, Wang KH, Woolf CJ, He Z. Touch and tactile neuropathic pain sensitivity are set by corticospinal projections. *Nature* 2018; 561: 547–550. DOI: 10.1038/s41586-018-0515-2
14. Melzack R, Casey KL. Sensory, motivational, and central control determinants of pain: A New Conceptual Model. *The skin senses*. Illinois, USA: Springfield, 1968, pp. 423–439.
15. Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and Molecular Mechanisms of Pain. *Cell* 2009; 139: 267–284. DOI: 10.1016/j.cell.2009.09.028
16. Todd AJ. Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci* 2010; 11: 823–836. DOI: 10.1038/nrn2947
17. Spiller WG, Martin E. The treatment of persistent pain of organic origin in the lower part of the body by division of the anterolateral column of the spinal cord. *Journal of the American Medical Association* 1912; LVIII: 1489–1490. DOI: 10.1001/jama.1912.04260050165001
18. Burchiel KJ, Raslan AM. Contemporary concepts of pain surgery. *J Neurosurg* 2019; 130: 1039–1049. DOI: 10.3171/2019.1.jns181620
19. Andrew D. Sensitization of lamina I spinoparabrachial neurons parallels heat hyperalgesia in the chronic constriction injury model of neuropathic pain. *J Physiol* 2009; 587: 2005–2017. DOI: 10.1113/jphysiol.2009.170290
20. Ikeda H, Heinke B, Ruscheweyh R, Sandkühler J. Synaptic Plasticity in Spinal Lamina I Projection Neurons That Mediate Hyperalgesia. *Science* 2003; 299: 1237–1240. DOI: 10.1126/science.1080659
21. Palecek J, Paleckova V, Dougherty PM, Carlton SM, Willis WD. Responses of spinothalamic tract cells to mechanical and thermal stimulation of skin in rats with experimental peripheral neuropathy. *J Neurophysiol* 1992; 67: 1562–1573. DOI: 10.1152/jn.1992.67.6.1562
22. Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA. Inhibition of Hyperalgesia by Ablation of Lamina I Spinal Neurons Expressing the Substance P Receptor. *Science* 1997; 278: 275–279. DOI: 10.1126/science.278.5336.275
23. Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, Finke MP, Li J, Lappi DA, Simone DA, Mantyh PW. Transmission of Chronic Nociception by Spinal Neurons Expressing the Substance P Receptor. *Science* 1999; 286: 1558–1561. DOI: 10.1126/science.286.5444.1558
24. Roome RB, Bourojeni FB, Mona B, Rastegar-Pouyani S, Blain R, Dumouchel A, Salesse C, Thompson WS, Brookbank M, Gitton Y, Tessarollo L, Goulding M, Johnson JE, Kmita M, Chédotal A, Kania A. Phox2a Defines a Developmental Origin of the Anterolateral System in Mice and Humans. *Cell Rep* 2020; 33: 108425. DOI: 10.1016/j.celrep.2020.108425
25. Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 2000; 87: 149–158. DOI: 10.1016/s0304-3959(00)00276-1
26. Bourquin A-F, Süveges M, Pertin M, Gilliard N, Sardy S, Davison AC, Spahn DR, Decosterd I. Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse. *Pain* 2006; 122: 14e1–14e14. DOI: 10.1016/j.pain.2005.10.036
27. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53: 55–63. DOI: 10.1016/0165-0270(94)90144-9
28. Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Chung JM, Devor M. Heritability of nociception I:

Responses of 11 inbred mouse strains on 12 measures of nociception. *Pain* 1999; 80: 67–82. DOI: 10.1016/s0304-3959(98)00197-3
29. Colburn RW, Lubin ML, Stone DJ, Wang Y, Lawrence D, D'Andrea MR, Brandt MR, Liu Y, Flores CM, Qin N. Attenuated Cold Sensitivity in TRPM8 Null Mice. *Neuron* 2007; 54: 379–386. DOI: 10.1016/j.neuron.2007.04.017
30. LaBuda CJ, Fuchs PN. A Behavioral Test Paradigm to Measure the Aversive Quality of Inflammatory and Neuropathic Pain in Rats. *Exp Neurol* 2000; 163: 490–494. DOI: 10.1006/exnr.2000.7395
31. Millecamps M, Shi XQ, Piltonen M, Echeverry S, Diatchenko L, Zhang J, Stone LS. The geriatric pain experience in mice: intact cutaneous thresholds but altered responses to tonic and chronic pain. *Neurobiol Aging* 2020; 89: 1–11. DOI: 10.1016/j.neurobiolaging.2019.12.018
32. Walsh RN, Cummins RA. The open-field test: A critical review. *Psychol Bull* 1976; 83: 482–504. DOI: 10.1037/0033-2909.83.3.482
33. Patel TP, Gullotti DM, Hernandez P, O'Brien WT, Capehart BP, Morrison B III, Bass C, Eberwine JE, Abel T, Meaney DF. An open-source toolbox for automated phenotyping of mice in behavioral tasks. *Front Behav Neurosci* 2014; 8: 349. DOI: 10.3389/fnbeh.2014.00349
34. Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: A new method for screening antidepressants in mice. *Psychopharmacology* 1985; 85: 367–370. DOI: 10.1007/bf00428203
35. Rexed B. The cytoarchitectonic organization of the spinal cord in the cat. *J Comp Neurol* 1952; 96: 415–495. DOI: 10.1002/cne.900960303
36. Karthik S, Huang D, Delgado Y, Laing JJ, Peltekian L, Iverson GN, Grady F, Miller RL, McCann CM, Fritzsch B, Iskusnykh IY, Chizhikov VV, Geerling JC. Molecular ontology of the parabrachial nucleus. *J Comp Neurol* 2022; 530: 1658–1699. DOI: 10.1002/cne.25307
37. Shields SD, Eckert WA, Basbaum AI. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. *J Pain* 2003; 4: 465–470. DOI: 10.1067/s1526-5900(03)00781-8
38. Michailidis V, Lidhar NK, Cho C, Martin LJ. Characterizing Sex Differences in Depressive-Like Behavior and Glial Brain Cell Changes Following Peripheral Nerve Injury in Mice. *Front Behav Neurosci* 2021; 15: 758251. DOI: 10.3389/fnbeh.2021.758251
39. Guida F, De Gregorio D, Palazzo E, Ricciardi F, Boccella S, Belardo C, Iannotta M, Infantino R, Formato F, Marabese I, Luongo L, de Novellis V, Maione S. Behavioral, Biochemical and Electrophysiological Changes in Spared Nerve Injury Model of Neuropathic Pain. *Int J Mol Sci* 2020; 21: 3396. DOI: 10.3390/ijms21093396
40. Bullitt E. Expression of C-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. *J Comp Neurol* 1990; 296: 517–530. DOI: 10.1002/cne.902960402
41. Intondi AB, Dahlgren MN, Eilers MA, Taylor BK. Intrathecal neuropeptide Y reduces behavioral and molecular markers of inflammatory or neuropathic pain. *Pain* 2008; 137: 352–365. DOI: 10.1016/j.pain.2007.09.016
42. Beggs S, Trang T, Salter MW. P2X4R+ microglia drive neuropathic pain. *Nat Neurosci* 2012; 15: 1068–1073. DOI: 10.1038/nn.3155
43. Abbadie C, Honoré P, Besson JM. Intense cold noxious stimulation of the rat hindpaw induces c-fos expression in lumbar spinal cord neurons. *Neuroscience* 1994; 59: 457–468. DOI: 10.1016/0306-4522(94)90609-2
44. Feil K, Herbert H. Topographic organization of spinal and trigeminal somatosensory pathways to the rat parabrachial and Kölliker-Fuse nuclei. *J Comp Neurol* 1995; 353: 506–528. DOI: 10.1002/cne.903530404
45. Bernard JF, Dallel R, Raboisson P, Villanueva L, Le Bars D. Organization of the efferent projections from the spinal cervical enlargement to the parabrachial area and periaqueductal gray: a PHA-L study in the rat. *J Comp Neurol* 1995; 353: 480–505. DOI: 10.1002/cne.903530403
46. Cheng L, Duan B, Huang T, Zhang Y, Chen Y, Britz O, Garcia-Campmany L, Ren X, Vong L, Lowell BB, Goulding M, Wang Y, Ma Q. Identification of spinal circuits involved in touch-evoked dynamic mechanical pain. *Nat Neurosci* 2017; 20: 804–814. DOI: 10.1038/nn.4549
47. Boadas-Vaello P, Castany S, Homs J, Homs J, Álvarez-Pérez B, Deulofeu M, Verdú E. Neuroplasticity of ascending and descending pathways after somatosensory system injury: reviewing knowledge to identify neuropathic pain therapeutic targets. *Spinal Cord* 2016; 54: 330–340. DOI: 10.1038/sc.2015.225
48. Menétrey D, Gannon A, Levine JD, Basbaum AI. Expression of c-fos protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular, and visceral stimulation. *J Comp Neurol* 1989; 285: 177–195. DOI: 10.1002/cne.902850203
49. Tansley S, Gu N, Guzmán AU, Cai W, Wong C, Lister K, Muñoz-Pino E, Yousefpour N, Roome RB, Heal J, Wu N, Castonguay A, Lean G, Muir EM, Kania A, Prager-Khoutorsky M, Zhang J, Gkogkas CG, Fawcett JW, Diatchenko L, Ribeiro-da-Silva A, De Koninck Y, Mogil JS, Khoutorsky A. Microglia-mediated degradation of perineuronal nets promotes pain. *Science* 2022; 377: 80–86. DOI: 10.1126/science.abl6773
50. Choi S, Hachisuka J, Brett MA, Magee A, Omori Y, Iqbal N-u-A, Zhang D, DeLisle MM, Wolfson RL, Bai L, Santiago C, Gong S, Goulding M, Heintz N, Koerber HR, Ross SE, Ginty DD. Parallel ascending spinal pathways for affective touch and pain. *Nature* 2020; 587: 258–263. DOI: 10.1038/s41586-020-2860-1
51. Wercberger R, Braz JM, Weinrich JA, Basbaum AI. Pain and itch processing by subpopulations of molecularly diverse spinal and trigeminal projection neurons. *Proc Natl Acad Sci U S A* 2021; 118: e2105732118. DOI: 10.1073/pnas.2105732118
52. Alsulaiman Wafa AA, Quillet Raphaelle, Bell Andrew M, Dickie Allen C, Polgár Erika, Boyle Kieran A, Watanabe Masahiko, Roome R Brian, Kania Artur, Todd Andrew J,

and Gutierrez-Mecinas Maria. Characterisation of lamina I anterolateral system neurons that express Cre in a Phox2a-Cre mouse line. *Sci Rep* 2021; 11(1): 17912, doi:10.1038/s41598-021-97105-w, 34504158.

53. Chiang MC, Bowen A, Schier LA, Tupone D, Uddin O, Heinricher MM. Parabrachial Complex: A Hub for Pain and Aversion. *J Neurosci* 2019; 39: 8225–8230. DOI: 10.1523/jneurosci.1162-19.2019
54. Löken LS, Braz JM, Etlin A, Sadeghi M, Bernstein M, Jewell M, Steyert M, Kuhn J, Hamel K, Llewellyn-Smith IJ, Basbaum A. Contribution of dorsal horn CGRP-expressing interneurons to mechanical sensitivity. *eLife* 2021; 10: e59751. DOI: 10.7554/elife.59751
55. Otsubo Y, Satoh Y, Kodama M, Araki Y, Satomoto M, Sakamoto E, Pagès G, Pouysségur J, Endo S, Kazama T. Mechanical allodynia but not thermal hyperalgesia is impaired in mice deficient for ERK2 in the central nervous system. *Pain* 2012; 153: 2241–2252. DOI: 10.1016/j.pain.2012.07.020
56. Polgár E, Hughes DI, Arham AZ, Todd AJ. Loss of Neurons from Laminas I-III of the Spinal Dorsal Horn Is Not Required for Development of Tactile Allodynia in the Spared Nerve Injury Model of Neuropathic Pain. *J Neurosci* 2005; 25: 6658–6666. DOI: 10.1523/jneurosci.1490-05.2005
57. Houghton AK, Hewitt E, Westlund KN. Dorsal column lesion prevents mechanical hyperalgesia and allodynia in osteotomy model. *Pain* 1999; 82: 73–80. DOI: 10.1016/s0304-3959(99)00034-2
58. Ma Q. A functional subdivision within the somatosensory system and its implications for pain research. *Neuron* 2022; 110: 749–769. DOI: 10.1016/j.neuron.2021.12.015