Research Paper

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Neuronal basis for pain-like and anxiety-like behaviors in the central nucleus of the amygdala

Wei-Hsin Chen[a], Cheng-Chang Lien[b,c], Chien-Chang Chen[a,*]

Abstract

Chronic pain is often accompanied by anxiety and depression disorders. Amygdala nuclei play important roles in emotional responses, fear, depression, anxiety, and pain modulation. The exact mechanism of how amygdala neurons are involved in pain and anxiety is not completely understood. The central nucleus of the amygdala contains 2 major subpopulations of GABAergic neurons that express somatostatin (SOM$^+$) or protein kinase Cδ (PKCδ$^+$). In this study, we found about 70% of phosphorylated ERK–positive neurons colocalized with PKCδ$^+$ neurons in the formalin-induced pain model in mice. Optogenetic activation of PKCδ$^+$ neurons was sufficient to induce mechanical hyperalgesia without changing anxiety-like behavior in naïve mice. Conversely, chemogenetic inhibition of PKCδ$^+$ neurons significantly reduced the mechanical hyperalgesia in the pain model. By contrast, optogenetic inhibition of SOM$^+$ neurons induced mechanical hyperalgesia in naïve mice and increased phosphorylated ERK–positive neurons mainly in PKCδ$^+$ neurons. Optogenetic activation of SOM$^+$ neurons slightly reduced the mechanical hyperalgesia in the pain model but did not change the mechanical sensitivity in naïve mice. Instead, it induced anxiety-like behavior. Our results suggest that the PKCδ$^+$ and SOM$^+$ neurons in the central amygdala exert different functions in regulating pain-like and anxiety-like behaviors in mice.

Keywords: SOM+, PKCδ+, pERK, CeA, Anxiety, Pain, Optogenetics, Chemogenetics, Formalin model

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1. Introduction

Chronic pain is a major global health issue that affects more than 25% of the world's population.[20] It is defined as pain lasts for more than 3 months.[51] Chronic pain also affects emotion, sleep, mental health, and quality of life. Clinically, approximately 40% of individuals with chronic pain have an anxiety disorder,[34] and up to 50% of patients have depression comorbidity.[4] Therefore, understanding chronic pain and its comorbidities have become an important issue. However, the correlation between emotional behaviors and chronic pain remains controversial in animal models. For example, the carrageenan-induced arthritic pain model in rats conferred increased anxiety-like behavior (ALB) through a corticotropin-releasing factor (CRF)-dependent pathway in the amygdala.[28] Complete Freund adjuvant–induced inflammatory pain also resulted in significant ALB in mice.[13] Several articles also showed increased ALB in neuropathic pain models.[36,39] Conversely, other studies found that chronic pain does not directly affect ALB.[21,33,40] These discrepancies might have been due to the use of different animal strains, sexes, age, environmental factors, pain models, and behavioral times investigated.[43] Recently, a comprehensive study showed that chronic pain does not induce ALB and depression-like behavior across the sexes and investigated time points between 1 to 14 days in the complete Freund adjuvant model and 3 to 84 days in the neuropathic pain model, respectively.[43] Also, another study showed that the activation of dopaminergic neurons within the periaqueductal gray produced an analgesic effect without changing anxiety behavior, whereas the activation of glutamatergic neurons produced an analgesic effect and induced anxiety behavior.[49] Therefore, understanding the mechanism causing chronic pain and its relationship to emotional behavior is a critical issue.

The amygdala, including the basolateral amygdala (BLA) and the central amygdala (CeA), is involved in regulating emotions, fear, depression, anxiety, and pain.[11,32,45,54] The CeA nucleus is anatomically divided into the lateral subdivision (CeL) and the medial subdivision (CeM).[23] The CeL contains 2 major subpopulations of GABAergic neurons that express somatostatin (SOM$^+$) or protein kinase Cδ (PKCδ$^+$).[9,23] Central amygdala neurons are involved in pain-induced neuronal plasticity change and promote hypersensitivity through an extracellular signal–regulated kinase (ERK)-dependent pathway.[11,16,50] Also, the transmission between the BLA and CeA is related to ALB.[27,52] Therefore, understanding how CeA neurons are involved in the development of chronic pain and ALB is essential for revealing the relationship between chronic pain and emotional behavior.

In this study, we used optogenetic and chemogenetic approaches to study the involvement of CeA PKCδ$^+$ and SOM$^+$ neurons in the development of mechanical hypersensitivity and ALB in mice. We found that the PKCδ$^+$ neurons were activated by the formalin-induced inflammatory pain model. Optogenetic activation of these neurons robustly induced prolonged hypersensitivity that lasted at least 3 days but did not significantly affect ALB. Conversely, the activation of SOM$^+$

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neurons induced ALB but did not change mechanical sensitivity in naïve mice. Chemogenetic inhibition of PKCδ$^+$ neurons significantly reduced the pain response in the formalin-induced inflammatory pain model. Our results suggest that the PKCδ$^+$ and SOM$^+$ neurons in CeA exert different functions in regulating pain-like and anxiety-like behaviors in mice.

2. Methods

2.1. Animal and pain model

All research conformed to the U.S. National Institutes of Health guidelines following the guidelines specified by the Institutional Animal Care and Utilization Committee, Academia Sinica (Taipei, Taiwan). C57BL6 or heterozygous Prkcd-cre (MMRRC_011559-UCD) or SOM-IRES-Cre mice (Jackson Laboratory_013044) at 8 to 12 weeks of age were used. All mice were housed in specific pathogen-free conditions in the Institute of Biomedical Sciences, Academia Sinica. Mice were randomly assigned to experimental groups. The formalin-induced inflammatory pain model was chosen for studying mechanical hypersensitivity: 10 μL of 5% formalin in phosphate-buffered saline (PBS) was injected subcutaneously into the plantar surface of the left hind paw of mice[10,11]

2.2. von Frey behavior tests

We used von Frey filaments (North Coast Medical, Morgan Hill, CA) to measure the mechanical hypersensitivity induced by formalin as described previously.[14,15] In brief, animals were placed on a wire mesh platform in an acrylic chamber and allowed 0.5 hours for acclimatization. The 1-g bending-force stimulus was applied when animals were calm but not sleeping or grooming. The withdrawal ratio was calculated by the number of times the hind limb withdrew in 10 applications of 1-g monofilament. There is at least a 30-second interval between each application.

2.3. Anxiety-like behavior test

Three standard behaviors were used to evaluate anxiety-like behavior in mice, including the open field test (OFT), elevated plus maze (EPM), and light or dark box (LDB). A square acrylic box (48 × 48 × 48 cm, 16 × 16 cm square center defined as the center) was used for the OFT. The mice were placed in the corner of the arena and allowed to explore for 10 minutes freely. The EPM test consists of 2 open arms (30 × 5 cm, with 1-cm ledges) and 2 closed arms (30 × 5 cm, with 15-cm walls) with a height of 50 cm to the floor. Mice were placed into the center of the EPM, and behavior was tracked for 5 minutes. For the LDB test, a square acrylic box consists of a dark (30 × 30 × 30 cm) and a light (30 × 30 × 30 cm) compartment; the dark and light compartments are connected by a central open hole (7 × 10 cm). Mice were placed in the light box, and behavior was tracked for 5 minutes. The results were analyzed with TopScan (Clever Systems, Reston, VA).[56] For the restraint stress-induced anxiety model, the mice were subjected to ventilated 50 mL tubes for 30 minutes. The mice were tested with different anxiety-related behaviors 1 day after the stress.[61]

2.4. Immunostaining

Mice were deeply anesthetized and then perfused transcardially with PBS, followed by 4% paraformaldehyde solution. All solutions were prepared in PBS buffer and kept at 4°C. Brains were postfixed overnight and dehydrated with 30% sucrose at 4°C until the brain sank. Continual cryosections of 50-μm thick were mounted on slides for immunofluorescence staining. After brain slice dehydration, the slides underwent a heat-induced epitope retrieval procedure with pH 6.4, 0.01 M sodium citrate. Then the slides were treated with blocking buffer (5% bovine serum albumin in PBS Triton X-100 0.01%) for 1 hour, then incubated with the primary antibodies for phosphorylated ERK1/2 (pERK1/2) (1:100, Cell Signaling, Danvers, MA), c-Fos (1:500, Santa Cruz Biotechnology, Dallas, TX), PKCδ (1:500, BD Biosciences, San Jose, CA), or somatostatin (SOM; 1:500, BMA Biomedicals, AG, Augst, Switzerland) in blocking solution overnight at 4°C.[59] Images were captured under the Olympus microscope BX51 or Zeiss LSM700 confocal scanning laser microscope and analyzed using ZEN software. For pERK$^+$ analysis, positive cells in CeA regions 1.2, 1.4, and 1.6 mm posterior to the bregma were counted and analyzed. All staining were repeated 3 times.

2.5. Virus infection and optical stimulation

Adeno-associated viruses (AAV) were obtained from the UNC Vector Core or Penn Vector Core. For virus infection, 0.2 μL AAV5-CaMKII-hChR2 (H134R)-enhanced yellow fluorescent protein (EYFP) (qPCR titer: 8.5 × 10^{12} GC/mL), AAV5-CaMKII-EYFP (qPCR titer: 6.3 × 10^{12} GC/mL), AAV5-EF1a-DIO-hChR2-EYFP (qPCR titer: 5.2 × 10^{12} GC/mL), AAV5-EF1a-DIO-EYFP (qPCR titer: 6.5 × 10^{12} GC/mL), AAV5-EF1a-DIO-eNpHR3.0-EYFP (titer: 1.29 × 10^{13} GC/mL), or AAV8-hSyn-DIO-hM4D-EYFP[55] (qPCR titer: 6.4 × 10^{12} GC/mL) were stereotaxically infused into the right CeA (in millimeters, midline, Bregma, and dorsal surface: 2.5, −1.46, and −4.5, respectively). We focused on the right CeA because the right CeA synaptic transmission and function are highly associated with pain modulation.[11,37,44] For the optogenetic study, after virus expression for 5 to 6 weeks, a 23-G guide cannula was implanted above the CeA. At 1 week after cannulation, animals were tested for mechanical sensitivity. An optical fiber (UM22-200, 0.22NA. ø200 mm; Thorlabs, Newton, NJ) connected to a Fiber Optic Rotary Joint directly linked to a light-emitting diode driver (Doric Lenses, Quebec, Canada) was inserted into the cannula. The mouse received 465-nm blue-light stimulation (10 Hz, 10-ms light pulse, 5-6 mW mm^{-2}) or 595-nm yellow-light stimulation for 10 minutes. Anxiety-like behavior was tested during light stimulation. Mechanical hypersensitivity was measured using the von Frey filament test after light stimulation for 0.5 hours. For the chemogenetic study, clozapine N-oxide (CNO) (3 mg/kg) was administered intraperitoneally (i.p.) and mechanical hypersensitivity was measured as described previously.[12,14,15]

2.6. Whole-cell recordings

At 4 to 6 weeks after AAV-ChR2 infection in CeA, mice were anesthetized with 1.5% isoflurane. Brains were quickly removed and immersed in ice-cold artificial cerebrospinal fluid (in mM: 119 NaCl, 2.5 KCl, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 1.3 $MgSO_4$, 11 glucose, and 2.5 CaCl2, pH 7.4, gassing with 5% CO2/95% O_2). Slices 300-μm thick were cut with a vibrating tissue slicer (D.S.K. Microslicer DTK-1000; Dosaka EM, Kyoto, Japan) and then allowed to recover at room temperature (24-25°C) for 1.5 hours. The slices were transferred to an immersion-type recording chamber mounted on an upright microscope (Scientifica) equipped with 40× water immersion objectives. The oxygenated ACSF was continuously perfused at 1 to 2 mL/min. Patch pipettes were pulled from borosilicate glass tubing (1.5-mm outer diameter and 0.86-mm inner diameter; G150F-4, Warner Instruments, resistance of 5-8 MΩ) and filled with an internal solution that consisted of the following (in mM): 131 K-gluconate, 20 KCl, 10 HEPES, 2 EGTA, 8 NaCl, 2 ATP, 0.3 GTP, and 6.7 biocytin, with pH adjusted to 7.2 by KOH and osmolality adjusted to 300 to 305 mOsm by distilled water. Recordings were made at room temperature (24-25°C) with a patch

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amplifier (MultiClamp 700 B; Axon Instruments, Molecular Devices, LLC, San Jose, CA). For voltage-clamp recordings, the membrane potential of YFP$^+$ cell was held at −70 mV and a series of light pulses were applied to elicit inward currents. Then the mode shifted to current-clamp recording to record the evoked action potential. The hM4d group's spontaneous action potentials were recorded before and after 10 μM CNO perfusion. Data were discarded when the Rs varied by >20% from the original value during the recording. All signals were low-pass filtered at a corner frequency of 1 kHz and digitized at 10 kHz by using a Micro1401 interface (Cambridge Electronic Design). Data were collected by using Signal software (Cambridge Electronic Design). The light source involved the OptoLED Light source system (CAIRN Research). Light pulses of 10 milliseconds, 5 Hz, and intensity 5 mW mm^{-2} were used.[12]

2.7. Data analysis

The number of samples (n) is shown in the text and figures. All experiments were replicated at least 3 times in the laboratory. All data are presented as mean ± SEM. Staining quantification was analyzed by the Student *t* test. For animal behavior data, statistical differences between groups were tested with 1-way ANOVA, followed by a post hoc Tukey test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Central amygdala protein kinase Cδ neurons are activated in the formalin-induced inflammatory pain model

To study the role of CeA neurons in the development of mechanical hypersensitivity, we used an optogenetic tool by expressing channelrhodopsin-2 (hChR2) in the CeA with AAV-CaMKIIα-hChR2 (H134R)-EYFP. CaMKIIα is widely expressed in the CeA nucleus,[53] so we can obtain a good expression of hChR2 in the CeA (Fig. S1, available as supplemental digital content at http://links.lww.com/PAIN/B420). Six weeks after AAV injection, optogenetic activation of CeA neurons for 10 minutes induced a transient mechanical hypersensitivity that lasted less than 1 day in naïve mice (**Fig. 1A**). CaMKIIα-EYFP$^+$ neurons colocalized with both PKCδ$^+$ (Fig. S1a, available as supplemental digital content at http://links.lww.com/PAIN/B420) and SOM$^+$ signals (Fig. S1b, available as supplemental digital content at http://links.lww.com/PAIN/B420). Thus, the direct activation of both PKCδ$^+$ and SOM$^+$ CeA neurons could induce a transient mechanical hypersensitivity. Next, we used an intraplantar injection of 5% formalin to evoke prolonged mechanical hypersensitivity that lasts more than 1 day in naïve animals (**Fig. 1B**). Extracellular signal–regulated kinase activity in the CeA is involved in synaptic transmission and central sensitization,[11,52] and increased pERK in the CeA, an indication of increased ERK activity, occurs 3 hours after formalin injection.[11] We examined pERK expression in PKCδ$^+$ and SOM$^+$ neurons at 90 minutes after formalin injection in female (**Fig. 1C**) and male mice (Fig. S2, available as supplemental digital content at http://links.lww.com/PAIN/B420). Approximately 70% of pERK$^+$ cells colocalized with PKCδ$^+$ neurons (**Figs. 1C and D**; Fig. S2a, available as supplemental digital content at http://links.lww.com/PAIN/B420), whereas only 5% of pERK$^+$ neurons were SOM$^+$ (**Figs. 1C and D**; Fig. S2b, available as supplemental digital content at http://links.lww.com/PAIN/B420). These data indicate that CeA PKCδ$^+$ neurons were activated in the formalin-induced inflammatory pain model in both genders. Therefore, we choose only the female mice for the following studies.

3.2. Activation of protein kinase Cδ neurons in the central amygdala induces prolonged mechanical hypersensitivity in naïve mice

We hypothesized that if 70% of the CeA pERK$^+$ neurons are PKCδ$^+$ in the formalin-induced inflammatory pain model, a sustained behavioral change could be induced by the direct activation of PKCδ$^+$ neurons. To test this idea, we expressed a Cre-dependent hChR2 (AAV-EF1a-DIO-hChR2-EYFP) in the CeA of a transgenic Prkcd-cre mouse line that expressed Cre enzyme only in PKCδ$^+$ neurons (**Fig. 2A**). To test whether the expressed ChR2 was functional, we conducted a single-cell recording on hChR2-EYFP$^+$ neurons in amygdala brain slices. Blue-light stimulation (5 Hz, 10 ms pulse) induced inward currents and action potential firing in PKCδ$^+$ neurons (**Fig. 2A**). We also examined the expression of pERK at 30 minutes after blue-light stimulation and found the number of pERK$^+$ neurons increased in ChR2 but not the EYFP group (**Figs. 2B and C**). We next investigated whether the optogenetic activation of CeA PKCδ$^+$ neurons could induce mechanical hypersensitivity under blue-light stimulation. Blue-light stimulation for 10 minutes in CeA-PKCδ-ChR2–expressing mice induced prolonged mechanical hypersensitivity that lasted for at least 3 days compared with EYFP control mice (**Fig. 2D**). Therefore, the activation of CeA PKCδ$^+$ neurons promoted the development of mechanical hypersensitivity.

3.3. Inhibition of protein kinase Cδ neurons in central amygdala attenuates mechanical hypersensitivity in the formalin-induced inflammatory pain model

Next, we asked whether inhibiting CeA PKCδ$^+$ neurons could attenuate mechanical hypersensitivity induced by formalin injection. We used the chemogenetic technique of designer receptors exclusively activated by designer drugs (DREADDs) to inhibit CeA PKCδ$^+$ neuronal activity remotely by using the AAV8-hSyn-DIO-hM4D-EYFP (hM4D) vector. We conducted the same experiments using a control eYFP vector (AAV5-EF1a-DIO-EYFP) to control for any off-target effect of CNO. We first confirmed hM4D is functional in brain slices. The spontaneous action potential showed slow inhibition after 10 μM CNO perfusion in hM4d$^+$ neurons (**Fig. 2E**). At 4 weeks after infection, administration of CNO (3 mg/kg, i.p.) immediately after intraplantar injection of formalin led to a transient reduction of mechanical hypersensitivity at 4 hours after formalin injection in the hM4D group (**Fig. 2F**). Administration of CNO had no effect on the mechanical hypersensitivity induced by formalin injection in the control EYFP group (**Fig. 2F**). The number of CeA pERK$^+$ neurons decreased at 90 minutes after CNO injection in hM4D as compared with the EYFP group (**Figs. 2G and H**). These results suggest that the PKCδ$^+$ neuronal activity in CeA participates in regulating prolonged hypersensitivity development.

3.4. Inhibition of central amygdala somatostatin neurons induces mechanical hypersensitivity

Protein kinase Cδ neurons are known to be regulated by the local inhibition of SOM$^+$ neurons in CeA. Optical activation of SOM$^+$ neurons increased the inhibitory control of PKCδ$^+$ neurons.[22] Therefore, we hypothesized that the manipulation of SOM$^+$ neuronal activity might also regulate the mechanical sensitivity of animals using PKCδ$^+$ neurons. To test this, we expressed Cre-dependent NpHR (AAV-EF1a-DIO-eNpHR3.0-EYFP) in the CeA of a transgenic SOM-IRES-Cre mouse line and confirmed it is functional. Yellow-light stimulation induced transient photocurrents in CeA SOM$^+$ neurons expressing eNpHR3.0 and continuously

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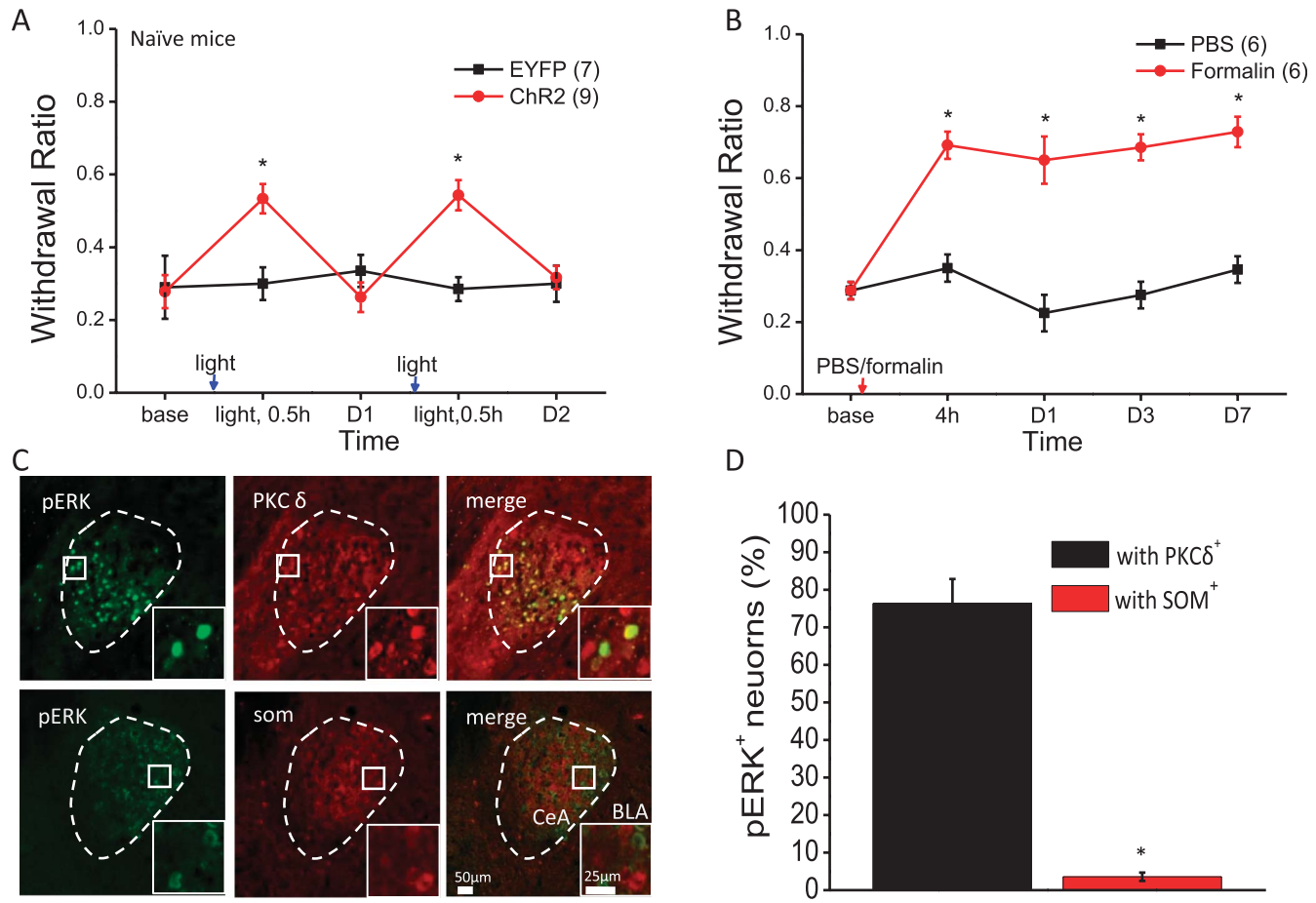

Figure 1. Activation of CeA neurons induce mechanical hypersensitivity in naïve mice, and CeA-PKCδ$^+$ neurons are activated by intraplantar injection of 5% formalin. (A) Mechanical responses with the optogenetic activation of CeA neurons (EYFP groups, n = 7, ChR2 groups, n = 9). (B) Mechanical responses after intraplantar injection of 5% formalin or PBS injection (PBS groups, n = 6, Formalin groups, n = 6). (C) Immunoreactivity of pERK and PKCδ or somatostatin in CeA after intraplantar injection of 5% formalin at 90 minutes and higher magnification (box area). (D) Quantification of colocalized pERK$^+$ PKCδ neurons or pERK$^+$ somatostatin (SOM) neurons (n = 3). (A and B) Blue arrow indicates the light stimulation time. Red arrow indicates the drug infusion time. *$P < 0.05$. CeA, central amygdala; ChR2, channelrhodopsin-2; PBS, phosphate-buffered saline; pERK$^+$, phosphorylated ERK; PKCδ$^+$, protein kinase Cδ.

photostimulation led to a sustained hyperpolarization (**Fig. 3A**). We next investigated whether optogenetic inhibition of CeA SOM$^+$ neurons had any effect on the mechanical sensitivity of naïve mice. Yellow-light stimulation of CeA-SOM-NpHR–expressing mice for 10 minutes induced mechanical hypersensitivity that lasted for at least 3 days compared with EYFP control mice (**Fig. 3B**). We examined the expression of pERK at 30 minutes after yellow-light stimulation and found few pERK$^+$ neurons in the EYFP group; by contrast, a significant number of pERK$^+$ neurons were detected in the NpHR group (**Figs. 3C and D**). When we costained these sections with anti-PKCδ antibody, ~70% of pERK$^+$ neurons were also PKCδ$^+$ (**Figs. 3C and E**). Therefore, the inhibition of CeA SOM$^+$ neurons induced prolonged mechanical hypersensitivity, possibly by activating PKCδ$^+$ neurons.

3.5. Activation of central amygdala somatostatin neurons has no effect on the mechanical sensitivity in naïve mice but slightly attenuates mechanical hypersensitivity in the formalin-induced inflammatory pain model

Next, we asked whether the activation of CeA SOM$^+$ neurons could affect the mechanical sensitivity of animals in the naïve and formalin-induced inflammatory pain model. We expressed AAV-EF1a-DIO-hChR2-EYFP in the CeA of a transgenic SOM-IRES-Cre mouse line. Blue-light stimulation induced inward currents and action potential in CeA SOM$^+$ neurons (**Fig. 4A**), which indicates functional expression of ChR2. We examined the expression of pERK at 30 minutes after blue-light stimulation and found few pERK$^+$ neurons after the activation of CeA SOM$^+$ neurons (data not shown). We also checked the expression of c-fos and found increased c-fos$^+$ cells after 10 minutes blue-light stimulation in the ChR2 vs EYFP group at 30 minutes after light stimulation (**Fig. 4B**), which indicates successful activation of SOM$^+$ neurons. Next, we found no effect on the mechanical sensitivity after 10 minutes of optogenetic activation of CeA SOM$^+$ neurons in naïve mice (**Fig. 4C**). We also optogenetically activated CeA SOM$^+$ neurons for 10 minutes and examined the effect on mechanical sensitivity in the formalin-induced inflammatory pain model. Activation of CeA SOM$^+$ neurons slightly reduced the mechanical hypersensitivity induced by formalin (**Fig. 4D**). These results suggest that CeA SOM$^+$ neurons could negatively regulate the mechanical hypersensitivity.

3.6. Activation of central amygdala protein kinase Cδ neurons has no effect on anxiety-like behavior

Central amygdala is involved in ALB.[3] However, the specific neuronal types involved in ALB are still controversial.[1,3,6,9] To answer this, we optogenetically activated PKCδ$^+$ and SOM$^+$ neurons and examined their effects on ALB in mice. We used 3 behavioral tests OFT, EPM, and LDB to assess ALB in mice.[56] We

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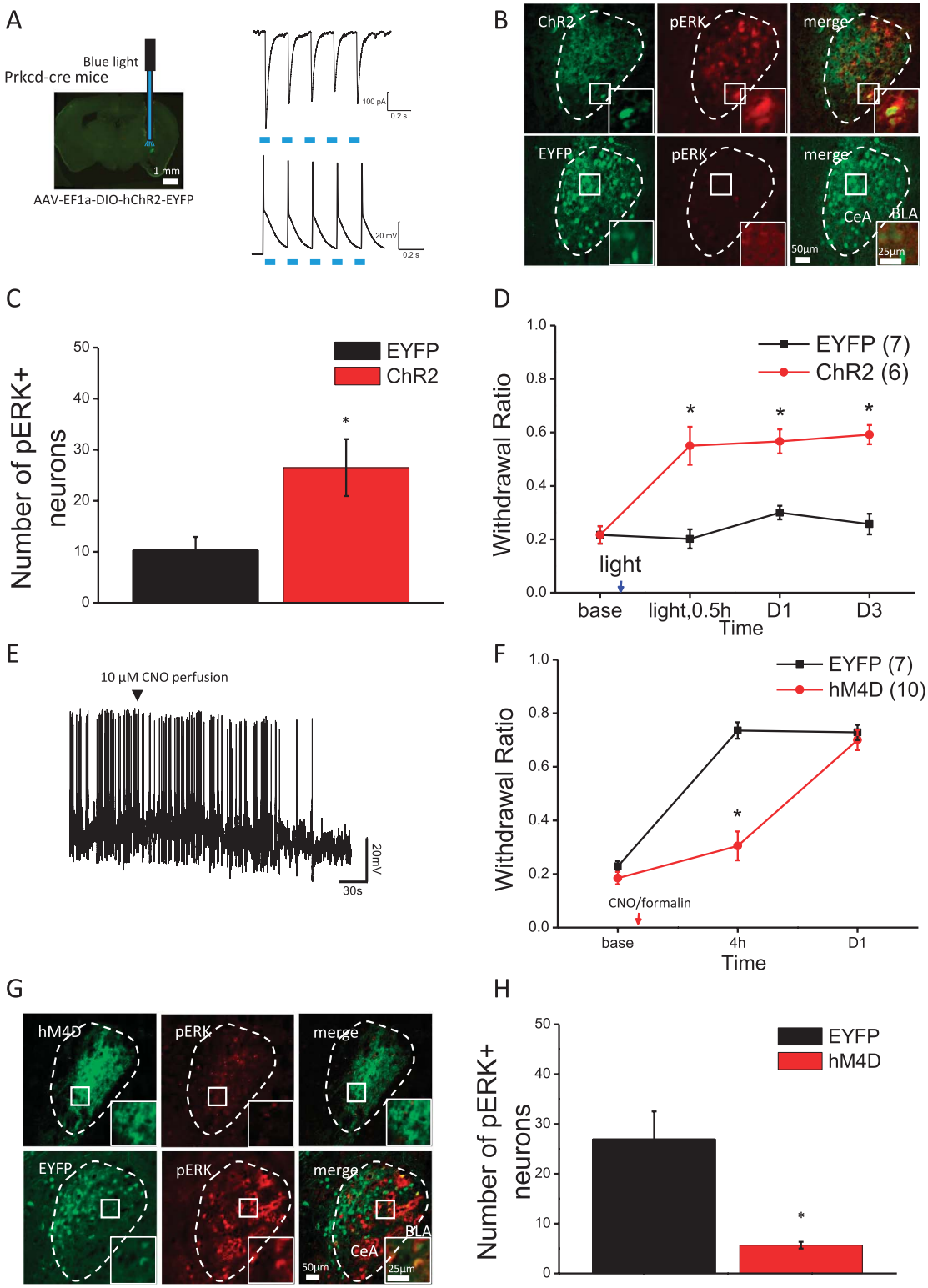

Figure 2. PKCδ$^+$ neurons are involved in chronic pain development. (A) Diagram illustrating the optogenetic activation experiments. ChR2 was expressed in CeA-PKCδ$^+$ neurons. The blue light is delivered to the CeA. Inward currents and action potential elicited by blue-light stimulation (5 Hz, 10 ms pulse) from PKCδ$^+$ neurons. (B) Prkcd-cre mice expressed hChR2 or EYFP were sacrificed at 30 minutes after blue-light stimulation and costained with pERK. Higher magnification (box area). (C) Quantification of pERK$^+$ neurons for EYFP or PKC-ChR2 groups (n = 3). (D) Mechanical responses with the optogenetic activation of CeA-PKCδ$^+$ neurons. (EYFP groups, n = 7 and ChR2 groups, n = 6). (E) Spontaneous action potentials recorded from hM4D-mCherry–positive neurons with CNO bath perfusion. (F) Mechanical responses with chemogenetic silencing of PKCδ$^+$ neurons after intraplantar injection of 5% formalin (EYFP groups, n = 7 and hM4d groups, n = 10). (G) Brain slices with PKCδ$^+$ neurons expressing hM4d or EYFP costained with pERK after formalin injection at 90 minutes. (H) Quantification of pERK$^+$ neurons of EYFP or PKC-hM4D groups (n = 3). (D and F) Blue arrow indicates the light stimulation time. Red arrow indicates the drug infusion time. *$P <$ 0.05. CeA, central amygdala; ChR2, channelrhodopsin-2; pERK$^+$, phosphorylated ERK; PKCδ$^+$, protein kinase Cδ.

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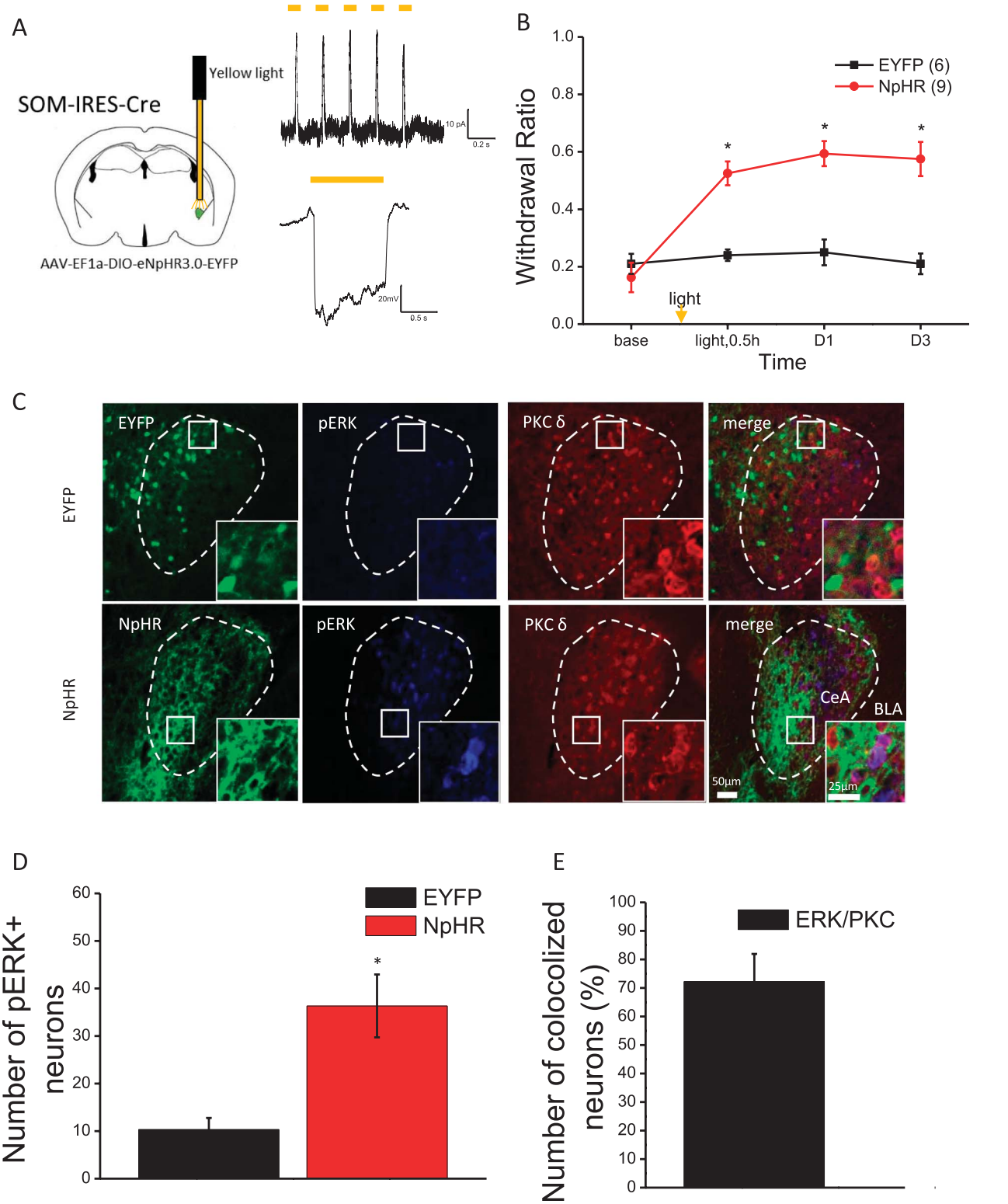

Figure 3. Inhibition of SOM$^+$ neurons induced chronic mechanical hypersensitivity. (A) Brain slice recordings from SOM$^+$ neurons expressing eNpHR3.0. Yellow light induced photocurrent from SOM$^+$ neurons (voltage clamp) and membrane hyperpolarization (current clamp). (B) Mechanical responses with optogenetic inhibition of CeA-SOM$^+$ neurons in naïve mice (EYFP groups, n = 6 and NpHR groups, n = 9). (C) SOM-IRES-Cre mice expressed eNpHR3.0 or EYFP were sacrificed at 30 minutes after yellow-light stimulation and costained with pERK and PKCδ. Higher magnification (box area). (D) Quantification of pERK$^+$ neurons of EYFP or NpHR groups (n = 3). (E) Quantification of colocalized pERK and PKCδ neurons (n = 3). (B) Yellow arrow indicates the light stimulation time. *$P < 0.05$. CeA, central amygdala; pERK$^+$, phosphorylated ERK; PKCδ$^+$, protein kinase Cδ; SOM$^+$, somatostatin.

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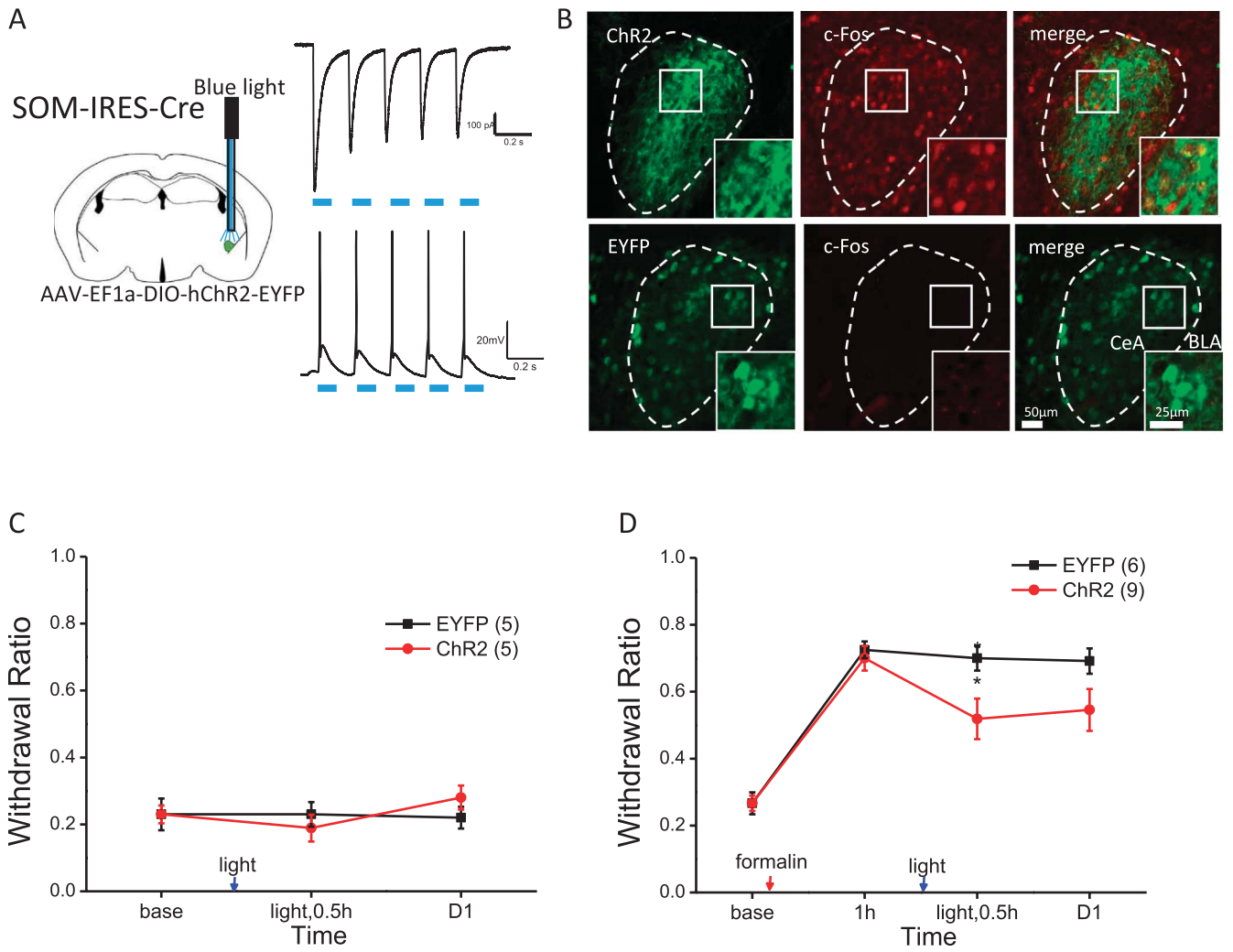

Figure 4. Activation of SOM$^+$ neurons slightly reduced mechanical hypersensitivity in mice. (A) Brain slice recordings from SOM$^+$ neurons expressing ChR2. Inward currents and action potential elicited by blue-light stimulation (5 Hz, 10 ms pulse) from SOM$^+$ neurons. (B) SOM-IRES-Cre mice expressed hChR2 or EYFP were sacrificed at 30 minutes after blue-light stimulation and costained with c-Fos. Higher magnification (box area). (C) Mechanical responses with the optogenetic activation of CeA-SOM$^+$ neurons (EYFP groups, n = 5 and ChR2 groups, n = 5). (D) Mechanical responses with the optogenetic activation of CeA-SOM$^+$ neurons after intraplantar injection of 5% formalin (EYFP groups, n = 6 and ChR2 groups, n = 9). (C and D) Blue arrow indicates the light stimulation time. Red arrow indicates the drug infusion time. CeA, central amygdala; ChR2, channelrhodopsin-2; SOM$^+$, somatostatin.

optogenetically activated PKCδ$^+$ neurons in the transgenic Prkcd-cre mouse line and simultaneously conducted behavioral tests in naïve animals. The ChR2 and EYFP groups did not differ in time spent in the center, total distance, and speed of movement during the 10-minute OFT (**Fig. 5A**). The groups did not differ in time spent in the open and closed arms, but time spent in the central region was significantly reduced in the ChR2 vs EYFP group during the 5-minute EPM test (**Fig. 5B**). The groups did not differ in time spent in the light or dark box during the 10-minute LDB test (**Fig. 5C**). These results suggest that CeA PKCδ$^+$ neurons most likely are not involved in ALB.

3.7. Activation of central amygdala somatostatin neurons induces anxiety-like behavior

Next, we optogenetically activated SOM$^+$ neurons in the transgenic SOM-IRES-Cre mouse line and simultaneously conducted behavioral tests in naïve animals. In contrast to mice with PKCδ$^+$ neurons activated, the activation of SOM$^+$ neurons significantly changed the mouse behavior. When SOM$^+$ neurons were activated, mice showed a significant reduction in time spent in the center, total distance, and speed of movement during the 10-minute OFT (**Fig. 6A**). Mice stayed longer in the closed arm and spent less time in the open arm and central region in the ChR2 vs EYFP group during the 5-minute EPM test (**Fig. 6B**). Mice also stayed longer in the dark box in the ChR2 vs EYFP group during the 10-minute LDB test (**Fig. 6C**). These results showed that the activation of CeA SOM$^+$ neurons induces ALB.

To study the role of SOM$^+$ neurons in an anxiety-like behavior model, we used the restraint stress-induced anxiety model.[18] We expressed AAV8-hSyn-DIO-hM4D-EYFP (hM4D) vector or EYFP control vector in the CeA of a transgenic SOM-IRES-Cre mouse line. The mice were subjected to restraint stress for 30 minutes in a restrainer.[61] Then, 1 day after the restraint stress, the mice received the CNO (3 mg/kg, i.p.) injection 2 hours before the behavior test (**Fig. 7A**). The EYFP group showed a significant reduction in the time spent in the center during the 10-minute OFT (**Fig. 7B**) but no difference in the EPM and LDB tests (**Figs. 7C and D**). However, the restraint stress-induced reduction in the time spent in the center in the OFT was abolished in the hM4d group (**Fig. 7B**). These data indicate that the inhibition of the SOM$^+$ neurons could reverse restraint stress-induced ALB in mice.

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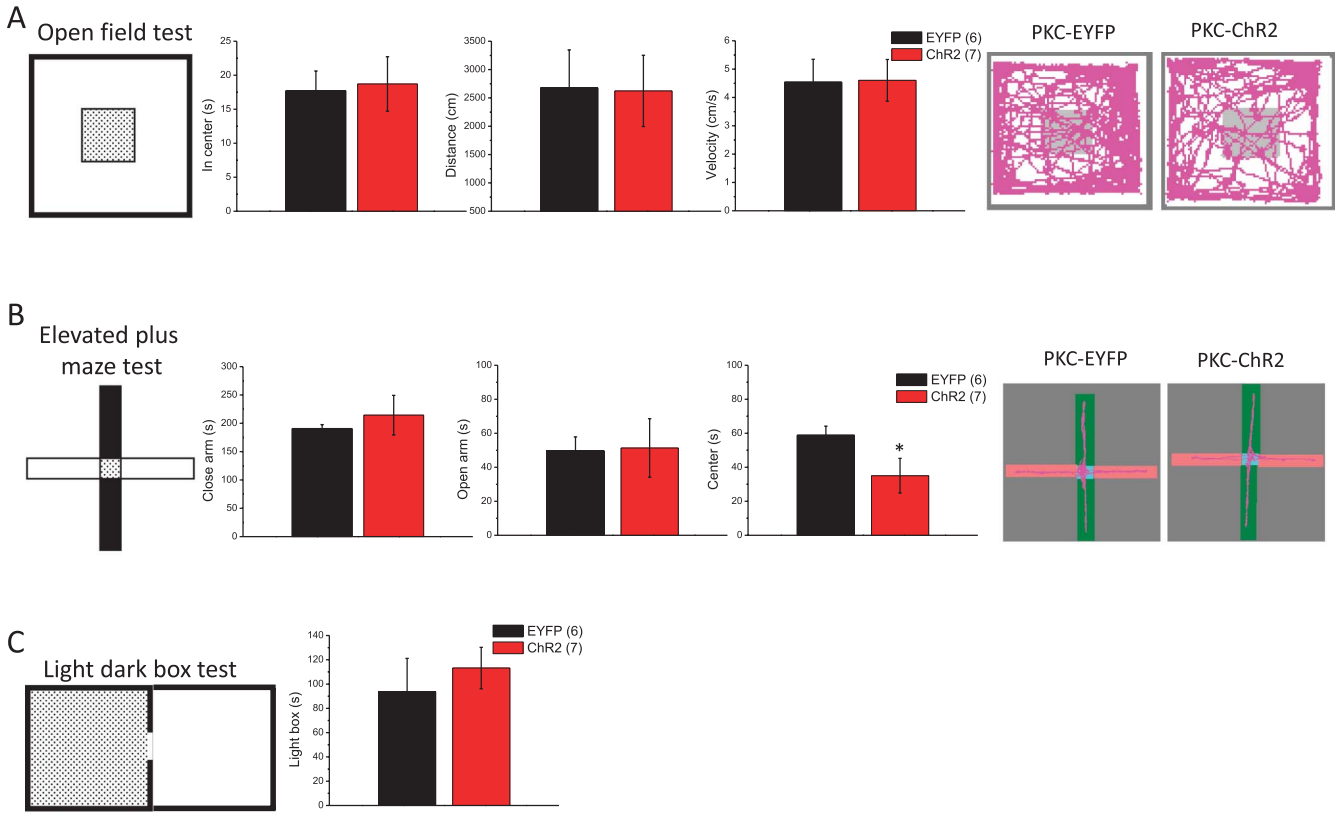

Figure 5. Activation of PKCδ$^+$ neurons does not directly alter mice anxiety-like behavior. Optogenetic activation of PKCδ$^+$ neurons during behaviors. (A) Open field test and quantification of behavioral parameters; black spot indicates the central area. (B) Elevated plus maze test and quantification of behavioral parameters; black bar indicates the closed arm, white bar indicates the open arm, and black spot indicates the central area. (C) Light dark box test and quantification of behavioral parameters; the left square indicates the dark box and the right square indicates the light box. EYFP groups, n = 6 and ChR2 groups, n = 7, *$P < 0.05$. PKCδ$^+$, protein kinase Cδ.

3.8. Formalin-induced inflammatory pain model does not evoke anxiety-like behavior in mice

We showed that CeA PKCδ$^+$ but not SOM$^+$ neurons were activated in the formalin-induced inflammatory pain model, and inhibition of PKCδ$^+$ neurons attenuated the mechanical hypersensitivity induced by formalin injection. We also demonstrated that the activation of CeA SOM$^+$ but not PKCδ$^+$ neurons induced ALB. We hypothesized that if formalin injection mainly activated PKCδ$^+$ but not SOM$^+$ neurons in CeA, formalin injection might induce only mechanical hypersensitivity but not ALB. To test this, we examined whether mice would develop ALB at 4 hours and 1 day after formalin injection. The PBS and formalin groups did not differ in time spent in the center, total distance, and speed of movement during the 10-minute OFT (**Fig. 8A**). For the EPM test, the groups did not differ in time spent in the open and closed arms at 4 hours but the formalin group showed a significant reduction in time spent in the center region at 1 day after formalin injection (**Fig. 8B**). The groups did not differ in time spent in the light or dark box during the 10-minute LDB test at both times (**Fig. 8C**). Hence, formalin-induced hypersensitivity did not directly alter mouse ALB at the times examined.

4. Discussion

The amygdala is involved in emotional responses, fear, depression, anxiety, and pain modulation.[3,11,32,45,54,55] The CeA receives nociceptive signaling by the spino-parabrachial (PB)-amygdala[5,7] or direct synaptic input from the BLA or thalamus.[27] Also, CeA directly projects to the bed nucleus of the stria terminalis (BNST), paraventricular thalamus, and periaqueductal gray.[2,42] Previously, we showed that the projection from the amygdala to the anterior part of the paraventricular thalamus is also involved in the pain circuit.[12] In this study, we demonstrated that 2 major population neurons in the CeA, SOM$^+$ and PKCδ$^+$, exert different functions in mediating pain-like and anxiety-like behaviors in mice. Activation of ERK in CeA mostly occurred in PKCδ$^+$ neurons after formalin injection. Activation of PKCδ$^+$ neurons for 10 minutes in naïve mice evoked prolonged mechanical hypersensitivity. Conversely, inhibition of PKCδ$^+$ neurons significantly reduced formalin-induced hypersensitivity transiently. However, activation of PKCδ$^+$ neurons did not affect ALB in mice. Furthermore, SOM$^+$ neurons in CeA had an inhibitory influence on the activity of PKCδ$^+$ neurons; inhibition of SOM$^+$ neurons for 10 minutes activated ERK in PKCδ$^+$ neurons and eventually led to mechanical hypersensitivity. By contrast, activation of SOM$^+$ neurons for 10 minutes slightly reduced mechanical hypersensitivity transiently in the formalin-induced inflammatory pain model. However, activation of SOM$^+$ neurons clearly induced ALB in mice. Finally, the formalin-induced inflammatory pain model did not significantly change ALB in mice.

Recently, with the advance of technologies such as optogenetics, chemogenetics, genetic tools, and neural circuit tracing, one can map the anatomical connections of specific cell types and link these connections to behavioral changes.[19,31,38] Use of electrophysiology and immunohistochemistry markers revealed 2 major subpopulations of neurons in CeA with opposing functions that inhibit one another: The PKCδ$^+$ neurons are inhibited by conditioned stimulus (CS) after fear conditioning (CeL$_{off}$ cells) and

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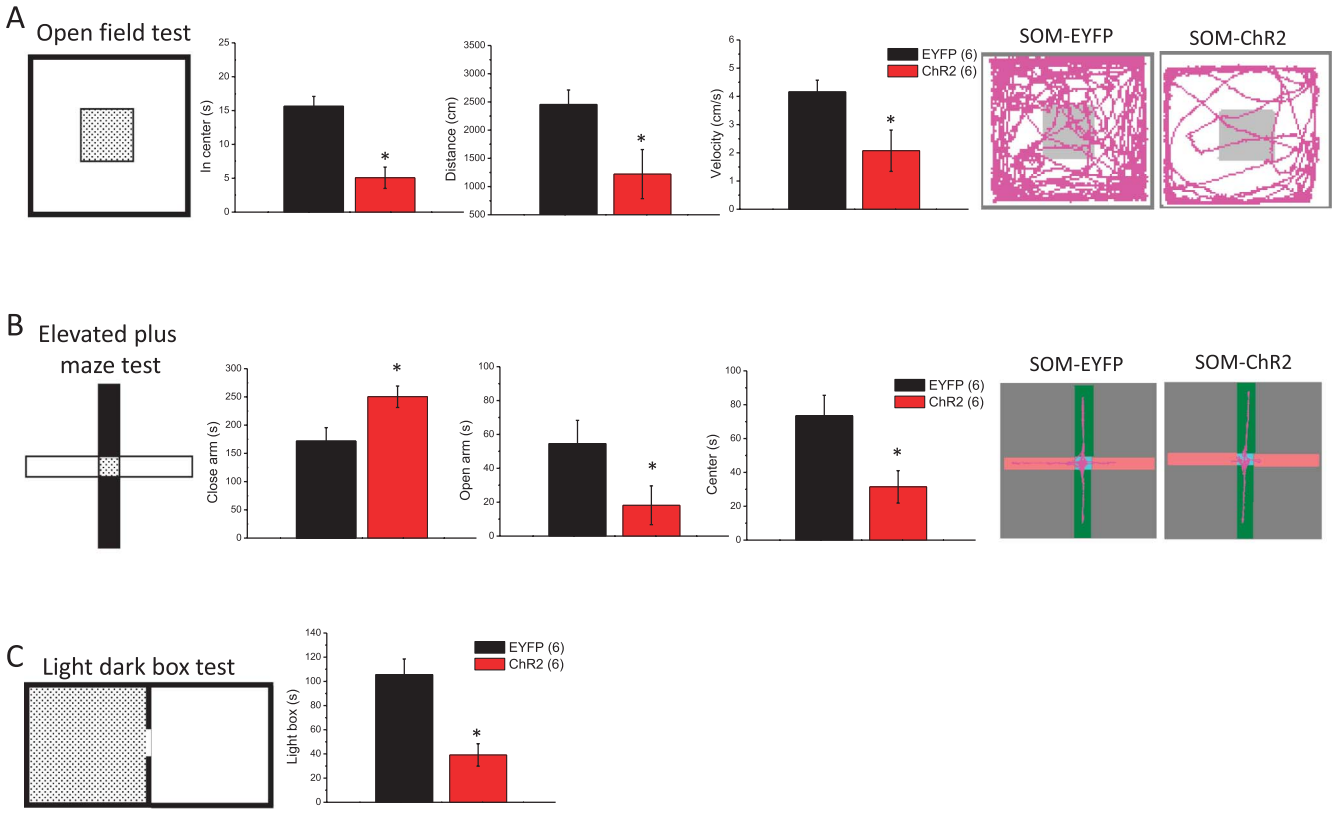

Figure 6. Activation of SOM$^+$ neurons increased anxiety-like behavior in mice. Optogenetic activation of SOM$^+$ neurons during behaviors. (A) Open field test and quantification of behavioral parameters; black spot indicates the central area. (B) Elevated plus maze test and quantification of behavioral parameters, black bar indicates the close arm, white bar indicates the open arm, and black spot indicates the central area. (C) Light dark box test and quantification of behavioral parameters, the left square indicates the dark box and the right square indicates the light box. EYFP groups, n = 6 and ChR2 groups, n = 6, $^*P < 0.05$. ChR2, channelrhodopsin-2; SOM$^+$, somatostatin.

CeL_{on} cells are predominantly SOM$^+$ neurons activated by CS after fear conditioning.[22] Also, a smaller population of neurons expressing CRF in the CeA is also critical for discriminative fear.[46] A recent study shows that the chemogenetic activation of GABAergic neurons in CeA could elicit mechanical hypersensitivity and lasted less than 1 day.[48] In our hand, we also found that nonselective activation of CeA neurons could elicit a transient mechanical hypersensitivity that lasted less than 1 day (**Fig. 1A**). However, specific activation of PKCδ$^+$ neurons for 10 minutes induced prolonged hypersensitivity (**Fig. 2D**). When we activated the CeA neurons globally for 10 minutes, both PKCδ$^+$ and SOM$^+$ neurons could be activated, which may cause the local inhibition of the PKCδ$^+$ neurons by SOM$^+$ neurons. Thus, only a transient pain response was induced. So how can a transient activation of PKCδ$^+$ neurons induce prolonged hypersensitivity? LTP is induced in the PB to CeA synapses in the inflammation or neuropathic pain model.[24,47] It is possible that 10 minutes light stimulation of PKCδ$^+$ neurons elicits the LTP formation in CeA or downstream circuits and thus causes prolonged hypersensitivity. Direct activation of PKCδ$^+$ neurons supersedes any inhibitory effects of SOM$^+$ activation, possibly because they are downstream of SOM$^+$ neurons. Of note, inhibition of PKCδ$^+$ neurons reduced only the mechanical hypersensitivity transiently in the formalin-induced inflammatory pain model (**Fig. 2F**), which suggests that short-term inhibition of PKCδ$^+$ neurons is inefficient to prevent formalin-induced chronic pain. The results also indicate CeA neurons have bidirectional roles in mediating the pain response.

It is still unclear how PKCδ neurons are activated in pathological pain conditions. Based on our and others' studies, it is most likely that there is a well-balanced local inhibitory network between PKCδ$^+$, SOM$^+$, CRF$^+$, and other CeA neurons in health conditions. The endogenous calcitonin gene–related peptide (CRGP) is involved in the PB-CeA synaptic plasticity in the inflammation pain model,[47] and the PKCδ$^+$ cells in CeA are surrounded by the CGRP$^+$ terminals.[57] The PB CGRP$^+$ neurons may modulate the CeA PKCδ$^+$ neurons activity in the inflammation model. We also found that the inhibition of SOM$^+$ neurons for 10 minutes induced prolonged hypersensitivity (**Fig. 3B**) and increased ERK activity in PKCδ$^+$ neurons. This result implies that PKCδ$^+$ neurons could be tonically and spontaneously active and continuously receiving inhibitory inputs from SOM$^+$ neurons in naïve animals. Direct inhibition of SOM$^+$ neurons bypasses the peripheral signal input to activate the PKCδ$^+$ neurons and leads to hypersensitivity.

The ERK signal pathway is involved in both acute and chronic pain development.[11,12,14,30] Extracellular signal–regulated kinase activity in the spinal cord dorsal horn is involved in hyperalgesic priming formation and pain hypersensitivity.[14,30] Also, increased ERK activity in the CeA could enhance synaptic transmission, which then leads to central sensitization and behavior hypersensitivity.[11,16] In this study, we demonstrated that ERK was activated in the CeA in the formalin-induced inflammatory pain model and about 70% of pERK$^+$ neurons were PKCδ$^+$ neurons; few were SOM$^+$ neurons. Some pERK$^+$ neurons were neither SOM$^+$ nor PKCδ$^+$. This group may also play a different role in pain modulation in CeA. Furthermore, direct activation of CeA neurons with optogenetics activated ERK in PKCδ$^+$ but not SOM$^+$ neurons. Also, our study reveals that optogenetic

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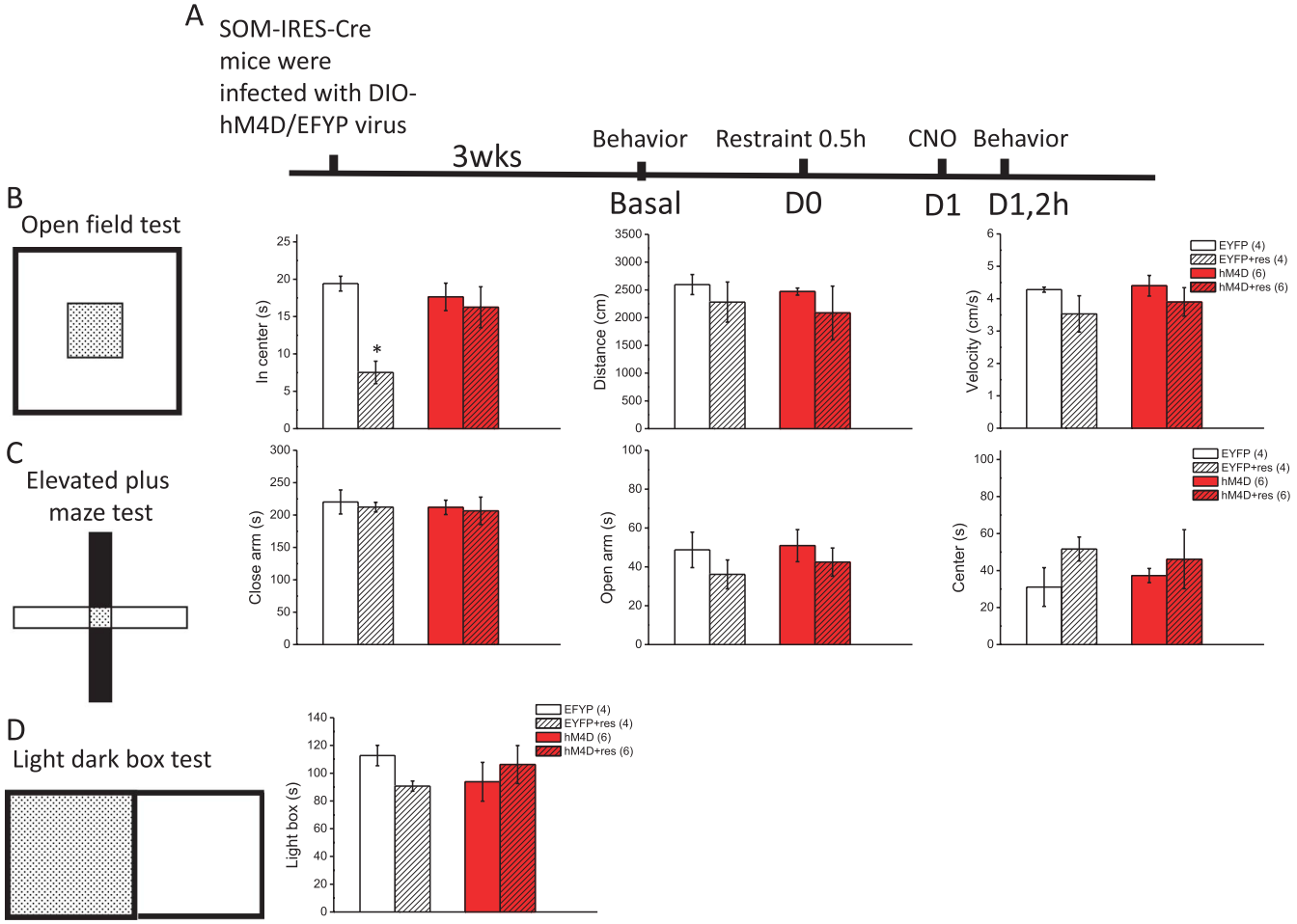

Figure 7. Inhibition of the SOM$^+$ neurons decreased restraint stress evoked anxiety-like behavior in mice. (A) Timeline illustrating the restraint stress experiments. 1 day after the restraint stress, the mice received the CNO (3 mg/kg, i.p.) injection 2 hours before the behavior test. (B) Open field test and quantification of behavioral parameters before and after the restraint stress. (C) Elevated plus maze test and quantification of behavioral parameters. (D) Light dark box test and quantification of behavioral parameters. EYFP groups, n = 4 and hM4D groups, n = 6, $^*P < 0.05$. CNO, clozapine N-oxide; SOM$^+$, somatostatin.

activation of SOM$^+$ neurons could induce c-fos expression. These data indicate that the activation of PKCδ$^+$ neurons induces pain-like behavior, whereas the activation of SOM$^+$ neurons induces ALB.

Recently, a study observed that increased CeA PKCδ$^+$ neuron activity at 1 to 2 weeks after cuff-induced neuropathic pain. The authors also showed that the chemogenetic activation of CeA PKCδ$^+$ neurons led to a transient mechanical hypersensitivity in naïve mice. Chemogenetic activation of CeA SOM$^+$ neurons reversed the cuff-induced neuropathic pain.[55] However, another study demonstrated increased CeA SOM$^+$ neuron activity at 6 weeks but not 2 weeks after spared nerve injury–induced neuropathic pain. The authors showed reduced inhibition from the dorsal raphe nucleus 5-hydroxytryptamine projection (5-HT^{DRN})-activated CeA SOM$^+$ neurons, which caused depression-like behavior at 6 weeks after spared nerve injury.[60] Of note, activation of 5-HT^{DRN} inputs reversed the depression-like behavior and attenuated mechanical hypersensitivity at 6 weeks after spared nerve injury. Interestingly, they found a subset of the CeA SOM$^+$ neurons mainly sends glutamatergic projections to the lateral habenula. These results imply that a subset of CeA SOM$^+$ neurons regulates both depression-like behavior and mechanical hypersensitivity.[60] Also, the 5-HT$_{2C}$ receptor activation in the basal lateral amygdala leads to CRF1 activation and increases CeA neuronal activity in the neuropathic pain model.[29] Here, we found that the optogenetic activation of CeA PKCδ$^+$ or inhibition of SOM$^+$ neurons led to prolonged but not transient hypersensitivity in naïve mice. Optogenetic activation of SOM$^+$ neurons induced ALB in naïve mice but only slightly reduced the mechanical hypersensitivity in the formalin-induced inflammatory pain model. The discrepancy between these studies may be due to differences in techniques used to activate neurons, pain models, neuronal heterogeneity, and the behavior time point. Recently, in vivo calcium imaging is used to link the neuronal activities to aspects of behavior and cognition in free-moving animals.[17,58] A study using in vivo calcium imaging showed that a distinct BLA neuron population is involved in pain affective-motivational behaviors in the neuropathic pain model.[17] Future work combining different techniques to evaluate the neuronal circuitry and function in free-moving animals will be required to fully understand the role of the amygdala in pain and emotional behaviors.

It has been shown that stress could elicit the pain relief by regulating the endogenous opioid peptides, monoamine, cannabinoid, g-aminobutyric acid, and glutamate systems.[8,41] Interestingly, acute and chronic restraint stress produces analgesia in the formalin model and hyperalgesia in naive mice, respectively.[25,26,35] In our hand, we did not observe altered mechanical sensitivity in naïve mice after restraint stress. However, we found that the 30 minutes restraint stress-induced

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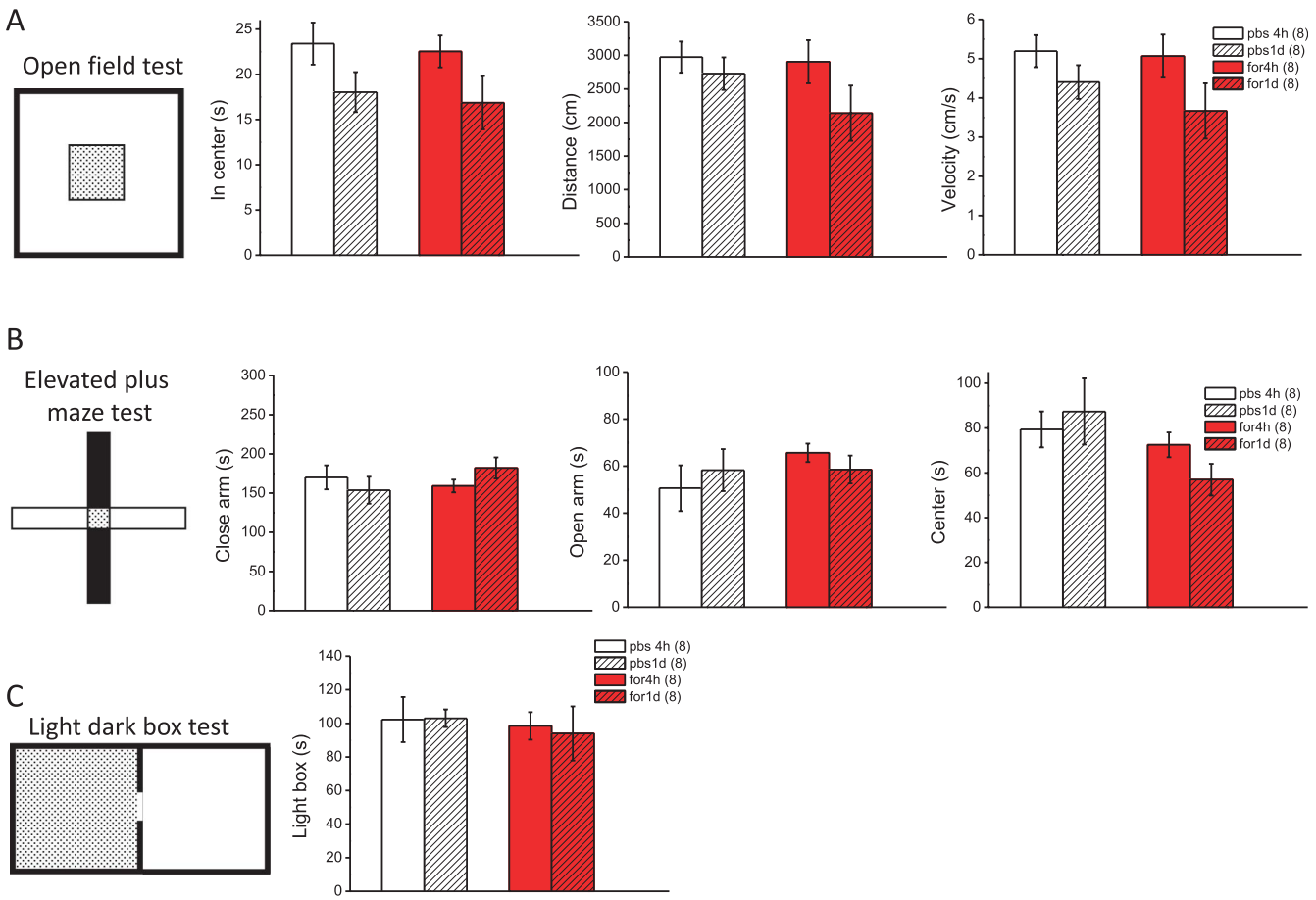

Figure 8. Formalin-induced inflammatory pain model does not increase anxiety-like behavior in mice. (A) Open field test and quantification of behavioral parameters; black spot indicates the central area. (B) Elevated plus maze test and quantification of behavioral parameters; black bar indicates the closed arm, white bar indicates the open arm, and black spot indicates the central area. (C) Light dark box test and quantification of behavioral parameters; the left square indicates the dark box and the right square indicates the light box. PBS groups, n = 8 and formalin groups, n = 8, $^{*}P < 0.05$. PBS, phosphate-buffered saline.

anxiety behavior could be attenuated by the inhibition of CeA SOM^{+} neurons activity. Also, we found that the activation of SOM^{+} neurons slightly reduces mechanical hypersensitivity in mice. Thus, it is conceivable that the analgesic effect induced by acute restraint stress in the formalin model is mediated by activating the SOM+ neurons in CeA. The CeA is involved in various emotional behaviors, including fear, anxiety, and depression.[6,22,52] Depending on the experimental setting, $PKC\delta^{+}$ neurons in CeL are reported to be anxiogenic or anxiolytic.[6,9] Also, increased excitatory synaptic inputs into SOM^{+} neurons through the CeA-BNST circuit leads to abnormal anxiety behavior.[1] In our study, the activation of $PKC\delta^{+}$ neurons did not alter ALB in mice, but the activation of SOM^{+} neurons had a significant impact on ALB. Furthermore, in the pain model, 70% ERK^{+} neurons colocalized with $PKC\delta^{+}$ neurons. These data support the notion that $PKC\delta^{+}$ and SOM^{+} neurons in CeA exert different functions in regulating the pain-like and anxiety-like behaviors. However, the clinical study still shows a high association between chronic pain and anxiety. Long-term chronic pain may cause emotional effects, increased stress, sleep disturbance, and impaired quality of life, etc., which may lead to anxiety and depression.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B420.

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