

Spared nerve injury differentially alters parabrachial monosynaptic excitatory inputs to molecularly specific neurons in distinct subregions of the central amygdala

Jun-Nan Li^{a,b}, Patrick L. Sheets^{a,b,*}

Abstract

Dissecting the organization of circuit pathways involved in pain affect is pivotal for understanding behavior associated with noxious sensory inputs. The central nucleus of the amygdala (CeA) comprises distinct populations of inhibitory GABAergic neurons expressing a wide range of molecular markers. CeA circuits are associated with aversive learning and nociceptive responses. The CeA receives nociceptive signals directly from the parabrachial nucleus (PBn), contributing to the affective and emotional aspects of pain. Although the CeA has emerged as an important node in pain processing, key questions remain regarding the specific targeting of PBn inputs to different CeA subregions and cell types. We used a multifaceted approach involving transgenic reporter mice, viral vector-mediated optogenetics, and brain slice electrophysiology to delineate cell-type-specific functional organization of the PBn–CeA pathway. Whole-cell patch clamp recordings of molecularly defined CeA neurons while optogenetically driving long-range inputs originating from PBn revealed the direct monosynaptic excitatory inputs from PBn neurons to 3 major subdivisions of the CeA: laterocapsular (CeC), lateral (CeL), and medial (CeM). Direct monosynaptic excitatory inputs from PBn targeted both somatostatin-expressing (SOM+) and corticotropin-releasing hormone expressing (CRH+) neurons in the CeA. We find that monosynaptic PBn input is preferentially organized to molecularly specific neurons in distinct subdivisions of the CeA. The spared nerve injury model of neuropathic pain differentially altered PBn monosynaptic excitatory input to CeA neurons based on molecular identity and topographical location within the CeA. These results provide insight into the functional organization of affective pain pathways and how they are altered by chronic pain.

Keywords: Parabrachial nucleus, Amygdala, Pain, Somatostatin, Corticotropin-releasing hormone

1. Introduction

Pain is an unpleasant and multidimensional experience that involves sensorimotor, emotional-affective, and cognitive components.^{52,55} Somatosensory pain is protective by alerting injury and/or threat.⁴ However, pathological pain commonly leads to affective disorders and cognitive impairment.^{77,78} Despite progress in understanding sensory dimensions of pain, mechanisms underlying pain affect, while emerging, remain unresolved. Dissecting circuits modulating pain affect is pivotal for understanding the functional organization of neural networks

mediating pain and for facilitating novel therapeutic strategies for patients with chronic pain.¹⁹

Amygdala dysfunction is implicated in many disorders including addiction, autism, and anxiety disorders,^{17,28,31,37} but it has also emerged as an important focus in pain research.^{55,58} The amygdala contains anatomically and functionally distinct nuclei: the basolateral complex, which includes the lateral (LA), basolateral, and basomedial nuclei and the central nucleus (CeA), which can be subdivided into the laterocapsular (CeC), lateral (CeL), and medial (CeM) subregions.^{27,46,62} Sensory information reaches the amygdala mainly through the LA, whereas the CeA houses major output pathways for amygdala function.^{46,62} The CeA is termed the “nociceptive amygdala” as it receives noxious sensory information from the spinal cord and brain stem through the parabrachial nucleus (PBn).^{6,9,29,30,39,55,66,67,72} Inputs from lateral PBn targeting the CeA are primarily excitatory and monosynaptic.^{56,71} Novel work has also shown that the PBn–CeA pathway transmits aversive signals important for threat and avoidance memory.^{16,33,68} However, the organization of PBn inputs to specific neural populations in different CeA subdivisions remains unresolved.

In the BLA, a majority (≈80%) of neurons are glutamatergic, and a minority (≈20%) of neurons are GABAergic.^{51,70} Conversely, the CeA houses distinct GABAergic neuronal populations expressing a wide range of molecular markers, including corticotropin-releasing hormone (CRH), somatostatin (SOM), protein kinase C- δ (PKC- δ), and neurotensin (Nts).^{27,43,50} Studies

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^a Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN, United States, ^b Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN, United States

*Corresponding author. Address: Indiana University School of Medicine, Neuroscience Research Building 400D, 320 West, 15th St, Indianapolis, IN 46202, United States. Tel.: 317-278-6383. E-mail address: plsheets@iupui.edu (P.L. Sheets).

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show that CeL-SOM+ neurons are crucial for expression of conditioned fear^{47,60} and that CeA-CRH+ neurons are involved in anxiety-like behavior and fear learning.^{59,65} Later work suggests that SOM+ and CRH+ neurons in the CeL mediate appetitive, but not defensive behaviors.⁴³ However, organization of PB inputs to these cell types, both in naive and chronic pain animals, remains unclear but is crucial for understanding the transition of noxious input to behavior.

Here, we used viral vector-mediated optogenetics and 2 transgenic Cre reporter mouse lines to selectively probe PBn inputs onto SOM+ and CRH+ neurons in distinct CeA subregions using whole-cell electrophysiological recordings in slice. These experiments expand on the notion that PBn input to the CeA is topographically organized^{6,9,29,39,66} and test the hypothesis that PBn input differentially targets CeA neurons based on molecular identity. We next tested neuropathic pain-induced alterations to plasticity of PBn–CeA inputs using the spared nerve injury (SNI) model. Results from our study provide insight into mechanisms of how ascending noxious inputs target specific CeA cell types implicated in various pain-related behaviors.

2. Methods

2.1. Ethical approval

The Institutional Animal Use and Care Committee of the Indiana University School of Medicine approved all procedures and experiments presented in this study. Animals were used in accordance with the animal care and use guidelines of Indiana University, the National Institutes of Health, and the Society for Neuroscience.

2.2. Animals

The heterozygous SOM-IRES-Cre; Ai14 mice were generated by mating female homozygous SOM-IRES-Cre (Jackson laboratory stock no. 013044) mice with male homozygous Ai14 (C57BL/6J-congenic version, Allen Institute line Ai14, Jackson Labs no. 007914) mice. The heterozygous CRH-ires-Cre; Ai14 mice were generated by mating female homozygous CRH-ires-Cre (Jackson laboratory stock no. 012704) mice with male homozygous Ai14 (C57BL/6J-congenic version, Allen Institute line Ai14, Jackson Labs no. 007914) mice. The heterozygous SOM-IRES-Cre; Ai14 or CRH-ires-Cre; Ai14 mice of both sexes were used in all experiments. Mice were housed on a 12:12-hour light:dark schedule (lights on at 7:00) with ad libitum access to food and water.

2.3. Intracranial injection of pACAGW-ChR2-Venus-AAV into the lateral PBn

Mice were anesthetized with 1.5% isoflurane in 100% O₂ with a flow rate of 0.8 L/minute (SurgiVet Isotech 4; Smith). The top of the head was shaved. The head was stabilized in a stereotaxic frame (900 series; Kopf instruments, Tujunga, CA). Betadine and ethanol were applied in alternating fashion 3 to 4 times to disinfect the shaved area. Body temperature was maintained at 37°C using a feedback-controlled heating pad (FHC). For PBn injection, the scalp was incised, a craniotomy was made, the dura was reflected, and pipettes were advanced to reach the stereotaxic coordinates of the desired target. The pipette was advanced to the intracranial target, and a submicroliter volume (100 nL) of pACAGW-ChR2-Venus-AAV (20071-AAV1; Addgene,

Watertown, MA) was injected at a rate of 25 nL/minutes using a Hamilton syringe connected to an UltraMicoPump 3 driven by a Micro 4 MicroSyringe Pump Controller (World Precision Instruments, Sarasota, FL). The pipette was kept in place for 5 to 7 minutes to limit virus reflux out of the injection site. The incision was closed with tissue adhesive (Vetbond). After surgery, meloxicam (5 mg/kg) was injected subcutaneously for pain relief during recovery. Stereotaxic details for PBn injections are as follows: The head was fixed at a 38° down angle, and coordinates were (relative to lambda) 4.42 mm caudal, 1.15 mm lateral, and 3.0 mm deep at a 54° angle off the horizontal plane. Targeting of pACAGW-ChR2-Venus-AAV into the lateral PBn was verified using a fluorescence stereo microscope (Leica M165 FC, W. Nuhsbaum Inc., McHenry, IL) to image slices of the PBn. Animals were allowed at least 14 days of recovery and adequate anterograde ChR2-YFP expression before slice electrophysiology experiments.

2.4. Spared nerve injury model

Spared nerve injury surgeries were performed based on published protocols from previous studies.^{12,18,22,53} Briefly, mice were anesthetized with 1.5% isoflurane in 100% O₂ with a flow rate of 0.8 L/minute (SurgiVet Isotech 4; Smith). After shaving the left hind leg area, an incision was made in the skin overlying the area where the sciatic nerve branches into 3 peripheral nerves (common peroneal, tibial, and sural). The overlying muscles were spread apart to obtain access to the trifurcation of the sciatic nerve. Once exposed, the common peroneal and the tibial branches of the sciatic nerve were ligated (silk 6-0) and cut, leaving only the sural nerve intact. The wound was closed using tissue adhesive (Vetbond).

2.5. Assessment of pain behavior

Mice were acclimatized to the pain testing behavior apparatus, behavioral suite, and experimenter before the von Frey filament paw withdrawal threshold was established. Acclimatization entailed placing the mouse inside a clear 6-inch vertical plastic tube (4-inch internal diameter) on top of a wire mesh platform (exposing the hind paws for testing). Mice were acclimatized for 2 nonconsecutive days for 30 minutes each day before recording baseline withdrawal thresholds. Baseline withdrawal threshold (for both hind paws) was established before the SNI surgery by following the “simplified up-down” or SUDO method.¹⁰ On postoperative day 10 (POD-10), the SUDO method was used to assess mechanical allodynia, both ipsilateral and contralateral to the injury. Experimenters were blinded to treatment groups (sham vs SNI) during behavioral testing. Using standard von Frey filaments 2 to 9 (filament 1: 0.008 g; filament 2: 0.02 g; filament 3: 0.07 g; filament 4: 0.16 g; filament 5: 0.4 g; filament 6: 1 g; filament 7: 2 g; and filament 8: 6 g) testing began with the middle filament (filament 4). The pressure from the filament was applied to the lateral aspect of the hind paw for 3 seconds, and behavior responses such as hind paw retraction, paw licking, or shaking were considered as nocifensive behavior and classified as a pain response.⁴⁹ If the applied filament did not elicit a response, the next highest filament was used, while if a response was elicited, the next lowest filament was used until the fifth and final filament was presented. This method minimized the number of filament presentations to the mouse and maximized score sensitivity. On the day of slice experiments, a final withdrawal testing was performed, with the mouse being killed immediately thereafter.

2.6. Acute brain slice preparation

After brief anesthetization by isoflurane, injected mice were decapitated and brains were rapidly extracted (<1 minute) and placed in ice-chilled cutting solution (in mM: 110 choline chloride, 25 NaHCO₃ (sodium bicarbonate), 25 D-glucose, 11.6 sodium ascorbate, 7 MgSO₄ (magnesium sulfate), 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH₂PO₄, and 0.5 CaCl₂). Coronal slices (300 μm) containing the amygdala were prepared by vibratome (VT1200S; Leica) and transferred to artificial cerebrospinal solution (ACSF, in mM: 127 NaCl, 25 NaHCO₃, 25 D-glucose, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, and 1.25 NaH₂PO₄, aerated with 95% O₂/5% CO₂) at 37°C for 30 minutes. Slices were subsequently incubated in ACSF at 21 to 22°C for at least 45 to 60 minutes before electrophysiological recordings and optogenetic experiments.

2.7. Optogenetic and electrophysiological recordings

Briefly, slices were transferred to the recording chamber of a SliceScope Pro 6000 (Scientifica, Clarksburg, NJ) containing an upright microscope (BX51; Olympus, Tokyo, Japan) and PatchStar micromanipulators (Scientifica) and held in place with short pieces of flattened gold wire (0.813-mm diameter; Alfa Aesar, Havertown, MA). Fluorescently labeled neurons were visualized using coolLED optics (Scientifica) and a Retiga-2000 camera (QImaging, Surrey, BC, Canada). Pipettes for whole-cell recordings were fabricated from borosilicate capillaries with filaments (G150-F; Warner, Hamden, CT) using a horizontal puller (P-97; Sutter, Novato, CA) and filled with intracellular solution composed of the following (in mM): 128 Cs-methanesulfonate, 10 HEPES, 1 EGTA, 4 MgCl₂, 4 ATP, and 0.4 GTP, 10 phosphocreatine, 3 ascorbate, and 0.05 AlexaFluor 488 (Molecular Probes, Eugene, OR), pH 7.3. EGTA was included both to facilitate seal formation and to reduce cytosolic calcium elevations induced by the various stimulus protocols used in these studies. ACSF was used as the extracellular recording solution. Recordings were targeted to tdTomato-expressing CeA neurons 60 to 100 μm deep in the slice. Pipette capacitance was compensated; series resistance was monitored but not compensated and required to be <35 MΩ for inclusion in the data set. Recordings were filtered at 4 kHz and digitized at 10 kHz. Slices were ideally used 1.5 to 3 hours after preparation, but some were used up to 6 hours after preparation. Recordings were performed at 30 to 34°C. The recording temperature was controlled by an in-line heating system (TC324B; Warner). The ACSF was refreshed every 2 hours. In voltage clamp configuration, excitatory (glutamatergic) and inhibitory (GABAergic) responses during photoactivation of ChR2-positive PBN projections were recorded at command voltages of −70 mV (near the GABAergic reversal potential) and +10 mV (near the glutamatergic reversal potential), respectively. Wide-field photoactivation (20 ms for recording excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs), 5 ms for recording paired-pulse ratio [PPR]) of ChR2-positive PBN axons was performed using a 470-nm wavelength LED (CoolLED pE excitation system) in line with a green fluorescent protein (GFP) filter (ET FITC/GFP; Olympus) and a 4X objective. For isolating monosynaptic inputs, tetrodotoxin (TTX, 1 μM) and 4-aminopyridine (4-AP, 100 μM) were added to the bath solution (ACSF) at least 5 minutes before starting to record. The application of this strategy is because that the TTX abolishes action potentials and thus eliminates polysynaptic transmission relying on action potential propagation, and 4-AP blocks potassium channels and enhances local depolarization of photostimulated ChR2-expressing axons, which results in local photoevoked depolarization of presynaptic terminals sufficient to induce neurotransmitter release.^{61,75} This approach was

applied in all experiments. Comparisons of monosynaptic strength were performed using sequential recordings of adjacent neurons within the same brain slice and CeA subregion to limit variability due to AAV infection variability and/or slice orientation. The order of recorded neurons was alternated between paired neurons at similar depths within the slice. To evaluate the SNI-induced changes to synaptic plasticity in the PBN–CeA pathway, we used a paired-pulse stimulation (100 ms between pulses) protocol using light to evoke transmission. The PPR is the ratio of the amplitude of the second EPSC to that of the first, reflecting presynaptic release probability; a lower PPR correlates with higher release probability.⁴⁷

2.8. Statistical analysis

Custom MATLAB (Mathworks, Natick, MA) routines were used to analyze the data off-line. A two-way analysis of variance (ANOVA) was conducted to determine the effects of the SNI model on mechanical allodynia using Prism (GraphPad Software, San Diego, CA). For all data, a Lilliefors test was performed before significance testing to determine whether the data were normally distributed. Comparative analyses for sequential recordings of neuron pairs were performed using the Wilcoxon signed-rank test. *W*-values represent the test statistic for the Wilcoxon signed-rank test. Pairwise comparisons before and after drug application were performed using the Student paired *t* test. Statistical comparisons between sham and SNI groups were performed using the Student unpaired *t* test for normally distributed data and the Wilcoxon rank-sum test for non-normally distributed data. Data are represented as mean ± SEM. Significance was set at *P* < 0.05, but *P*-values less than 0.01 are reported.

3. Results

3.1. Topographical expression of PBN axons overlaps with SOM+ and CRH+ CeA neurons

To test monosynaptic input from PBN to molecularly defined neurons in distinct subregions of the CeA, we injected the pCAGW-ChR2-Venus-AAV into the right lateral PBN of mice transgenically expressing tdTomato red fluorescent protein in SOM+ neurons (Figs. 1A–C). At least 2 weeks after the viral delivery, acute slices containing the right CeA were prepared (Figs. 1D and E). The expression of SOM+ neurons was detected throughout CeA with strong expression in the CeL (Figs. 1F and G). Sparse expression of SOM+ neurons was detected in lateral amygdala/basolateral amygdala (LA/BLA) as previously shown.⁴⁵ We find yellow fluorescent protein (YFP)-labeled PBN axons throughout the CeA with the strongest expression in the CeC (Figs. 1H and I). However, we did not detect YFP expression in LA/BLA (Fig. 1H). This pattern of PBN axon terminals in the CeA is consistent with previous reports.^{26,33,68} We implemented the same injection strategy in mice transgenically expressing tdTomato red fluorescent protein in CRH+ neurons (Figs. 1J–L). In acute slices containing the CeA, robust expression of CRH+ neurons was detected within CeL with weaker expression in the CeM and scarce expression in the CeC (Figs. 1M–P). CRH+ neurons were also detected in the LA/BLA (Fig. 1P). The pattern of PBN axon terminals in CRH-tdTomato mice was consistent with SOM-tdTomato mice (Figs. 1Q and R). This topographic expression for SOM+ and CRH+ CeA neurons is consistent with previous studies.^{43,50} Our approach here allowed us to target molecularly defined SOM+ and CRH+ neurons in the right CeA for whole-cell recordings while optogenetically driving long-range synaptic inputs originating in PBN. We chose to target the right CeA based on compelling evidence for amygdalar lateralization in nociception.^{13,14,40,64}

3.2. Parabrachial input differentially targets SOM+ and SOM- neurons in specific subregions of the CeA

We first targeted the LA/BLA neurons as negative control for our optogenetic strategy. Wide-field photoactivation of Chr2+ PBn axons with 470-nm blue LED light did not evoke any monosynaptic EPSCs nor IPSCs (Figs. 2A and B) in 9 BLA and 2 LA neurons, which is consistent with our anatomical results showing no YFP-labeled PBn axons in the LA/BLA region (Fig. 1). Next, we targeted 6 CeA neurons (1 SOM+, 1 SOM-, 1 CRH+, and 3 CRH- neurons) to measure monosynaptic EPSCs and IPSCs (Figs. 2C and D). Because NMDA receptors at resting membrane potential are completely blocked by Mg²⁺,^{25,42} the EPSCs measured in our condition (1 mM Mg in ACSF and -70-mV holding membrane potential) were mediated by AMPA receptors. We observed light-evoked monosynaptic EPSCs in all 6 CeA neurons recorded, and 10- μ M 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), an AMPA receptor antagonist, blocked the excitatory postsynaptic response in all 6 recorded neurons ($t(5) = 2.51$; $P = 0.05$; paired t test; Fig. 2D), confirming that the light-evoked EPSCs are AMPA-mediated.

Our recordings revealed that wide-field photoactivation of Chr2+ PBn axons with 470-nm blue LED light elicited monosynaptic EPSCs in both SOM+ and SOM- neurons recorded from all distinct subregions (CeC, CeL, and CeM) of the CeA (Fig. 3). Pairs of SOM+ and SOM- CeA neurons at approximately the same depth were sequentially recorded within the same brain slice and CeA subregion to normalize for adeno-associated virus (AAV) infection variability

and/or slice orientation (see Methods). We found no statistical difference between amplitude and PPR of evoked EPSCs from recorded SOM+ (amplitude: 100 ± 23 pA; PPR: 0.41 ± 0.04) and SOM- (amplitude: 127 ± 38 pA; PPR: 0.41 ± 0.04) neurons in the CeL (amplitude: $W = 33$; $P = 1$; PPR: $W = 31$; $P = 0.9$, Wilcoxon signed-rank test; Figs. 3A-C). In the CeC, amplitude of monosynaptic EPSCs in the SOM- neurons (325 ± 104 pA) was significantly larger than SOM+ neurons (74 ± 9.5 pA; $W = 13$; $P = 0.01$, Wilcoxon signed-rank test), while analysis of PPR showed no significant differences (SOM-: 0.39 ± 0.06 ; SOM+: 0.48 ± 0.05 ; $W = 35$; $P = 0.3$, Wilcoxon signed-rank test; Figs. 3D-F). We detected light-evoked monosynaptic EPSCs in 12 of 13 SOM+ and 8 of 13 SOM- CeM neurons recorded in the CeM. Amplitude of monosynaptic EPSCs in the SOM+ CeM neurons (175 ± 53 pA) was significantly larger than EPSCs recorded in SOM- CeM neurons (64 ± 59 pA; $W = 14$; $P = 0.03$, Wilcoxon signed-rank test; Figs. 3G and H), but PPR was not significantly different (SOM-: 0.49 ± 0.04 ; SOM+: 0.40 ± 0.06 ; $W = 9$; $P = 0.13$, Wilcoxon signed-rank test; Fig. 3I). These data suggest that efficiency of presynaptic glutamate release from PBn inputs does not account for differences in EPSC amplitude between SOM+ and SOM- neurons in the CeL and CeM. For all PPR recordings, amplitude of monosynaptic EPSCs elicited by the second light pulse was smaller than the first light pulse indicating high release probability for PBn synapses targeting CeA neurons.

We used the same approach to test the organizational principle for monosynaptic inputs from the PBn to the CeA in the CRH-tdTomato mice. Our results showed that wide-field

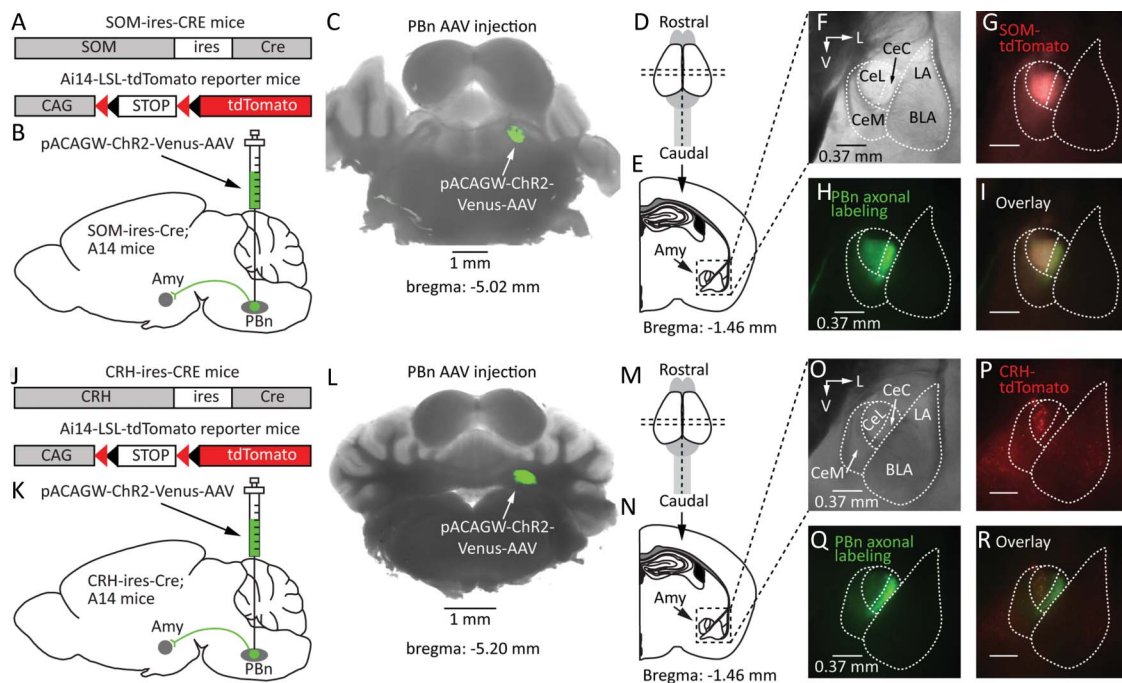


Figure 1. Coexpression of PBn axons and SOM+ and CRH+ CeA neurons. (A) SOM-IRES-CRE mice were mated with Ai14-LSL-tdTomato reporter mice to obtain SOM-IRES-Cre; A14 mice. (B) Delivery of pACAGW-ChR2-Venus-AAV into the PBn of SOM-IRES-Cre; A14 mice. (C) A representative coronal brain section showing pACAGW-ChR2-Venus-AAV injection into the right parabrachial nucleus (PBn) of a SOM-IRES-Cre; A14 mouse. (D and E) At least 2 weeks after the AAV injection, acute coronal brain slices containing the central amygdala (CeA) were prepared. (F) Representative bright-field image of slice containing the lateral amygdala (LA), basolateral amygdala (BLA), and subregions of the CeA (CeC: laterocapsular; CeL: lateral; and CeM: medial). (G) Epifluorescence image showing the expression of SOM+ neurons in the CeA. (H) Epifluorescence image showing the expression of the PBn axons in the CeA. (I) Merged image showing the coexpression of tdTomato-expressing SOM+ neurons and Venus-expressing PBn axons in the CeA. (J) CRH-IRES-CRE female mice were mated with Ai14-LSL-tdTomato reporter mice to obtain CRH-IRES-Cre; A14 mice. (K) Delivery of pACAGW-ChR2-Venus-AAV into the right PBn from a CRH-IRES-Cre; A14 mouse. (L) Coronal brain section showing pACAGW-ChR2-Venus-AAV injection into the right PBn from a CRH-IRES-Cre; A14 mouse. (M and N) At least 2 weeks after the AAV injection, acute coronal brain slices containing the CeA were prepared. (O) Bright-field image showing a representative acute coronal brain slice containing the CeA. (P) Epifluorescence image showing the expression of CRH+ neurons in the CeA and LA/BLA. (Q) Epifluorescence image showing the expression of the PBn axons in the CeA. (R) Merged image showing the coexpression of CRH+ neurons and PBn axons in the CeA. L, lateral; V, ventral.

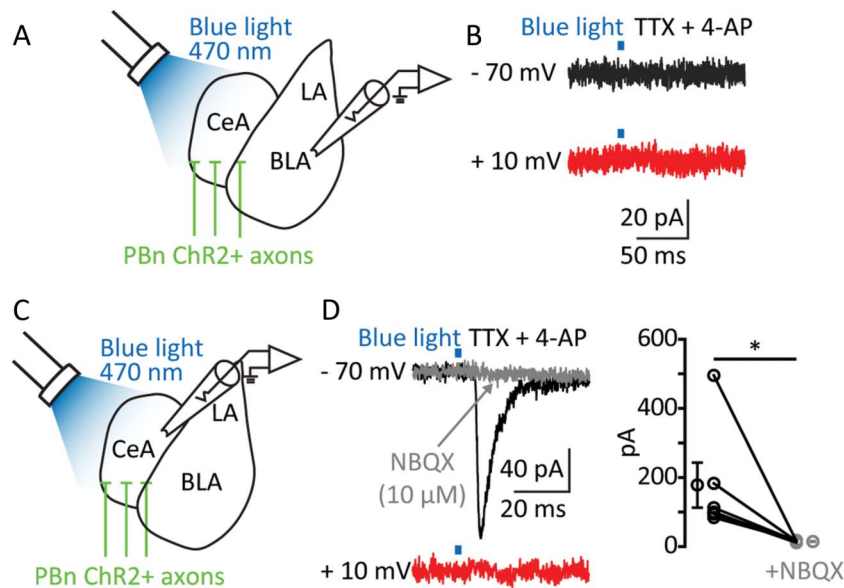


Figure 2. Photoactivation of PBN axon terminals elicited a glutamatergic monosynaptic EPSC in the CeA, but not BLA. (A) Schematic of wide-field stimulation of PBN axons while recording in the LA/BLA. (B) Photoactivation of PBN axon terminals with blue light did not evoke glutamatergic monosynaptic EPSCs (top) nor monosynaptic IPSCs (bottom) in the LA/BLA neurons ($n = 9$; BLA = 7, LA = 2). (C) Schematic of wide-field stimulation of PBN axons while recording in the CeA. (D) Photoactivation of PBN axon terminals with blue light–evoked glutamatergic monosynaptic, AMPA-mediated EPSCs (top), but not IPSCs in CeA neurons (bottom). (E) The AMPA receptor antagonist NBQX blocked PBN-evoked EPSCs in CeA neurons (1 SOM+, 1 SOM–, 1 CRH+, 3 CRH– neurons, 4 mice, $*P = 0.05$).

photoactivation of ChR2+ PBN axons elicits monosynaptic EPSCs in subsets of CRH+ and CRH– neurons recorded in the CeL and CeM (**Fig. 4**). We did not detect expression of CRH+ neurons in the CeC. We did not find any statistical differences in EPSC amplitude or PPR between CRH+ (amplitude: 61.5 ± 16

pA; PPR: 0.30 ± 0.06) and CRH– (amplitude: 92.1 ± 33 pA; PPR: 0.42 ± 0.07) neurons in the CeL (amplitude: $W = 33$; $P = 0.68$; PPR: $W = 7$; $P = 0.07$, Wilcoxon signed-rank test; **Figs. 4A–C**). Only 4 of 8 CRH+ neurons and 5 of 8 CRH– neurons recorded in the CeM exhibited EPSCs after stimulation of PBN

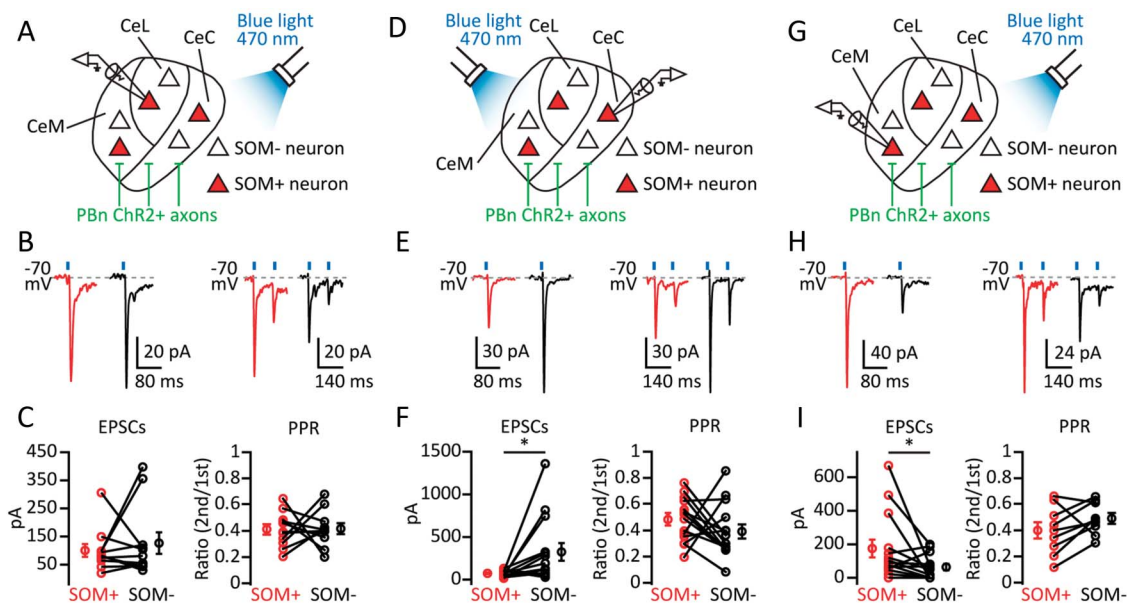


Figure 3. Organization of parabrachial monosynaptic excitatory input to SOM+ and SOM– neurons in subdivisions of the CeA. (A) Schematic recording configuration in the CeL of SOM-tdTomato mice. (B) Representative traces of light-evoked (blue ticks) monosynaptic EPSCs (left, at -70 -mV holding potential, $1\text{-}\mu\text{M}$ TTX + $100\text{-}\mu\text{M}$ 4-AP) and PPR (right, at -70 -mV holding potential, $1\text{-}\mu\text{M}$ TTX + $100\text{-}\mu\text{M}$ 4-AP; interval 100 ms) in SOM+ (red) and SOM– (black) CeL neurons. (C) Comparison of monosynaptic EPSCs and paired-pulse ratio (PPR) between SOM+ and SOM– CeL neurons ($n = 11$ pairs/5 mice). (D) Schematic recording configuration in the CeC. (E) Representative traces of light-evoked monosynaptic EPSCs and PPR in SOM+ and SOM– CeC neurons. (F) Comparison of monosynaptic EPSCs and PPR between SOM+ and SOM– CeC neurons ($n = 14$ pairs/5 mice; $*P < 0.05$). (G) Schematic recording configuration in the CeM. (H) Representative traces of light-evoked monosynaptic EPSCs and PPR in SOM+ and SOM– CeM neurons. (I) Comparison of monosynaptic EPSCs and PPR between SOM+ (red) and SOM– (black) CeM neurons ($n = 14$ pairs/5 mice; $*P < 0.05$). Pairs are connected by lines; mean \pm SEM of SOM– values (normalized to SOM+ pair) is plotted to the right. Comparison of the monosynaptic EPSCs and PPR between SOM+ and SOM– CeM neurons ($n = 14$ EPSC pairs; 9 PPR pairs/5 mice; $*P < 0.05$).

inputs. As in the CeL, we did not find significant differences in EPSC amplitude or PPR between CRH+ (amplitude: 61.5 ± 16 pA; PPR: 0.30 ± 0.06) and CRH- (amplitude: 92.1 ± 33 pA; PPR: 0.42 ± 0.07) in the CeM (amplitude: $W = 7$; $P = 0.3$, Wilcoxon signed-rank test; PPR: $t(7) = -0.30$; $P = 0.77$, Student unpaired t test; Fig. 4G and H).

3.3. Spared nerve injury differentially alters presynaptic release probability of parabrachial monosynaptic excitatory input to the SOM+ and SOM- neurons in distinct subregions of the CeA

We next tested whether the SNI model of neuropathic pain alters the dynamics of PBn input to SOM+ and SOM- CeA neurons. We first injected the pACAGW-ChR2-Venus-AAV into the lateral PBn of SOM-IRES-Cre; A14 mice (Fig. 5A). After adequate recovery time (at least 4 days), the common peroneal and tibial nerves were cut, leaving the sural intact to establish the SNI pain model (Fig. 5B). Pain behavior was evaluated by applying the von Frey fiber to the lateral aspect of the hind paw (Fig. 5C). Mice undergoing the SNI surgery displayed mechanical hypersensitivity 10 days after the surgery compared with the sham mice (50% gram threshold: SNI = 0.003 ± 0.002 g; sham = 1.3 ± 0.46 g; two-way ANOVA; $F_{1,9} = 6.3$, $P = 0.023$, Fig. 5D). In brain slices made after behavioral testing, we stimulated PBn ChR2+ axons while recording from SOM+ and SOM- neurons in distinct CeA subregions (Fig. 5E). Our results reveal that SNI significantly

increased PPR of PBn input to CeC-SOM+ neurons when compared with sham mice (SNI: 0.56 ± 0.05 ; sham: 0.33 ± 0.05 ; $t(10) = -2.43$; $P = 0.035$, Student unpaired t test, Fig. 5F). Conversely, SNI significantly reduces PPR of PBn input to CeC-SOM- neurons (SNI: 0.25 ± 0.04 ; sham: 0.54 ± 0.06 ; $t(11) = 3.79$; $P = 0.003$, Student unpaired t test, Fig. 5G). Recordings in the CeL show that SNI does not alter PPR input to SOM+ (SNI: 0.46 ± 0.07 ; sham: 0.42 ± 0.06 ; $t(11) = -0.415$; $P = 0.69$, Student unpaired t test, Fig. 5H) nor SOM- neurons (SNI: 0.47 ± 0.06 ; sham: 0.52 ± 0.09 ; $t(8) = 0.461$; $P = 0.66$, Student unpaired t test, Fig. 5I). In the CeM, our analyses showed that SNI did not alter PPR of PBn input to SOM+ neurons (SNI: 0.41 ± 0.1 ; sham: 0.41 ± 0.09 ; $t(11) = 0.024$; $P = 0.98$, Student unpaired t test, Fig. 5J), but increased PPR of PBn input to SOM- neurons (SNI: 0.64 ± 0.11 ; sham: 0.31 ± 0.03 ; $t(11) = -2.88$; $*P = 0.015$, Student unpaired t test, Fig. 5K). These results suggest that SNI attenuates synaptic efficacy of PBn input to CeC-SOM+ and CeM-SOM- neurons while increasing synaptic efficacy of PBn input to CeC-SOM- neurons.

3.4. Spared nerve injury decreased the presynaptic release probability of parabrachial monosynaptic excitatory input to the CRH+ and CRH- CeL neurons

We next ran a set of experiments to test effects of SNI on PBn-CeA connections in CRH-IRES-Cre; A14 mice (Fig. 6). Consistent with SOM-IRES-Cre; A14 mice, CRH-ires-Cre; A14

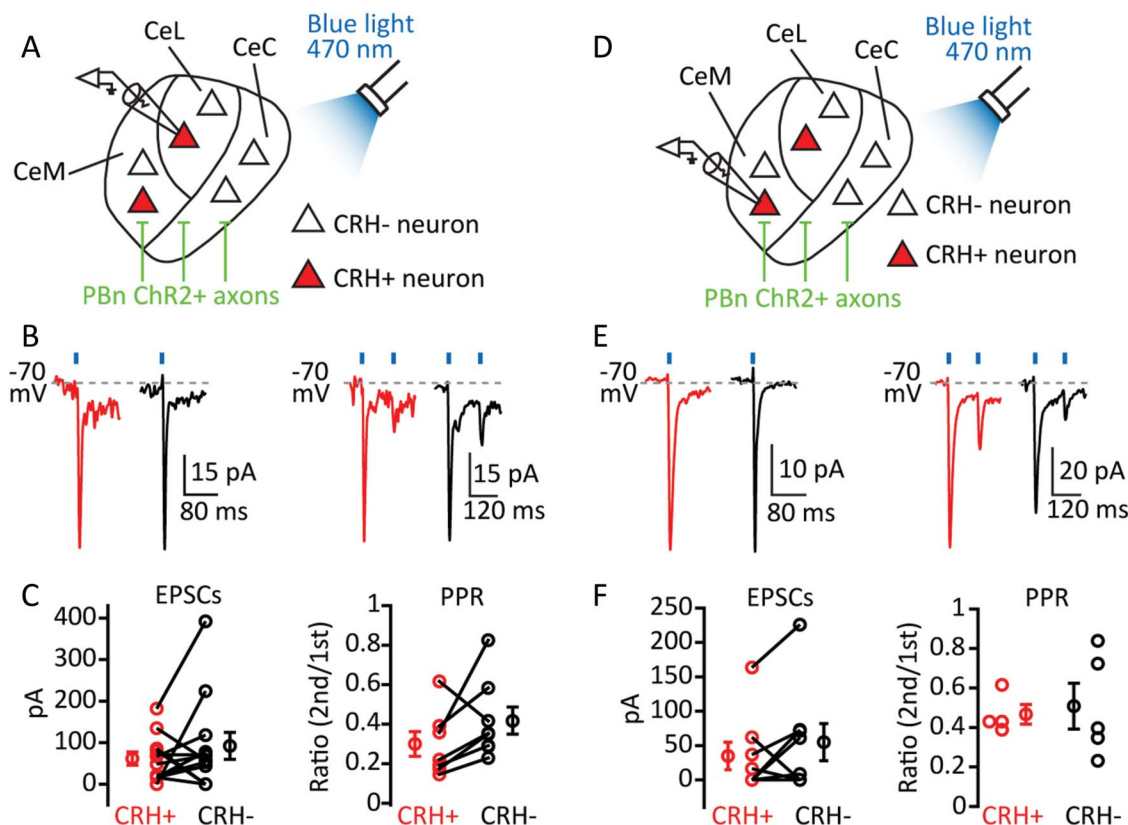


Figure 4. Organization of parabrachial monosynaptic excitatory input to the CRH+ and CRH- neurons in subdivisions of the CeA. (A) Schematic recording configuration in the CeL of CRH-tdTomato mice. (B) Representative traces of light-evoked monosynaptic EPSCs and PPR (interval 100 ms) recorded from a CRH+ (red) and CRH- (black) CeL neurons. (C) Comparison of monosynaptic EPSCs ($t(11) = -1.05$; $P = 0.32$, Student paired t test) and PPR ($t(6) = -1.84$; $P = 0.11$, Student paired t test) between CRH+ and CRH- CeL neurons ($n = 12$ EPSC pairs; 7 PPR pairs/5 mice). (D) Schematic recording configuration in the CeM of CRH Cre mice. (E) Representative traces of light-evoked monosynaptic EPSCs and PPR (interval 100 msec) recorded from a CRH+ (red) and CRH- (black) CeM neuron. (F) Comparison of monosynaptic EPSCs ($t(7) = -1.23$; $P = 0.26$, Student paired t test) and PPR (CRH+: 4; CRH-: 5; $t(7) = -0.30$; $P = 0.77$, Student unpaired t test) between CRH+ and CRH- CeM neurons ($n = 8$ EPSC pairs/5 mice). PPR, paired-pulse ratio.

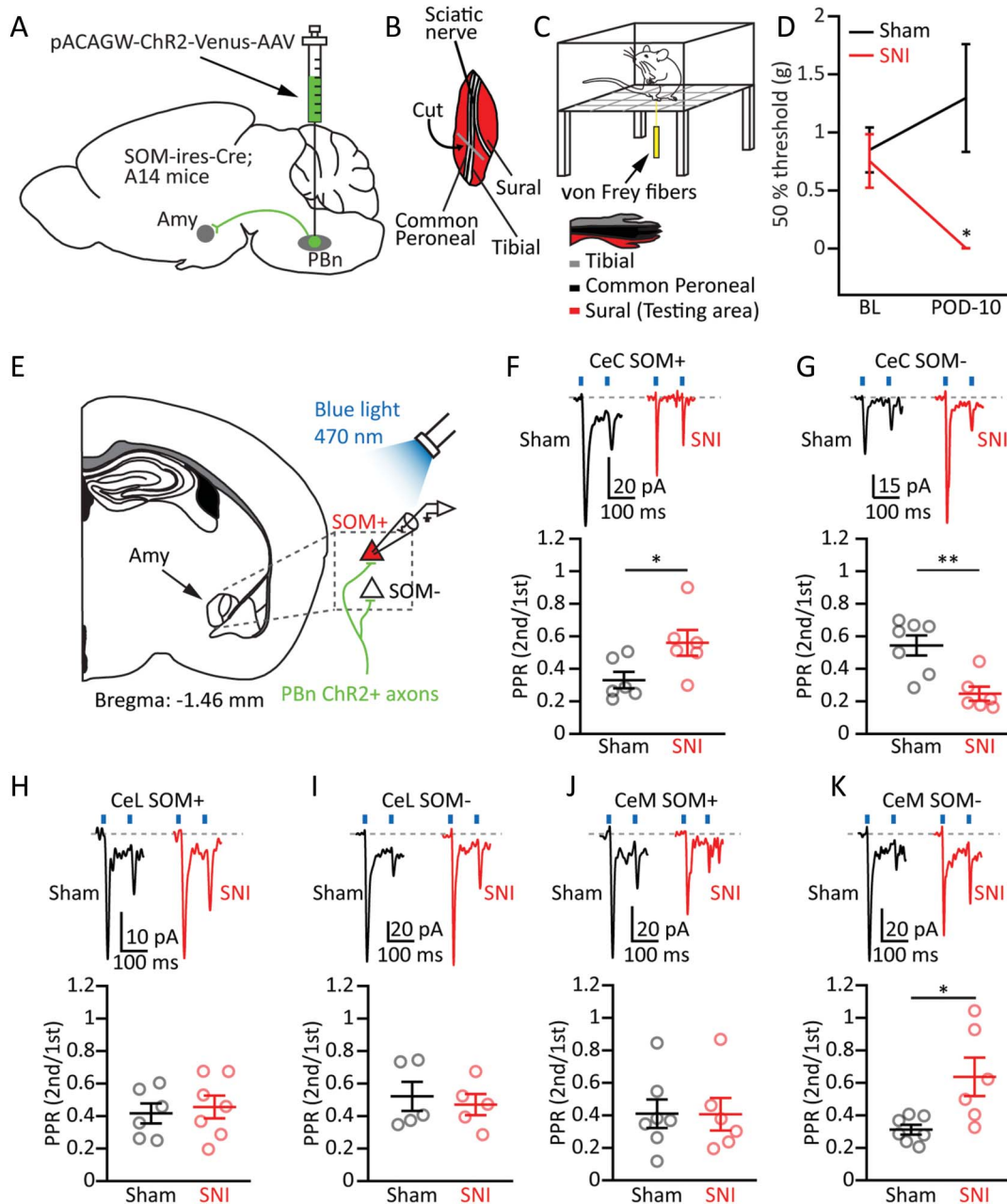


Figure 5. SNI distinctly alters PBn input to SOM+ and SOM− neurons based on subdivision of the CeA. (A) Delivery of pACAGW-ChR2-Venus-AAV into the PBn of SOM-IRES-Cre; A14 mice. (B) Schematic of SNI surgery involving transection (grey line) of the tibial and peroneal branches of the sciatic nerve, leaving the sural nerve intact. (C) SNI and sham-operated mice were placed in cages on an elevated mesh platform for assessment of pain behavior using von Frey filaments. The hind paw region innervated by the sural nerve (red) skin was targeted the von Frey hair while regions innervated by common peroneal (black) and tibial (gray) nerve were avoided. (D) 50% withdrawal threshold response (mean ± SEM) to von Frey fibers of SNI (n = 5) and sham (n = 5) mice (*P = 0.023) on postoperative day 10 (POD-10). (E) SOM+ and SOM− CeA neurons were sequentially recorded during blue (470-nm) LED wide-field stimulation of ChR2-expressing PBn axons. (F) Representative traces (top) and statistical comparison (bottom) of PPR in SOM+ CeC neurons from SNI mice compared with sham mice (SNI: 6 neurons; sham: 6 neurons; *P = 0.035). (G) Representative traces (top) and statistical comparison (bottom) of PPR in SOM− CeC neurons from SNI mice compared with sham mice (SNI: 6 neurons; sham: 7 neurons; **P = 0.003). (H) Representative traces (top) and statistical comparison (bottom) of PPR in SOM+ CeL neurons from SNI and sham mice (SNI: 7 neurons; sham: 6 neurons; P = 0.69). (I) Representative traces (top) and statistical comparison (bottom) of PPR in SOM− CeL neurons from SNI and sham mice (SNI: 5 neurons; sham: 5 neurons; P = 0.66). (J) Representative traces (top) and statistical comparison (bottom) of PPR in SOM+ CeM neurons from SNI mice and sham mice (SNI: 6 neurons; sham: 7 neurons; P = 0.98). (K) Representative traces (top) and statistical comparison (bottom) of PPR in SOM− CeM neurons from SNI mice compared with sham mice (SNI: 6 neurons; sham: 7 neurons; t(11) = −2.88; *P = 0.015, Student unpaired t test). PPR, paired-pulse ratio; SNI, spared nerve injury.

mice displayed mechanical hypersensitivity 10 days after SNI (50% gram threshold: SNI = 0.006 ± 0.002 g; sham = 0.7 ± 0.19 g; repeated-measures ANOVA; $F_{1,7} = 10.35$, *P = 0.015; **Fig. 6A**). We targeted CRH+ and CRH− neurons in distinct subregions of the CeA for whole-cell patch clamp recording

while optogenetically stimulating ChR2+ PBn axons (**Fig. 6B**). Our results reveal that SNI significantly increases PPR of PBn inputs to both CeL–CRH+ (SNI: 0.48 ± 0.05; sham: 0.26 ± 0.05; t(10) = −2.85; *P = 0.017, Student unpaired t test; **Fig. 6C**) and CeL–CRH− neurons (SNI: 0.51 ± 0.05; sham: 0.33 ± 0.02; t(25) = −2.96;

** $P = 0.007$, Student unpaired t test; **Fig. 6D**). However, SNI significantly reduces PPR of PBN input to CeM–CRH+ neurons (SNI: 0.26 ± 0.03 ; sham: 0.37 ± 0.02 ; $t(11) = 3.00$; $*P = 0.012$, Student unpaired t test; **Fig. 6E**). We did not observe any change of PPR in CeM–CRH– neurons (SNI: 0.53 ± 0.07 ; sham: 0.48 ± 0.07 ; $t(11) = 3.00$; $P = 0.69$, Student unpaired t test; **Fig. 6F**). These results indicate that SNI decreases the synaptic efficacy of PBN inputs targeting to CeL–CRH+ and CeL–CRH– neurons while increasing the synaptic efficacy of PBN inputs targeting the CeM–CRH+ neurons.

4. Discussion

Accumulated anatomical and physiological evidence clearly demonstrates a PBN–CeA circuit that plays an essential role in the emotional-affective dimension of pain.^{5–9,30,33,55,57,66,67,74} Excitatory synapses from PB to CeA neurons are potentiated in arthritic, visceral, neuropathic, inflammatory, and muscle pain models^{32,36,54,55,57,69,74} and after nociceptive stimuli with no ongoing injury.⁴⁴ These previous studies focused mainly on PBN–CeC pathways leading to the labeling of the CeC as the nociceptive-specific subnuclei of the CeA.^{11,56,58} However, other reports demonstrate that the PBN–CeL pathway is also involved in nociceptive transmission.^{9,24,38} A recent study reveals that CeL

neurons expressing the calcitonin gene-related peptide receptor (CGRPR) play a key role in consolidating PBN input into threat memory,³³ uncovering a molecularly specific affective pain circuit within the PBN–CeL pathway.

Here, we expand upon these findings by revealing that PBN differentially targets neurons in the CeA based on both molecular identity and topographical location within CeA. Although PBN innervation of CeL and CeC subdivisions is well established, we show that PBN inputs to SOM– neurons are stronger compared with SOM+ neurons in the CeC. It is likely that CeC–SOM– neurons express protein kinase C- δ (PKC- δ) based on work showing that nonoverlapping PKC- δ + and SOM+ neurons constitute 80% to 90% of the entire CeL/C population.^{34,35,47,50} This is consistent with a recent study showing greater density of CGRP+ terminals surrounding PKC- δ + neurons compared with SOM+ neurons in CeL/C.⁷⁶ Pivotal work shows that PKC- δ + neurons inhibit CeM output to the periaqueductal gray (PAG).^{34,47} The PAG is a midbrain structure that integrates motivational/limbic and sensory input, including pain, to initiate specific outputs including coping behavior.^{2,3,15,21} Therefore, our data suggest PBN–CeC circuits preferentially and indirectly influence PAG activity, which is relevant for behavioral responses to pain input. In the CeM, we show that PBN inputs to SOM+ neurons are stronger compared with SOM– neurons. Although

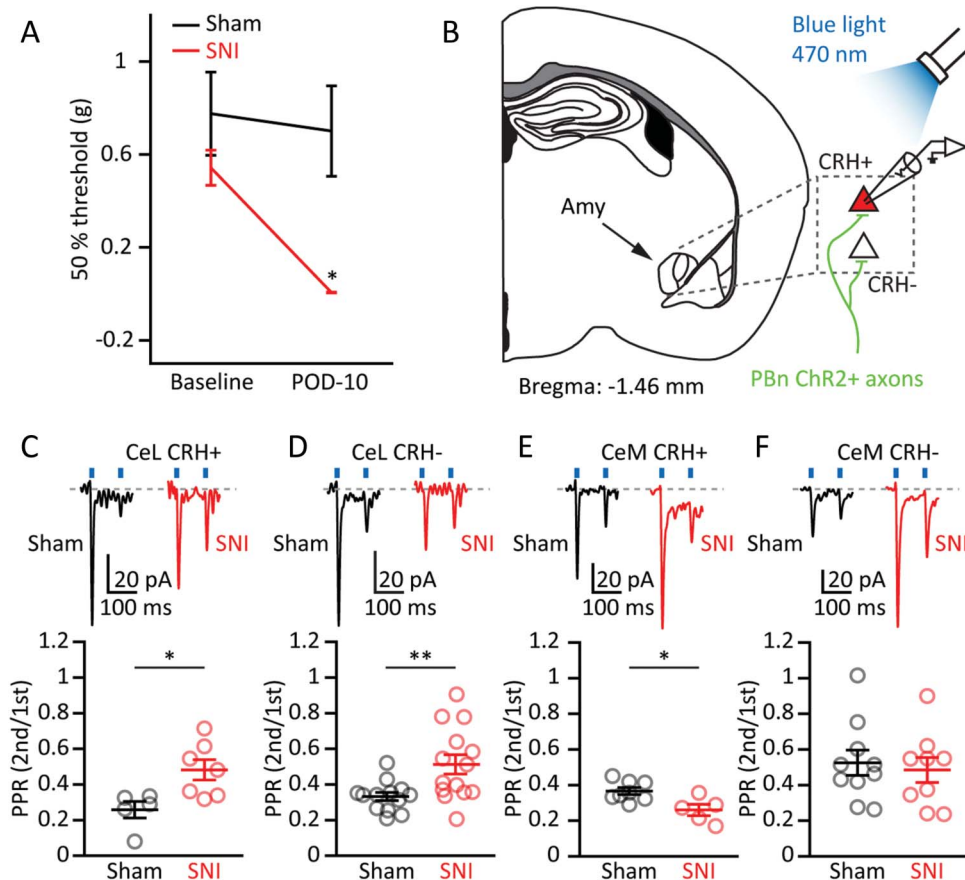


Figure 6. SNI differentially alters PBN input to CRH+ and CRH– neurons in distinct subregions of the CeA. (A) Fifty percent withdrawal threshold response (mean \pm SEM) to von Frey fibers of SNI ($n = 5$) and sham ($n = 4$) mice ($*P = 0.015$) on postoperative day 10 (POD-10). (B) CRH+ and CRH– CeA neurons were recorded during blue (470-nm) LED wide-field stimulation of ChR2-expressing PBN axons. (C) Representative traces (top) and statistical comparison (bottom) of PPR in CRH+ CeL neurons from SNI compared with sham mice (SNI: 7 neurons; sham: 5 neurons; $*P = 0.017$). (D) Representative traces (top) and statistical comparison (bottom) of PPR in CRH– CeL neurons in the SNI mice (SNI: 14 neurons; sham: 5 neurons; $**P = 0.007$). (E) Representative traces (top) and statistical comparison (bottom) of PPR in CRH+ CeM neurons in SNI compared with sham mice (SNI: 5 neurons; sham: 8 neurons; $*P = 0.012$). (F) Representative traces (top) and statistical comparison (bottom) of PPR in CRH– CeM neurons between SNI and sham mice (SNI: 9 neurons; sham: 10 neurons; $P = 0.69$). PPR, paired-pulse ratio; SNI, spared nerve injury.

CeM projections to PAG are known,^{34,47,48} molecular profiles of these CeM–PAG neurons remain unknown. A subset of PAG-projecting neurons in the CeL are SOM+,⁶⁰ but further work is needed to confirm whether this is also true in the CeM. Nonetheless, this imbalanced targeting of PBn inputs suggests that SOM+ neurons play a significant role in the PBn–CeM pathway.

We find no differential targeting or differences in synaptic efficacy of PBn input to CRH+ vs CRH– neurons in either the CeL or CeM. However, probability of detecting EPSCs in CRH+ and CRH– neurons in the CeL was less than observed in SOM+ and SOM– neurons. This suggests there is a small subset of CeL neurons that do not receive direct input from PBn. Only half of the CeM–CRH+ neurons produced EPSCs after stimulation of PBn inputs. This is considerably less than the probability of evoking EPSCs in CeM–SOM+ neurons, which suggests that PBn differentially targets SOM+ neurons vs CRH+ neurons in the CeM. Still, current limitations of transgenic strategies does not allow us to directly compare PBn inputs with SOM+ and CRH+ neurons in the CeM, which would give us the proper control for viral efficiency.

We further find that the SNI model of neuropathic pain both potentiated and attenuated the synaptic efficacy of PBn inputs to CeA neurons, as measured by PPR, and that the nature of these contrasting PPR changes is based on both the molecular identity of target neurons and topographical location within CeA. Amplitude of EPSCs evoked by monosynaptic inputs from PBn to late-firing CeC neurons is potentiated in the formalin model of inflammatory pain.⁷¹ One study shows that a majority of late-firing neurons in the CeL express PKC- δ ,³⁴ which suggests that inflammatory pain enhances PBn input to PKC- δ + neurons in the CeC. However, this remains unclear due to another study reporting that subpopulations of both PKC- δ + and SOM+ neurons in the CeL display a late-firing phenotype.³⁵ Our data show that SNI potentiates synaptic efficacy of PBn inputs targeting CeC–SOM– neurons, which are likely PKC- δ + neurons.^{34,35,47,50} Interestingly, we find PPR of PBn input to CeC–SOM+ neurons increases after SNI, which implies an attenuated synaptic efficacy. Given the established reciprocal inhibitory relationship between SOM+ and PKC- δ + neurons,^{34,35,47} our observed SNI-induced decrease and increase in PPR of PBn inputs targeting CeC–SOM– and CeC–SOM+ neurons, respectively, suggest that neuropathic pain shifts excitatory balance toward PKC- δ + (ie, SOM–) neurons. Pivotal work has shown that a substantial number PKC- δ + neurons in caudal CeL/C express the CGRPR and that these CeL/C CGRPR+ neurons play a critical role in encoding pain input from PBn.³³ Based on these findings and our current data, we speculate that SNI evokes a long-lasting and enhanced excitatory tone to PKC- δ +/CGRPR+ neurons in the CeC involving changes to synaptic plasticity of inputs from CGRPR+ PBn neurons. A related study supports this notion by showing that knocking out CGRPR in mice prevents decreased PPR observed in CeC neurons after induction of inflammatory pain.⁶⁹ Therefore, it is likely that we are detecting a similar PBn–CGRPR effect on CeC–SOM– neurons in our SNI model of neuropathic pain.

Surprisingly, we did not detect SNI-induced changes to PPR of PBn input to either CeL–SOM+ or CeL–SOM– neurons. This shows that SNI not only evokes changes to topographically distinct PBn–CeA pathways (ie, PBn–CeC) but also induces contrasting changes to synaptic efficacy of PBn inputs based on the molecular identity of CeC neurons (ie, SOM+ vs SOM–). We found that SNI significantly decreases synaptic efficacy of PBn inputs targeting CeM–SOM– neurons. Functions

corresponding to specific molecular markers in the CeM are not well defined. Therefore, the functional significance of PPR changes to the PBn–CeM–SOM– pathway after SNI is inconclusive. Nonetheless, the CeM is the major output of the CeA, and our previous data show that a significant subpopulation of CeM neurons send projections throughout multiple regions of the PAG,⁴⁸ suggesting SNI may affect pain-related behaviors through a PBn–CeM–PAG pathway.

In the CeA, CRH+ neurons modulate fear- and anxiety-like behavior^{1,23,59,65} as well as negative effects of alcohol withdrawal.²⁰ We find that SNI reduced synaptic efficacy to both CRH+ and CRH– neurons in CeL, which contrasts our data showing no effects of SNI on PPR of PBn inputs to SOM+ and SOM– in CeL. One study reports negligible expression of SOM and PKC- δ in CeL–CRH+ neurons,⁶⁵ while others demonstrate substantial (~70%) coexpression of SOM in CeL–CRH+ neurons.^{43,50} However, the same studies also reported that only 35% to 50% of CeL–SOM+ neurons express CRH,^{43,50} which suggest that we did not detect effects of SNI on synaptic efficacy of PBn inputs to CeL–SOM+ neurons because a majority we recorded did not coexpress CRH. Why we see reduced synaptic efficacy to CRH– neurons in the CeL is unclear. It is reported that approximately 19% of CeL neurons are CRH+,⁵⁰ meaning that the molecular heterogeneity of CeL–CRH– neurons is considerable. Approximately 90% of CeL neurons are either SOM+ or PKC- δ +, but there are a number of other molecular markers that can discriminate between CeL subpopulations including CGRPR, tachykinin 2, oxytocin receptor, neurotensin, prodynorphin, and dopamine receptor 2.^{33,34,41,43,50} Our data suggest that SNI reduces synaptic efficacy of PBn input to a subpopulation of CeL–CRH– neurons that was not represented in our CeL–SOM– recordings. In the CeM, we found that SNI enhances synaptic efficacy of PBn input to CRH+ neurons. The CeM contains a large number of CRH receptive neurons,^{65,73} which likely respond to local release of CRH implicated in contextual memory consolidation.⁶³ Therefore, enhanced PBn input to CeM–CRH+ neurons may facilitate memory associated with pain after nerve injury. Whether this enhancement in synaptic efficacy happens acutely or over the development of pain behavior induced by SNI remains to be elucidated.

In summary, this study is the first to establish an organizational principle of PBn monosynaptic input to molecularly defined neurons within the 3 major subregions of the CeA: laterocapsular (CeC), lateral (CeL), and medial (CeM). It further establishes that plasticity of distinct PBn–CeA pathways is differentially altered in the SNI model of neuropathic pain. Together, these findings provide updated insight for dissecting functional changes to CeA circuit pathways in pain models as both topography and targeting to neuronal subtypes need to be considered.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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