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Persistent pain intensifies recall of consolidated fear memories

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A B S T R A C T

Ensembles of principal neurons in the basolateral amygdala (BLA) generate the initial engrams for fear memories, while projections from the BLA to the medial prefrontal cortex (mPFC) are essential for the encoding, transfer and storage of remote fear memories. We tested the effects of chronic pain on remote fear memories in mice.

Male mice underwent classic fear conditioning by pairing a single tone (conditional stimulus, CS) with a single electric foot shock (unconditional stimulus, US). Sciatic nerve constriction was used to induce neuropathic pain at various time points before or after the fear conditioning. The mice with sciatic nerve cuffs implanted 48 h after the fear conditioning showed an increased freezing response to CS when compared to mice without cuffs or when compared to mice in which the nerve cuffing was performed 48 h before the fear conditioning. The enhancing effect of pain on consolidated fear memory was further tested and mice in which the nerve cuffing was performed 14 days after the fear conditioning also showed an increased fear response when tested 56 days later.

We used immunostaining to detect morphological changes in the BLA that could suggest a mechanism for the observed increase in fear response. We found an increased number of calbindin/parvalbumin positive neurons in the BLA and increased perisomatic density of GAD65 on projection neurons that connect BLA to mPFC in mice with nerve cuffs. Despite the strong increase of c-Fos expression in BLA and mPFC that was induced by fear recall, neither the BLA to mPFC nor the mPFC to BLA projection neurons were activated in mice with nerve cuffs. Furthermore, non-injured mice had an increased fear response when BLA to mPFC projections were inhibited by a chemogenetic method.

In conclusion, this study provides evidence that persistent pain has a significant impact on consolidated fear memories. Very likely the underlying mechanism for this phenomenon is increased inhibitory input onto the BLA to mPFC projection neurons, possibly from neurons with induced parvalbumin expression. Conceivably, the increased fear response to consolidated fear memory is a harbinger for the later development of anxiety and depression symptoms associated with chronic pain.

1. Introduction

Studies in rodents have shown that ensembles of principal neurons in the BLA generate the initial engrams for fear memories (Kitamura et al., 2017). The memory information is then transferred by BLA projection neurons to the mPFC, where the engrams for long-term memories are stored (Kitamura et al., 2017). The mPFC controls both amygdala-dependent fear expression and the extinction of aversive memories (Sierra-Mercado et al., 2011) via its reciprocal connections with the BLA. With respect to fear expression, BLA principal neurons can be divided into two functionally distinct, non-overlapping populations. Activation of “fear” neurons is triggered by the conditioned stimulus, while “extinction” neurons become active only after repetitive presentations of the conditioned stimulus that are not followed by the unconditioned stimulus (Herry et al., 2008). Both types of neurons project to the mPFC but only extinction neurons receive reciprocal input from the mPFC, which makes their activity susceptible to mPFC modulation (Herry et al., 2008). During recall of remote aversive memories, activation of memory engrams in the mPFC and subsequently in the BLA is funneled via the central amygdala (CeA) to brainstem regions, which trigger a freezing response (Kitamura et al., 2017). In addition to long-term aversive memories, the mPFC regulates an array of other cognitive functions including attention, response inhibition and working memory and it exerts a top-down control over numerous subcortical regions including the amygdala. Amygdala-dependent Pavlovian fear conditioning is one of the most widely used behavior paradigms in the laboratory for studying the neurobiology of aversive memories.

The fear-conditioning test relies on freezing, a natural response of rodents to danger. The amplitude and duration of this freezing response can be modulated simply by manipulating experimental conditions. The malleability of the fear response is shown by laboratory protocols that elicit phenomena such as fear incubation, fear generalization, fear habituation and stress-enhanced fear (Fanselow, 1980; Kamprath and Wotjak, 2004; Poulos et al., 2015, 2016). These fear-conditioning models allow for investigation of mechanisms that may underlie many clinically important anxiety disorders. However, all of these models rely on forward causation, in which altered conditioning parameters lead to changes in an animal's behavioral response. Laboratory models that rely on retrograde effects or where perturbations such as stressful events

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alter the manifestation of previously consolidated aversive memories have not been used very much. Chronic pain is a good candidate for such a perturbation because it is a well studied physical stressor that can be timed precisely, and leads to complex and profound changes in animal and human behavior, cognitive abilities and memories (Berryman et al., 2013; Mutso et al., 2012). Pain associated changes in behavior and cognitive performance are rooted in the plasticity changes that are caused by the condition (Ji et al., 2010). In the amygdala, the net result is neuronal hyperactivity while neuronal deactivation occurs in the mPFC, and is suspected to underlie the cognitive impairment observed in patients with chronic pain (Ji et al., 2010; Thompson and Neugebauer, 2017). However, how the pain-induced overactivation of the amygdala and concurrent deactivation of the mPFC affects already consolidated aversive memory engrams is not currently known. We hypothesized, based on the robust changes in the amygdala and the mPFC associated with chronic pain that pain will affect the recall of fear memories by interfering with the activity of the reciprocal connections between the BLA and mPFC.

We investigated the effects of sciatic nerve constriction, which produces a pain-related state that includes mechanical allodynia, thermal hypersensitivity and anxiodepressive-like behavior, on consolidated fear memories in mice. First, we show that nerve constriction changes the expression of long-term fear memories and second, we describe associated morphological changes in BLA inhibitory circuitry that affect the BLA projections to mPFC, which control the recall of remote aversive memories. Finally, we used chemogenetics to replicate the effects of nerve injury on consolidated fear memories in mice without pain.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice of 7–8 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME) and were group housed at 10/14-h light/dark cycle. The surgeries and behavior experiments were carried out in accordance to the Guide for the Care and Use of Laboratory Animals and the guidelines of the Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science.

2.2. Sciatic nerve constriction

The surgery was done as described previously (Dimitrov et al., 2014). Briefly, the mice were anesthetized and the shaved skin of the upper left thigh was treated with antiseptic. A pair of small forceps was used to expose the main branch of the sciatic nerve and a 4 mm long piece of sterile polyethylene tubing or “cuff” (PE 90, i.d. 86 mm and o.d. 1.27 mm, Becton Dickinson Intramedic, Franklin Lakes, NJ), split lengthwise was placed onto the nerve. The surgical incision was closed with wound clips and analgesia with NSAID was given for the following three days. The sham mice underwent the same general anesthesia as the experimental mice. The upper thigh was shaved and a skin incision was made, but the sciatic nerve was only exposed and not exteriorized. The sham mice received the same pain treatment for three days after the surgery. The development of neuropathic pain was assessed by testing the mice for thermal hypersensitivity with the Hargreaves apparatus (Stoelting, Wood Dale, Il) and mechanical allodynia with von Frey filaments (North Coast Medical, Morgan Hill, CA) every tenth day from the fifth day to the end of the experiments, as described previously (Dimitrov et al., 2014).

2.3. Stereotaxic surgery

The stereotaxic surgeries were done following aseptic technique and under isoflurane anesthesia. After anesthesia induction, the mice were positioned in Stoelting stereotaxic frame and the top of the skull was exposed by a longitudinal incision. A 10-μl gastight Hamilton syringe with a 32-gauge needle was placed into an infusion pump (Microsyringe pump, World Precision Instruments, Sarasota, FL) that was set to inject 200 nl volume at the rate of 1.5 nl per second. A microdrill was used to make 1 mm holes into the skull and the needle was positioned with coordinates for BLA - 1.8 mm, ± 3.0 mm, - 4.5 mm and for mPFC +1.8 mm, ± 0.4 mm, - 2.5 mm. The Cre activated adeno-associated virus AAV-hSyn-DIO-hM3D(Gi)-mCherry (titer ≥ 5.4×10^{12}, UNC Vector core, Chapel Hill, NC) was injected bilaterally into the BLA followed by bilateral injections of rAAV-CAG-eGFP-F2A-Cre, (titer ≥ 1×10^{12}, NINDS Viral Production Core Facility, Bethesda, MD) into the mPFC. The DREADD receptors were activated with Clozapine N-Oxide (CNO) (Tocris, Ellisville, MO), which was injected in dose of 1 mg/kg, i.p. 30 min before testing for fear recall. The control animals were injected into the mPFC with the retrograde AAV-CAG-tdTomato (titer ≥ 7×10^{12}, Addgene, Cambridge, MA). The same virus was also injected into the mPFC and BLA respectively and used for assessment of the activation of the projections from the BLA to the mPFC and from the mPFC to the BLA during fear recall in separate groups of mice, which were fear conditioned (FC) three weeks after the viral injections.

2.4. Fear conditioning

Mice were habituated to the test box (Box A) in three separate sessions, each lasting 180 s. The box was wiped clean with veterinary disinfectant after each animal. The fear conditioning took place in Box B, with different wall patterns, floor, light intensity and background sound from box A, where after 3 min in the box, the mice were exposed for 30 s to a 75 dB, 6 kHz tone, the end of which coincided with a 2 s 1.5 mA electric shock after which the mice were left in the box for another 2 min. The box was cleaned with 70% ethanol after each conditioning instead of veterinary disinfectant. The control animals, sham or cuffed, were also habituated to Box A and spent an equal amount of time in Box B where they were exposed to the CS but did not receive a foot shock. The mice were tested for fear recall in box A, where the CS was presented 3 min after the animal was placed in the box. The mice were left in the box for an additional 5 min so the entire fear recall testing lasted 8 min. The mouse behavior was video recorded and Any-Maze tracking software (Stoelting, Wood Dale, IL) was used to quantify the percent of time the animals were immobile in 30-s bins. The total freezing time in the 5 min after the presentation of the CS was used for statistical analysis.

2.5. Novelty suppressed feeding

Mice were food deprived overnight. The testing arena consisted of an open field box positioned under bright light with 250 Lux intensity. A piece of white paper was placed in the middle of the arena with a single pellet of regular rodent chow on the paper. After an hour of acclimatization to the testing room, the mice were placed in the arena and their behavior was recorded with a ceiling video camera for 10 min. The time until the first bite with an audible crunch was accepted as “Latency to Feed”, while sniffing, pushing the food pellet around or holding it without biting were not considered positive signs. The mice were moved after the test to individual boxes, which contained predetermined amounts of rodent chow. The weight of food consumed was recorded after 1 h in the box and then the mice were returned to their home cages.

2.6. Immunohistochemistry

Mice were deeply anesthetized and perfused transcardially with 0.01M phosphate buffered saline (PBS) followed by perfusion with 4% paraformaldehyde in PBS. The perfusions were timed 1 h after the fear recall experiments. The right side of the brains was marked and the tissue was cut into 40 μm thick coronal sections. The brain sections

were incubated in 3% H_2O_2 for 15 min, washed with PBS, and then incubated in blocking solution (0.05% Triton X-100, 3% normal donkey serum in PBS) for 2 h at room temperature and then the sections were incubated with a primary rabbit antibody against c-Fos (Cell Signaling Technology, Danvers, MA) diluted 1:10K or a rabbit antibody against Kv2.1 (Cedarline Labs, Burlington, NC) diluted 1:5K at 4 °C for 48 h on a rotating platform. Following incubation with the primary antibody, sections for c-Fos and Kv2.1 were incubated with biotinylated secondary antibodies (Jackson ImmunoResearch Inc. West Grove, PA), 1:2 K dilution for 2 h, followed by incubation in avidin-biotin complex (ABC kit, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The fluorescent signal was developed by incubation for 12 min in 20 nmols of tyramide conjugated Alexa Fluor 488 for c-Fos-ir (immunoreactivity) and Alexa Fluor 405 for Kv2.1-ir respectively. For GAD65 staining incubation with a mouse monoclonal antibody (Abcam, Cambridge, MA) diluted 1:2 K was followed by incubation with a secondary antibody conjugated to Alexa 488 diluted 1:400 for 4 h at room temperature. A similar immunostaining protocol was used for visualization of calbindin and parvalbumin in the BLA using rabbit anti-calbindin D28 antibody (Synaptic Systems, Goettingen, Germany) diluted 1:10 K and monoclonal mouse antibody against parvalbumin (Sawnt, Marly, Switzerland) diluted 1:5 K. The next step was incubation with secondary anti-rabbit Alexa 488 and anti-mouse Alexa 594 antibodies (Jackson ImmunoResearch Inc. West Grove, PA) for 4 h at room temperature. The mCherry signal after the activation of the DREADD virus was amplified by immunostaining with chicken anti-mCherry (EnCore Biotechnology, Geinsville, Fl) in dilution 1:2 K and 594 anti-chicken antibody as described above. The viral GFP and tdTomato fluorescencent expression was observed without additional immunostaining.

2.7. Microscopy

An epifluorescent Leica DM5500 microscope was used to obtain images of the entire BLA, at three levels, - 1.5 mm, −1.8 mm and −2.1 mm to bregma and mPFC between +1.5 mm and +1.8 mm to bregma. Two sections (50–100 μm apart) per bregma level were used for each mouse. The images were analyzed with Image J by splitting the composite image into single channel images and labeling the individual cells with a marking tool, which provided the cell numbers for single labeled neurons. Once all cells were marked in one channel, the channels were merged again and the colocalized cells were counted. No qualitative differences were observed in the cell numbers between the left and right side and the final cell counts were averaged per hemisphere based on six sections per animal.

A Zeiss LSM510 confocal scanning microscope was used to obtain images for analysis of the perisomatic inhibitory marker GAD65 in the BLA. We followed a protocol that closely resembles a published method (Trouche et al., 2013). Five to six Z-stacks containing 15 to 20 optical sections with size 71.4 × 71.4 μm and separated by 0.5 μm steps were collected from the BLA of each mouse. The images contained principal neurons as defined by Kv2.1 labeling, which is a marker for the somatic membrane and proximal dendrites of the principal neurons (Vereczki et al., 2016). Each image contained both retrogradely labeled BLA to mPFC projection neurons and BLA principal neurons that were not retrogradely labeled. The z-stacks were analyzed with Image J. A single optical section with the cell's largest diameter was first selected, for both tdTomato positive and negative cells, next, the cellular membrane was outlined based on Kv2.1-ir and then the membrane overlay was applied to the thresholded image of GAD65 immunostaining. All pixels above threshold inside the cell silhouette were summed as optical density and the results from 6 to 10 cells of each type were averaged per mouse.

2.8. Statistical analysis

Graph Prism software was used for all statistical analysis. Data are presented as mean ± SEM. Development of mechanical allodynia and thermal hypersensitivity was assessed by Two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test. T-tests were used to compare cell counts, while Two-way ANOVA was used for analysis of all behavior experiments. The level of significance α was set at 0.05.

3. Results

3.1. Sciatic nerve constriction decreases mechanical and thermal thresholds and elicits anxiodepressive-like behavior

Two days after fear-conditioning paradigm, randomly selected unconditioned and conditioned mice underwent the cuffing surgeries to establish neuropathic pain. The mice were left undisturbed except for a few tests for mechanical allodynia and thermal hypersensitivity, which were taken every 10 days and verified the development of neuropathic pain in the cuffed groups. The average mechanical threshold at the 10th postsurgical day was 3.5 ± 1.4 g for Sham/Control group and 3.9 ± 1.5 g for Sham/FC group but was much lower for the Cuffed/Control group with 0.3 ± 0.2 g and Cuffed/FC group with 0.4 ± 0.2 g respectively; the difference in the mechanical thresholds was well expressed at the 40 postsurgical day when the were Sham/Control group with 3.6 ± 1.4 g and Sham/FC group with 4.2 ± 1.5 g were different from the Cuffed/Control group with 1 ± 0.2 g and Cuffed/FC group with 1.4 ± 0.5 g, Two-way Repeated Measures ANOVA, significantly different for time, $F_{4,\ 148} = 17.4$, $P < 0.001$, cuffing, $F_{3,\ 148} = 46.9$, $P < 0.001$ and interaction, $F_{12,\ 148} = 3.7$, $P < 0.001$(Fig. 1 **A**). Similar results were obtained for the thermal hypersensitivity where the Sham/Control group with response latency of 3.3 ± 0.4 s and Sham/FC group with 2.8 ± 0.2 s were much higher than the cuffed groups with 1.1 ± 0.1 s response latency for Cuffed/Control group and 1.2 ± 0.1 s

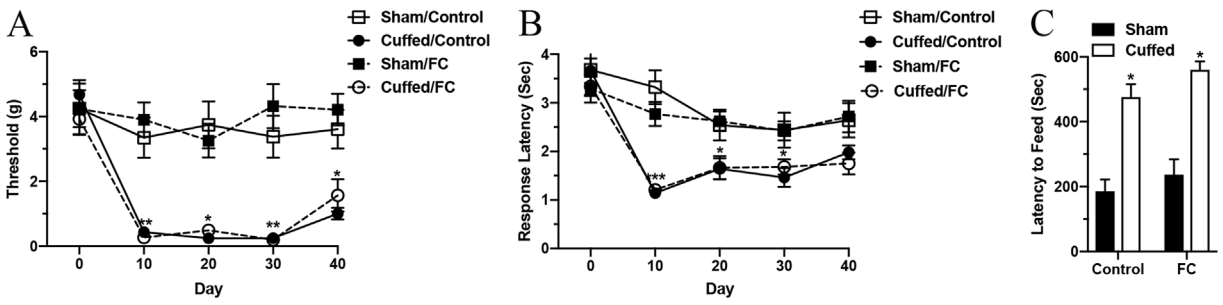

Fig. 1. Sciatic nerve constriction surgery decreased the mechanical and thermal thresholds, in both control and fear conditioned groups and caused anxiodepressive-like behavior. Panel **A** shows mechanical allodynia and panel **B** shows thermal hypersensitivity in cuffed groups and control groups from the day of surgery to postsurgical day 40. Panel **C** shows increased latency to feed in the cuffed groups regardless of their fear conditioning (FC) status. The time courses in A and B were evaluated by Two-way repeated measures ANOVA followed by Bonferroni's post test for time versus cuffing while the latency to feed in C was evaluated by Two-way ANOVA for cuffing versus FC; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, bars represent mean ± SEM, n = 10 per group.

response latency for Cuffed/FC group at the 10th postsurgical day. The significant difference in the thermal threshold between the groups persistent until the 30th postsurgical day when the thresholds of the cuffed groups were 1.5 ± 0.1 s response latency for Cuffed/Control group and 1.7 ± 0.2 s response latency for Cuffed/FC group while the control groups were respectively 2.6 ± 0.3 s for Sham/Control group and 2.4 ± 0.3 s respond latency for Sham/FC group, Two-way Repeated Measures ANOVA found significant difference for time, $F_{4, 104} = 25.1$, $P < 0.001$, cuffing, $F_{3, 104} = 11.2$, $P < 0.001$ and interaction, $F_{12, 104} = 2.9$, $P < 0.01$ (Fig. 1 **B**).

We also confirmed the development of anxiodepressive-like behavior in the mice with cuffs by novelty suppressed feeding test that was applied 40 days after cuffing. The average latency to feed was 186 ± 32 s for the Sham/Control and 236 ± 41 s for Sham/FC groups respectively but notably higher for the cuffed groups with 478 ± 46 s latency to feed for Cuffed/Control and 560 ± 28 s latency to feed for Cuffed/FC, Two-way ANOVA, significantly different for cuffing, $F_{1, 20} = 64.7$, $P < 0.001$ but not significant for FC, $F_{1, 20} = 3$, $P > 0.05$ and interaction, $F_{1, 20} = 0.1$, $P > 0.05$ (Fig. 1 **C**). Thus, nerve cuffing leads to mechanical allodynia and thermal hypersensitivity and increases anxiety-related behavior while the fear conditioning appears not to affect the pain sensitivity, which develops after nerve cuffing.

3.1.1. Ongoing pain augments the fear response to consolidated fear memories

Fear acquisition or simultaneous presentation of CS and US triggers a cascade of plastic events in the BLA, which alter the synaptic strength between the activated neurons. The process of fear memory consolidation is completed in less than 6 h in rodents (Schafe and LeDoux, 2000). We asked the question: what would be the effect of pain on already consolidated fear memories? Fear conditioning was followed by nerve constriction two days later and the mice were tested for fear recall 28 days after the fear conditioning (Fig. 2 **A**). Non-conditioned sham and non-conditioned cuffed mice had indistinguishable freezing behavior after introduction of the CS. However, in the conditioned groups, mice with nerve constriction spent significantly more time freezing than mice without nerve cuffs. Two-way ANOVA with fear conditioning and nerve cuffing as factors showed a significant effect of fear conditioning, $F_{1, 81} = 22.6$, $P < 0.001$, a significant effect of nerve cuffing, $F_{1, 81} = 4.92$, $P < 0.05$ and a significant interaction between the two factors, fear conditioning and nerve cuffing, $F_{1, 81} = 6.9$, $P < 0.01$ (Fig. 2 **B**).

This first experiment showed that sciatic nerve cuffing shortly (2 days) after fear conditioning lead to enhanced long-term fear memory performance. Memory storage is a dynamic process and the initial consolidation in the amygdala is followed by generation of fear memory engrams in mPFC for long-term storage. The mPFC engrams become active about twelve days after the fear event (Kitamura et al., 2017). Next, we tested if neuropathic pain that is initiated after the fear memory engram is already established in the mPFC will affect fear recall. In this experiment we inserted sciatic nerve cuffs 14 days following a single presentation of the CS/US. We used different groups of mice to evaluated fear recall at different time points after FC. Mice with cuffs and without cuffs did not show any difference in their freezing behavior 28 days ($F_{1, 25} = 0.9$, $P > 0.05$ for interaction of fear conditioning versus nerve cuffing) and 42 days ($F_{1, 23} = 0.04$, $P > 0.05$ for interaction of fear conditioning versus nerve cuffing) after fear conditioning (Supplementary Figure 1) but the group that was tested 56 days after conditioning, which was 42 days following nerve cuffing, showed increased freezing after the CS (Fig. 2 **C and D**). Two-way ANOVA showed a significant effect for fear conditioning, $F_{1, 50} = 46.04$, $P < 0.001$, no effect of nerve cuffing alone, $F_{1, 50} = 0.64$, $P > 0.05$ and a significant effect for interaction of fear conditioning and nerve cuffing, $F_{1, 50} = 5.8$, $P < 0.05$ (Fig. 2 **D**).

3.1.2. Ongoing pain does not augment new fear memories

We tested if the magnitude of fear recall depends on the relative timing of pain onset and fear conditioning. The nerve cuffs were inserted two days before a fear conditioning session or two days after fear conditioning (Fig. 2 **E**). The mice that were fear conditioned before the cuffing surgery spent more time freezing after the CS than the mice that were already cuffed during the fear conditioning when the groups were tested 28 days later (Fig. 2 **F**). Two-way ANOVA for the two variables, fear conditioning and time of nerve cuffing showed a significant effect for fear conditioning, $F_{1, 36} = 30.7$, $P < 0.001$, a significant effect for time of nerve cuffing, $F_{1, 36} = 4.9$, $P < 0.05$ and significant effect for interaction between the two variables, fear conditioning and time of nerve cuffing, $F_{1, 36} = 4.2$, $P < 0.05$, (Fig. 2 **F**).

3.1.3. Ongoing pain changes inhibitory neurotransmission within the amygdala

Pain associated neuronal plasticity has been described for numerous brain regions and involves changes in the expression of an array of transmitters, receptors and cell type markers. Despite being fewer in number than excitatory neurons, inhibitory interneurons in the BLA are essential for acquisition of fear memories (Krabbe et al., 2018). We immunostained brain sections for a variety of inhibitory neuron markers (GABA, calbindin, parvalbumin, somatostatin, NPY) and compared their expression between mice with and without nerve cuff induced neuropathic pain. The only difference that we found was in the number of Parv-ir positive neurons. The averages were 23 ± 2 Parv-ir neurons for controls and 35 ± 3 Parv-ir neurons for cuffed mice in the BLA, T-test, $t = 4.4$, $P < 0.001$ (Fig. 3). Almost all of these Parv-ir neurons also contained Calb-ir, but the two groups of mice showed the same average number of Calb-ir positive neurons in the BLA, control group 77 ± 18 Calb-ir and cuffed group 78 ± 16 Calb-ir, T-test, $t = 0.06$, $P > 0.05$ (Fig. 3). The numbers of cells that were double labeled for Parv-ir and Calb-ir were significantly increased in the brain sections of the nerve-cuffed mice, control group average 22 ± 2 Parv/Calb-ir cells versus cuffed group average 29 ± 2 Parv/Calb-ir cells, T-test, $t = 2.5$, $P < 0.05$ (Fig. 3), suggesting a phenotypic change in a subpopulation of the Calb-ir neurons that is associated with Parv expression.

The behavior experiments showed that neuropathic pain might affect fear memories that were already stored in the mPFC at the time of pain onset. We also observed changes associated with nerve cuffing in the number of Parv-ir neurons in the BLA. These two observations were the basis for our next experiment in which we investigated whether nerve cuffing changes the inhibitory signaling onto the pyramidal neurons that project from the BLA to the mPFC, and are responsible for transfer and consolidation of fear memory in the cortex (Kitamura et al., 2017). Because the axonal targets of the GABAergic Parv interneurons are the soma and proximal dendrites of the excitatory pyramidal neurons, we compared the association of the putative inhibitory presynaptic marker GAD65 with the soma of the general pyramidal population and the pyramidal neurons that project to the mPFC. We injected retrograde AAV-CAG-tdTomato into mPFC of mice and inserted sciatic nerve cuffs three weeks later. The mice were sacrificed after one month and brain sections were immunostained for Kv2.1 (a marker for the soma and proximal dendrites of pyramidal neurons) and GAD65 (a presynaptic maker for inhibitory synapses). We evaluated the density of GAD65 on the membranes of pyramidal neurons without the retrograde tracer and on the membranes of pyramidal neurons labeled with the retrograde tracer AAV-CAG-tdTomato (Fig. 4 **A** to **D**). There was a clear increase in GAD65-ir associated with cell bodies and proximal dendrites of neurons that project to the mPFC in nerve cuffed mice (Fig. 4 **E** to **G**). The effect of nerve cuffing on overall GAD65-ir did not reach statistical significance, $F_{1, 25} = 2.2$, $P > 0.05$, but there were significant effects of neuronal type (tdTom-versus tdTom+), $F_{1, 25} = 7.2$, $P < 0.05$ and a significant effect for interaction between nerve cuffing and neuronal type, $F_{1, 25} = 5.5$, $P < 0.05$, (Fig. 3 **H**).

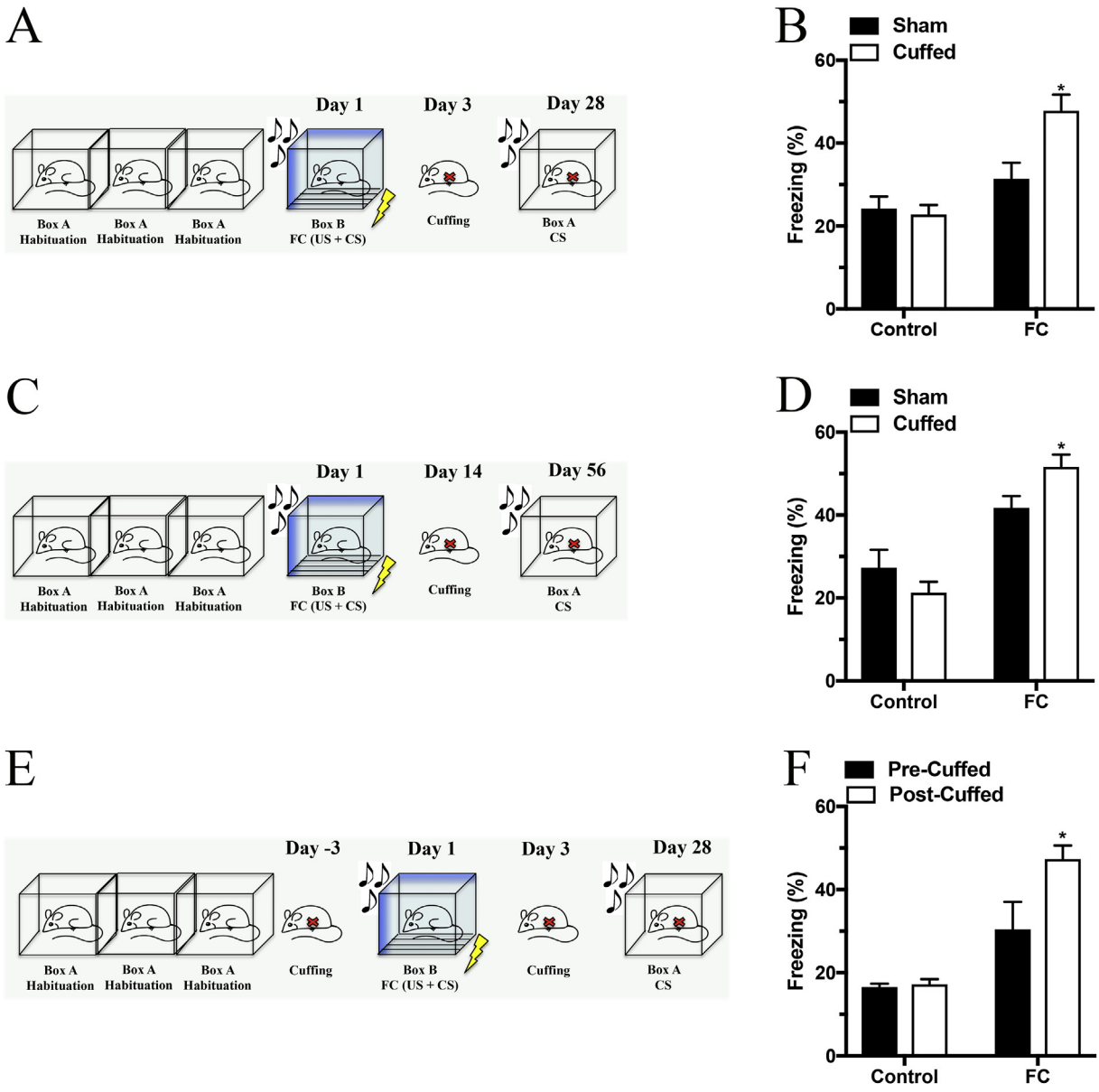

Fig. 2. A and B: **Ongoing pain augments the fear response to consolidated fear memories.** Panel **A** shows the time line of the fear conditioning (FC) and sciatic nerve constriction surgery (Cuffed). The mice were FC to a single combination of tone (CS) and mild electric shock (US) two days before cuffing surgery, which induced neuropathic pain, and were tested for fear recall 28 days later. Panel **B** shows the total freezing time for 5 min after the presentation of CS. The mice with persistent pain exhibit a much stronger fear response to CS when compared to mice without pain, Two-way ANOVA showed significant difference for fear conditioning versus cuffing, $*p < 0.05$, bars represent mean ± SEM, n = 19 to 23 per group.

Fig. 2 C and D**: Ongoing pain augments the fear response to long-term fear memories.** The mice were FC fourteen days before the cuffing surgery and tested for fear recall fifty-six days later (**C**). The cuffed group froze more after the CS than non-cuffed controls, Two-way ANOVA showed a significant difference for fear conditioning versus cuffing, $*p < 0.05$, bars represent mean ± SEM, n = 12 to 20 per group (**D**).

Fig. 2 E and F: **Ongoing pain does not augment the fear response to new fear memories.** Panel **E** shows the time line of the fear conditioning and cuffing surgery. The mice were FC to a single tone two days before cuffing (FC/Cuffed) or two days after cuffing (Cuffed/FC) and were tested for fear recall 28 days later. Panel **F** shows the total freezing time for 5 min after the presentation of CS. Cuffing after FC led to a greater fear response to the CS than cuffing before FC. Two-way ANOVA showed significant difference for fear conditioning versus time of cuffing factor, $*p < 0.05$, bars represent mean ± SEM, n = 9 to 12 per group.

3.1.4. The BLA to mPFC projection neurons are not activated during fear recall in mice with ongoing pain

If the nerve cuff increases the inhibitory input onto the BLA to mPFC projections then it is possible that fewer of these neurons will be activated during fear recall. We used c-Fos as a marker for neuronal activation and retrograde AAV-CAG-tdTomato virus to label the BLA to mPFC projection neurons as in the previous experiment. However, c-Fos is widely expressed in the mouse brain and potentially induced by small perturbations such as handling, so we first verified that the CS presentation causes an increase in amygdala c-Fos expression that can be detected by our immunostaining procedure. A separate cohort of mice that were all fear conditioned, was divided into a control group that spent 5 min in Box A without CS and an experimental group that was exposed to the CS in Box A. CS presentation led to detectable differences in both the freezing time and c-Fos-ir in the BLA. The average amount of freezing expressed as a percentage of the testing period was 33.5 ± 5.9% for the control group and 53.3 ± 5.5% for the experimental group, T-test, t = 2.2, P < 0.05. There was an average of 43 ± 5 c-Fos-ir positive cells in the BLA of the control group while the BLA of the experimental group contained an average of 53 ± 3 c-Fos-ir

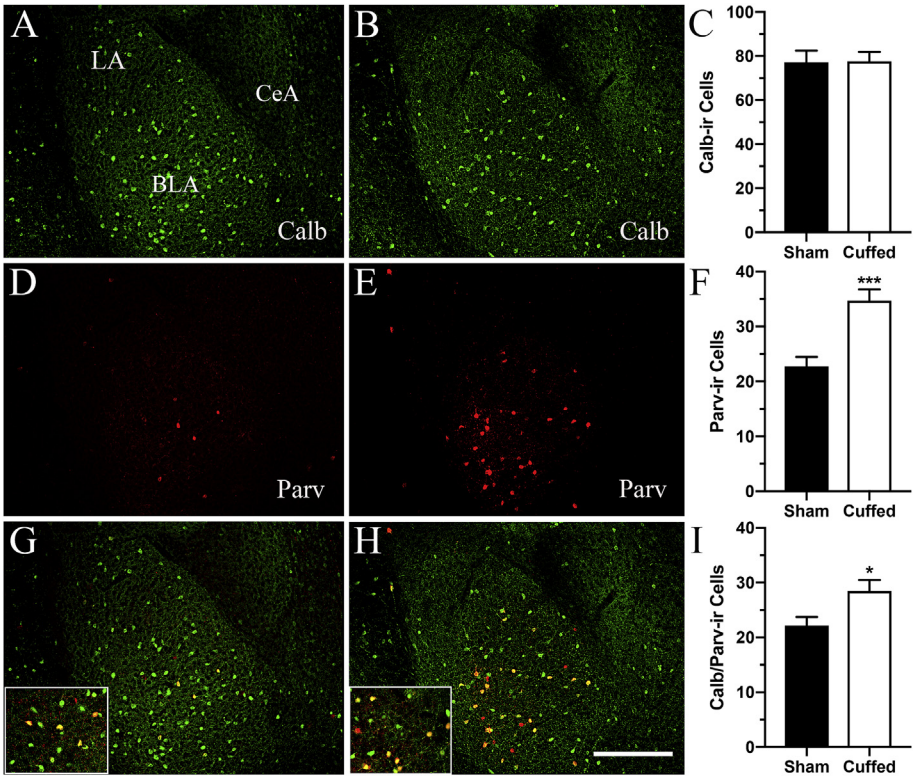

Fig. 3. Ongoing pain induced morphological changes in the BLA interneuronal population. Panels **A** and **B** show calbindin immunoreactivity (Calb-ir, green) and panels **D** and **E** show parvalbumin immunoreactivity (Parv-ir, red) expression in sham mice (**A** and **D**) and in cuffed mice (**B** and **E**). Panels **G** and **H** show the combined expression of the two markers in sham (**G**) and cuffed (**H**) mice, where the Calb/Parv-ir co-localization appears as yellow. The number of Calb-ir cells did not differ between the groups (**C**) but the number of Parv immunopositive neurons increased in the cuffed mice (**F**) and most of these Parv-ir neurons were colocalized with Calb-ir, or the yellow labeled neurons in the **G** and **H** inserts; The graph in panel **I** compares the number of Calb/Parv-ir neurons of sham and cuffed mice. The cell counts were evaluated by T-test, $*p < 0.05$ and $***p < 0.001$, bars represent mean ± SEM, n = 12 to 14 per group. BLA - basolateral amygdala, CeA - central amygdala, LA - lateral amygdala. Scale bar = 200 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

positive cells, T-test, t = 2.1, P < 0.05 (Fig. 5 **A** to **C**). The results of this experiment are congruent with published reports that c-Fos expression in BLA provides a dependable index of fear expression (Rajbhandari et al., 2016).

Once we verified that exposure to the CS and not simply exposure to the training box increases c-Fos in the BLA, we repeated the experiment with nerve-cuffed mice and controls, in which the BLA to mPFC neurons were labeled with tdTomato after an injection of retro AAV-CAG-tdTomato into mPFC (Fig. 5 **D** to **I**). The total number of c-Fos positive neurons did not differ between the controls (34 ± 4 cells) and the cuffed mice (31 ± 5 cells), T-test, t = 0.4, P > 0.05 (Fig. 5 **F**) but the control mice had more c-Fos/tdTom double labeled cells (22 ± 4) than the cuffed mice (5 ± 2), T-test, t = 4.5, P < 0.001, (Fig. 5 **I**).

3.1.5. The mPFC is overactive in mice with ongoing pain but the projection neurons from mPFC to BLA are not activated by fear recall

It is believed that mPFC stores long-term memories and that the activity of mPFC neurons is responsible for the freezing behavior of fear conditioned rodents (Burgos-Robles et al., 2009). We assessed and compared the expression of c-Fos-ir 1 h after fear recall in the mPFC and the mPFC projection neurons that were labeled by an injection of retrograde tracer AAV-CAG-tdTomato into the BLA of mice with and without pain. Mice with ongoing pain showed an increased number of c-Fos-ir positive neurons in mPFC and the increase was confined to the prelimbic (PrL) region of the mPFC but no difference in the expression of c-Fos-ir was observed in the infralimbic (IL) region of the mPFC (Fig. 6 **A** and **B**). An average of 95 ± 12 c-Fos-ir positive cells were counted in the PrL of the control group while the cuffed mice averaged

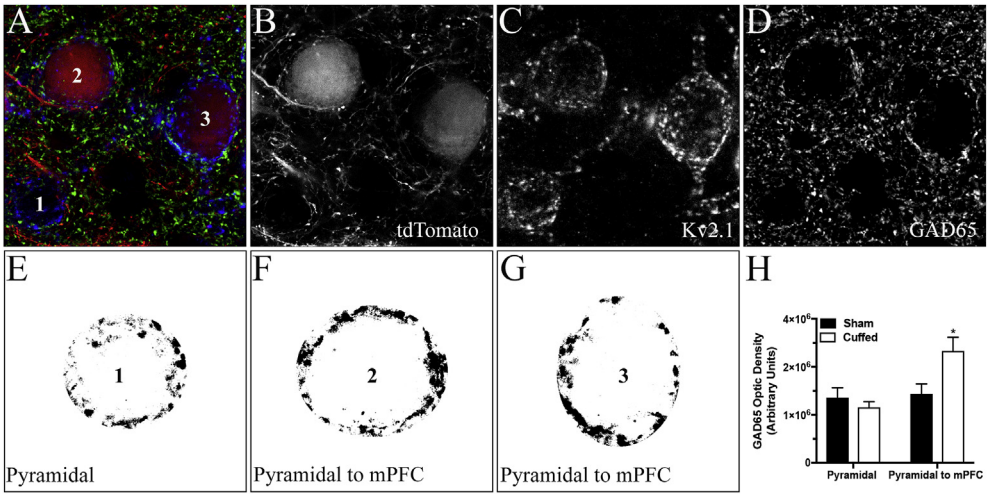

Fig. 4. Ongoing pain increases a presynaptic inhibitory marker on BLA to mPFC projection neurons. Panel **A** shows a cluster of pyramidal neurons (red and blue) and GAD65 (green) immunostaining in the BLA of a mouse 30 days following sciatic nerve cuffing. A retrograde tracer AAV-CAG-tdTomato was used to label the pyramidal neurons that project from the BLA to the mPFC (**B**), while labeling of the potassium channel Kv2.1 was used to identify the somatic membranes and proximal dendrites of all pyramidal neurons in the BLA (**C**). The optical density of GAD65 expression (**D**) was measured after outlining the soma of the pyramidal neurons using Kv2.1 staining for guidance. When compared to pyramidal neurons that project elsewhere in the CNS (labeled as "**1″** in panels **A** and **E**), the pyramidal neurons that project to the mPFC showed increased optical density of GAD65-ir on their somatic membranes (labeled as "**2″** and **"3″** in panels **A, F** and **G** respectively) with a significant difference for interaction, neuron type versus cuffing; Two-Way ANOVA, $*p < 0.05$, bars represent mean ± SEM, n = 5 to 10 per group (**H**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

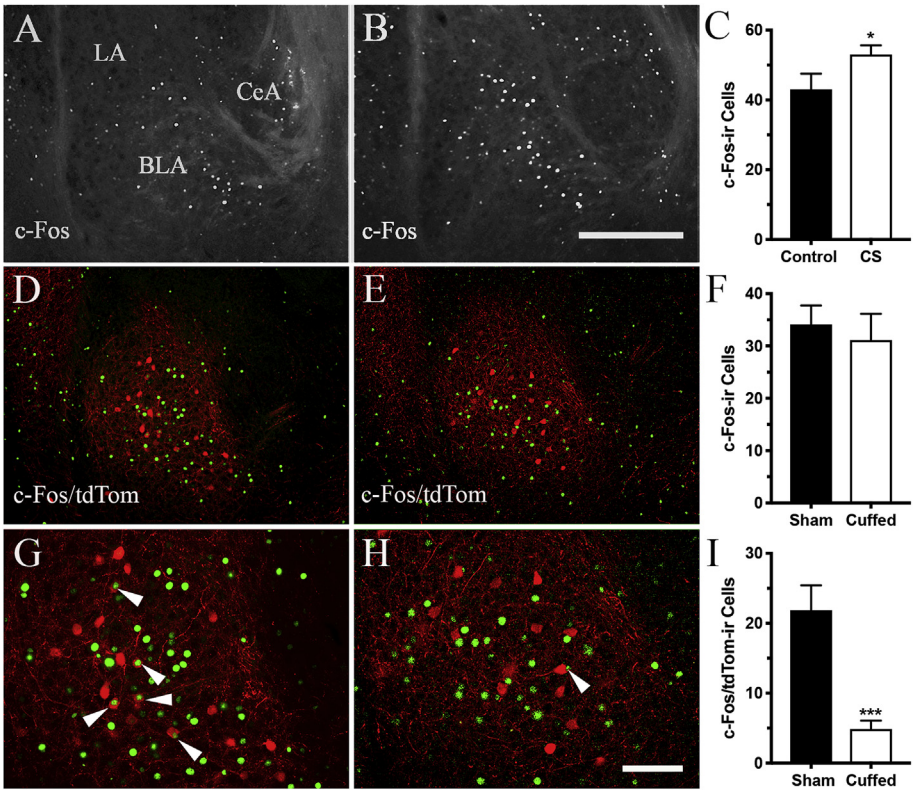

Fig. 5. Presentation of the CS increases c-Fos-ir in the BLA of fear-conditioned mice but principal neurons that project from the BLA to the mPFC are not activated in mice with ongoing pain. The figure illustrates the results of two sequential experiments. In the first experiment two groups of mice were FC but only the experimental group was exposed to the CS during the test. The number of c-Fos-ir positive cells in the BLA was lower in the control group (**A**) than the experimental group (**B**); T-test, $^{*}p < 0.05$, bars represent mean ± SEM, n = 6 per group, Panel **C**.

Panels **D, E, G and H** illustrate the results of the second experiment where the projection neurons from BLA to mPFC were labeled by the retrograde AAV-CAG-tdTomato (red) and the neuronal activation was evaluated by c-Fos (green) expression in control mice (**D**) and cuffed mice (**E**). Panels **G** and **H** are higher magnification images of the sections shown in panels **D** and **E** and demonstrate that fear recall 28 days after fear conditioning triggered robust expression of c-Fos in both groups of mice without a significant difference between them in the total number of c-Fos-ir cells (**F**). However, a much smaller fraction of the projection neurons in the cuffed mice contained c-Fos-ir (**I**). The cell counts were evaluated by T-test, $^{***}p < 0.001$, bars represent mean ± SEM, n = 5 to 6 per group. Arrowheads point to double labeled neurons expressing c-Fos-ir in green and tdTomato in red. BLA - basolateral amygdala, CeA - central amygdala, LA - lateral amygdala. Scale bar = 200 μm in B and scale bar = 20 μm in H. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

158 ± 14 c-Fos-ir cells, T-test, t = 4.7, P < 0.001 (Fig. 6 **C**). The expression of c-Fos in the IL subdivision of the mPFC was similar between the groups, controls with 56 ± 15 and cuffed mice with 43 ± 10 c-Fos-ir positive cells, T-test, t = 0.6, P > 0.05.

Similar to other studies that use viral vectors as retrograde tracers (Arruda-Carvalho et al., 2017), the tdTom labeled neurons were found in the deep layers of PrL and to a smaller extent in the IL after viral injection into the BLA (Fig. 6 **A** and **B**). Despite a much higher expression of c-Fos-ir in the PrL of the cuffed mice, a very few of the projections to the BLA showed c-Fos-ir (Fig. 6 **D** and **E**). The control group showed an average of 17 ± 4 c-Fos/tdTom double labeled neurons, while the cuffed mice had only 4 ± 2 c-Fos/tdTom double labeled neurons, T-test, t = 2.9, P < 0.05, (Fig. 6 **F**).

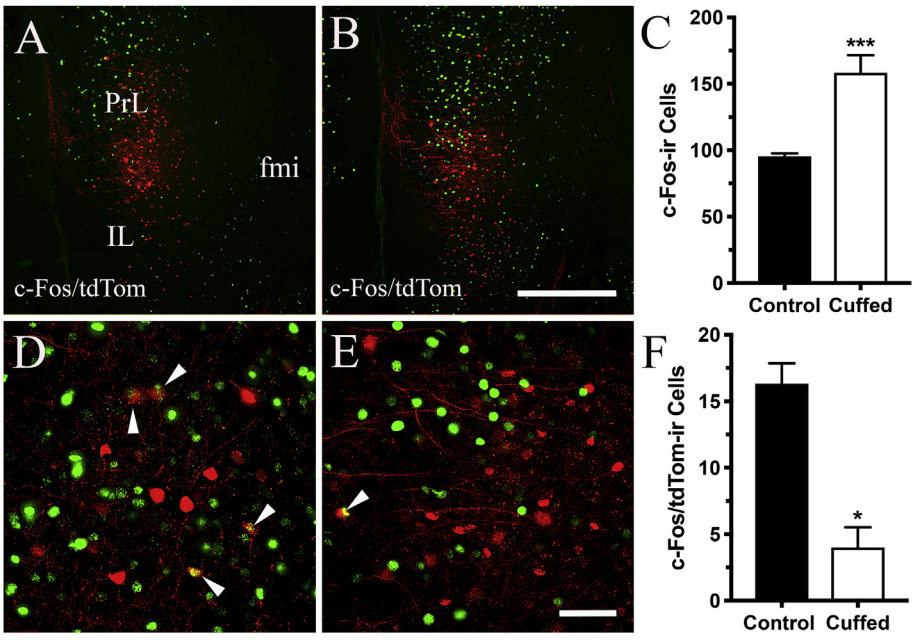

Fig. 6. The overall increased expression of c-Fos-ir in the mPFC 1 h after fear recall in mice with persistent pain did not include increased c-Fos expression in the projection neurons from mPFC to BLA. Panels **A** and **B** show the projection neurons from mPFC to BLA labeled by the retrograde AAV-CAG-tdTomato (red) and the marker for neuronal activation c-Fos-ir (green) in control mice (**A**) and cuffed mice (**B**). Fear recall 28 days after fear conditioning significantly increased the expression of c-Fos-ir in the PrL subdivision of the mPFC in mice with pain in comparison to pain-free mice (**C**), however, a very few of these projection neurons contained c-Fos-ir (**F**), The cell counts were evaluated by T-tests, $^{*}p < 0.05$ and $^{***}p < 0.001$, bars represent mean ± SEM, n = 6 per group. Panels **D** and **E** are higher magnification images of the sections shown in **A** and **B.** Arrowheads point to double labeled neurons expressing c-Fos-ir in green and tdTomato in red. fmi - forceps minor of the corpus callosum, IL - infralimbic cortex, PrL - prelimbic cortex. Scale bar = 200 μm in B and scale bar = 20 μm in D. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

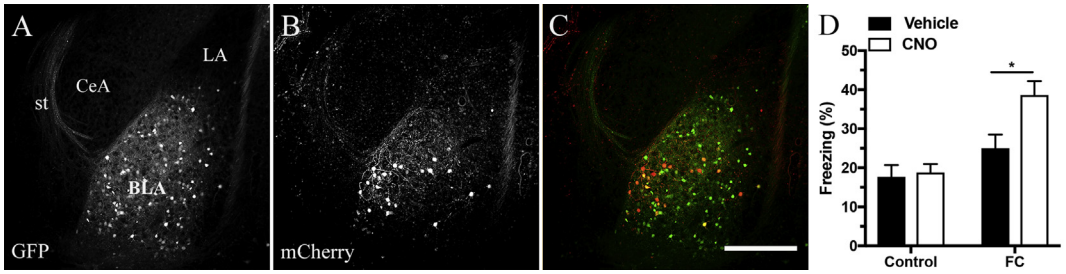

Fig. 7. Chemogenetic inhibition of BLA projections to mPFC during fear recall increases the freezing response. Panel **A** shows the expression of the retrograde rAAV-CAG-eGFP-F2A-Cre, panel **B** shows the expression of cre-activated pAAV-hSyn-hM4D(Gi)-mCherry and panel **C** shows a combined image of the two viruses, where the retro virus is in green and the DREADD virus is in red. The stria terminalis (st; panel **A)** is the main efferent pathway of the BLA projection neurons and it is clearly labeled by both viruses, which indicates activation of the DREADD construct by the retrogradely delivered cre recombinase. Panel **D** shows a comparison of the average freezing time of the controls and fear conditioned (FC) groups after the presentation of conditional stimulus, where the CNO treatment significantly increased the freezing time; Two-way ANOVA, $*p < 0.05$, bars represent mean ± SEM, n = 10 per group. BLA - basolateral amygdala, CeA - central amygdala, LA - lateral amygdala, st - stria terminalis. Scale bar = 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.1.6. Inhibition of BLA to mPFC neurons during recall increases the fear response

If increased inhibition of the neurons that project from the BLA to mPFC is the mechanism by which pain augments the fear response, then inhibiting this population of projection neurons should increase the fear response in mice in the absence of a pain input. To test this hypothesis, we injected the retrograde cre virus (rAAV-CAG-eGFP-F2A-Cre) into the mPFC and the cre-activated DREADD virus (pAAV-hSyn-hM4D(Gi)-mCherry) into the BLA. We fear conditioned the mice three weeks after the virus injections and tested for fear recall 26 days later. The control mice treated with CNO showed the same baseline freezing as the vehicle treated controls but fear conditioned mice that received CNO spent much more time freezing that the vehicle treated control, Two-way ANOVA indicated significance for fear conditioning factor $F_{1, 36} = 19.2$, $P < 0.001$, significance for CNO treatment factor $F_{1, 36} = 5.6$, $P < 0.05$ and a trend for interaction of the two factors, $F_{1, 36} = 4.0$, $P = 0.052$, (Fig. 7).

4. Discussion

We demonstrated that ongoing pain affects the expression of consolidated fear memories. A change in inhibitory transmission within the BLA may be the mechanism by which pain that is initiated after fear conditioning augments the learned fear response. We suggest that increased inhibitory signaling onto the BLA to mPFC projections prevents the activation of these projection neurons during fear recall, therefore effectively uncoupling the BLA activity from the mPFC. The top-down control that mPFC exerts over the amygdala is very likely diminished as a result of that uncoupling.

At least two possible interactions between the BLA and mPFC during fear recall are suggested by electrophysiology experiments. While one study reports that the amygdala inhibits the mPFC during fear recall (Garcia et al., 1999), others report the opposite interaction or that the amygdala activates mPFC during fear recall (Sotres-Bayon et al., 2012). Anatomical studies combined with optogenetics demonstrate that BLA projections fibers innervate both cortical excitatory pyramidal neurons that reciprocally connect to BLA and cortical inhibitory interneurons. Therefore there is evidence for BLA projections that are capable of activating the mPFC and also ones capable of driving a robust feed-forward inhibition of the mPFC, and the interplay between excitatory and inhibitory drive may determine the net effect of BLA on mPFC activity (McGarry and Carter, 2016). Furthermore, the BLA projection neurons can be categorized as “fear neurons” and “extinction neurons” according to their activity in response to the conditioning stimulus (Herry et al., 2008), which adds to the complexity of the circuitry described above. Our techniques do not allow precise assignment of the BLA to mPFC projections neurons to the “fear” or “extinction” category but the results of our experiments showed that these neurons are active during fear recall in mice without pain, and that they are less activated in mice with pain. Even further, the inhibition of the BLA to mPFC projections augmented fear response in mice without pain. All together, our results indicate that the direct BLA to mPFC projection neurons, which are necessary for normal fear response, receive an increased inhibitory input driven by ongoing pain and the increased inhibitory input onto these BLA to mPFC projections impairs the normal fear response.

Our experiments also showed changes associated with pain that affect a subpopulation of inhibitory interneurons of the BLA. The increase of Parv immunopositive cells in the BLA of mice with chronic pain is somewhat unexpected but change in Parv expression is not that unusual. Recent papers reported an increase of Parv expression in the mPFC as a consequence of chronic stress (Shepard and Coutellier, 2018; Shepard et al., 2016). Other studies have shown changes in the cortical Parv expression with age (Caballero et al., 2014). These reports indicate that Parv expression is malleable; it is activity dependent and responds to external stimulation. The BLA cellular composition is similar to the cortex, in essence BLA is a cortical structure, and it very likely possesses similar capacity for plasticity changes as the cortex. While the Parv positive neurons in the BLA are classified as basket cells (Bienvenu et al., 2012) that target the soma and proximal dendrites of the pyramidal neurons (Vereczki et al., 2016) and respond to a variety of noxious stimuli including auditory stimulation during fear conditioning (Wolff et al., 2014), the Calb interneurons target the dendrites of the pyramidal cells and synchronize their activity to hypothalamic inputs (Bienvenu et al., 2012). However, according to the authors that provide the above description, a number of Parv positive cells also expresses Calb (Bienvenu et al., 2012), which makes it possible that Parv levels in some of the Calb cells with normally low levels may be stimulated by chronic pain and increase their Parv content to levels that become easily detectable by immunostaining. The same activity dependent expression very likely explains the results of our next experiment in which we observed increased presynaptic GAD65 expression near the somatic membranes of BLA to mPFC projection neurons. An elegant paper by Rannals and Kapur demonstrated an increase of GAD65 presynaptic puncta as a result of high activity by the targeted neurons (Rannals and Kapur, 2011). According to the authors, this phenomenon illustrates the mechanism of homeostatic plasticity or if the firing rate of a neuron increases above base level for more than 48 h, a compensatory increase of GAD65 transmission onto this neuron helps to bring down the neuronal activity. A heightened activity of the BLA neurons as a consequence of chronic pain is well-known (Thompson and Neugebauer, 2017) and that supports the idea that the increase of Calb/Parv-ir cell number and GAD65-ir around the somata of the BLA projection neurons is a compensatory response to overactive pyramidal cells.

Reports that chronic pain leads to overactive BLA neurons appears somewhat incongruent with our results that show a very low c-Fos expression in BLA cells that project to mPFC cells during fear recall. Both, experiments based on electrophysiological recordings from BLA projection neurons and experiments that include the expression of neuronal markers for sustained activation show an increased neuronal activity of BLA as a consequence of chronic pain (Ji et al., 2010; Dimitrov et al., 2014). However, these studies do not provide information on the activity of these projection neurons during fear recall. Here, we used c-Fos-ir as a marker for acute neuronal activation and our results indicate that while there is no overall difference of c-Fos expression in BLA between pain-free mice and mice with pain, the presentation of the CS failed to induce c-Fos-ir specifically during the narrow time interval of fear recall in the neuronal population that projects from the BLA to the mPFC.

The deleterious effects of chronic pain on the cognitive performance of patients are well known (Berryman et al., 2013; Nadar et al., 2016) and experiments with rodents using different types of pain models show similar effects of pain on the memory of laboratory animals, including impairment of contextual fear memory, fear extinction and novel object recognition (Mutso et al., 2012; Dimitrov et al., 2014; Kodama et al., 2011; Ren et al., 2011). However, there is very little published on the effects of chronic pain on consolidated fear memories. A study in which inflammatory pain was initiated only a few hours after the fear conditioning detected a deficit in contextual recall but not in cued recall of rats (Johnston et al., 2012) and a second study that used bone fracture as a pain model detected a difference in contextual fear recall but only in female mice and not in males (Tajerian et al., 2015). The last study also used multiple pairings of CS and US and subsequently did not find a difference in cued fear recall between mice with and without pain. Our experimental protocol is based on extensive habituation to the test chamber, a single presentation of CS-US and induction of pain after a long period of time, from 48 h to 14 days after a FC session. Therefore, we believe that we have avoided phenomena such as fear incubation (Poulos et al., 2016; Tsuda et al., 2015), fear sensitization (Poulos et al., 2015), fear generalization (Fanselow, 1980) and non-associative learning (Kamprath and Wotjak, 2004) that might obscure the results. The fact that pain by itself did not increase freezing and that only the presentation of CS but not the placement of the mouse in the test box increased c-Fos-ir in the BLA support our argument that the effects of pain on the fear response and neuronal activation in the BLA reflect only the effects of pain on the expression of specific fear memory recall and it is not simply a result of heightened anxiety-like behavior.

The published literature allows a parallel to be drawn between the fear response after chronic pain and the fear response after chronic stress. Surprisingly, while chronic stress tends to augment newly formed aversive memories and inhibits fear extinction (Zhang and Rosenkranz, 2013); it does not affect already consolidated fear extinction memory (Rahman et al., 2018). Furthermore, the authors of the last study found a discrepancy between the activity of the mPFC and amygdala or that the stressed animals failed to synchronize the activity of amygdala with the activity of mPFC (Rahman et al., 2018). Our experiment also point to disengagement of the BLA activity from the mPFC during fear recall in mice with chronic pain. The conclusion of those authors is that pre-stress and post-stress memories are regulated differently, which is similar to our interpretation of the current results.

It is plausible that the augmented effect of chronic pain on consolidated fear memories in mice described here is may be somewhat akin to the mechanism of intense ruminations that are a hallmark symptom of major depressive disorder (Gotlib and Joormann, 2010; Burwell and Shirk, 2007) or to negative cognitive bias. Systematic review of the literature shows that, similar to the patients with clinical depression, patients with chronic pain lack control over negative thoughts, leading to intense recollection of past aversive events (Mazza et al., 2018). In other words, the ongoing pain continuously reinforces aversive emotional memories for past incidents. Despite the clinical importance of negative ruminations for diagnosis of major depression, the neuronal mechanisms that underlie this phenomenon are not well understood. We showed here a plausible neuronal circuitry that replicates the phenomenon in laboratory mice. Future investigations of the circuitry between BLA and mPFC using this mouse model may improve our understanding of amygdalo-prefrontal cortex interactions in the development of depressive symptomatology.

Supporting information

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2019.100163.

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