

# Alteration of the neurogenesis and long term potential of olfactory bulb in an animal model of PTSD

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Failure of extinction of fear-conditioned traumatic memory is the main pathology behind post-traumatic stress disorder (PTSD). Functional and structural dysfunctions in the olfactory system are implicated by studies in PTSD patients. However, little is known regarding the neurobiological networks of trauma-related odor sensitivity in PTSD. Male Wistar rats were exposed with a female cat for 10 min and long-term stress was evaluated by behavioral tests, containing open field (OF) and elevated plus maze (EPM). To prove the PTSD model, the serum level of cortisol was evaluated and compared with the control group. Local field potential (LFP) was applied to compare the electrophysiology of the OB in two groups. To assess neurogenesis, the expression of nestin, and doublecortin were evaluated. Data from EPM revealed a significant increase in spent time in the closed arms in PTSD group. We observed a significant reduction in OF parameters in terms of the total distance traveled, the time spent in the central zone, and the number of crossing the central zone in PTSD group compared to the control group. The mean serum cortisol level was significantly higher in the PTSD group than the control group. In LFP recording, the slope and the amplitude of field excitatory postsynaptic potential (fEPSP) in the PTSD group were significantly higher than that of the control group. Our results also showed that the mRNA expression level of nestin as a neural progenitor marker and doublecortin, as an immature neuron marker, significantly decreased in the PTSD group compared to the control group. This study has shown that PTSD can disrupt the OB function through decreasing neurogenesis. More information on PTSD and OB would help us to establish a greater degree of accuracy on this matter.

**Key words:** post-traumatic stress disorder, neurogenesis, behavioral assessment

## INTRODUCTION

To understand the pathophysiology of emotional disturbance in mental disorders, evaluation of smell memory can provide valuable insight into the underlying mechanism. Failure of extinction of fear-conditioned emotional or traumatic memory is the core pa-

thology behind post-traumatic stress disorder (PTSD) (Vermetten and Lanius, 2012; Yehuda et al., 2015), which makes it a potential target for assessment of olfactory memory dysfunction. Specific trauma-related odors (like the odor of burning or diesel) have long been considered by researchers to be precipitants of anxiety and fear-related memories in PTSD patients (Vermetten

and Bremner, 2003). In addition, comparing to visual, acoustic, or tactile stimuli, olfactory–limbic pathways are synapsing more direct with the amygdala–hippocampal complex (Otto et al., 1991). Therefore, olfactory stimuli are relatively more effective at triggering aversive memories related to trauma (Wiemers et al., 2014; Parsons et al., 2018). Due to close link of odor memory to intense emotions, its assessment seems to be very promising in PTSD studies (Daniels and Vermetten, 2016). However, in contrast to the vast empirical literature on cognitive processing of visual or verbal memory, comparatively few studies have evaluated olfactory memory in trauma-related disorders (Daniels and Vermetten, 2016).

Functional as well as structural dysfunctions in the olfactory system are implicated by studies in PTSD patients (Cortese et al., 2018). Comparing grey matter volume (GMV) of veterans with and without PTSD revealed significant reduced GMV in the anterior piriform (primary olfactory) and orbitofrontal (secondary olfactory) cortices of PTSD veterans to non-PTSD ones (Cortese et al., 2015). Comparing olfactory and visual cues by fMRI analysis of PTSD patients have shown dramatically higher activation of the hippocampal and amygdala regions during recall to the personally significant odor (Herz et al., 2004). In addition, fMRI study of PTSD patients in response to olfactory stimuli indicated an increased activation in several brain area, such as precentral frontal lobe, inferior and middle frontal structures, posterior parietal lobe, occipital lobe, and the posterior cingulate cortex (Croy et al., 2010). However, up to now, far too little attention has been paid to the neurobiological networks of trauma-related odor sensitivity or how they relate to other objective and subjective measures of olfaction and PTSD (Cortese et al., 2018).

In the last decade, an increasing interest has been paid to neurogenesis, and in particular to the plasticity of the olfactory bulb (OB). In the adult mammalian brain, neural stem cells (NSCs) have found in subgranular zone of the dentate gyrus and the walls of the lateral ventricles that generate new neurons for hippocampus and olfactory bulb, respectively (Hayashi et al., 2018). It is suggested that the main function of adult neurogenesis is pattern separation, defined as the ability of hippocampal circuits to discriminate highly similar inputs, through generating different neuronal activity patterns (Chavlis and Poirazi, 2017). This is completely necessary for memory since the human being has different experiences that are similar to each other but each one must be remembered as distinct (Snyder, 2013). Regarding PTSD, dysfunctional pattern separation in the dentate gyrus (DG)-CA3 circuit has been found (Besnard and Sahay, 2016). It could resolve interference between

ambiguous or uncertain threats and maintain episodic content of aversive memories in hippocampal-cortical networks of PTSD patients (Besnard and Sahay, 2016; Ishikawa et al., 2016). Despite the promising finding in the assessment of hippocampal neurogenesis, evaluation of neurogenesis dysregulation in the OB of PTSD patients is completely neglected.

A sophisticated investigation of the role of olfactory bulb dysfunction in the pathophysiology of PTSD can provide a better understanding to the underlying brain circuits of this chronic disorder, which opens new lines for further basic and clinical studies. To achieve this purpose, after confirming our animal model of PTSD, we applied electrophysiological and molecular techniques to evaluate the possible alteration of physiology as well as neurogenesis in OB.

## METHODS

### Animals

Twenty-eight 8 weeks old male Wistar rats weighing 200–250 g were obtained from the animal house of Mashhad University of Medical Sciences, Mashhad, Iran. Rats were housed in plexiglass cages under standard condition (12–12 h light/dark schedule, the humidity of 54±2% and temperature 22±2°C) and free accessibility to food and water (*ad libitum*). Animal examinations were carried out in accordance with procedures approved by the Committee on Animal Research of Mashhad University of Medical Sciences.

Rats were randomly divided into two groups: experimental (PTSD) and control group (14 rats in each group). Elevated plus maze (EPM), open field (OF), and electrophysiological studies were evaluated (8 rats in each group). Six rats in each group were assessed for molecular evaluation.

### Establishment of animal model of PTSD

For this purpose, exposure to a female cat was applied. In each day, two rats were selected from the local Animal Research Center and weighed. In order to acclimate to the room and removing the environmental anxiety, rats were brought to the exposure room, which was located in a different area of the animal facility than the laboratory, 15 min prior to the exposure. Rats were individually immobilized in transparent circular plexiglass container (radial=3.75 cm, height=15 cm) smeared up with the cat's favorite food. It directed cat activity toward the rats. The circular plexiglass enclosure averted any contact between the cat and rats but

exposed the rats to all non-tactile sensory stimuli related to the cat. For oxygen providing and detecting the cat's smell, the door of the circular plexiglass container was punched several times. In the experiment room, an adult, female cat was placed inside the metal cage (60 cm × 100 cm × 50 cm) and kept hungry for 12 h. The immobilized rats, inside the specific circular plexiglass container, were exposed to the cat for 5 min. After this period, the rat was taken out and placed in his own cage (Fig. 1).

## Elevated plus maze

EPM is a routine and common test for measuring anxiety in rodents. This apparatus consists of two open (50 cm × 10 cm) and two enclosed (50 cm × 10 cm × 40 cm) arms that intersect to each other and form plus sign. Rats were placed by gloved hands on the center of EPM and their ambulation was controlled for 5 min. A camera was set above the apparatus and recorded

rat movements. This camera was connected to a computer application that processed all of the movements. In each day, 2 rats were brought to the behavioral test room and evaluated. After one trial for each rat, the procedure was repeated one more time. The dependent measures were the total time spent in the open and closed arms. Entering an arm was considered only when the entire rat's body was moved to that arm. In the present study, EPM was performed on day 7 after the exposure.

## Open field test

The OF was a well-known test to measure anxiety levels in accordance with exploratory behavioral changes. The apparatus consists of a dark gray square box (100 cm × 100 cm) enclosed with 50 cm high walls. The square was divided into 10 smaller square (10 cm × 10 cm) by means of thin white lines. The open-field test was conducted in a room with controlled noise, temperature, and light. In each trial, a rat was placed in the center of the apparatus and rat's behavior was recorded by a camera above the apparatus for 5 min. The computer program scored entry to a square only when the whole rat's body was moved. After completing one trial for each rat, this procedure repeated one more time and spent time in the central zone, total ambulation distance, and central square entries were assessed. In the present study, the OF test was performed on day 7 after the exposure.

## Electrophysiological studies

The animals were anesthetized with urethane (1.6 g/kg) deeply. Then, rat's heads were fixed in the stereotaxic apparatus. After removing the skin and exposing the skull, bregma and lambda points were detected. In order to place recording and stimulating electrodes, two small holes were drilled, under sterile conditions. For recording field excitatory postsynaptic potential (fEPSP), a bipolar stimulating stainless-steel electrode with 0.125 mm in diameter was fixed in the lateral olfactory tract (LOT) of the right hemisphere (7.5 mm deep, 2.3 anterior and 3.5 mm posterior). The coordinates were based on the atlas of Paxinos and Watson and a unipolar recording electrode with the same characteristics of the stimulating electrode was inserted into the olfactory bulb of the ipsilateral hemisphere. The proper location of the electrodes was determined using physiological and stereotaxic indicators. The stimulating electrode and recording electrode were attached to a stimulator and an amplifier respec-

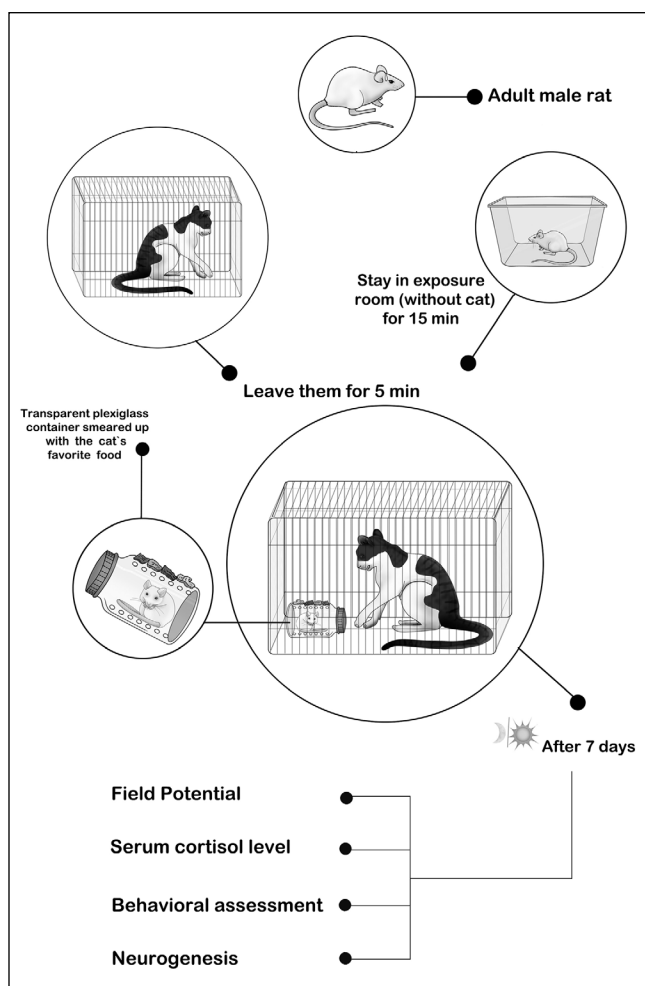


Fig. 1. Establishment of animal model of PTSD.

tively. A screw was implanted into the skull above the left cortical surface and used a ground electrode.

After surgery, the animal was allowed to rest for 30 min. Then, for evaluating synaptic potency before induction of stimulation, the input-output (I/O) protocol was applied. For this purpose, the intensity of stimuli was increased step by step with a constant current as input (100-1000  $\mu$ A) and fEPSP was recorded as output. Afterward, fifty percent of the current which provided maximum response was considered for recording baseline during 15 min. The stimuli were then administered with the same current by the high-frequency stimuli (HFS) protocol of 300 Hz, and extracellular field potential was detected from the olfactory bulb in following stimulation of the LOT. The recording of fEPSP was continued 15 min after high-frequency stimuli. The result was then amplified (100 $\times$ ) and filtered (1 Hz to 3 kHz bandpass) applying differential amplifier. For computer-based stimulating and recording, Neurotrace software version 9 and Eletromodule 12 (Science Beam Institute, Iran) were used. The analysis of responses was carried out using specific software from the same institute. Using the provided software the slope between the baseline and the peak of the negative wave in each trace was measured and considered as fEPSP slope. The amplitude of fEPSP was measured as the difference in voltage between the negative peak of the fEPSP wave and the baseline. An average of slope and also amplitude of fEPSPs during 15 min before HFS was calculated. The value of the slope and amplitude of the fEPSP at each point in the graph was averaged from 10 consecutive traces. The ratio of slope and amplitude of fEPSPs in each point to the calculated average of the values during 15 min before HFS was calculated and provided as percent.

### Corticosteroid test

Although the merit of the evaluation of serum cortisol level for the validation of animal model of PTSD has been argued, generally it is applied as an index of long-term anxiety biomarkers. To assess concentration of serum cortisol level, after recording field potential,

while the rats were in the 4<sup>th</sup> grade of deep urethane anesthesia, carotid artery was cut and blood samples were collected. These samples were centrifuged (2200-2500 RPM) and the serum was immediately frozen at -80°C.

### Assessment of neurogenesis in OB

To determine mRNA values, qRT-PCR was performed. Total RNA was extracted from tissues in control and treatment groups using the RNeasy Mini kit. To eliminate genomic DNA, samples were treated with the RNase-Free DNase Set (Qiagen, Germany). The RNA concentration was assessed at 260 nm absorbance using a spectrophotometer (Nanodrop 1000, Thermo Scientific, USA). First-strand cDNA was synthesized with 500 ng of RNA using the cDNA Synthesis Kit (Thermo Scientific, Germany). qRT-PCR amplification was performed with a CFX96 real-time PCR detection system (Bio-Rad, Germany) using Eva Green dye (Invitrogen, Germany). Nestin (neural progenitor marker) and Dcx (immature neuron), as well as the internal control  $\beta$ -actin, were evaluated (Table 1).

### Statistical analysis

Analyses were conducted by GraphPad Prism software (version 7.0). Statistically significant differences between two groups were assessed using paired sample t test (for amplitude and slope of LFP signal before and after stimulation) and independent samples T test. Data were presented as the mean  $\pm$  standard deviation and the significance level was considered at  $P < 0.05$ .

## RESULTS

### Establish PTSD model

To prove the PTSD model, we evaluated the cortisol serum level. Our findings demonstrated that the cor-

Table I. The sequence of primers and annealing temperature of applied neurogenesis markers.

Gene	Length (bp)	Primers Forward primer 5' to 3' Reveres primer 3' to 5'	T A (°C)
Nestin	226	GTATGATGCCAAGAAGCCAACAT GGACTGGCTGTGTCTTGAATC	63
Dcx	190	GCTGACCTGACTCGATCCTT CCGACCAGTTGGGATTGACAT	59
Act $\beta$	98	AAGTCCCTCACCCCTCCAAAAG AAGCAATGCTGCACCTTCCC	60

tisol level significantly increased in the PTSD group compared to the control group (Fig. 2A,  $t_{14}=-7.093$ ,  $P<0.001$ ). Our results showed that time spent in closed arm as a marker for anxiety like behavior significantly increased in PTSD group compared to the control group (Fig. 2B,  $t_{62}=-2.347$ ,  $P<0.05$ ). In comparison to control group, significant decrease have been observed in PTSD group in the result OF test including total distance traveled ( $t_{23,758}=3.477$ ,  $P<0.01$ ), time in the center square ( $t_{15,757}=2.626$ ,  $P<0.05$ ), and the number of crossing in the central zone ( $t_{15,679}=2.671$ ,  $P<0.05$ ) (Fig. 2C).

### Electrophysiological assessment

There was no significant difference between baseline amplitude of LFP recording in PTSD and control groups, which were averagely 97.57 and 98.91 mV in the same order. Applying of HFS increased 10-90% slope of fEPSP in PTSD ( $t_{23}=-14.634$ ,  $P<0.001$ ) as well as control ( $t_{23}=-6.592$ ,  $P<0.001$ ) groups compared to before applying stimulation. After applying of HFS, the 10-90% slope of fEPSP in PTSD group was significantly higher than that of control group ( $t_{30,349}=-11.416$ ,  $P<0.001$ ). High frequency

stimulation also increased amplitude of fEPSP in both groups compared to before applying stimulation (PTSD group:  $t_{23}=-22.292$ ,  $P<0.001$ , Control group:  $t_{23}=-7.347$ ), but the amplitude of fEPSP in PTSD group was significantly higher than that of the control group (Fig. 3,  $t_{46}=-15.332$ ,  $P<0.001$ ).

### Neurogenesis

Our results showed a significant decrease in the mRNA expression level of nestin, as a neural progenitor marker ( $t_{8,429}=4.321$ ,  $P<0.001$ ) and Dcx, as an immature neuron marker ( $t_5=2.618$ ,  $P<0.001$ ) in PTSD group compared to the control group (Fig. 4).

### DISCUSSION

To best of our knowledge, this paper is the first study to evaluate the role of olfactory bulb in the pathophysiology of PTSD by assessment of OB electrophysiology and evaluation of its neurogenesis related markers in the animal model of PTSD. For establishment of proper

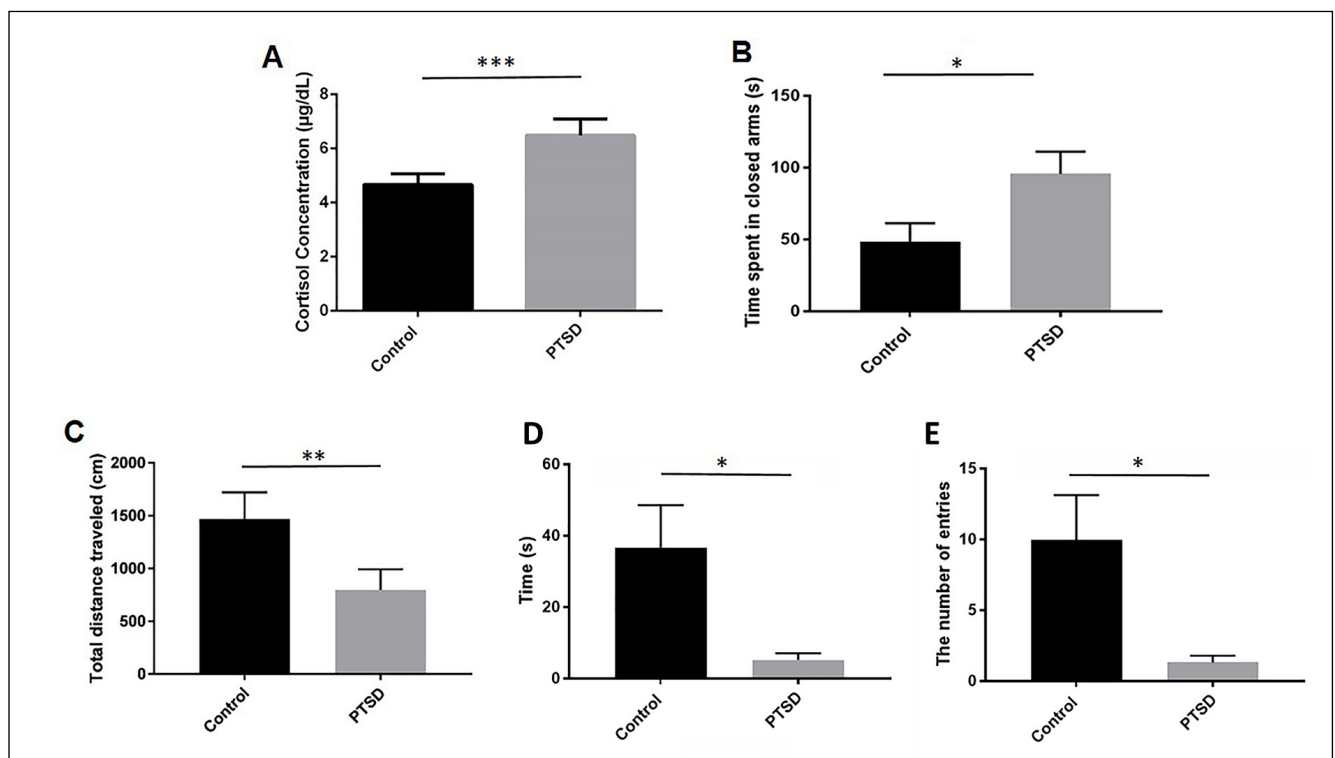


Fig. 2. (A) Comparison of serum cortisol level between two groups (n=6 in each group); \*\*\* $p<0.001$  compared to control group. (B) Comparison of total spent time in the closed arms of EPM test between two groups (n=8 in each group); \* $p<0.05$  compared to control group. (C) Comparison of total distance traveled between two groups (n=8 in each group); \*\* $p<0.01$  compared to control group; (D) Comparison of time spent in the central area between two groups (n=8 in each group); \* $p<0.05$  compared to control group; (E) Comparison of number of central crossing between two groups (n=8 in each group); \* $p<0.05$  compared to control group.

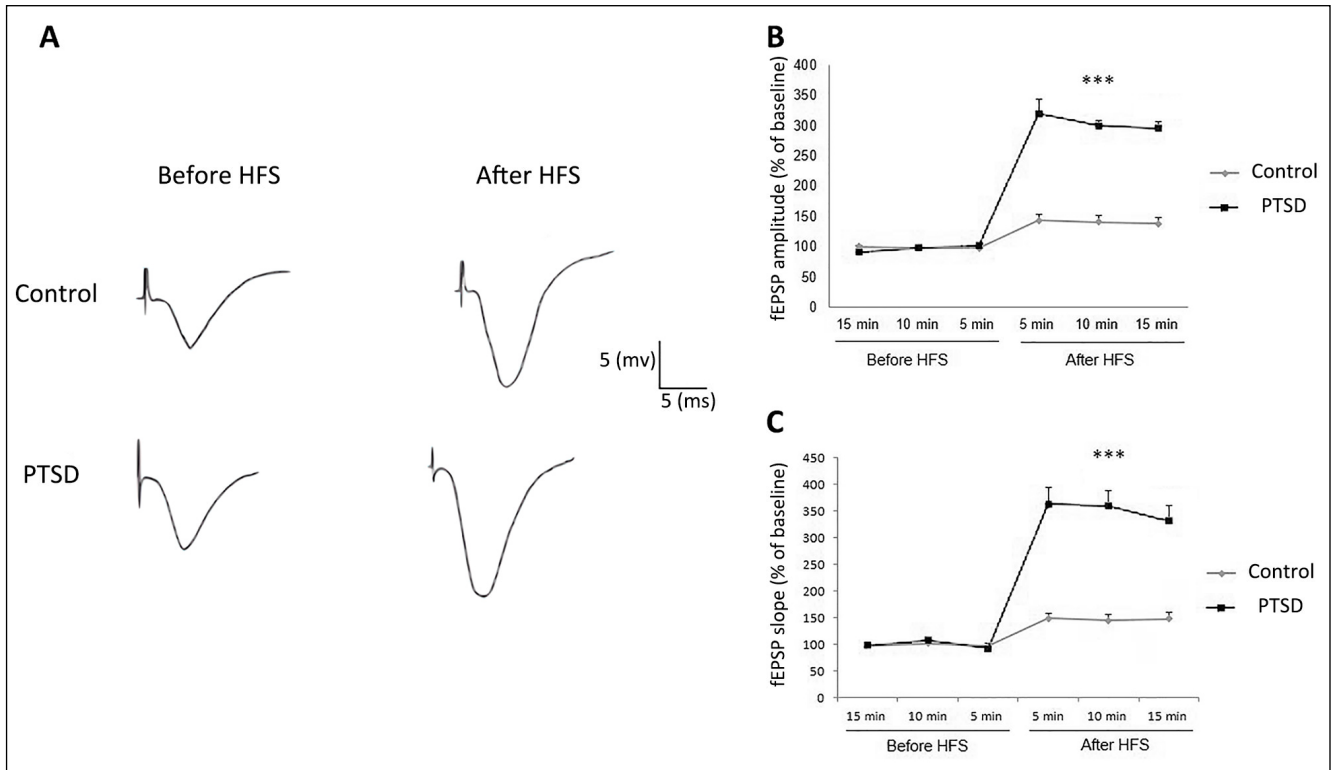


Fig. 3. (A) Representative changes in LFP after application of high frequency stimulation (HFS). (B) Comparison of fEPSP amplitude recorded from the olfactory bulb before and after HFS in two groups. Data are presented as the average percentage change from baseline responses (n=8 in each group); \*\*\**p*<0.001 compared to control group. (C) Comparison of fEPSP 10-90% slope recorded from the olfactory bulb before and after HFS in two groups. Data are presented as the average percentage change from baseline responses (n=8 in each group); \*\*\**p*<0.001 compared to control group.

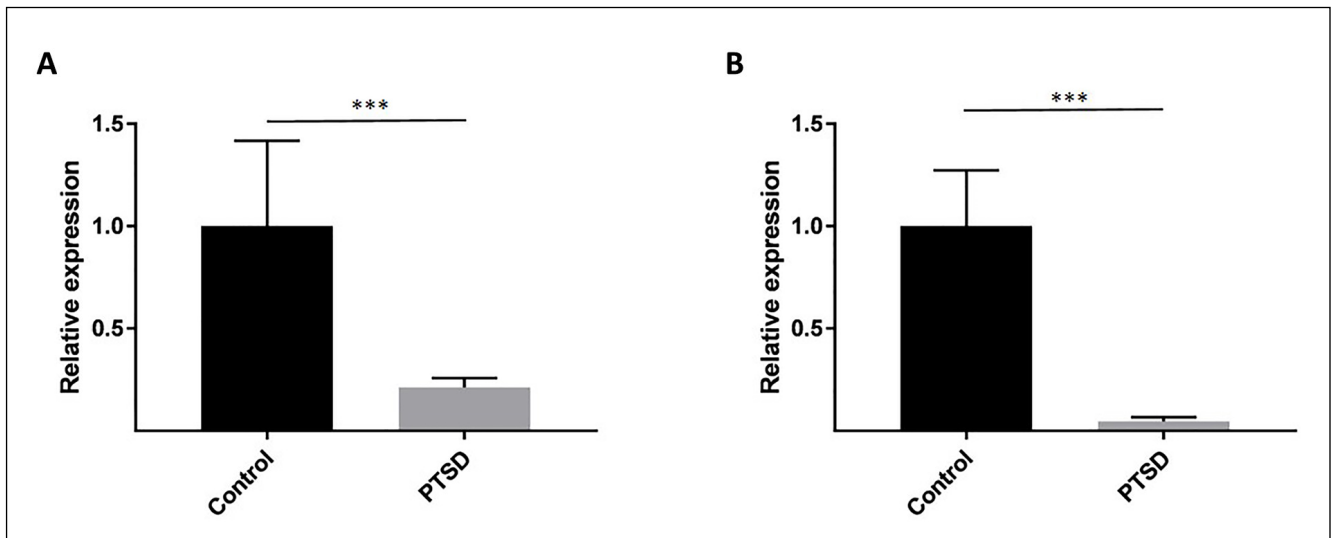


Fig. 4. Comparison of mRNA expression level of nestin (A) and Dcx (B) between two groups (n=6 in each group) \*\*\**p*<0.001 compared to control group.

animal model, rats were exposed with a female cat for 10 min and long-term stress, the characteristic feature of PTSD, was evaluated by behavioral test (EPM as well as open field) and assessment of serum level of cortisol after seven days. Our results showed that serum corti-

sol level significantly increased in PTSD group. Local field potential (LFP) was applied to compare the electrophysiological parameter of OB in two groups. We observed a dramatic increase in baseline amplitude during field excitatory post synaptic potential (fEPSP)

in PTSD rats. To detect neurogenesis, nestin and Dcx were applied and a significant decrease of nestin and Dcx in PTSD group was found, revealing the dysregulation of neurogenesis in OB.

To understand underlying neuronal mechanisms of PTSD, animal models are the vital scientific tools, but they may face with relative lack of translatability for PTSD symptoms (Richter-Levin et al., 2018). For our study, modified version of Zoladz's psychosocial predator stress (PPS) model (Zoladz et al., 2008) was applied. The main concept of this model is to combine multiple risk factors of PTSD to maximize the likelihood of generating PTSD-like symptoms in animals (Zoladz and Diamond, 2016). PTSD is often triggered by a life threatening event, triggering intense fear and helplessness, which has mimicked in this model by immobilizing (loss of control) and placing rat in very close proximity to a cat (Zoladz and Diamond, 2016). Although there is no physical contact between the rats and cat, it was revealed that the experience produces a profound physiological stress response in the rats, including increased heart rate, ascended blood pressure, reduced basal glucocorticoid levels, increased glucocorticoid suppression following dexamethasone administration, heightened anxiety and a robust fear memory in response to cues that were paired with the cat exposures (Zoladz et al., 2008; 2012). However, in comparison to original PPS model in which rats were exposed to a cat on two times separated by 10 days, further studies revealed that one exposure is enough and even has a more significant effect (because of adaptation effect of multiple exposure) (Genovese et al., 2014). Therefore, we exposed rats to a cat for 10 min one time and proved PTSD-like symptoms by evaluating EPM, OF tests, and serum cortisol level in PTSD group.

Due to correlation with different brain activities related to memory, learning, cognition and sense, local field potentials (LFPs) is considered pivotal in neuroscience studies (Mao et al., 2018). In OB, LFPs is generally applied to evaluate gamma (40-100 Hz) and beta (15-30 Hz) oscillations, ascending at the peak of inhalation and by odorants in learning or odor sensitization paradigms respectively (Osinski et al., 2018). However, despite important findings from this pool of studies (Lepousez and Lledo, 2013) their mechanism and function are still poorly understood (Osinski et al., 2018). In the current study, we assessed long term potentiation (LTP) of OB in a PTSD animal model for the first time, drawing attention to different aspect of OB electrophysiology. One interesting finding was a significant rising in the 10-90% slope as well as amplitude of fEPSP in PTSD group after HFS application. There is no comparable study, applying this method in OB, but this observation can reveal a new cognitive role for OB.

Despite the different functional connectivity of hippocampus and OB, if increased LTP considered as a sign of more enriched memory (like hippocampus), it could be interpreted as impaired extinction of avoidance learning. Excessive avoidance is a critical feature of all anxiety disorders and is a core component of PTSD diagnosis (American Psychiatric Association, 2013). Impaired extinction of avoidance learning was reported in LTP study of hippocampus Sprague Dawley rats (Cominski et al., 2014) by examining the effect of hippocampal damage in avoidance learning. Both early phase LTP (15 min and 1 h after HFS) and late phase LTP (2 and 3 h after HFS) were observed, which interpreted as prominent risk factor in development of PTSD. Therefore, it seems that the dysfunction of the OB and its related brain circuit may increase the rate of avoidance acquisition and even caused persistent avoidant responding.

A strong relationship between hippocampal neurogenesis and PTSD has been reported in the literature (Zhou et al., 2019). Recently, it has been shown that mementine as a neurogenesis enhancers recover PTSD by assisting the forgetting of traumatic memory (Ishikawa et al., 2019). Furthermore, treadmill exercise facilitates adult hippocampal neurogenesis and behavioral improvement in PTSD model *via* regulating Akt signaling (Sun et al., 2020). PI3K/Akt pathway plays an important role in hippocampal neurogenesis through several growth factors (e.g. brain derived neurotrophic factor (BDNF) and epithelial growth factor (EGF) signaling) (Yin H. et al., 2019; Yin L. et al., 2019). However, few researchers have been able to draw on the role of OB neurogenesis in pathophysiology of mental disorders. In the present study, two neurogenesis markers, such as nestin and Dcx were assessed in an animal model of PTSD, and a significant decrease were observed in the PTSD group for the first time. It is proposed that the main function of embryonic-born OB interneurons is in fundamental olfactory responses such as simple odor detection and postnatal- and adult-born OB interneurons are more vital in the learning of more complicated olfactory behaviors (Takahashi et al., 2018).

Recent studies have also hypothesized pattern separation as crucial function of adult neurogenesis in dentate gyrus, a process by which similar experiences or events are transformed into discrete representations (Kheirbek et al, 2012; Chavlis and Poirazi, 2017). It is suggested that blunt pattern separation causes the overgeneralization, which is seen in all anxiety disorders, in particular as cardinal feature of PTSD (Kheirbek et al., 2012). According to the result of present study, we found overgeneralization of fear in terms of impair discrimination between similar smell memory. We observed an association between dysregulation of neurogenesis in OB and PTSD related symptoms, im-

plying a fundamental role for OB pattern separation in pathophysiology of PTSD, which is completely compatible with (Besnard and Sahay, 2016).

## CONCLUSION

It seems that the role of OB in underlying neuronal mechanism of psychiatric disorders is underestimated, and present study revealed a strong connection between defective OB pattern separation and PTSD, which should be evaluated by other neurogenesis factors in the further studies. On the other hand, LTP assessment of OB, which is conducted in the present study in animal model of mental disorders, demonstrated very promising results and opened new lines for further studies. Together, dysfunctional neurogenesis in OB and boosted LTP can result in an impair distinction between harmful and non-harmful cues and prevent aversive memory to be extinct over time. However, the results of present study should be compared to further studies on different rat strains and other animal models of PTSD.

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