

## Original Article

# Retrieval of olfactory fear memory alters cell proliferation and expression of pCREB and pMAPK in the corticomedial amygdala and piriform cortex

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## Abstract

The brain forms robust associations between odors and emotionally salient memories, making odors especially effective at triggering fearful or traumatic memories. Using Pavlovian olfactory fear conditioning (OFC), a variant of the traditional tone-shock paradigm, this study explored the changes involved in its processing. We assessed the expression of neuronal plasticity markers phosphorylated cyclic adenosine monophosphate response element binding protein (pCREB) and phosphorylated mitogen-activated protein kinase (pMAPK) 24 h and 14 days following OFC, in newborn neurons (EdU+) and in brain regions associated with olfactory memory processing; the olfactory bulb, piriform cortex, amygdala, and hippocampus. Here, we show that all proliferating neurons in the dentate gyrus of the hippocampus and glomerular layer of the olfactory bulb were colocalized with pCREB at 24 h and 14 days post-conditioning, and the number of proliferating neurons at both time points were statistically similar. This suggests the occurrence of long-term potentiation within the neurons of this pathway. Finally, OFC significantly increased the density of pCREB- and pMAPK-positive immunoreactive neurons in the medial and cortical subnuclei of the amygdala and the posterior piriform cortex, suggesting their key involvement in its processing. Together, our investigation identifies changes in neuroplasticity within critical neural circuits responsible for olfactory fear memory.

**Key words:** olfactory, fear memory, neurogenesis, plasticity, pMAPK, pCREB

## Introduction

Post-traumatic stress disorder (PTSD) relies on consolidated traumatic memories that precipitate debilitating psychological symptoms upon their retrieval. Exposure therapy, medications, and other forms of psychotherapy used to treat PTSD are only successful in a subset of patients and occasionally produce side effects (Lokshina and Liberzon 2017). Effective long-term treatment of PTSD is based on the complete knowledge of the neurocircuitry involved in fear memory acquisition and consolidation. However, much of the research surrounding PTSD and its neuroanatomical changes has been predominantly with auditory and visual fear conditioning paradigms (Ledoux et al. 1986; Campeau and Davis 1995a, 1995b; Johnson et al. 2012; Bergstrom, McDonald, Dey, Fernandez, et al., 2013; Bergstrom and Johnson 2014; Daldrup et al. 2015). Odors, a less discussed stimuli, are especially effective cues for triggering memories of high emotional saliency and intensity, much more so than other sensory cues

(Herz 1998; Chu and Downes 2002; Herz and Schooler 2002; Willander and Larsson 2006). Furthermore, olfactory fear memory differs considerably in its underlying neuroanatomical pathways in comparison to other fear memories (Hakim et al. 2019). This study, therefore, aimed to better understand the neurological basis for olfactory fear conditioning, as it is a rarely acknowledged process in the memory reconsolidation that underpins PTSD pathogenesis.

Neuroplasticity studies have shown that unlike auditory and visual fear conditioning, the olfactory conditioning pathway does not project directly and exclusively to the basolateral (BLA) or lateral (LA) amygdala (Sevelinges et al. 2007; Keshavarzi et al. 2015; Luchkina and Bolshakov 2018). Evidence shows that the medial amygdala (MeA) is the prime region of interest in olfactory fear conditioning (OFC) and that the corticomedial group (CMe) receives direct projections from the olfactory bulb (OB) (Schettino and Otto 2001; Walker et al. 2005). However, other studies have

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confirmed that the central amygdala (CeA), cortical amygdala (CoA), and BLA are also activated, suggesting a role in OFC (Hitchcock et al. 1989; Sananes and Campbell 1989; Cousens and Otto 1998; Rosenkranz and Grace 2002; Kilpatrick and Cahill 2003; Sevelinges et al. 2004). Overall, results have varied regarding the involvement of sub regions of the amygdala in OFC, in comparison to auditory and visual fear conditioning, which are more popular models for investigating fear memory. Further investigation on OFC and identification of key subregions of the amygdala is necessary to refine the neuroanatomy involved.

Learning-dependent neurogenesis plays a vital role in acquiring olfactory information. 5-Bromo-2'-deoxyuridine positive (BrdU+) newborn olfactory neurons have shown to increase in number as a result of olfactory associative learning (So et al. 2008; Sultan et al. 2010). This increase is proportional to the strength of learning, therefore the stronger the learning the more BrdU + olfactory neurons, with more emotional memories resulting in stronger associative learning. Interestingly, So et al. (2008) showed that OFC had no significant effect on the number of BrdU + neurons in the dentate gyrus (DG) of the hippocampus despite abundant evidence suggesting an intimate link between hippocampal neurogenesis and contextual fear conditioning (Gould et al. 1999; Jaako-Movits and Zharkovsky 2005; Saxe et al. 2006; So et al. 2008). What remains unclear is whether or not newborn neurons are involved in olfactory fear memory processing and its related neuroplasticity in key brain regions.

Fear memory reconsolidation involves plasticity in a variety of brain regions; however, such plasticity has yet to be assessed in olfactory structures. To address the question, we adopted olfactory Pavlovian fear conditioning in order to investigate the changes in plasticity and neurogenesis following olfactory fear memory reconsolidation. We utilized the proliferative marker 5-ethyl-2'-deoxyuridine (EdU) to label proliferative cells, combined with immunohistochemistry against transcription factor pCREB (phosphorylated cAMP response element binding protein) and pMAPK (phosphorylated mitogen-activated protein kinase) to investigate the effects of OFC on plasticity and neurogenesis. More specifically, we wanted to investigate the survival of labeled newborn neurons 14 d after fear conditioning and its associated neuroplasticity. Approximately 50% of newborn olfactory cells are consolidated and integrated into neural circuits following synaptogenesis at 14 d, which ultimately determines their survival (Winner et al. 2002; Kelsch et al. 2008). Birth-dating cells with EdU immediately following conditioning and counting the number of labeled cells at 14 d demonstrates the survival of those newborn neurons.

Synaptic and neuronal plasticity associated with fear memory conditioning is strongly dependent on pMAPK, which then transactivates CREB via phosphorylation (Xing et al. 1996; Bozon et al. 2003; Sindreu et al. 2007). Phosphorylated CREB (pCREB) follows by activating transcription of target genes in response to a wide range of external stimuli. Ultimately, coordinated activation of pMAPK and pCREB modulates neuronal activities, as well as learning and memory-related plasticity, making them appropriate proteins for investigation of olfactory fear memory reconsolidation (Finkbeiner 2000; Barco et al. 2003; Bozon et al. 2003; Borlikova and Endo 2009). We demonstrate that in response to olfactory fear memory, the MeA, CoA, piriform cortex (PC), and the DG showed an increase in the number of

pCREB- and pMAPK-positive neurons. OFC also increased neurogenesis of the glomerular layer of the OB and the DG.

## Materials and methods

### Subjects

Subjects were experimentally naive adult male Sprague-Dawley rats (supplied by Animal Resource Center [ARC], Western Australia). They were selected by weight, falling between 200 and 400 g. Animal housing was provided by The University of Queensland Biological Resources (UQBRI) at the Translational Research Institute and was based on 12-h light/dark cycle, 7 AM–7 PM. The subjects were housed in standard group cages (2×/cage) in a temperature (≈ 24 C) and humidity-controlled (35%) vivarium and were given ad libitum access to food and water. For the purpose of consistency, all subjects had the same environmental enrichment. All subjects were acclimated with the facility's climate for 7 d prior to handling, handled for 10 d, and then habituated to the conditioning chamber and enclosure for 15 min 1 d prior to fear conditioning.

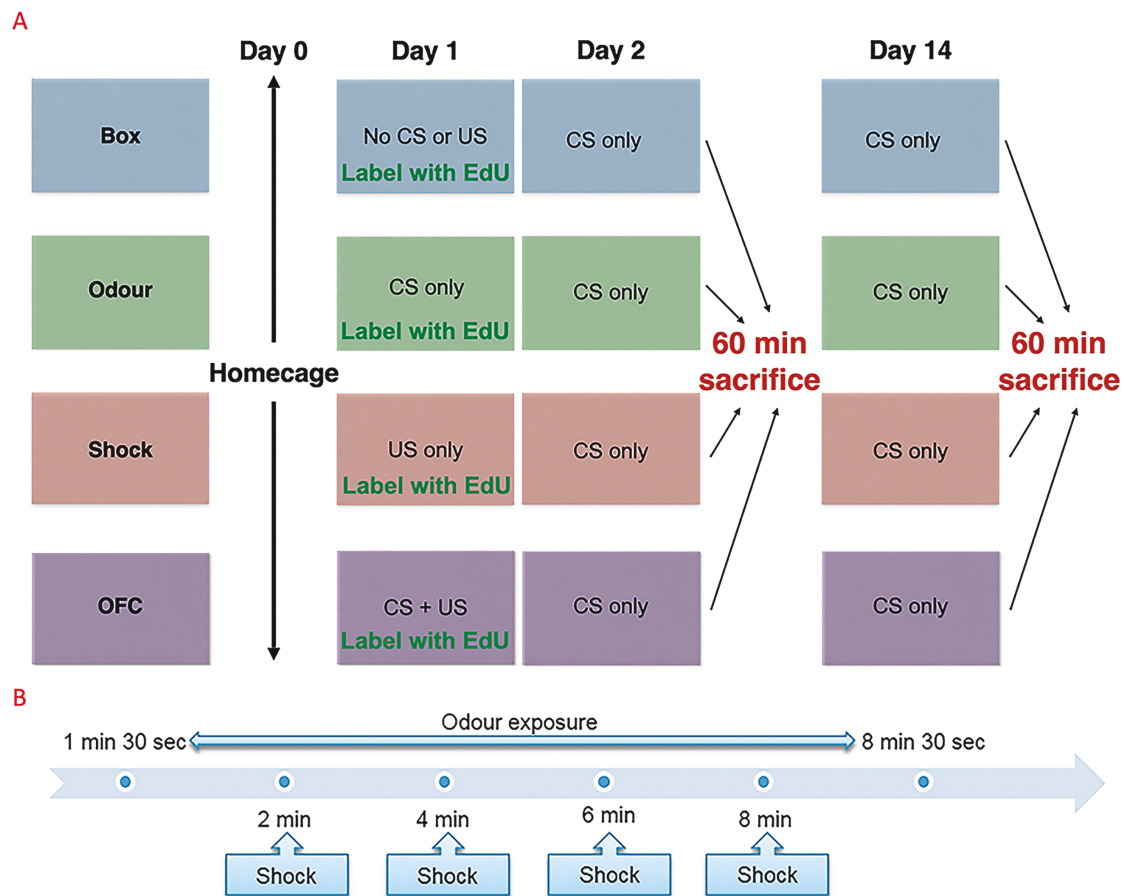
All procedures and protocols were approved by The University of Queensland Animal Ethics Committee (Approval No. 282/17) and Queensland University of Technology Animal Research Ethics Committee (Approval No. 1700000734).

### Behavioral procedures

The behavioral studies were conducted in 2 different contexts. Context A contained a Coulbourn fear conditioning chamber constructed of plexiglass walls and a stainless-steel rod floor connected to a shock generator, situated in a sound-attenuating enclosure. The chamber was equipped with an infrared camera which was connected to a computer to record behavior. Context B had a similar set up but was made distinct with visual and olfactory cues containing a plastic floor covered with fresh bedding and internal colored decoration on the walls and ceiling. 0.25 mL of 100% amyl acetate (neutral odor) was equally distributed onto a piece of filter paper and introduced into the chamber by sliding underneath the rod floor on a waste catcher. The subjects were continually exposed to the odor, after which the odourised air was eliminated by a vacuum. The chamber and enclosure were cleared with a cleaning agent and ethanol (70%) following the testing of each subject.

### Olfactory fear conditioning and EdU delivery

Subjects ( $n = 40$ ) were randomized into one of 4 experimental conditions (olfactory fear conditioning, OFC,  $n = 10$ ; shock alone, Shock,  $n = 10$ ; odor alone, Odor,  $n = 10$ ; box alone, Box,  $n = 10$ ). All subjects were habituated to the conditioning chamber for 15 min, 1 d prior to conditioning. On the day of conditioning, following 90 s of acclimation OFC rats were presented with an olfactory conditioned stimulus (CS), amyl acetate, and 30 s later the unconditioned stimulus (US), a mild foot shock (0.1 mA, 1 s), was introduced, with 4 presentations at 2-min intertrial interval. The CS was removed 30 s after the final US presentation and the rats were removed 90 s later (Fig. 1A and B). Shock rats were presented the US without the CS, while odor rats were presented with the CS without the US. Box rats underwent the same handling and habituation and were exposed to the chamber for the same duration



**Fig. 1.** Conditioning schedule (A) and olfactory fear conditioning protocol (B). All subjects were acclimatized in home cages for 7 + 10 d (habituation and handling) prior to conditioning. On conditioning day (day 1) all subjects underwent their respective conditioning protocols. The box group was in the conditioning chamber for 10 min with neither the shock nor odor. The odor group was in the chamber for 90 s before the odor was presented. After 7 min, the odor was removed and 90 s after that the subjects were removed from the chamber. Shock subjects were in the chamber for 10 min with mild shocks presented every 2 min. Subjects that were olfactory fear conditioned were in the chamber for 10 min and had combined exposure to both the odor and shock (A). Seven minutes of odor exposure after 90 s and mild shock presentations every 2 min (B). Twenty-four hours following conditioning all subjects underwent a fear memory test where they were in a separate context to the conditioning chamber for 10 min and exposed to the odor alone for 7 min. Sixty minutes later, half of the subjects were sacrificed while the rest were returned to their home cages. Two weeks later the remaining subjects underwent the same fear memory test followed by sacrifice 60 min later (B).

as the other experimental conditions; however, they were not exposed to either the CS or US. Following olfactory fear conditioning, all subjects received a single intraperitoneal injection of EdU (Invitrogen) at a dose of 50 mg/kg body weight in 20 mg/mL of PBS to label proliferative cells (Fig. 1A).

Twenty-four hours following fear conditioning, all subjects underwent a fear memory test (FMT), which consisted of exposure to the CS only in context B for the same duration of time as the conditioning procedure. Freezing exhibited by the rats was defined as fearful behavior. Freezing is defined as a lack of all movement except that which is required for respiration (LeDoux 1998, p. 199). The animal was exposed to the odor for a total of 7 min. Of these, only 6 min (360 s) was quantified leaving 30 s at the beginning and at the end to allow for the animal to completely perceive the odor. The total duration of freezing was transformed into a percentage freezing. Mean freezing percentage was the dependent variable for all behavioral analyses. To ensure experimental integrity, scoring was completed by a single observer, and the observer was blinded to the experimental conditions when scoring freezing from recorded videos.

### Tissue preparation

The subjects were sacrificed 24 h or 14 d postolfactory fear conditioning. A 14-d time point was chosen to effectively allow for memory reconsolidation to occur prior to tissue preservation (Nader et al. 2000). The subjects were deeply anesthetized by an intraperitoneal injection of pentobarbital at a dose of 40–50 mg/kg body weight exactly 60 min after the end of the fear memory test (Fig. 1A) and were then transcardially perfused. The latency (60 min) between end of the FMT and perfusion was specified to maximize plasticity protein expression (Schafe et al. 2000). Perfusion was conducted with cold saline (0.9%) followed by cold paraformaldehyde (4% PFA) in 0.1 M phosphate buffer (1× PBS) at pH 7.4. Brains were extracted and postfixed at 4 °C in 4% PFA for 24 h and then transferred to 0.1% sodium azide in 1× PBS for storage. Free-floating serial coronal brain sections containing the olfactory bulb, amygdala, and hippocampus were prepared on a vibratome at 40 μm. All sections were thoroughly washed PBS prior to processing for immunohistochemistry.

### Immunohistochemistry and EdU detection

In order to detect the EdU-labeled cells (prior to immunolabeling), the protocol provided by Click-iT Plus EdU Alexa Fluor 488 Imaging Kit (Cat#: C10637; Life Technologies, Invitrogen) was used, and the sections were incubated in a reaction cocktail for 30 min at room temperature on a slow speed rocker, protected from light. After Edu Click-iT chemistry, the sections were washed in the 3% normal donkey serum in 1× PBS for 5 min, then transferred into blocking solution (3% normal donkey serum, 0.1% Triton X-100 in 1× PBS) for 1 h at room temperature. This was followed by an overnight incubation of the primary antibody polyclonal rabbit anti-pCREB (Cat#: 06-519; 1:5,000; Merck, Germany), which was diluted in the blocking solution. The following day, after 3 washes in blocking solution, the sections were incubated with anti-rabbit Alexa Fluor 594 secondary antibody (1:500, A21207, Invitrogen, USA) for 30 min at room temperature. After 3 washes in 1× PBS, the sections were counterstained using Vectashield 4',6-diamidino-2-phenylindole mounting medium (Vector Labs, CA) to label cell nuclei. Slides were imaged with a Nikon Spectral Spinning disk confocal microscope.

For pMAPK immunostaining, the sections were first thoroughly washed with 1× PBS and then permeabilized with 1% Triton X-100 in 1× PBS for 1 h at room temperature. The sections were then washed thoroughly with 1× PBS before transferred into blocking solution for 1 h at room temperature. This was followed by an overnight incubation of the primary antibody phospho-p44/42 MAPK (Cat#: 9101S; 1:100; Cell Signaling Technology, Australia) diluted in the blocking solution. The following day, after 3 washes, the sections were incubated with anti-rabbit Alexa Fluor 488 secondary antibody (1:500, A21207, Invitrogen, USA) for 30 min at room temperature. After 3 washes, the sections were counterstained using Vectashield 4',6-diamidino-2-phenylindole mounting medium (Vector Labs, CA) to label cell nuclei.

### Image capture and image preparation

Slides were scanned using a Nikon Spectral Spinning disk confocal microscope (Nikon Instruments Inc., NY) to capture 20× magnified tile-scan mosaics of the OB, PC, amygdala, and hippocampus. The scans were set with the following parameters: 7 z-stacks with 2.5 μm step size. All OB scans contained all layers of the bulb. All amygdala scans contained the PC, MeA, and CoA. All hippocampus scans contained the DG. The exposure setting for each antibody was the same across all animal groups. Individual scans (each z-stack and wavelength/channel as a separate image) were saved as separate. tiff files and manually merged using Fiji ImageJ (Schindelin et al. 2012). Merged z-stacks and channels were then stitched in Fiji ImageJ. Images were color balanced uniformly across the field of view with Adobe Photoshop CC 2018 and compiled into panels with Adobe Illustrator CC 2018.

### Neuron quantifications

Neuroplasticity quantification was performed by a single experimenter blind to the experimental conditions. The sections were chosen from 3 matched locations (Bregma  $-2.92$  mm  $\pm$  40 μm). This distance was selected to ensure that the MeA and CoA were present, as well as the DG of the hippocampus and the PC. At this location, only the posterior PC was included. The OB sections were obtained from the middle portion of

the bulb rostrocaudally. There were 3 sections to represent each region per animal and 3 animals per group selected for anatomical assessment. Sections were identically matched across each animal at specific bregma locations ( $-2.92$  mm) by identification of the lateral ventricle and PC size and shape (Chaaya et al. 2019; Jacques et al. 2019). Animals were excluded from a specific location if the section required was missing, visibly torn or damaged.

To segregate the various amygdala subdivisions, a digital rat brain atlas (Paxinos and Watson 2007) was scaled, superimposed, and aligned with a representative immunolabeled section of the amygdala. The anatomical boundaries of the DG, amygdala subnuclei (CoA and MeA), PC and OB were traced to create a generic contour on Fiji Image J (Schindelin et al. 2012). For the quantification of neurons from each subject and at each bregma coordinate, the respective digital contours were positioned over the BLA using various amygdala-centric anatomical features for alignment including the distance/presence of the rhinal fissure, central amygdala, and external capsule. Therefore, the dimensions and position of the generic contour was identical between experimental groups.

Neurons were automatically counted using the particle analyzer function on Fiji ImageJ (Schindelin et al. 2012). An average diameter was obtained for cells labeled with EdU, pCREB and pMAPK. Representative areas of each region were selected and the numbers of cells were counted. To count colocalized cells, the same method was used; however, the channel and intensity filters were adjusted prior to particle analysis to show EdU and pCREB together.

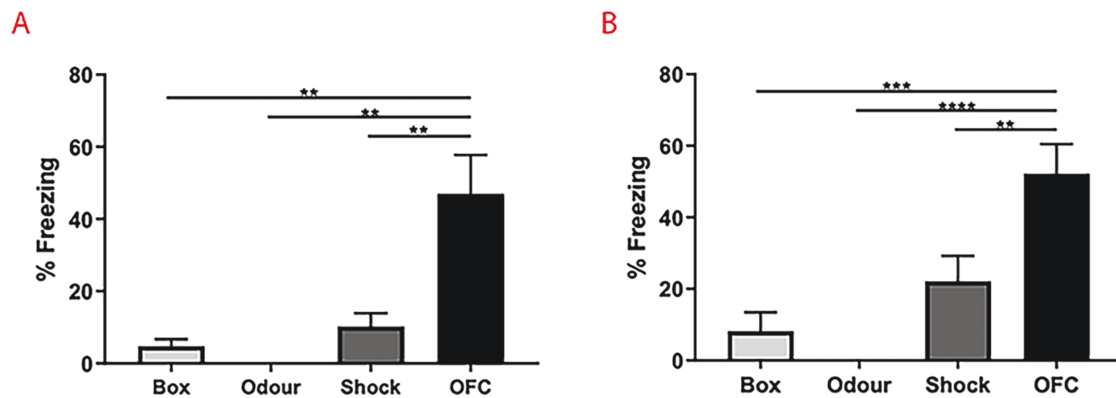
### Statistical analysis

In order to assess the overall difference across all experimental conditions at individual cellular marker, individual subregion and individual Bregma coordinate (e.g. difference in pCREB expression between experimental groups in the DG at Bregma  $-2.92$  mm), a 1-way analysis of variance (ANOVA) was performed. Post hoc Bonferroni correction was used to reduce type 1 errors synonymous with multiple comparisons. Outliers were removed from neuron counts using the ROUT method with the maximum false discovery rate ( $q$ ) set at 1%. A  $P$  value  $\leq 0.05$  was stated as significant, \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ . All statistical analysis was generated with GraphPad Prism 8, and values are expressed as the mean  $\pm$  standard error of the mean (SEM).

## Results

### Olfactory fear conditioning results in more freezing in the conditioned animals in comparison to the control animals

In order to assess whether olfactory fear conditioning results in behavioral changes and distinction between OFC and controls, we studied the freezing response after 24 h following fear conditioning by presenting of the olfactory CS. These data indicated a significantly higher freezing response in OFC animal group ( $F(3, 36) = 8.720$ ,  $P = 0.0002$ ) than the 3 control conditions (Box, Odor, and Shock; Fig. 2A). Furthermore, a fear memory recall was tested at 14 d post-conditioning, which demonstrated the same differences between groups ( $F(3, 22) = 14.21$ ,  $P < 0.0001$ ) (Fig. 2B). These data indicated the fear conditioning protocol employed was sufficient



**Fig. 2.** Fear-related freezing 24 h (A) and 14 d (B) post-conditioning. The fear memory test (FMT) at 24 h and 14 d post-conditioning showed that subjects that were olfactory fear conditioned (OFC) expressed significantly higher levels of fear-related freezing behavior than the box, odor, and shock controls at both time points. There was no significant difference in fear-related freezing behavior between the box, odor, and shock groups in both groups. Asterisks denote level of statistical significance between groups \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ .

to produce a robust and lasting olfactory conditioned fear memory not observed in controls.

### Olfactory fear conditioning increased pCREB and pMAPK expression in the amygdala and piriform cortex

In order to investigate whether the olfactory fear memory has effects on the neuroplasticity of the amygdala and PC, we immunolabeled coronal sections of the amygdala and PC against pMAPK and pCREB. The quantification of pCREB-positive cells in the MeA, CoA, and PC revealed that 24 h after olfactory fear conditioning, pCREB expression increased significantly in these regions (MeA:  $F(3, 32) = 6.706$ ,  $P = 0.0012$ , CoA:  $F(3, 32) = 8.553$ ,  $P = 0.0003$ , PC:  $F(3, 32) = 6.872$ ,  $P = 0.0011$ ) (Fig. 3A and B). There was no significant difference between the 3 control conditions ( $P > 0.05$ ; Fig. 3A and B). We then studied the effect of the olfactory fear memory on pCREB activation 14 days post-conditioning which revealed similar results to 24 h post-conditioning (MeA:  $F(3, 32) = 24.26$ ,  $P < 0.0001$ , CoA:  $F(3, 32) = 45.29$ ,  $P < 0.0001$ , PC:  $F(3, 31) = 7.012$ ,  $P = 0.0010$ ) (Fig. 3C and D). Similarly, pMAPK activity was significantly increased in the MeA, CoA, and PC 24 h post-conditioning (MeA:  $F(3, 8) = 6.352$ ,  $P = 0.0164$ , CoA:  $F(3, 8) = 14.05$ ,  $P = 0.0015$ , PC:  $F(3, 8) = 15.55$ ,  $P = 0.0011$ ) (Fig. 4B, C, and D, respectively). Together, these results suggest that the MeA, CoA and PC play a vital role in olfactory fear memory reconsolidation and expression.

### Olfactory fear conditioning increased the number of pCREB-positive proliferating neurons in the hippocampus and olfactory bulb

Next, the effects of olfactory fear conditioning on the neurogenesis of the olfactory pathway and the hippocampus was examined by quantifying the number of EdU-positive cells co-labeled with pCREB 24 h post-conditioning. EdU and pCREB colabeling in the DG revealed that there was a significant ( $F(3, 8) = 9.656$ ,  $P = 0.0001$ ) increase in the number of co-labeled neurons in the OFC group compared with the control groups (Fig. 5B). There was a similar significant ( $F(3, 8) = 8.266$ ,  $P = 0.0004$ ) increase observed in the glomerular layer of the olfactory bulb where the primary olfactory neurons make synapse with the second order neurons (Fig.

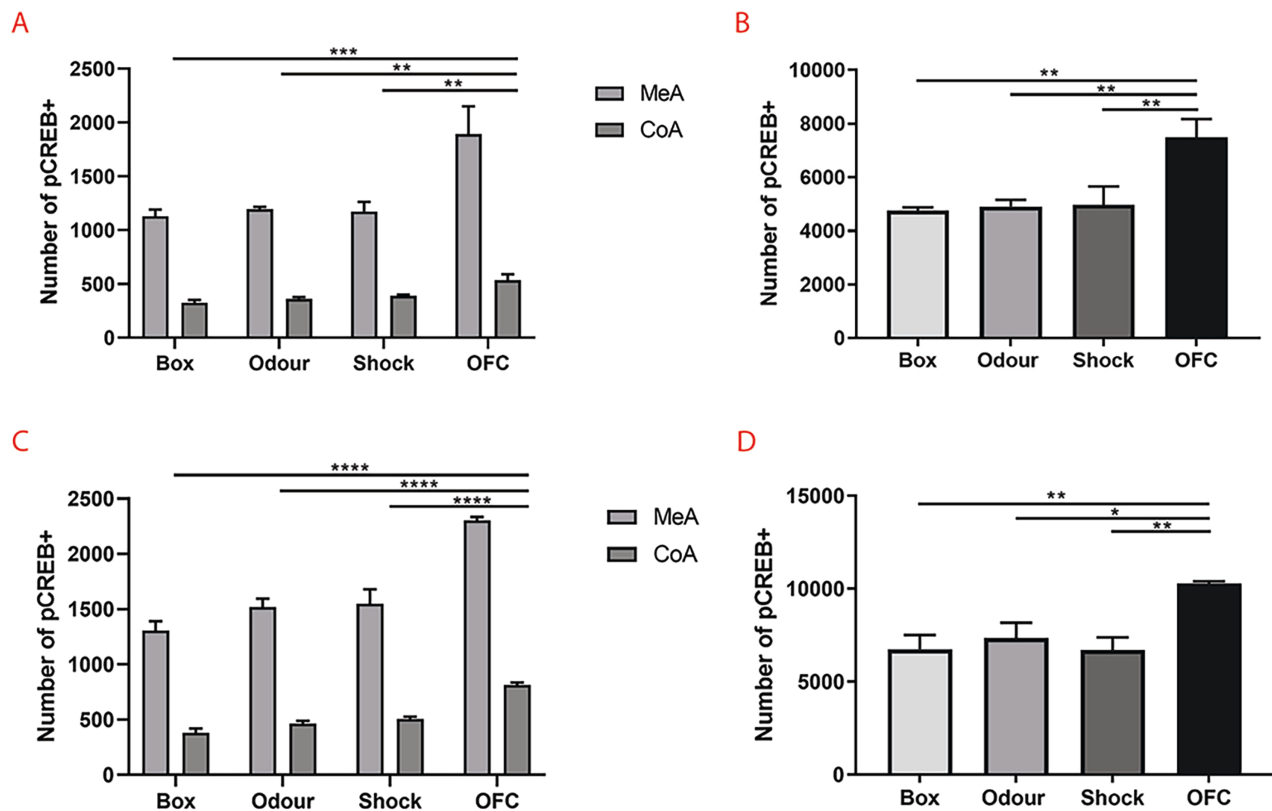
5B). However, there was no statistical difference between the 3 control conditions ( $P > 0.05$ ). These differences between the groups were similar at 14 d post-conditioning; however, the number of co-labeled cells had decreased in the DG and increased in the OB (Fig. 5C). This suggests that an aversive associative memory of shock and odor (OFC) increased the number of newborn neurons in the DG of the hippocampus and the glomerular layer of the OB.

## Discussion

Memory consolidation is a process by which unstable short-term memory is stabilized and integrated into the long-term memory trace (Dudai 2004). In contrast, memory reconsolidation involves retrieval of a consolidated memory by presentation of the original stimulus or a “reminder” cue, making the memory labile again (McKenzie and Eichenbaum 2011). Therefore, by testing olfactory fear memory recall after 24 h, our results show the behavioral and cellular changes of long-term reconsolidated memory. Behavioral data showed successful acquisition of fear to odor, but not to other parameters (shock or context) in the conditioned group.

The understanding that reactivated memories return to a labile state has many implications. One being, that the malleability may allow it to be manipulated for treatment of PTSD by reactivating fear memories and creating conditions to prevent reconsolidation. Some studies have inhibited protein synthesis essential for synaptic plasticity during reconsolidation with the aim of causing amnesia for fear conditioned memories (Mactutus et al. 1979; Judge and Quartermain 1982; Przybylski and Sara 1997; Nader et al. 2000). Unfortunately, these inhibitors have considerable toxicity and have not been optimized for human use. Our study identifies active markers and potential targets for olfactory fear memory reconsolidation, which may help to guide further studies and clinical translation.

Our data demonstrated that an olfactory CS can successfully elicit a Pavlovian conditioned response such as freezing that is consistent with previous studies in which the fear conditioned group displayed significantly higher levels of fear (freezing) than the 3 control groups under the presence of the odor (Schettino and Otto 2001; Sevelinges et al. 2007). This was evident at both time points, 24 h and 14



**Fig. 3.** pCREB expression in the MeA, CoA (A) and PC (B) 24 h post-conditioning. pCREB expression in the MeA, CoA (C), and PC (D) 14 days post-conditioning. Evaluation of the number of pCREB + cells revealed statistically significant differences between the OFC subjects compared to the box, odour, and shock control groups at both time points. There was no significant difference between the box, odour, and shock control groups at both time points. pCREB, cyclic adenosine monophosphate response element-binding protein; OFC, olfactory fear conditioned; DG, dentate gyrus; MeA, medial amygdala; CoA, cortical amygdala; PC, piriform cortex. Asterisks denote level of statistical significance between groups \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ .

d post-conditioning, indicating that discrete olfactory fear memory is strongly reconsolidated 24 h after acquisition and able to be recalled up to 14 d after acquisition.

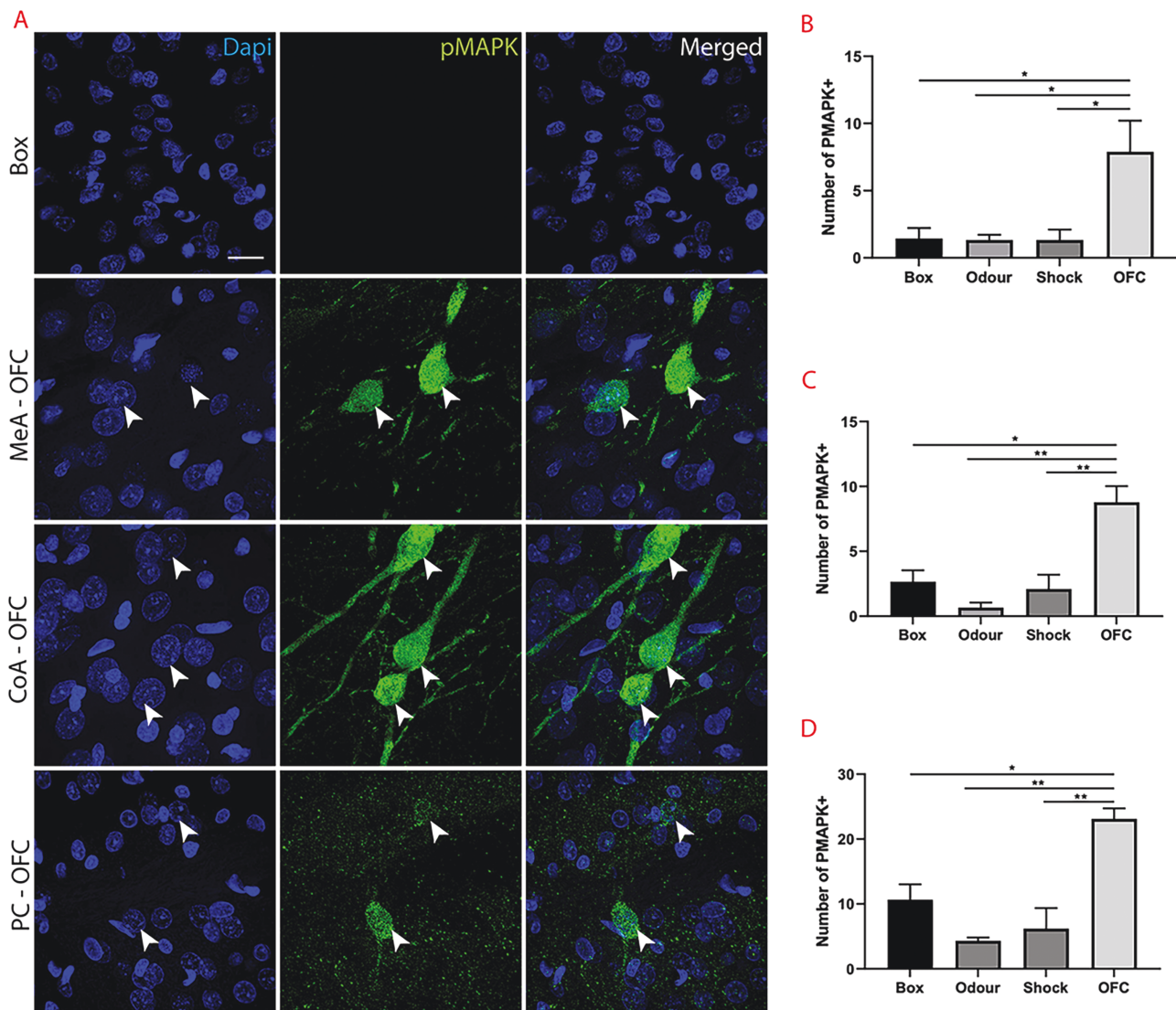
Our data indicate that OFC can increase neuroplasticity in the MeA, CoA, and posterior PC 24 h and 14 d post-conditioning, as shown by immunolabeling against pCREB and pMAPK. Immediate early genes (IEGs), such as pCREB, are genes that rapidly transcribe in response to neuronal activity and lead to synaptic plasticity and synaptogenesis (Flexner et al. 1963; Lynch and Baudry 1987), and they are a requirement for long-term potentiation (LTP) or long-term memory (Abraham et al. 1993; Silva et al. 1998; Tischmeyer and Grimm 1999). A previous study showed that the expression of IEG's has only been observed beyond 6 weeks after neuron birth (Jessberger and Kempermann 2003); however, our study showed that pCREB is readily expressed at 24 h and 2 weeks after OFC in the EdU-positive neurons of the glomerular layer of the OB and DG of the hippocampus. This data provides evidence to support the idea that IEG's may be stimulated as early as 24 h following OFC.

We consolidated these results by investigating the activity of pMAPK (a protein involved in activity-dependent modulation of synaptic plasticity), which has a well-characterized role in consolidation of fear memory in the hippocampus and amygdala (Schafe et al. 2000; Arendt et al. 2004; Bergstrom et al. 2011). Our findings showed a significant increase in region-dependent pMAPK expression as a result of olfactory fear memory recall 24 h after OFC. Studies with auditory and

visual fear conditioning localized pMAPK expression predominantly to the LA (Benedetto 2008; Schafe et al. 2000; Bergstrom et al. 2011; Bergstrom, McDonald, Dey, Tang, et al. 2013; Jacques et al. 2019). However, our data indicate that the neuroplasticity differs considerably for OFC and is predominantly localized to the CoA, MeA of the amygdale, and the posterior PC.

The CoA and MeA receive direct input from the olfactory system, as well as somatosensory inputs, allowing them to be activated by both odor and shock (Kevetter and Winans 1981; Schettino and Otto 2001; Hakim et al. 2019). The combination of information lead to the high pCREB expression observed following OFC, as compared to single stimulus controls. Similarly, a study conducted by Schettino and Otto (2001) found elevated neuronal activity in the CMe, which contains the CoA and MeA, following OFC. Other studies have found higher expression of other activity protein markers such as C-FOS in the CeA and BLA; however, the results were not consistent and the markers are known to be nonspecific to LTP (Sevelinges et al. 2004; Walker et al. 2005). Although our study suggests their involvement, an ablation or silencing investigation of the CoA and MeA in OFC would be necessary to confirm their role in the mediation of such memories.

Our data showed that OFC led to elevated expression of pCREB at both time points and pMAPK at 24 h post-conditioning in the posterior PC; however, the odor alone did not evoke significant expression of these substrates in



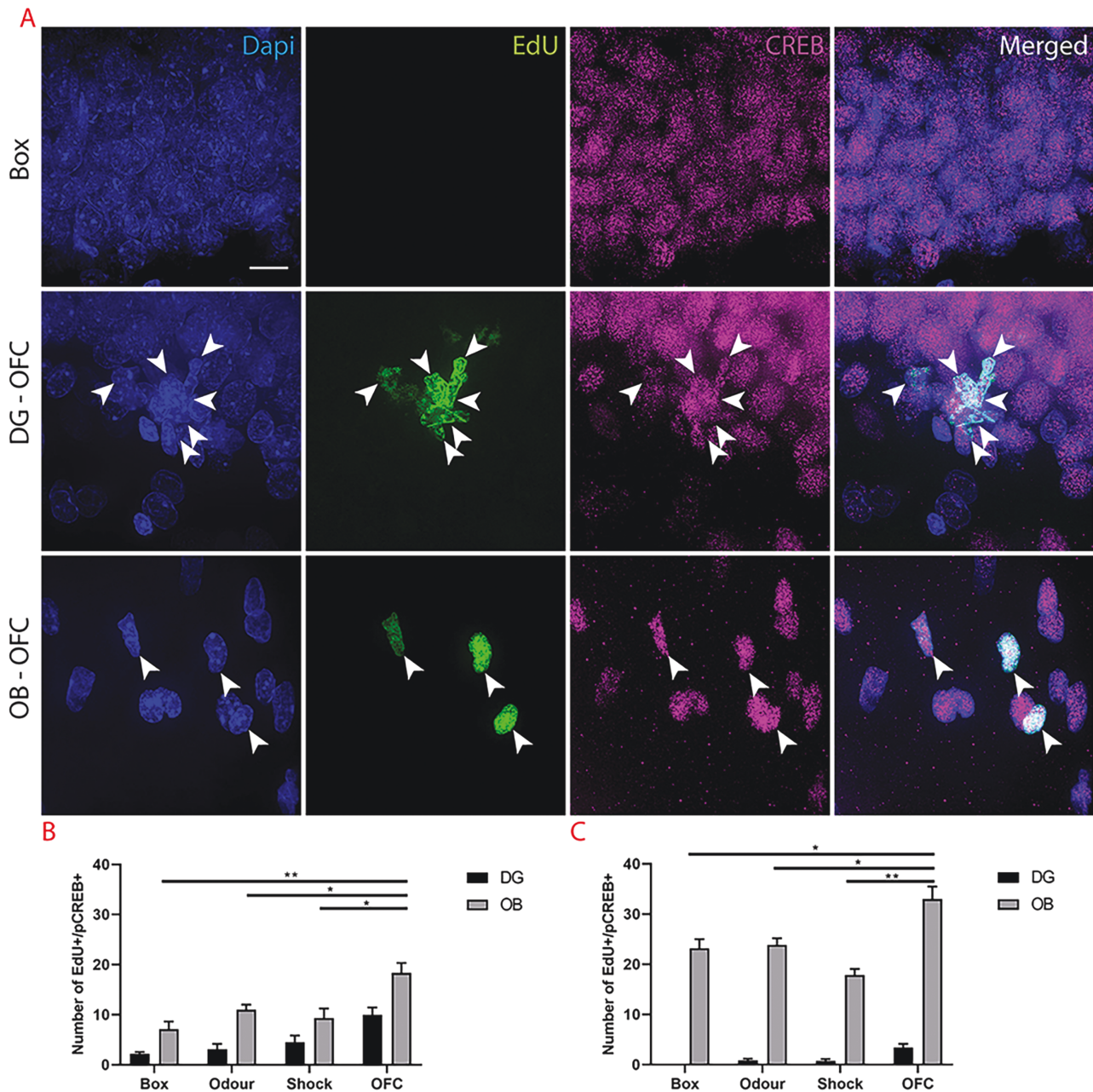
**Fig. 4.** pMAPK expression in the MeA (B), CoA (C), and PC (D) 24 h post-conditioning. Representative images of labeled pMAPK from a box group and OFC group of each region at 24 h post-conditioning (A). Evaluation of the number of pMAPK + cells revealed statistically significant differences between the groups in all 3 regions. Subjects that underwent OFC had significantly more pMAPK + cells when compared with the box, odor, and shock control groups in all 3 regions (B, C, D). Arrowheads are referring to co-labeled DAPI and pMAPK cells. pMAPK, phosphorylated mitogen-activated protein kinase; MeA, medial amygdala; CoA, cortical amygdala; PC, piriform cortex; OFC, olfactory fear conditioning; DAPI, 4',6-diamidino-2-phenylindole. Scale bar represents 50  $\mu$ m in all images. Asterisks denote level of statistical significance between groups \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ .

the PC, despite it being a novel odor. This indicates that fear-associated olfactory input has a higher potential to be perceived in the PC than a neutral olfactory input. This result is interesting as the PC is the largest direct cortical recipient of odor information from the olfactory bulb (Woolsey and Van der Loos 1970; Henkin et al. 1977; West and Doty 1995; Illig and Haberly 2003; Rennaker et al. 2007; Chen et al. 2014). It plays an important role in odor processing by integrating cognition and experience into odor information (Wilson and Sullivan 2011). Due to its salient role in the olfactory system, the posterior PC expectantly showed a vast increase in pCREB-positive cells following OFC compared to other regions. The posterior PC then has direct projections to the MeA and CoA, making it a vital input source for olfactory information reaching the amygdala (Krettek and Price 1978). The PC has long been thought to store associative olfactory memories, yet the cellular substrates for the function remained unknown. Our study has revealed that posterior PC

neurons expressing both pCREB and pMAPK are positively associated with the neural circuit involved in facilitating olfactory fear learning and memory.

Our results regarding the effect of olfactory fear memory reconsolidation on neurogenesis showed a significant increase in proliferative neurons in the DG of the hippocampus and the glomerular layer of the OB at 24 h and 14 d post-conditioning. The increase in the number of EdU-labeled neurons in the DG of the hippocampus suggests that OFC may be a form of hippocampal-dependent learning (Kempermann et al. 2004; Ma et al. 2009; Sakamoto et al. 2014). Note that a previous study has also showed that hippocampal-dependent learning affects the number of proliferating neurons in the hippocampus (Gould et al. 1999).

Similarly, we found that the glomerular layer of the OB showed elevated numbers of EdU-positive neurons co-labeled with pCREB 24 h after OFC, which increased 2 weeks post-conditioning. This increase was observed in all groups;



**Fig. 5.** Expression of pCREB in proliferating neurons in the DG and OB 24 h (B) and 14 d (C) post-conditioning. Representative images of co-labeled pCREB and EdU neurons from a box group and OFC groups from each region at 24 h post-conditioning (A). Evaluation of the number of co-labeled pCREB + and EdU + neurons revealed statistically significant differences between the groups in the granular layer of the DG and glomerular layer of the OB. Subjects that underwent OFC had significantly more co-labeled cells when compared with the box, odour, and shock control groups at both regions and both time points (B, C). Arrowheads are referring to co-labeled pCREB + and EdU + cells (A). There was no significant difference between the box, odour, and shock groups in either region at both time points (B, C). pCREB, cyclic adenosine monophosphate response element-binding protein; EdU, 5-ethyl-2'-deoxyuridine; DG, dentate gyrus; OB, olfactory bulb; OFC, olfactory fear conditioning. Scale bar represents 50  $\mu$ m in all images. Asterisks denote level of statistical significance between groups \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ .

however, OFC remained significantly higher. It has previously been suggested that OFC stimulates neurogenesis in the subventricular zone (SVZ) from which cells migrate rostrally toward the granular layer of the OB (So et al. 2008; Sultan et al. 2010). However, the glomerular layer of the OB also continuously receives newborn neurons migrating from the SVZ (Lois and Alvarez-Buylla 1994; Ma et al. 2009). One study found that 15 d after injection of BrdU in the SVZ, most migrated neurons in the OB were found around the glomeruli (Lois and Alvarez-Buylla 1994). Furthermore, the survival of

these neurons significantly increases in response to olfactory discrimination learning (Alonso et al. 2006; Mandairon et al. 2006).

Our results showed that independent spatial, odour, and shock learning in the box, odour, and shock control conditions, respectively, had minimal effect on olfactory neurogenesis and on olfactory fear memory expression. However, when associated with fear, there was a significant improvement in memory recall in the OFC group, which was also positively associated with enhanced number of EdU + periglomerular



neurons. A study conducted by Rochefort et al. (2002) also found that an increased number of newborn neurons in the OB lead to improved olfactory memory, independent of hippocampal learning and neurogenesis. This could further attest to the importance of olfactory neurogenesis in memory formation.

## Conclusion

Our findings, combined with previous works, have provided many valuable insights into the molecular changes in the neurocircuitry of OFC. Investigation of neurogenesis showed that olfactory fear conditioning increased neurogenesis in the periglomerular neurons of the OB. The OB has direct projections to the posterior PC, where fear-associated olfactory information is perceived at a higher potential than neutral olfactory information. Information is then sent to the amygdala via the direct connection from the posterior PC to the CMe group (containing the CoA and MeA), as well as directly from the OB. The CMe presumably functions to evoke a behavioral fear response. Increased pCREB expression and EdU-labeled cells in the DG indicated that olfactory fear memory recall leads to enhanced neuroplasticity and neurogenesis, and newborn neurons are most likely being re-consolidated via LTP and integrated into the olfactory fear memory trace. Further investigations of the trajectory of these newborn neurons may give insight into the specific function of this event. As the neurobiological mechanisms of normal fear memory expression are thought to be compromised in PTSD, a thorough understanding of these mechanisms and the neuroanatomy involved is necessary for providing potential new microanatomical pharmacological therapeutic target sites.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data Availability

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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