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Chemogenetic activation of the mPFC alleviates impaired fear memory extinction in an animal model of PTSD

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ABSTRACT

Background and aim: Although impaired extinction of fear memory (EFM) is a hallmark symptom of posttraumatic stress disorder (PTSD), the mechanisms underlying the impairment are unknown. Activation of the infralimbic cortex (IL) in the medial prefrontal cortex (mPFC) has been reported to predict successful fear extinction, whereas functionally disrupting this region impairs extinction. We examined whether chemogenetic activation of the IL could alleviate impaired EFM in a single prolonged stress (SPS) rat model of PTSD.

Methods: Chemogenetic activation of IL and prelimbic (PL) excitatory neurons was undertaken to evaluate EFM using a contextual fear conditioning paradigm. Neuronal activity in the IL was recorded using a 32-multichannel silicon electrode. To examine histological changes in the mPFC, apoptosis was measured by TUNEL staining.

Results: Chemogenetic activation of excitatory neurons in the IL, but not the PL, enhanced EFM in sham rats and resulted in alleviation of EFM impairment in SPS rats. The alleviation of impaired EFM in SPS rats was observed during the extinction test session. Neuronal activity in the IL of SPS rats was lower than that of sham rats after clozapine-n-oxide administration. Increased apoptosis was found in the IL of SPS rats.

Conclusions: These findings suggest that a decreased excitatory response in the IL due, at least in part, to an increase in apoptosis in SPS rats leads to impaired EFM, and that neuronal activation during extinction training could be useful for the treatment of impaired EFM in PTSD patients.

1. Introduction

While the lifetime prevalence of posttraumatic stress disorder (PTSD) varies according to social background and country of residence, ranging from 1.3% to 12.2%, it is the most prevalent psychopathological consequence of exposure to traumatic events (Karam et al., 2014), (Shalev et al., 2017). Since long-lasting emotional and psychomotor disturbances due to PTSD can induce functional difficulties at work, school, or home, the costs of PTSD to society are substantial (Kessler, 2000). In addition, PTSD is highly comorbid with depression, other anxiety disorders, and substance abuse, and it is one of the leading causes of suicide (Sareen et al., 2007). Whereas several different types of treatments, such as prolonged exposure, cognitive processing therapy, eye movement desensitization, reprocessing, and selective serotonin reuptake inhibitors, have been reported to be effective in the treatment of PTSD, a significant percentage of patients with PTSD show a poor response to these treatments or relapse after an initially promising response (Committee on the Assessment of Ongoing Efforts in the Treatment of Posttraumatic Stress, D, et al., 2014). Thus, novel therapeutic approaches for the treatment of PTSD are needed.

Patients with PTSD who are treatment resistant usually have long-lasting intrusion of traumatic memory, which may be due to impaired extinction of fear memory (EFM). Since EFM leads to the formation of a new unthreatened memory that is incompatible with preexisting elements in the fear structure, EFM is broadly recognized as an important factor underlying PTSD (Careaga et al., 2016) (Norrholm and Jovanovic, 2018) (Pitman et al., 2012). The importance of EFM in PTSD is also indicated by the effectiveness of behavioral therapies based on this memory process. Numerous preclinical and clinical studies elucidating the underlying mechanisms of EFM have been undertaken to develop novel approaches to the treatment of PTSD (Careaga et al., 2016) (Smith et al., 2017).

Although the neural circuits (and mechanism) underlying EFM are not fully understood, brain regions such as the hippocampus, amygdala, and prefrontal cortex (PFC) are closely involved (Sotres-Bayon et al., 2004) (Sotres-Bayon et al., 2006) (Ross et al., 2017). In particular,

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pharmacological and electrolytic manipulations to activate the infralimbic cortex (IL), a subregion of the medial PFC (mPFC), have been shown to induce EFM successfully, while functionally disrupting this region leads to impaired EFM (Sierra-Mercado et al., 2011) (Gourley and Taylor, 2016) (Kataoka et al., 2019). Recent experiments using optogenetics and chemogenetics have further elucidated the involvement of the IL, namely, the activity of glutamatergic pyramidal neurons in this region, in the development of EFM (Do-Monte et al., 2015b) (Bloodgood et al., 2018). Furthermore, the size and activity of the ventral portion of the mPFC (vmPFC) were reported to be associated with the extent of EFM deficits in individuals with PTSD (Rauch et al., 2003), and changes in functional connectivity between the left vmPFC and amygdala in individuals with PTSD have also been reported (Stevens et al., 2013). Although there is some debate among comparative neuroanatomists regarding the similarities of the human and rodent PFC, these findings suggest that it could be worthwhile to examine whether neuronal activation of mPFC excitatory neurons can alleviate EFM impairment in a rat model of PTSD to facilitate the development of a novel therapeutic strategy for PTSD.

In the present study, we first examined whether neuronal activation of mPFC excitatory neurons by chemogenetic activation could induce EFM in a contextual fear conditioning paradigm in naïve rats (sham group). Recent advances in techniques to manipulate neuronal activity (optogenetics and chemogenetics [designer receptors exclusively activated by designer drugs: DREADDs]) have made it possible to establish causal links between the activity of specific brain circuits and particular behavioral and physiological outcomes (Flores et al., 2018). In particular, DREADDs are more helpful in manipulating circuits in a sustained manner (Roth, 2016), and findings from animal studies using DREADDs might have translational implications because DREADDs act through G-proteins similar to G-protein coupled receptors, which are the most common targets of current therapeutic drugs in humans (Rask-Andersen et al., 2011) (Zhu and Roth, 2014). Second, we examined whether chemogenetic activation of mPFC excitatory neurons could alleviate EFM impairment in a single prolonged stress (SPS) rat model of PTSD. A series of our studies demonstrated that SPS rats mimic many aspects of the pathophysiological and behavioral abnormalities of PTSD such as enhanced anxiety-like behaviors, enhanced glucocorticoid negative feedback, stress-induced analgesia, and impaired EFM (Yamamoto et al., 2009) (Souza et al., 2017) (Knox et al., 2012) (Keller et al., 2015). Third, we examined the difference in mPFC neuronal activity in response to chemogenetic activation between sham and SPS rats using multi-unit extracellular recording. Lastly, we examined whether SPS could induce apoptosis in the mPFC by TUNEL staining to uncover the mechanism underlying the impairment of EFM.

2. Materials and methods

All experimental procedures are shown in Fig. 1.

2.1. Animals

A total of 110 (Experiment 1–5: 41, 17, 32,10,10, respectively) male Sprague-Dawley rats weighing 300–400 g (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used in this study. Rats were housed 3 per cage under a temperature-controlled environment with a 12-h light-dark cycle and ad libitum food and water. All animal procedures were conducted in strict accordance with the Hiroshima University School of Medicine Animal Care Committee Guiding Principles on Animal Experimentations in Research Facilities for Laboratory Animal Science, School of Medicine, Hiroshima University.

We used only male rats because of the well-known bidirectional (facilitation and suppression) influence of the estrous cycle and gonadal hormone on learned fear (Day and Stevenson, 2019).

2.2. Fear conditioning test

On Day 1, rats were placed in a conditioning chamber (W500 × D280 × H325mm) and then exposed to a 180-s conditioning context without any stimulation. Then, they received two footshocks (4 s, 0.8 mA) with an inter-trial interval of 30 s through a stainless steel grid by a shock generator-scrambler (SGS-003: Muromachi, Tokyo, Japan). Following the footshocks, rats remained in the chamber for an additional 1 min. On Day 2, rats were subjected to extinction training, which is an exposure without footshock for 10 min to the same chamber where they received the footshocks on Day 1. Extinction recall was tested on Day 3 under the same conditions as extinction training.

Freezing behavior of rats was scored every 1 min for 5 min (2–7 min after the exposure to chamber) and expressed as a percentage; i.e., the proportion of freezing time divided by the total observation time period.

2.3. Single prolonged stress (SPS)

For the evaluation of the effects of chemogenetic activation of IL excitatory neurons on the impaired EFM in the SPS rat model of PTSD, animals were randomly assigned to 2 groups (sham or SPS). SPS was conducted after 7 days of recovery from surgery in 3 stages as previously described (Kataoka et al., 2019) (Matsumoto et al., 2013): restraint for 2 h, forced swim for 20 min, and ether anesthesia.

2.4. Viral vectors and surgery

For chemogenetic activation, viral vector expressing DREADD receptors (rAAV2/CaMKIIa-hM3Dq(Gq)-mCherry, 3.1×10^{12} vp/mL) and a control viral vector expressing EYFP (rAAV2/CaMKIIα-EYFP, 4.1×10^{12} vp/mL) were obtained from Vector Core at the University of North Carolina at Chapel Hill.

Rats were anesthetized with pentobarbital (40 mg/kg, i.p.) in combination with inhaled isoflurane, and a small bilateral craniotomy was performed. Using a 5-μL Hamilton syringe, 0.5 μL of virus was delivered at a rate of 0.1 μL/min into the infralimbic cortex (IL: +3.0 mm AP; ± 0.6 mm ML; 5.1 mm DV), or prelimbic cortex (PL: +3.0 mm AP; ± 0.6 mm ML; 3.5 mm DV) (Paxinos and Watson, 1998). The syringe tip was left in place for 5 min to allow for diffusion of virus. After surgery, a triple antibiotic was applied and an analgesic (carprofen; 5 mg/kg) was injected s.c. for 3 days.

All behavioral and electrophysiological studies were conducted 2 weeks after surgery. Behavioral testing and electrophysiological recording were performed at 30–120 min after i.p. administration of clozapine-n-oxide (CNO) (0.5 mg/kg, Enzo Life Sciences) or saline to viral-infected rats. The dose of CNO was determined according to previous studies demonstrating significant effects on neuronal activity and behavior using hM3Dq receptors (Alexander et al., 2009) (Vazey and Aston-Jones, 2014).

2.5. Histology

Immediately after the completion of extinction testing, transcardial perfusion was performed using 10% formalin under chloral hydrate anesthesia (250 mg/kg, i.p.), and brains were then removed and post-fixed in the same fixative for 48–72 h at 4 °C. Subsequently, brains were cryoprotected in 30% sucrose-TBS for 48–72 h. Section were cut at a thickness of 40 μm using a cryostat, and the expression of mCherry or control EYFP was verified in the sections. Animals with incorrect viral expression were not included in the study. Representative mCherry and control EYFP expression in the IL [Fig. S1A] and PL [Fig. S1B] are shown.

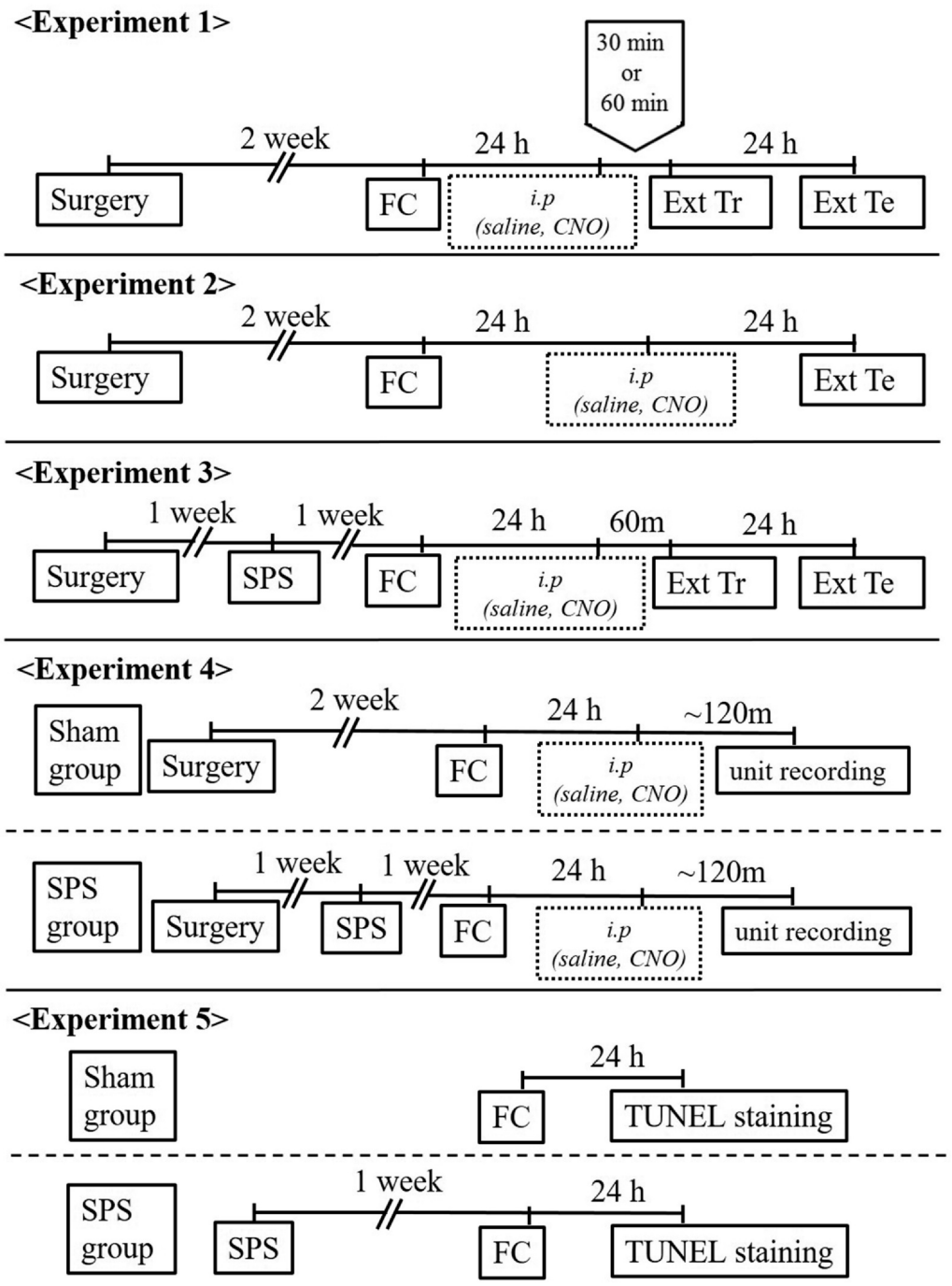

Fig. 1. Timelines for behavioral and biochemical tests.
FC, fear conditioning; Ext Tr, extinction training; Ext Te, extinction test; CNO, clozapine-n-oxide; SPS, single prolonged stress.

2.6. Electrophysiology (multi-unit recording)

Two weeks after surgery, rats were anesthetized with urethane (1 g/kg, i.p.) and placed into a stereotaxic apparatus (Fig. 1, Experiment 4). A 32-multichannel silicon electrode (A1x32-6 mm-50-177-Z32, NeuroNexus, Ann Arbor, MI, USA) were placed into the IL (+3.0 mm AP; ± 0.6 mm ML; −6.0 mm DV) for multi-unit extracellular recording. Recording was performed using a 32-channel neurophysiology workstation (TDT System-3, Tucker & Davis Technologies, Inc., Alachua, FL) controlled by OpenEX software (Tucker & Davis Technologies, Inc.). The neuronal signals were sampled at 24.4 kHz and the band-pass filter was set at 300/5000 Hz (low/high). The threshold for spike detection was set at 4 standard deviations below the background activity. Spike sorting was then performed by principal component analysis followed by K-means clustering algorithm using Open Sorter software (Tucker & Davis Technologies, Inc.). In this step, clusters with typical spike waveforms were considered as unit activity.

To assess the effects of chemogenetic activation of neurons expressing hM3D(Gq) on the neuronal activity of the IL, we first extracted the activity of putative pyramidal neurons. The concentration of CNO was identical to that used in the behavioral experiments (0.5 mg/kg). It has been demonstrated by intracellular studies that pyramidal neurons are regular-spiking neurons, while inhibitory interneurons are fast spiking neurons, and one striking difference between these 2 kinds of neurons is that the extracellular waveform of regular-spiking neurons has a longer and shallower peak following the initial trough (McCormick et al.,

1985) (Henze et al., 2000) (Nowak et al., 2003) (Hasenstaub et al., 2005). We therefore extracted the activity of putative pyramidal neurons with cut-off values with half-amplitude width of 0.3 ms and trough to peak time of 0.5 ms, as reported by Bartho and associates (Bartho et al., 2004) [Fig. S2A, B]. Only the activities of putative pyramidal neurons were used to evaluate temporal changes in the firing rate among sham rats due to chemogenetic activation. We then extracted only the activated pyramidal neurons following CNO administration. Unit recording was started 5 min prior to CNO administration and continued until 120 min post administration. The average firing rate over 5 min before CNO administration (designated as baseline) and 30, 60, 90 min after CNO administration was calculated. The average firing rate of each neuron at each timepoint was compared with that at baseline. Changes in neuronal activity at each timepoint are shown in Fig. S3, and the activity of neurons with a significant increase in firing rate compared to that at baseline were used for subsequent analyses.

2.7. TUNEL staining

To detect apoptotic cells in the mPFC, TdT-mediated dUTP nick end labeling (TUNEL) staining was performed using an In Situ Cell Death Detection Kit, POD (Roche Diagnostic GmBH, Mannheim, Germany). Perfusion and preparation of 40-μm thick slices were performed in the same manner as the histological verification. Slices were prepared at approximately 3.2 mm anterior to bregma (Paxinos and Watson, 1998). The slices were incubated with: 4% paraformaldehyde and Histochoice tissue fixative (Sigma-Aldrich, St. Louis, USA) mixed 3:1 for 60 min at room temperature; 0.6% PBST for 60 min at room temperature; proteinase K for 30 min at 37 °C; 100 μL of 3% BSA for 20 min at room temperature; 50 μL of TUNEL reaction mixture for 60 min at 37 °C; and 50 μL of converter-POD for 30 min at 37 °C. Subsequently, sections were stained using a DAB Peroxidase Substrate Kit, ImmPACT (Vector Laboratories, Burlingame, USA) and then counterstained with methyl green solution (Fujifilm Wako Pure Chemical Industries, Osaka, Japan). Four microscopic fields in the IL or PL were captured using a Keyence BZ-8000 microscope (Keyence Corp., Osaka, Japan). ImageJ software was used to identify TUNEL-positive and methyl green-positive cells. The ratio of apoptotic cells was determined as the number of TUNEL-positive cells divided by the total number of cells (TUNEL-positive cell plus methyl green-positive cells).

2.8. Statistical analyses

All values shown represent the mean ± SEM. In the experiments of fear conditioning test, the results were analyzed separately for each day (extinction training and test).

The results of experiments containing 2 groups of rats were analyzed by independent *t*-test (Experiment 1 [Fig. 2B, C], Experiment 2 [Fig. 3], Experiment 4 [Fig. 5B, D], Experiment 5 [Fig. 6]).

In Experiment 1, the results of experiments with 4 groups of rats were analyzed by two-way ANOVA (factors: drug, time after admin) [Fig. 2A].

In Experiment 3 [Fig. 4], freezing in the 4 groups was compared by two-way ANOVA (factors: drug, stress) followed by appropriate post hoc comparisons.

In Experiment 4 [Fig. 5A], temporal changes in the firing rate among sham rats due to chemogenetic activation was analyzed by one-way ANOVA followed by appropriate post hoc comparisons. The difference of mPFC neuronal activity in response to chemogenetic activation in sham and SPS rats was analyzed by two-way ANOVA (factors: stress, time after admin) [Fig. 5C, E] followed by appropriate post hoc comparisons.

Results were considered statistically significant at $p < .05$.

3. Results

3.1. Experiment 1: The effect of chemogenetic activation of mPFC excitatory neurons on EFM in sham rats

To examine whether chemogenetic activation of mPFC excitatory neurons could affect EFM, we first administered CNO at 30 min or 60 min prior to extinction training in rats that expressed DREADDs in the IL (Fig. 1, Experiment 1). At the extinction training session, statistical analysis revealed significant main effects of drug [$F(1,40) = 7.49$, $p < .01$], but not of (time after admin) [$F(1,40) = 0.51$, $p = .48$]. There was no significant drug × (time after admin) interaction [$F(1,40) = 1.89$, $p = .18$]. At the extinction test session, statistical analysis revealed significant main effects of drug [$F(1,40) = 7.24$, $p = .011$], but not of (time after admin) [$F(1,40) = 1.22$, $p = .28$]. There was no significant drug × (time after admin) interaction [$F(1,40) = 1.79$, $p = .19$] [Fig. 2A].

To examine whether the chemogenetic activation in the PL of rats that expressed DREADDs in the PL also affect EFM, we next examined the effect of CNO administration 60 min prior to extinction training in rats that expressed DREADDs in the PL (Fig. 1, Experiment 1). Statistical analysis revealed no significant effects at the extinction training session ($t = 0.64$, $df = 15$, $p = .53$), or at the extinction test session ($t = 0.37$, $df = 15$, $p = .72$). [Fig. 2B].

We then examined the effect of administration of saline or CNO 60 min prior to extinction training in rats that expressed EYFP under the control of CaMKIIα promoter in the IL (Fig. 1, Experiment 1). Statistical analysis revealed no significant effects at the extinction training session ($t = 0.66$, $df = 11$, $p = .52$), or at the extinction test session ($t = -0.21$, $df = 11$, $p = .84$). [Fig. 2C].

3.2. Experiment 2: Extinction test performance in rats receiving chemogenetic activation of IL excitatory neurons without extinction training

We next examined extinction recall 24 h after chemogenetic activation of IL excitatory neurons without extinction training (Fig. 1, Experiment 2). Statistical analysis revealed no significant effect of chemogenetic activation in the extinction test session ($t = -0.13$, $df = 15$, $p = .90$) [Fig. 3].

3.3. Experiment 3: The effect of chemogenetic activation of IL excitatory neurons on impaired EFM in SPS rats

We next examined the effect of chemogenetic activation of IL excitatory neurons on EFM impairment, which has been reported to be a typical behavioral abnormality in SPS rats (Keller et al., 2015; Knox et al., 2012) (Fig. 1, Experiment 3). For the extinction training session, two-way ANOVA revealed significant main effects of stress ($F(1, 42) = 5.323$, $p = .026$) and drug ($F(1, 42) = 15.35$, $p < .01$), but there were no significant stress × drug interaction ($F(1, 42) = 0.475$, $p = .495$). Post-hoc analyses revealed that the administration of CNO significantly decreased freezing time in the sham group based on a comparison of freezing time in the sham-CNO and sham-saline group ($p = .019$), but showed no effect on freezing time in the SPS group ($p = .124$) [Fig. 4]. For the extinction test session, two-way ANOVA demonstrated significant main effects of stress ($F(1, 42) = 5.323$, $p < .01$) and drug ($F(1, 42) = 15.354$, $p < .01$), but there was no significant stress × drug interaction ($F(1, 42) = 0.475$, $p = .454$). Post hoc analyses revealed that CNO administration decreased the freezing time in both the sham group ($p = .029$) and the SPS group ($p < .01$) compared with the saline-treated group. [Fig. 4].

3.4. Experiment 4: Differences in neuronal activity of the mPFC in response to chemogenetic activation in sham and SPS rats

We next examined the effects of chemogenetic activation on

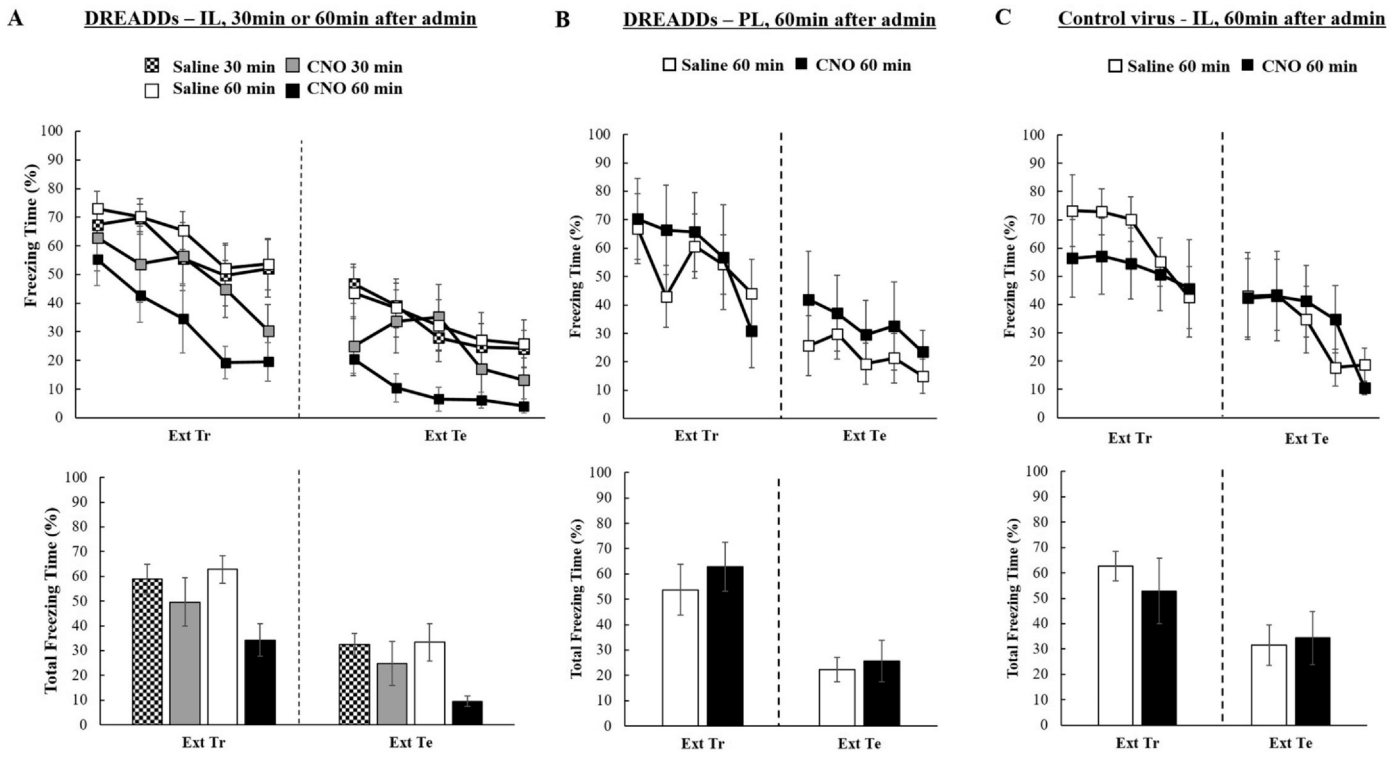

Fig. 2. Effect of chemogenetic activation of mPFC excitatory neurons on EFM in sham rats.
The effects on freezing time of (A) DREADDs in the IL – extinction training 30 min or 60 min after CNO administration, (B) DREADDs in the PL – extinction training 60 min after CNO administration, and (C) Control virus in the IL – extinction training 60 min after CNO administration.
Freezing behavior at every 1-min interval (upper panel) and for the total observation period (lower panel) are shown.
Data are expressed as mean ± SEM (n = 8–12 per group) and were analyzed by two-way ANOVA (A) or by independent *t*-test (B, C).

neuronal activity in the mPFC. With respect to temporal changes in firing rates caused by chemogenetic activation in sham rats, statistical analysis revealed a significant main effect [$F(3,679) = 7.74$, $p < .01$] and post-hoc analysis showed a significant increase in the firing rate at 60 min ($p < .01$) and 90 min ($p < .01$) after CNO administration (mean firing rate: baseline, 7.12 ± 0.81; 30 min, 11.66 ± 1.38; 60 min, 14.62 ± 1.62; 90 min, 15.86 ± 1.63) [Fig. 5A].

We then compared baseline neuronal activity of the IL between sham and SPS rats. Statistical analysis revealed no significant difference between the 2 groups ($t = 1.97$, $df = 389$, $p = .12$) [Fig. 5B]. Subsequent statistical analysis of the fold change (relative to baseline) revealed a significant main effect of stress ($F(1, 955) = 24.78$, $p < .01$), of time after administration ($F(3, 955) = 52.49$, $p < .01$), and an interaction between stress and time after administration (F(3,

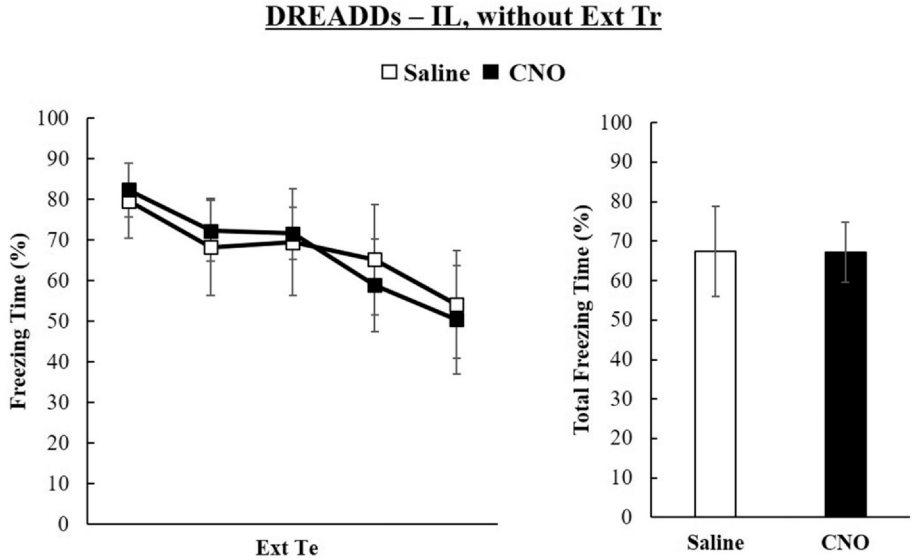

Fig. 3. Extinction test performance in rats receiving chemogenetic activation of IL excitatory neurons without extinction training.
Data are expressed as mean ± SEM of 8–9 rats per group. Freezing behavior at every 1- min interval and for the total observation period are shown. Analysis by independent t-test.

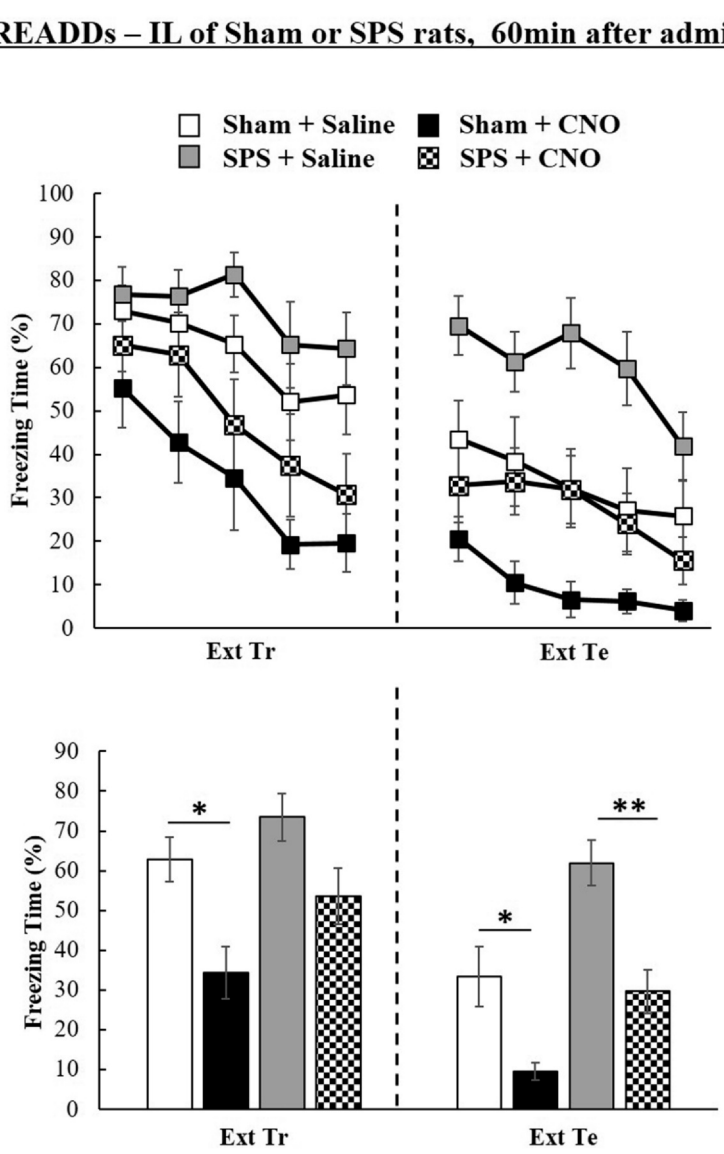

Fig. 4. Effect of chemogenetic activation of IL excitatory neurons on impaired EFM in SPS rats.
Data are expressed as mean ± SEM of 10–14 rats per group. Freezing behavior at every 1-min interval (upper panel) and for the total observation period (lower panel) are shown.
*p < .05, **p < .01, two-way ANOVA followed by Tukey's test.

955) = 8.11, p < .01). Post-hoc analysis revealed significant increases in the fold change of both groups 30 min, 60 min, and 90 min after CNO administration compared with the respective baselines of each group. With regard to the difference in the fold change between Sham and SPS rats, post-hoc analysis revealed significant differences at 60 min (p < .01) and 90 min (p < .01), but not at 30 min (p = .508) after CNO administration [Fig. 5C].

Statistical analysis of data for the PL revealed no significant differences between sham and SPS rats at baseline (t = 1.64, df = 283, p = 1.97) [Fig. 5D]. Subsequent statistical analysis of the fold change revealed a significant main effect of time after administration (F(3, 732) = 34.70, p < .01), but not of stress (F(1, 732) = 3.74, p = .053), and no significant interaction between stress and (time after admin) (F (3, 732) = 0.53, p = .66) [Fig. 5E].

3.5. Experiment 5: Effect of SPS on neuronal apoptosis in the mPFC of SPS rats

We next performed TUNEL staining of the IL and PL in the mPFC. Statistical analysis revealed that SPS significantly increased the percentage of TUNEL-positive cells compared with sham rats in the IL (t = 5.89, df = 126, p < .01) [Fig. 6A] but there was no significant difference in the PL (t = 1.77, df = 126, p = .08) [Fig. 6B].

4. Discussion

In this study, we first examined whether chemogenetic activation of mPFC excitatory neurons could affect EFM. The results demonstrated enhancement of EFM by chemogenetic activation of mPFC excitatory neurons in region-dependent (i.e., for DREADDs in the IL but not the PL) manner. Our result are in good agreement with several previous studies using molecular mapping through immediate-early gene expression, in vivo single-unit recordings, lesions, and IL-targeted pharmacological and optogenetic manipulations, demonstrating that fear extinction was accompanied by activation of the IL, whereas functional disruption of the IL impaired extinction (Milad and Quirk, 2012) (Tovote et al., 2015) (Rozeske et al., 2015) (Likhtik and Paz, 2015).

Although not statistically significant, 60 min of activation trended

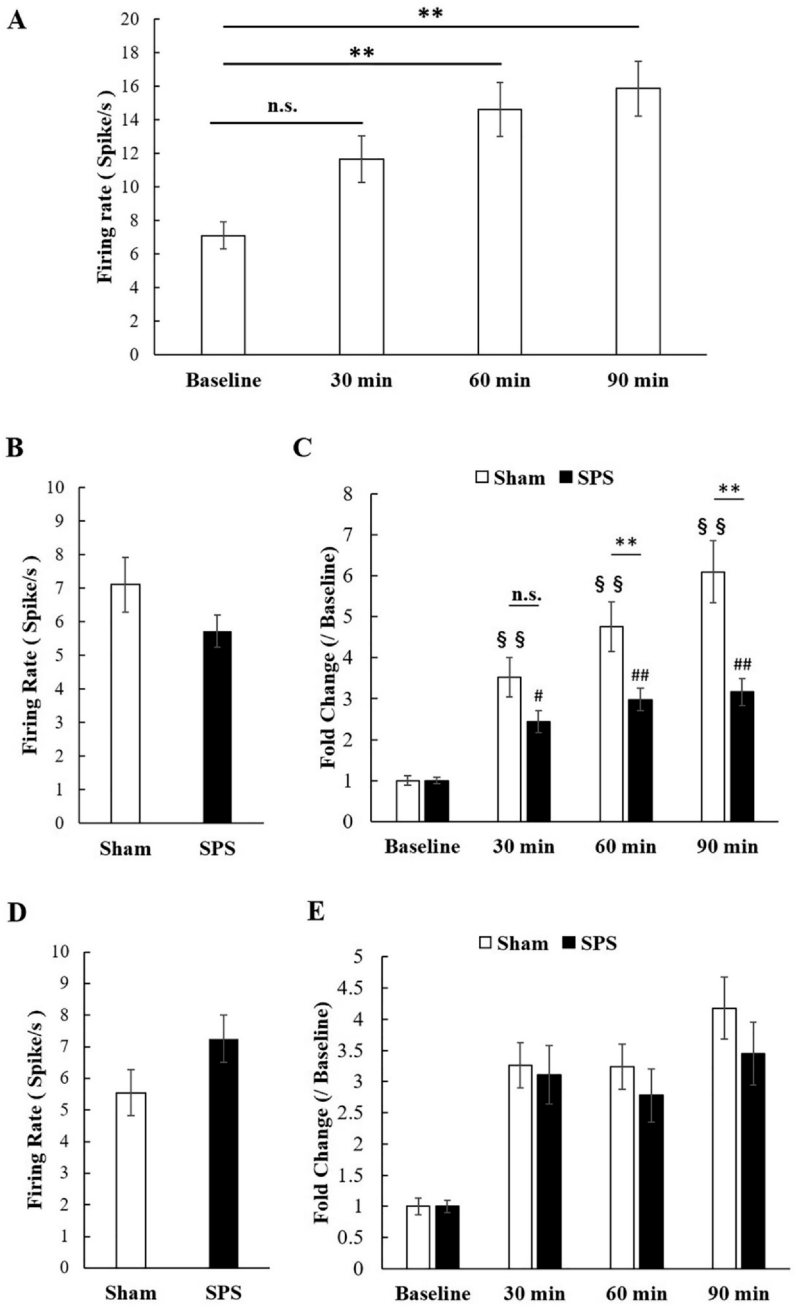

Fig. 5. Effects of chemogenetic activation on neuronal activity in sham and SPS rats.
(A) Temporal change in firing rates of the IL in sham rats following chemogenetic activation. Data are expressed as mean ± SEM (n = 170 from 4 sham rats). **p < .01, one-way repeated ANOVA followed by Tukey's test.
(B) Baseline neuronal activity of the IL in sham and SPS rats. Data are expressed as mean ± SEM (n = 170–221 per group) and were analyzed by independent t-test.
(C) Fold change in neuronal activity of the IL in sham and SPS rats 30 min, 60 min, and 90 min after CNO administration. Data are expressed as mean ± SEM (n = 160–208 per group). *p < .05, **p < .01 for Sham vs SPS, §§p < .01 for the comparison vs baseline of Sham group, $^{#}$p < .05, $^{##}$p < .01 for the comparison vs baseline of the SPS group, two-way ANOVA followed by Tukey's test.
(D) Baseline neuronal activity of the PL in sham and SPS rats. Data are expressed as mean ± SEM (n = 134–151 per group) and were analyzed by independent t-test.
(E) Fold change in neuronal activity of the PL in sham and SPS rats 30 min, 60 min, and 90 min after CNO administration. Data are expressed as mean ± SEM (n = 136–162 per group) and were analyzed by two-way ANOVA.

to strengthen the enhancement of EFM by chemogenetic activation of IL as compared to 30 min of activation in sham rats. To our knowledge, a firing rate of IL neurons sufficient to enhance EFM has not been reported. In a recent study by Do-Monte and colleagues, optogenetic activation of IL pyramidal neurons at rates of 10–50 Hz, but not at 5 Hz, was reported to enhance EFM in a contextual cued fear conditioning test (Do-Monte et al., 2015b). The mean firing rate of IL neurons chemogenetically activated in our study was approximately 12 Hz at 30 min and 15 Hz at 60 min after CNO administration [Fig. 5A]. It is plausible that differences in experimental procedures such as the method of activation (optogenetic vs. chemogenetic), differences in the electrophysiological recording of the cell population (non-specified neurons vs. pyramidal neurons), or differences in the fear conditioning test method (context with cue vs. context only), may be associated with the discrepant relationship between the cell firing rate and enhancement of EFM.

Although CNO is considered to be an inactive metabolite of the antipsychotic drug clozapine (Alves-Rodrigues et al., 1996) (Salmi and Ahlenius, 1996) (Wong et al., 1996), recent studies demonstrating the retroconversion of CNO to clozapine and multiple dose-dependent behavioral effects of CNO underscores the necessity of using non-DREADD-expressing animals to control for off-target activity in DREADD-based experiments (MacLaren et al., 2016) (Manvich et al., 2018). Although there were no differences in the freezing of total observation period during the extinction training between CNO-treated and saline-treated rats expressing EYFP in the IL (Experiment 1, Fig. 2C), the freezing behavior during the early stage of total observation period in CNO-treated rats trended to be shorter than that in saline-treated rats (Experiment 1 and 3, Figs. 2 and 4). In this context, it cannot be ruled out that CNO administration may, at least in part, enhance the overall activity and acquisition of extinction during the extinction training.

The chemogenetic activation of IL excitatory neurons enhanced the EFM in both sham and SPS rats. Further, whereas the enhancements in

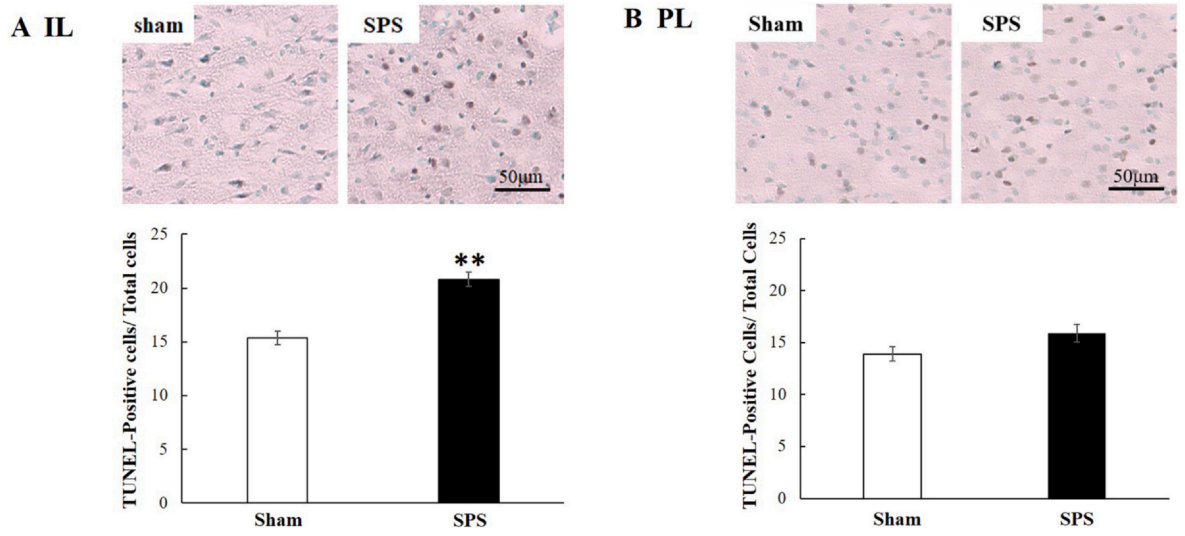

Fig. 6. Effect of SPS on neuronal apoptosis of the mPFC in SPS rats.
Representative images of TUNEL staining in the mPFC are shown in the upper panel. (A) IL, (B) PL, scale bar = 50 μm.
Data are expressed as the ratio of TUNEL-positive cells to total cells (TUNEL-positive cells/total cells) and shown as the mean ± SEM of 64 sections per group (4 sections per rat). **p < .01, independent t-test.

sham rats were seen in both extinction training and during the test session, the enhancements in SPS rats were only seen in the extinction test session. Our electrophysiological study revealed that the chemogenetic activation significantly increased the neuronal activity of mPFC in both sham and SPS rats, but the degree of the activation was smaller in SPS rats only in the IL, but not the PL. Knox and associates reported a similar region-specific attenuation in the increases in IL neural activity during extinction training in SPS rats using c-fos mapping (Knox et al., 2016).

With regard to the neural circuitry of EFM, hippocampal inputs to IL, IL inputs to intercalated cell masses (ITC) in the amygdala, and projections from ITC to central nucleus (CeA) are reported to be necessary (Sierra-Mercado et al., 2011) (Hill and Martinowich, 2016). On the other hand, the necessity of IL activity during extinction training for later retrieval has been advocated (Quirk et al., 2003) (Likhtik et al., 2008) (Bloodgood et al., 2018), supported by the findings that IL inputs exert a powerful depolarizing influence on ITC cells, inducing high-frequency bursts (Amir et al., 2011) and that strong depolarization may facilitate induction of NMDA-dependent plasticity at BLA inputs onto ITC cells (Royer and Paré, 2002). Do-Monte and colleagues also suggested the importance of the activity of the IL during extinction training for later retrieval, but not during retrieval using the optogenetic activation and silencing of IL (Do-Monte et al., 2015a). Furthermore, *N*-methyl-D-aspartate receptors (NMDAR) - dependent bursting in the IL was reported to initiate calcium-dependent molecular cascades that stabilize extinction memory, thereby allowing for successful recall of extinction (Burgos-Robles et al., 2007). This evidence together suggests a possible explanation for the enhancements of EFM of sham rats that were seen during both extinction training and the extinction test session, and the chemogenetic activation of IL excitatory neurons may be insufficient to reduce freezing behavior during extinction training because of increased apoptosis and insufficient increases in IL neural activity in SPS rats.

Although the precise mechanisms by which SPS disrupts neural function within the IL are not known, increased neuronal apoptosis in the IL of SPS rats found in the present study may be, at least in part, responsible. It has been reported that enhanced vmPFC apoptosis induced by SPS may be due to changes in signaling pathways in the endoplasmic reticulum that are critical for inhibiting apoptosis (Zhao et al., 2014) (Wen et al., 2016). Of particular relevance to the current findings, Piggott and colleagues recently reported that SPS decreased neural activity in the IL, but not the PL, as shown by manganese-enhanced magnetic resonance imaging, and that it decreased glutamate levels in the mPFC as shown by proton magnetic resonance spectroscopy (Piggott et al., 2019). These SPS-induced changes in vmPFC function could underlie the difference in the response to chemogenetic activation of the IL between sham and SPS rats.

In the light of the decreased IL neural activity in SPS rats, it is noteworthy that some clinical studies have observed hypoactivity in the vmPFC, which has been reported to be functionally analogous to the IL in the rat brain (Milad and Quirk, 2012), during fear extinction in patients with PTSD, as shown by positron emission tomography (Bremner et al., 2005) as well as functional MRI (Etkin and Wager, 2007) (Rougemont-Bucking et al., 2011). These results together with the present finding that chemogenetic activation alleviated impaired extinction in SPS rats implicate the potential of neuronal activation of the vmPFC as a novel therapeutic strategy for PTSD.

In summary, chemogenetic activation of excitatory neurons in the IL, but not the PL, 60 min before extinction training resulted in enhancement of EFM. Consecutive chemogenetic activation with extinction training was required for the enhancement. Chemogenetic activation of the IL could alleviate the impaired EFM of SPS rats, but EFM impairment was alleviated only in the extinction test session due, at least in part, to an SPS-induced increase in apoptosis and a decrease in the excitatory response of IL neurons.

Neuromodulation approaches such as deep brain stimulation, may represent a promising treatment option for patients with PTSD who remain symptomatic even after conventional psychotherapy and/or medications (Gouveia et al., 2019), and the combined approach of neuromodulation with exposure-based therapy may lead to better therapeutic responses in PTSD (Marin and Milad, 2015). We postulate, based on these findings, that a decreased excitatory response in the vmPFC leads to impaired fear extinction, and that neuronal activation with an adjunctive combination of exposure therapy could be useful for treating the impaired fear extinction characteristic of patients with PTSD.

Ethical statement

This manuscript is in accordance with the Authorship statement of ethical standards for manuscripts submitted to Progress in Neuro-Psychopharmacology & Biological Psychiatry. All authors declare that

they have no conflicts of interest. All authors have read and approved the submission of the manuscript; the manuscript has not been published and is not being considered for publication elsewhere, in whole or in part, in any language, except as an abstract.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2020.110090.

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