RESEARCH ARTICLE

Deep Brain Stimulation of the Ventromedial Prefrontal Cortex Disrupts Consolidation of Fear Memories

Shawn Zheng Kai **TAN**, Chi Him **POON**, Ying-Shing **CHAN**, Lee Wei **LIM**

School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China.

Corresponding author:

Lee Wei Lim MD, PhD, AM
School of Biomedical Sciences,
Li Ka Shing Faculty of Medicine,
The University of Hong Kong,
L4, 21 Sassoon Road,
Hong Kong SAR. China.
Tel: (852) 91572575
Email: limlw@hku.hk

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ABSTRACT

Anxiety disorders pose one of the biggest threats to mental health worldwide, yet current therapeutics have been mostly ineffective due to issues with relapse, efficacy, and toxicit. Deep brain stimulation (DBS) is a promising therapy for treatment-resistant psychiatric disorders including anxiety, but very little is known about the effects of DBS on fear memories. In this study, we used a modified plus-maze discriminative and showed that DBS of the ventromedial prefrontal cortex (vmPFC) was able to disrupt consolidation, but not acquisition or retrieval of fear memories. We validated these results using a standard tone-footshock fear conditioning paradigm. We further demonstrated short-term changes in dopaminergic receptor and c-Fos expression in the ventral hippocampus (vHPC) and established a partial casual role of dopamine 2 receptors in this effect. Lastly, we showed changes in neurotransmitter levels in the vHPC. This study highlights the potential therapeutic effect of vmPFC DBS to treat anxiety disorders.

INTRODUCTION

Anxiety disorders are highly prevalent and pose one of the biggest threats to mental health worldwide (Ehlers, 1997; WHO, 2001). Anxiety disorders are commonly treated using exposure therapy, a form of cognitive-behavior therapy (CBT) that targets maladaptive associated fear memories. However, such exposure therapy involves new learning that attempts to inhibit or update the previous maladaptive learning rather than erase it (Khalaf et al., 2018), resulting in many patients unable to maintain the benefits of CBT and often relapsing (Barad, 2006; Baum, 1988; Bouton, 2002; Bouton and Bolles, 1979; McNally, 2007; Rescorla and Heth, 1975). Current attempts to enhance CBT using pharmacological or paradigm changes have met with complications such as low efficacy and drug toxicity (Farach et al., 2012; Klucken et al., 2016). Furthermore, improper administration of these techniques can lead to exacerbation of the condition (Eisenberg et al., 2003; Merlo et al., 2014; Pedreira and Maldonado, 2003).

Deep Brain Stimulation (DBS) is a surgically invasive technique that involves implanting electrodes in specific regions of the brain to modulate firing of neurons through electrical stimulation. It has been shown to be a promising non-pharmacological treatment for depression and anxiety disorders (Denys et al., 2010; Mayberg et al., 2005; Mian et al., 2010; Puigdemont et al., 2012). However, little work has been done to systematically investigate its effects on fear memory. In this study, we examined the hypothesis that DBS of the ventromedial prefrontal cortex (vmPFC), a structure we have previously argued to be an ideal target (Tan et al., 2019), is able to disrupt fear memories. Both innate and conditioned fear were investigated in rats using a modified avoidance plus maze in which bob cat urine (aversive odour) was placed in one closed arm of an elevated plus maze and rabbit urine (neutral odour) was placed in the opposite closed arm. The results were verified using a standard tone-footshock conditioning paradigm.

We then probed the role of the hippocampus in the effects seen, and showed that ventral hippocampal dopaminergic transmission plays a central role in vmPFC DBS ability to disrupt consolidation of fear memories.

METHODS AND MATERIALS

Subjects

The study was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of The University of Hong Kong (Ref.: 4159-16). Male Sprague-Dawley rats (n=173; 7-8 weeks old at the time of surgery) were individually housed in standard cages with food and water available *ad libitum*. The environmental condition was maintained at temperature (21±1 °C) and humidity (60-65%) under a reversed 12/12 h light/dark cycle.

Surgical and Deep Brain Stimulation Procedures

Surgery and DBS procedures were performed as previously described (Lim et al., 2015; Liu et al., 2015). In brief, a construction of bilateral platinum-iridium electrodes (Synergy Engineering Pte Ltd, Singapore) was implanted in the vmPFC (AP: +3.0 mm; ML: +/-0.6 mm; DV: -3.6 mm) based on the Paxinos & Watson Rat Brain Atlas (Paxinos and Watson, 2006). Animals that received cannulation were also bilaterally implanted with guide cannulas in the ventral hippocampus (AP: -5.3 mm; ML: +/-5.0 mm; DV: -5.6 mm). Rats were connected to cables and stimulation was performed using a digital stimulator (Model 3800 MultiStim: 8-Channel Stimulator; A-M Systems, Carlsborg, USA) and two stimulus isolators (Model 3820; A-M Systems). Rats were stimulated according to the experimental parameters (100 Hz, 200 μA and 100 μs pulse width) as previously described (Lim et al., 2015; Liu et al., 2015). For verification of electrode/cannula localization tip, hematoxylin-eosin (Merck, Darmstadt, Germany) was performed to

examine the implantation site. Detailed information about surgical and DBS procedures are provided in the supplementary methods.

Administration of Drugs

Rats were infused with either Quinpirole–HCl (10 µg/side of salt, equivalent to 39.09 µmoles/side), Raclopride (1.67µg/side of salt, equivalent 3.36 µmoles/side) or artificial cerebral spinal fluid (aCSF) at dosages previously shown to be effective in the vHPC (Wilkerson and Levin, 1999). Drugs were infused into the vHPC through two Hamilton syringes (10 µl) connected to an internal cannula via polyethylene tubing (Protech International, Texas, USA). The infusion volume (2 µl) was delivered over approximately 3min and the internal cannula was left in for an additional 3min.

Modified Elevated Plus Maze

On day 1, a container with 5 ml of Bobcat urine (aversive odor; PredatorPee, Maine, USA) was placed in one closed arm of the modified elevated plus maze (EPM) and a container with 5 ml of rabbit urine (neutral odor) was placed in the opposite closed arm. Urine was changed every 4-5 animals and testings were counter-balanced to ensure a proper dispersion of odor across tests and subjects. On day 2, empty containers without odor were placed in the two closed arms. Rats were stimulated (or sham stimulated) accordingly (see protocol section) and placed in the central platform individually and tested for 10min. More details can be found in the supplementary methods.

Real-time PCR

Immediately after experiments, animals were sacrificed, and brains were removed. The dorsal hippocampus (dHPC), ventral hippocampus (vHPC), and medial prefrontal cortex (mPFC) were micro-dissected (300 µm thickness) on a cryostat CM3050 (Leica

Microsystems, Wetzler, Germany). Total RNA was extracted, reverse transcribed, and real-time PCR was performed. All primers used were previously published (Calabrese et al., 2012; Covacu et al., 2009; Dick et al., 2015; Ermolinsky et al., 2008; Rogers et al., 2004; Tan et al., 2015), and amplification efficiency was reassessed as previously described (Livak and Schmittgen, 2001). Relative gene expression analysis was performed using the $2^{-\Delta\Delta CT}$ method, and DBS animals were normalized to the sham animals, as previously described (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The list of primer sequences and the details of real-time PCR experiments can be found in the Supplementary Table 1 and supplementary methods.

Mass Spectrometry

Metabolites were extracted from mPFC, dHPC, and vHPC slices, and derivatized using a standard protocol. Gas chromatography–mass spectrometry (GC/MS) analysis was performed on an Agilent 7890B GC - Agilent 7010 Triple Quadrapole Mass Spectrometer system (Agilent, California, USA). Dopamine (DA), Serotonin (5-HT), γ-Aminobutyric acid (GABA), Glutamic acid (Glu), 3,4-Dihydroxyphenylacetic acid (DOPAC), Homovanillic acid (HVA), and 5-Hydroxyindole acetic acid (5-HIAA) were measured with norvaline as the internal standard. More details can be found in the supplementary methods.

Fear Conditioning

Fear conditioning was performed using a startle and fear conditioning system (Panlab Harvard Apparatus, Massachusetts, USA). For acquisition, the conditioned stimulus (CS) was a tone (Volume: 80 dB, Frequency: 5000 Hz) and the unconditioned stimulus (US) was a 0.6 mA footshock. The protocol consisted of 2min of adaptation, followed by three tone-footshock pairings, and then 2min of rest before being removed from the

chamber. For the context test, 24h after conditioning, rats were placed in the chamber for 5min. Percentage freezing was reported based on the 5min test duration. For the tone test, 24h after the context test, rats were placed in the chamber and tested in a different context to the one received during conditioning. The tone tests consisted of 2min of adaptation, followed by five tone presentations (10s) with 10s inter-trial interval (ITI) in the absence of footshock, and 2min of rest before being removed from the chamber. Percentage freezing was reported as the average of the five CS presentations.

To assess fear learning and memory, freezing was used as the dependent variable, which is a species-specific defense response defined as the absence of all movement except that required for respiration (Blanchard and Blanchard, 1969). Freezing values were calculated using a high sensitivity Weight Transducer System (StartFear System, Harvard Apparatus, Holliston, Massachusetts, USA). Open Field Test was conducted 24h after the tone test to control for locomotion differences. Animals were allowed to explore the arena for 10min. The behavior of rats was recorded and analyzed using a digital video camera and Any-maze video tracking system 5.0 (Stoelting Co).

Statistics

All statistical analyses were performed using GraphPad Prism 7.00. Statistical models used are stated in the various results sections. Outliers were removed using the ROUT method. Results were considered significant for $p<0.05$.

RESULTS

DBS of vmPFC disrupts consolidation, but not acquisition or retrieval of fear memory

To investigate the effect of vmPFC DBS on fear memory, rats implanted with bilateral electrodes in the vmPFC were tested in a modified EPM with aversive odor in one

closed arm, and then subjected to the retrieval task without odor 24h later. Rats were either stimulated for 5min in the home cage before continual stimulation in the modified EPM (Acquisition, Fig. 1A); stimulated for 5min in the home cage before continual stimulation in the EPM during the retrieval task (Retrieval, Fig. 1F); or stimulated for a further 15min at 15min after the acquisition task (Consolidation, Fig. 1K).

For the acquisition DBS group (n=20), unpaired t-test of the Discrimination Index (DI) on day 1 showed no significant difference ($t_{(18)}$=0.24, p=0.81), indicating that vmPFC DBS had no effect on acquisition of fear memory (Fig. 1B). Similarly, DI on day 2 showed no significant difference ($t_{(18)}$=1.44, p=0.17), indicating that vmPFC DBS during acquisition had no effect on retention of fear memory (Fig. 1C). There were no significant differences in the time spent (t<0.82, all p>0.05) (Fig. 1D,E), or distance (t<0.62, all p>0.05) (Supp. Fig. 1A,B), indicating that vmPFC DBS did not affect innate fear or locomotion.

For the retrieval DBS group (n=27), unpaired t-tests showed no significant differences in both acquisition ($t_{(23)}$=0.05, p=0.96) and retrieval ($t_{(24)}$=1.97, p=0.06) tasks (Fig. 1G,H), indicating that vmPFC DBS had no effect on retrieval of fear memory. Similarly, no significant differences were seen in the time spent (t<1.14, all p>0.05) (Fig. 1I,J) or distance moved (t<0.95, all p>0.05) in the open arms on either day (Supp. Fig. 1D,E)

For the consolidation DBS group (n=18), unpaired t-test of the DI in the acquisition task showed no significant difference ($t_{(14)}$=0.37, p=0.72), indicating the baseline fear between the two groups was similar (Fig. 1L). Unpaired t-test of the DI on the day 2 retrieval task showed a significant difference ($t_{(15)}$=3.25, p=0.005), suggesting that vmPFC DBS was able to disrupt consolidation of fear memory (Fig. 1M). There was no

significant difference in time spent in the open arms on either day (t<1.83, all p>0.05) (Fig. 1N,O), suggesting the difference seen was not due to differences in innate fear. Unpaired t-test of the distance moved on day 1 showed no significant difference ($t_{(14)}$=0.45, p=0.66) (Supp. Fig. 1H), whereas the distance moved on day 2 showed a significant difference ($t_{(16)}$=2.17, p=0.045) (Supp. Fig. 1I). Taking into account all previous experiments, these results seem to indicate that DBS did not cause any locomotion differences, but rather vmPFC DBS animals had higher exploratory drive.

Single vmPFC stimulation during consolidation disrupts both tone and contextual fear memory

To investigate if disruption of consolidation of memory affects both tone and contextual fear and to validate the previous results, rats (n=19) implanted with bilateral electrodes in the vmPFC were subject to the well-established tone-footshock fear conditioning paradigm. 15min after the acquisition task, rats were stimulated (or sham stimulated) in the home cage for 15min. 24h later, animals were placed back in the same context to test contextual fear memory. 24h after the context test, animals were subjected to a new context of five tones without footshock to test tone-footshock memory.

Two-way repeated measures ANOVA of the percentage of freezing in the conditioning trial and the inter-trial intervals (ITIs) revealed an effect in the CS-US trial ($F_{(2,34)}$=80.9, p<0.001; $F_{(3,51)}$=121.8, p<0.001), but not treatment or interaction (F<3.0, all p>0.05) (Fig. 2A,B). This indicates proper acquisition of tone and context fear memory with no difference between groups observed prior to DBS. Unpaired t-test of the context test showed a significant difference between sham and DBS groups ($t_{(16)}$=2.38, p=0.03) (Fig. 2C). Unpaired t-test of the exploration period for tone test context showed no significant differences between groups ($t_{(15)}$=0.32, p=0.75), indicating similar baseline levels for

new context. In the tone test, unpaired t-test of the average percentage of freezing for all five tones showed a significant difference between sham and DBS groups ($t_{(13)}$=3.11, p=0.008) (Fig. 2D). Animals were subsequently placed in an open field 24h after the tone test to ensure the effects were not due to differences in locomotion. Unpaired t-test of distance travelled showed no significant differences between sham and DBS groups ($t_{(17)}$=1.27, p=0.22), indicating no effects on locomotion (Fig. 2E).

Single stimulation during consolidation alters expressions of Drd2, Grm5, GluN2A receptor, and c-Fos, expression in the vHPC

To understand the molecular mechanisms of vmPFC DBS on the hippocampus, rats were sacrificed immediately after the trials on day 2. RT-qPCR was performed to detect genes related to synaptic plasticity, learning and memory in dHPC and vHPC sections. The t-tests showed no significant differences in any of the detected genes in the dHPC and vHPC (t<1.74, all p>0.05) (Supp. Fig. 2A,B), indicating 15min of vmPFC DBS did not cause long-term changes in gene expressions.

To examine the immediate changes in receptor expressions in the hippocampus upon vmPFC DBS, rats (n=20) subjected to a similar trial as in the consolidation DBS group were immediately sacrificed after DBS (Fig. 3A). RT-qPCR was performed to detect genes in dHPC and vHPC sections (Fig. 3B). t-tests of the fold changes of genes in the dHPC showed no significant differences (t<1.65, all p>0.05) (Fig. 3C,D). t-tests of fold changes in the genes of the vHPC showed significant changes in the expressions of Drd2 ($t_{(18)}$=2.37, p=0.029), Grm5 ($t_{(18)}$=2.12, p=0.048), and GluN2A ($t_{(18)}$=2.19, p=0.041), but not Drd1, Grm2, Grm3, or GluN2B (t<0.17, all p>0.05) (Fig. 3D).

To detect the changes in neuronal activity of vmPFC stimulation in the hippocampus, immediate early gene, c-Fos in mPFC, dHPC, and vHPC sections was examined by RT-qPCR. The *t*-tests of the fold changes in c-Fos expression showed no significant differences in the mPFC and dHPC ($t<1.12$, all $p>0.05$), but there was a significant decrease in the vHPC ($t_{(17)}=2.16$, $p=0.045$) (Fig. 3E).

vHPC dopamine 2 receptors are involved in the effects of vmPFC DBS on consolidation of memory

To establish the causal role of vHPC D2 receptor in the effects of vmPFC DBS on consolidation, rats (n=69) were implanted with electrodes in the vmPFC and with guide cannulas in the vHPC. Rats subjected to the modified EPM with an aversive odor in one closed arm were then administered either aCSF, Quinpirole (a D2R agonist), or Raclorpide (a D2R antagonist) via the guide cannula in the vHPC. At 15min after the acquisition task, rats were stimulated (or sham stimulated) in the home cage for 15min. Rats underwent the same EPM without odor 24h later to test retention of fear memory (Fig. 4A).

Two-way ANOVA of the DI in the acquisition task showed no significant differences ($F<1.8$, all $p>0.05$) indicating the baseline fear between the two groups were similar (Fig. 4B). Two-way ANOVA of the DI in the retrieval task showed an effect for interaction ($F_{(2,58)}=3.95$, $p=0.025$), stimulation ($F_{(1,58)}=21.6$, $p<0.001$), and drugs ($F_{(2,58)}=7.84$, $p=0.001$). Tukey post-hoc test revealed significant differences in the aCSF sham group compared to aCSF DBS ($p=0.002$) and Raclopride DBS ($p = 0.026$) groups, but not Quinpirole DBS group. Within the DBS groups, aCSF and Raclopride groups showed a significant difference compared with the Quinpirole group (aCSF: $p=0.001$; Raclopride: $p=0.016$), but not with each other ($p=0.91$). All sham groups showed no

significant differences with each other (lowest p=0.93) (Fig. 4C). Two-way ANOVA of the time spent in the open arms in the acquisition task showed an effect for drugs ($F_{(2,61)}$=5.07, p=0.01), but not interaction or stimulation (F<1.26, all p>0.05). Tukey post-hoc test revealed a significant difference in only the aCSF sham group compared with Raclopride sham group (p=0.01) (Fig. 4D). However, this effect disappeared in the retrieval task, with the two-way ANOVA of the time spent in open arms showing no effects (F<1.52, all p>0.05) (Fig. 4E). The differences seen in the acquisition task could be attributed to either batch or random effects, although given the small differences in the actual mean time (around 40s) and no differences in the retrieval task, we believe the results are still valid. Lastly, no significant difference was seen in the distance travelled in both the acquisition and retrieval tasks (F<2.50, all p>0.05) (Fig. 4F,G).

vmPFC DBS modulates neurotransmitters in the vHPC

To understand the effects of vmPFC DBS on neurotransmission, GC/MS was performed for Glu, GABA, HVA, DOPAC, DA, 5-HIAA, and 5-HT in mPFC, dHPC, and vHPC slices. All targets were within the linear ranges of the standard curves, except for DA, which was excluded from the analysis as it was only detected in the vHPC of vmPFC DBS group. t-test of the relative fold changes (target/average sham) in the vHPC sections revealed significant differences in all targets (t>3.49, p<0.05) with decreases in GABA, Glu, and 5-HIAA, and increases in HVA, DOPAC, and 5-HT (Fig. 5C). The t-test of the relative fold changes in the dHPC sections revealed a significant decrease in only 5-HT with an increase in its metabolite 5-HIAA (t>4.22, all p<0.05) (Fig. 5B). The t-test of the relative fold changes in mPFC sections revealed a significant decrease in only 5-HIAA ($t_{(4)}$=3.68, p=0.021), although the difference in the mean of only 0.07 suggests no clinically relevant significance (Fig. 5A). Chromatographs are shown in supplementary materials (mPFC: Supp. Fig. 3; dHPC: Supp. Fig. 4; vHPC: Supp Fig. 5).

DISCUSSION

In this study, we systematically investigated the effects of acute vmPFC DBS on fear memory. Using our experimental parameters, we showed vmPFC DBS was able to disrupt consolidation, but not acquisition or retrieval of fear memory. The results from the open arms and distance travelled further suggest that this is an effect of memory rather than innate fear or locomotion. We found changes in the expression of dopaminergic and glutamatergic receptors in the vHPC and established a partial causal role of vHPC D2 receptors in this effect. Lastly, we found changes in neurotransmitters in the vHPC.

Although using DBS to manipulate fear memory is not novel (Do-Monte et al., 2013; Milad and Quirk, 2002; Reznikov et al., 2016; Rodriguez-Romaguera et al., 2012), few studies have systematically studied its effects on the individual stages of the memory process. Furthermore, most studies have focused on increasing extinction efficacy rather than directly affecting the original memory (Do-Monte et al., 2013; Milad et al., 2004; Milad and Quirk, 2002; Rodriguez-Romaguera et al., 2012). To the best of our knowledge, this is the first study to isolate the effects on the consolidation of memory by stimulating post acquisition. We also showed consistent results across several models and repeat experiments. Our findings are of great importance as the effects of neuromodulation on memories has been highly controversial, with some studies showing improvements in memory (Fell et al., 2013; Miller et al., 2015; Suthana et al., 2012; Titiz et al., 2017), and others showing disruption of memory (Coleshill, 2004; Halgren et al., 1985; Halgren and Wilson, 1985; Jacobs et al., 2016; Lacruz et al., 2010; Merkow et al., 2017).

We chose to target the vmPFC for its bidirectional connections in both the hippocampus and the amygdala (Jin and Maren, 2015; Marek et al., 2013), structures which are heavily implicated in fear memory (Kim et al., 1993; Ledoux, 1995; Maren, 2001). Although microstimulation of the prelimbic cortex (PrL), and infralimbic cortext (IL) have been shown to have opposing effects on the expression of conditioned fear (Vidal-Gonzalez et al., 2006), we cannot comment on this specificity, as our results pertain to the stimulation of the vmPFC as a whole. The proximity of PrL and IL means that functional overlap of these structures cannot be ruled out (Giustino and Maren, 2015).

We investigated the downstream effects of vmPFC DBS on the hippocampus and showed effects on the vHPC, but not the dHPC. This is perhaps not surprising due to the direct monosynaptic projection from the vHPC to the vmPFC, which plays a crucial role in anxiety (Padilla-Coreano et al., 2016). More surprising, our results suggest effects on conditioned rather than innate fear - the functionality of the hippocampus is thought to be split across the dorso-ventral axis, with the dHPC involved in spatial encoding and the vHPC involved in innate fear (Bannerman et al., 1999; Fanselow and Dong, 2010; Kheirbek et al., 2013; Kjelstrup et al., 2002; Moser et al., 1995). On the other hand, the results could be related to how the dHPC and vHPC were microdissected (Fig 3B), as vHPC slices were richer in CA3 compared to dHPC slices. Obliteration of dopaminergic systems in CA3 has been shown to affect consolidation, but not acquisition of memory (Wen et al., 2015). We cannot, however, entirely exclude effects on innate fear, but rather suggest the effect is on memory. Furthermore, the impairment of the “innate fear circuit” during consolidation might in turn lead to disruption of the consolidation of the fear aspect of the memory, which could explain the effects observed. Similarly, effects on the amygdala cannot be excluded, which probably has a role in disrupting consolidation, as indicated by Klavir et al. (Klavir et al.,

2017), who found that high frequency optogenetic stimulation of amygdala inputs to the PFC disrupted consolidation, but not acquisition of fear memory. Although optogenetic stimulation is mechanistically different from DBS, it shares the same concept of axonal activation (Abulseoud et al., 2012), that could suggest another mechanism by which DBS can disrupt fear memories. Future work on the amygdala is needed to verify this.

Based on our gene expression results, we further studied the role of vHPC dopamine 2 receptor (D2R) on the effects of DBS. It has been shown vHPC D2R is involved in spatial working memory, and there was a dose-related improvement with D2 agonist Quinpirole and a dose-related deficit with D2 antagonist Raclopride (Wilkerson and Levin, 1999). This is in line with our current results that showed a decrease in D2R gene expression in the vHPC together with a deficit in memory. To establish a causal rather than a correlative role of vHPC D2R, we infused Quinpirole and Raclopride separately into the vHPC before DBS. Quinpirole was able to reverse the effects of DBS, whereas Raclopride alone was not able to disrupt consolidation of memory. This suggests that although vHPC D2Rs play a key role in the effects of vmPFC DBS on consolidation of memory, it alone is not sufficient to disrupt consolidation, or fully explain the action of vmPFC DBS.

One possible mechanism of how DBS exerts its action is through its modulatory effects on neurotransmitters such as monoamines (Hamani et al., 2010; Van Dijk et al., 2012) and glutamate (Agnesi et al., 2010; Jiménez-Sánchez et al., 2016; Tawfik et al., 2010). We investigated how vmPFC stimulation during consolidation could be modulated by various neurotransmitters. GC/MS results showed changes in all tested neurotransmitters in vHPC, whereas dHPC showed only changes in the serotonin system and mPFC showing no significant differences. As both glutamate and GABA

levels were lowered in vHPC, a “disruption hypothesis” might explain the observed effects, in which information flow was disrupted (Chiken and Nambu, 2015). Lowered glutamate level together with downregulated c-Fos might further suggest that vmPFC DBS inhibits activity in the vHPC that in turn affects memory. However, the lower GABA levels may complicate this theory and further work is needed to test this hypothesis. Similar to the study by Volle et al. (Volle et al., 2018), we showed that vmPFC DBS increased 5-HT levels in the vHPC. Contrary to another study (Hamani et al., 2010), we showed 5-HT was lower in the dHPC, but this could be due to differences in length of the stimulation (15min in our study compared to 4h in other study). The increase in 5-HT in the vHPC was accompanied by lower 5-HIAA, which appears to be similar to the actions of a MAO-inhibitor (Kaehler and Philippu, 1999) and hints at increased 5-HT availability, although this requires further study. More pertinently and consistent with our current study, we found significant changes in dopaminergic metabolites in the vHPC, but not in dHPC or mPFC. Unfortunately, dopamine levels were too low to detect reliably, making it difficult to accurately measure the dopamine turn-over. Interestingly, we found increased dopamine metabolites in the vHPC of vmPFC DBS groups, and dopamine was detectable only in the vmPFC DBS group, but not in the sham group. This initially seems to contradict our results (in that dopamine agonist reversed the effects of vmPFC DBS), however, it should be noted that Quinpirole has been shown to lower dopamine and DOPAC (Santiago et al., 1993), which could explain the reversing effects on vmPFC DBS. Further studies are needed to fully understand how vmPFC DBS affects the complex play of dopamine in modulating memory processes.

To the best of our knowledge, this is the first study to show vmPFC DBS can disrupt consolidation of fear memories. Our results were reproducible over multiple experiments and paradigms, consistently showing the involvement of the vHPC and dopaminergic

system (particularly D2 receptors). However, the effects of DBS are complex and the precise mechanisms, including the involvement of serotonergic and glutamatergic systems, are still very much unknown and need to be further explored. Overall, we provide strong evidence and a strong basis for further research into the use of neuromodulation to disrupt fear memory processes as a possible treatment for anxiety disorders.

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Figure Legends

Figure 1. vmPFC DBS affects consolidation but not acquisition or retrieval of fear memory. Rats were trained in a modified elevated plus maze with aversive odor in one closed arm, and neutral odor in the opposite closed arm. Rats were tested for arm preference 24h after acquisition. Rats were stimulated either during acquisition (A), retrieval (F), or consolidation (K). Both acquisition stimulation (A, B) and retrieval stimulation (G, H) showed no significant differences in the discrimination index of the closed arm with aversive odor vs neutral odor during the acquisition and retrieval tests, indicating no effect on memory. For the consolidation stimulation, L shows there was no

significant difference in the discrimination index during acquisition, whereas B shows significant differences in the retrieval test, indicating disruption of consolidation of memory. No significant differences were seen in the time spent in the open arms in both the acquisition and retrieval tasks for the acquisition DBS group (D, E), and retrieval DBS group (I, J). For the consolidation stimulation, N shows there was no significant difference in the time spent in the open arms, whereas O shows a significant difference in the time spent in the open arms during the retrieval task. * $p<0.05$, ** $p<0.01$.

Figure 2. vmPFC DBS disrupts consolidation of both contextual and tone-footshock memory. Rats were fear conditioned in a tone-footshock paradigm. vmPFC DBS was carried out 15min after acquisition. Rats were then tested for contextual fear memory and tone-footshock memory. A & B shows there was effective learning of both CS-US and context-US fear memory, with no differences between groups at baseline. Figure C shows there was significantly less freezing in the context test, whereas D shows significantly less freezing in the tone test. E shows there was no significant difference in the distance travelled in the open field test, indicating no difference in locomotion. F shows electrode implantation sites. * $p<0.05$, ** $p<0.01$.

Figure 3. qPCR of various synaptic plasticity- and neuronal activity-related genes immediately after stimulation. Rats were trained in a modified elevated plus maze with aversive odor in one closed arm and neutral odor in the opposite closed arm. vmPFC DBS was carried out 15 min after acquisition and rats were immediately sacrificed (A). mPFC, dHPC, and vHPC sections were micro-dissected for qPCR (B). C shows there were no significant differences in the dHPC. D shows downregulation of Drd2, Grm5, and Grin2A in the vHPC. C-Fos gene expression was not significantly

changed in the mPFC or dHPC (E and F) but was significant downregulated in the vHPC (G). * $p<0.05$.

Figure 4. vHPC Dopamine 2 receptor is involved in the effects of DBS on consolidation of memory. Rats were trained in a modified elevated plus maze with aversive odor in one closed arm and neutral odor in the opposite closes arm. aCSF, Quinpirole, or Raclopride were immediately administered to the vHPC. vmPFC DBS was carried out 15min after acquisition (A). B shows there was no significant difference in the discrimination index during acquisition, whereas C shows significant difference between DBS and sham groups for aCSF and Raclopride, but not Quinpirole group. None of the sham groups showed significant differences with each other. Quinpirole DBS group was significantly different from the aCSF and Raclorpide groups (C). There was a significant difference between the aCSF and Raclopride sham pretreatment groups (D), but no significant difference was observed in the retrieval task (E), which suggests minor batch differences, but overall no effect on innate fear. No significant difference was seen in the distance travelled indicating no effects on locomotion (F, G).

Figure 5. Mass spectrometry analysis of various neurotransmitters and metabolites. mPFC, dHPC, and vHPC slices were analyzed by GC/MS for Glutamate, GABA, HVA, DOPCA, 5-HIAA, and 5-HT. No Significant differences were observed in the mPFC (A), while dHPC showed significant differences with an increase in 5-HIAA and decrease in 5-HT. Significant differences were also observed in the vHPC with decreases in Glutamate, GABA, and 5-HIAA, and increases in HVA, DOPAC, and 5-HT. * $p<0.05$.

Supplementary Table 1. List of primers used for qPCR. Table of primer sequences for qPCR with references. All primers were tested for efficiency before use.

Supplementary Figure 1. vmPFC DBS does not affect locomotion. No significant differences were seen in the distance travelled for both the acquisition and retrieval tasks in the acquisition DBS group (A, B) and retrieval DBS group (D, E). For consolidation stimulation, H shows there was no significant difference in the distance travelled, whereas I shows a significant difference in distance travelled, suggesting higher exploratory drive rather than a difference in locomotion. C, F, & J show electrode implantation sites. * $p<0.05$.

Supplementary Figure 2. qPCR of various synaptic plasticity- and neuronal activity-related genes 24h after stimulation. Rats from the consolidation experiment (Fig. 1K) were sacrificed, and dHPC and vHPC sections were micro-dissected for qPCR. No significant differences were seen in the detected gene expressions in both dHPC and vHPC (A, B).

Supplementary Figure 3. Mass spectrometry chromatographs of various neurotransmitters and metabolites in the mPFC. mPFC slices were analyzed by GC/MS for Glutamate, GABA, HVA, DOPCA, 5-HIAA, and 5-HT. A shows chromatographs of Sham group, while B shows chromatographs of vmPFC DBS group.

Supplementary Figure 4. Mass spectrometry chromatographs of various neurotransmitters and metabolites in the dHPC. dHPC slices were analyzed by GC/MS for Glutamate, GABA, HVA, DOPCA, 5-HIAA, and 5-HT. A shows chromatographs of Sham group, while B shows chromatographs of vmPFC DBS group.

Supplementary Figure 5. Mass spectrometry chromatographs of various neurotransmitters and metabolites in the vHPC. vHPC slices were analyzed by GC/MS for Glutamate, GABA, HVA, DOPCA, 5-HIAA, and 5-HT. A shows chromatographs of Sham group, while B shows chromatographs of vmPFC DBS group.

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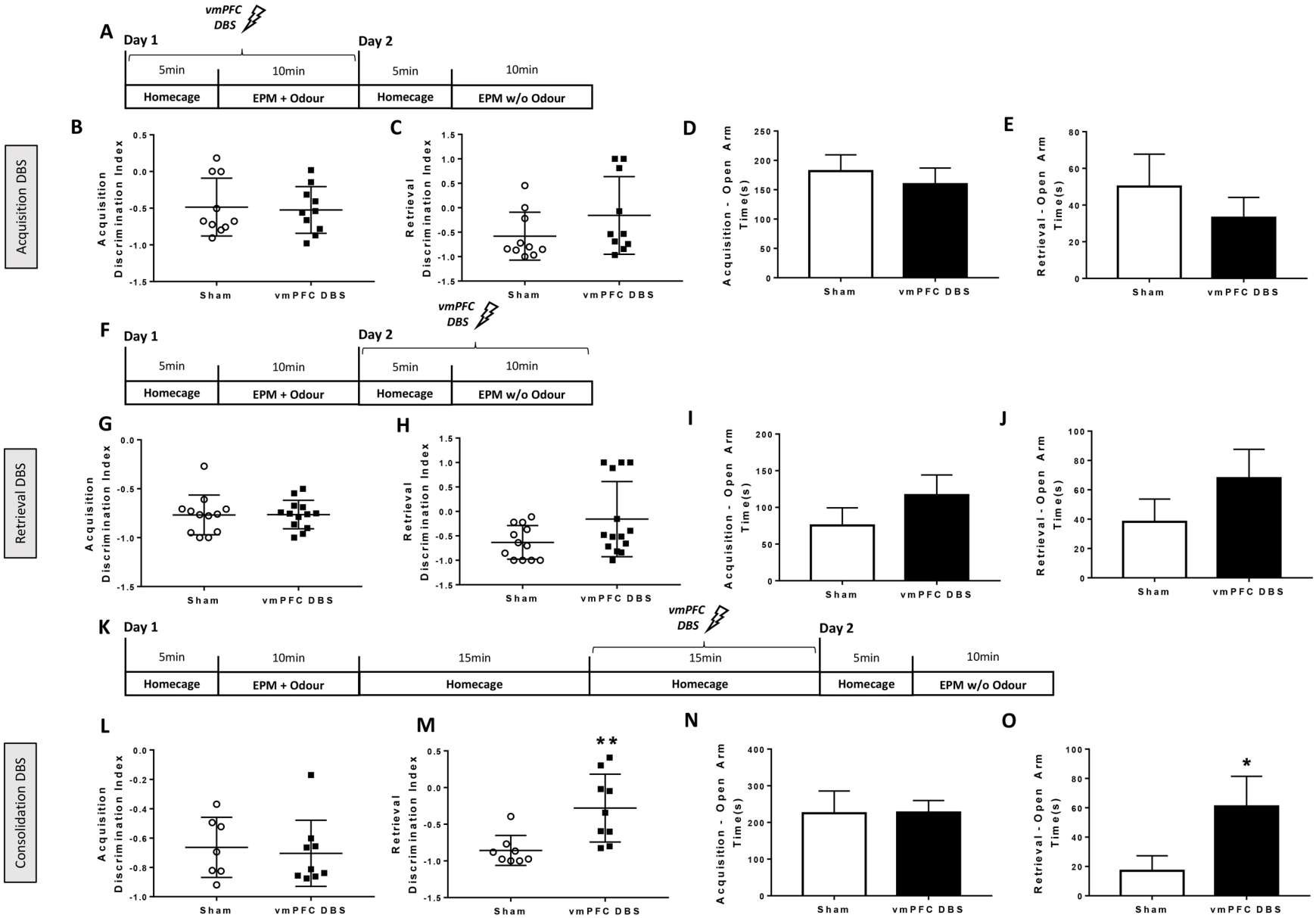
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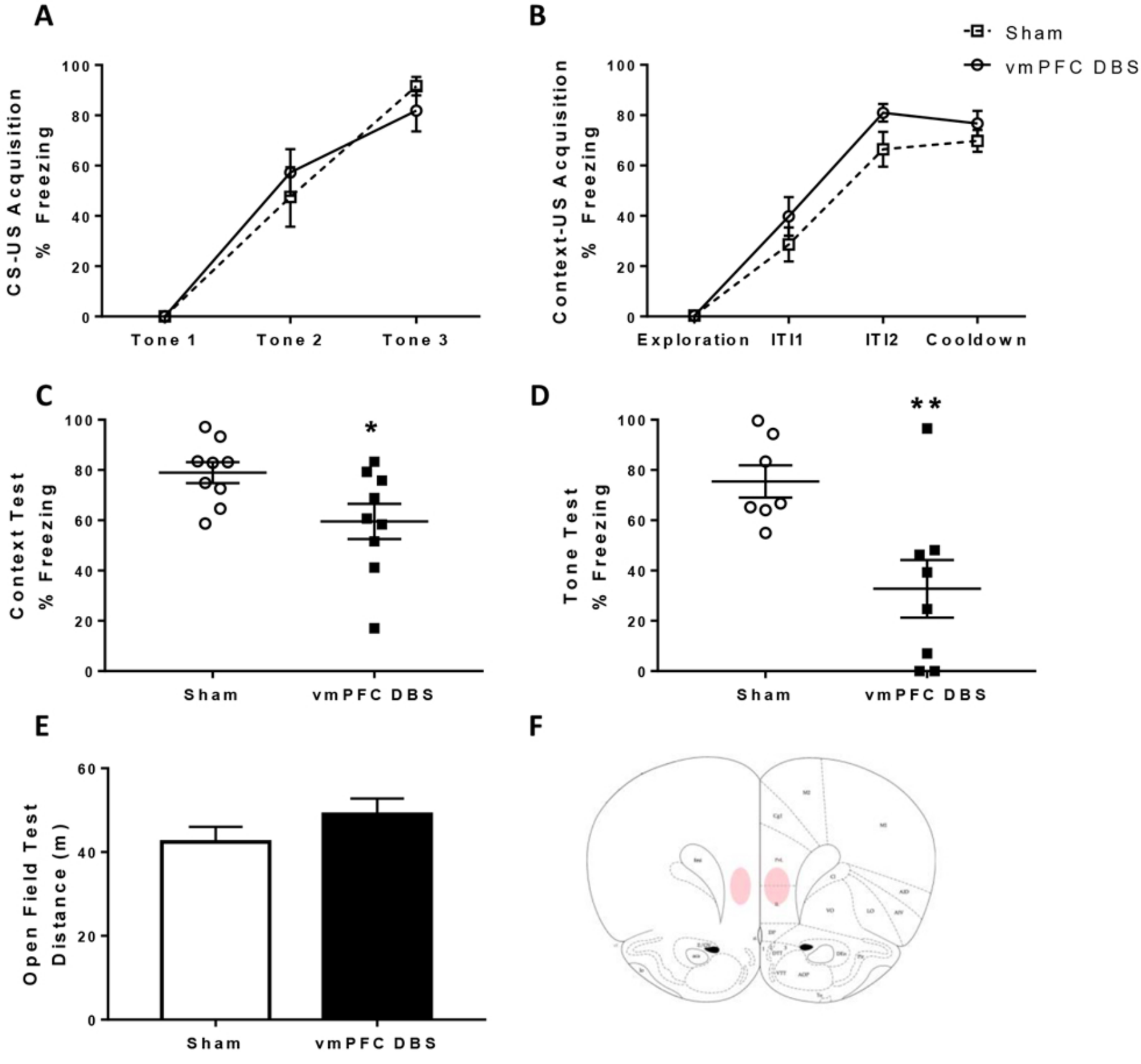
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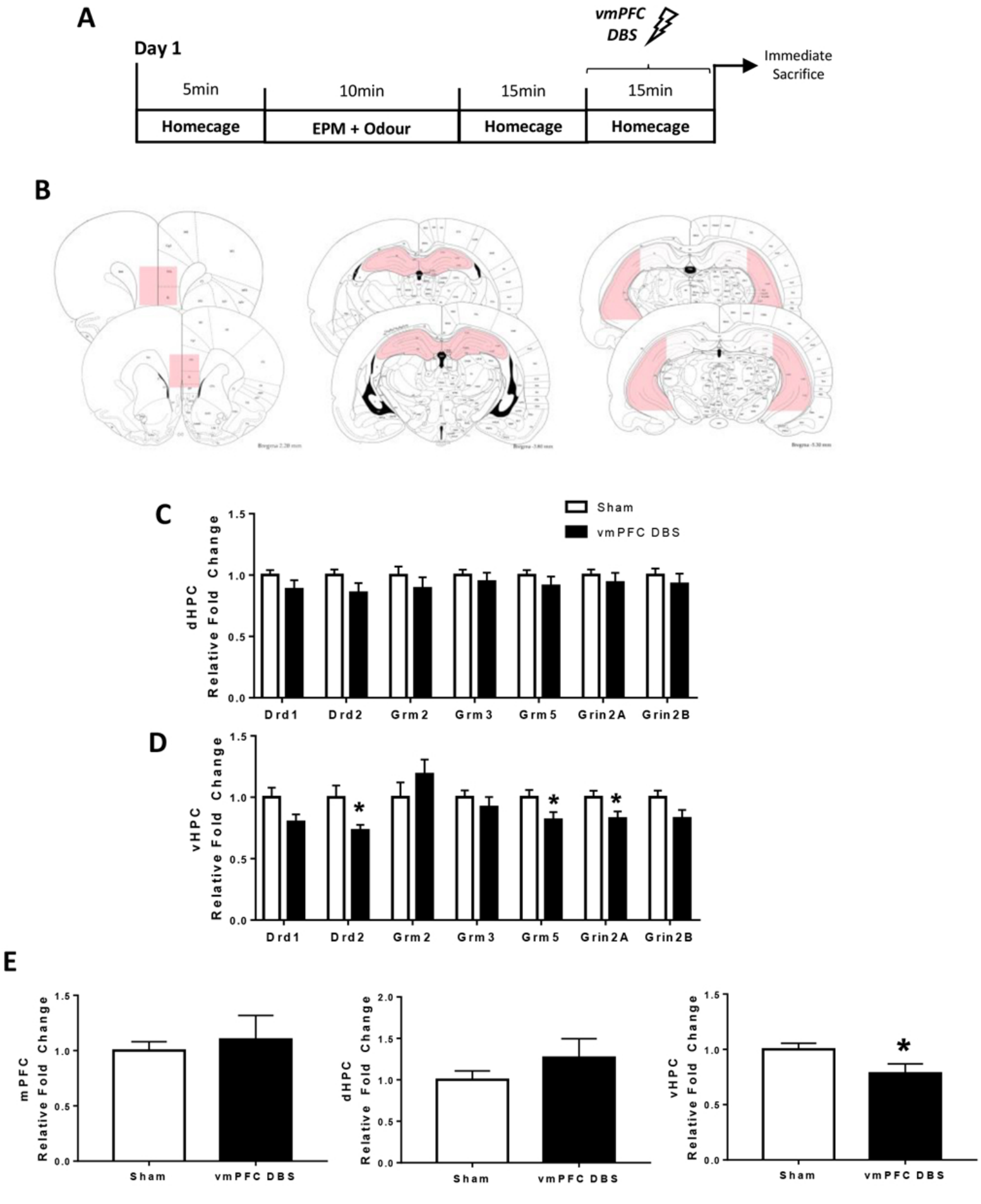
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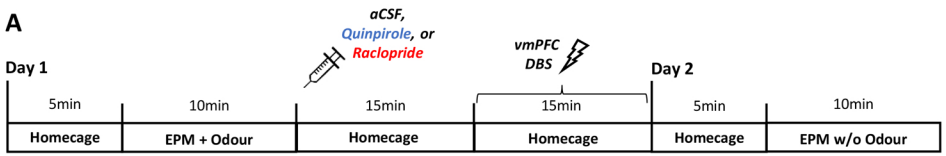
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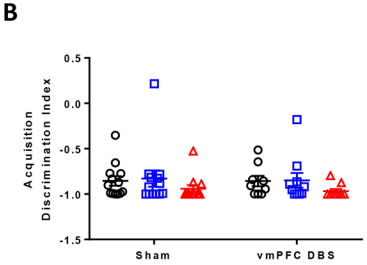
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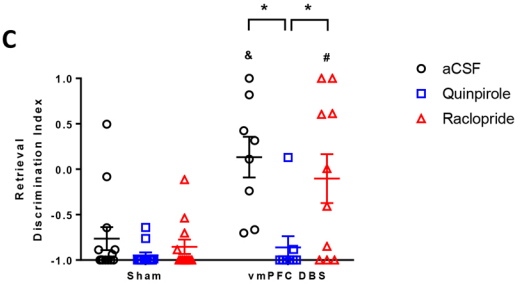
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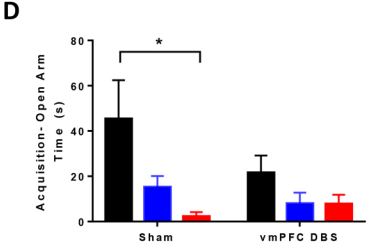
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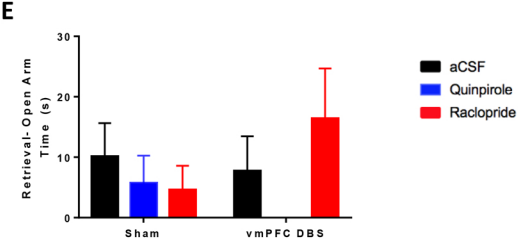
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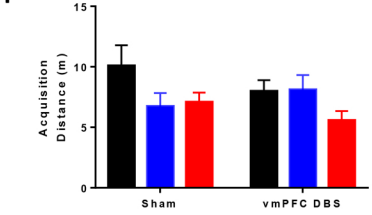
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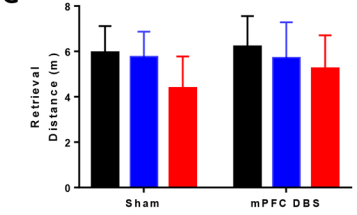
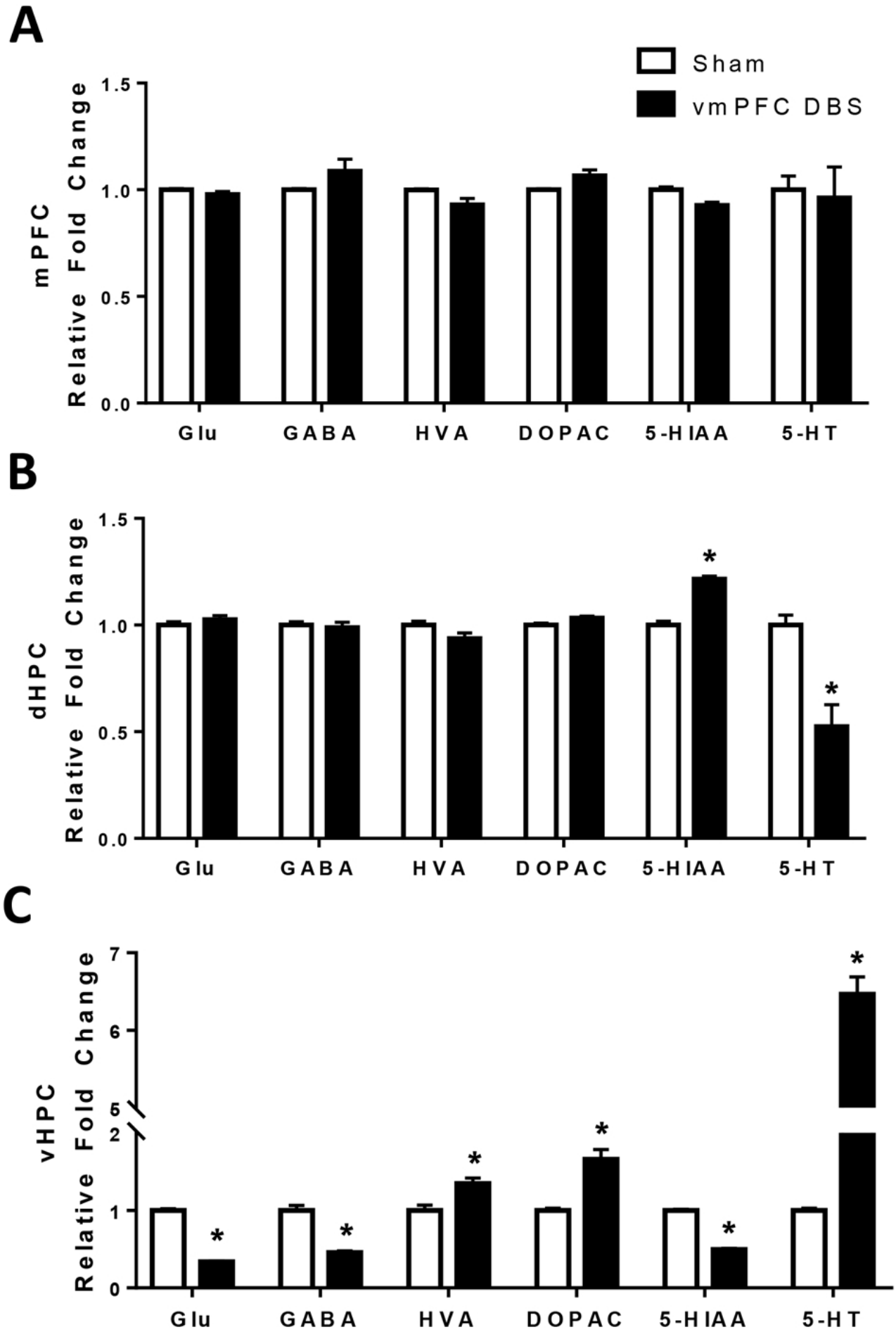
Sham
vmPFC DBS


G
Retrieval
Distance (m)
8
6
4
2
0
Sham
mPFC DBS


A
Sham
vmPFC DBS
mPFC Relative Fold Change
1.5
1.0
0.5
0.0
Glu
GABA
HVA
DOPAC
5-HIAA
5-HT
B
dHPC Relative Fold Change
1.5
1.0
0.5
0.0
*
*
Glu
GABA
HVA
DOPAC
5-HIAA
5-HT
C
vHPC Relative Fold Change
7
6
5
2
1
0
*
*
*
*
*
*
*
Glu
GABA
HVA
DOPAC
5-HIAA
5-HT