Original Article

Prelimbic Cortical Stimulation Disrupts Fear Memory Consolidation through Ventral Hippocampal Dopamine 2 Receptors

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.

[#] These authors contributed equally

Corresponding author:

Lee Wei Lim MD, PhD, AM
Neuromodulation Laboratory,
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: drlimleewei@gmail.com

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List of Abbreviations

5-HIAA - 5-Hydroxyindole acetic acid

5-HT – Serotonin

aCSF – Artificial Cerebrospinal Fluid

CS - Conditioned Stimulus

DA - Dopamine

DBS – Deep Brain Stimulation

dHPC – Dorsal Hippocampus

Drd2 – Dopamine 2 Receptor

EPM – Elevated Plus Maze

GABA - γ-Aminobutyric acid

Glu – Glutamic acid

HVA - Homovanillic acid

ITIs - Inter-trial Intervals

PrL – Prelimbic Cortex

US – Unconditioned Stimulus

vHPC – Ventral Hippocampus

vmPFC – Ventromedial Prefrontal Cortex

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 toxicity of the medications. 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 employed a standard tone-footshock fear conditioning paradigm and modified plus maze discriminative avoidance task to probe the effects of Prelimbic Cortex (PrL) DBS on various stages of memory. We identified memory consolidation stage as a critical time point to disrupt fear memory via PrL DBS. The observed disruption was partially modulated by the inactivation of the ventral hippocampus (vHPC) and the transient changes in vHPC dopamine 2 receptors expression upon PrL DBS. We also observed wide-scale changes of various neurotransmitters and their metabolites in vHPC, confirming its important role in response to PrL DBS. These findings highlight the molecular mechanism in the vHPC in response to PrL stimulation, and may have translational value, indicating that targeting the PrL in the memory consolidation stage via non-invasive neuromodulation techniques may be a feasible therapeutic strategy against anxiety disorders.

Keywords: Anxiety; Deep Brain Stimulation; Dopamine; Fear; Memory; Neuromodulation

INTRODUCTION

Anxiety disorders are highly prevalent and are among the biggest threats to mental health worldwide (Ehlers, 1997). Anxiety disorders are characterized by pervasive feelings of anxiety and fear that lead to maladaptive behaviour. Fear responses can be triggered by various stimuli including predator, pain, or environmental dangers such as height. Such stimuli induce defensive behaviours that neither require previous experience of direct harm nor involve learning—this is referred to as “innate fear” (Lim et al., 2010; Lim et al., 2009). An experience of innate fear can also involve the formation of a memory of that fearful event (e.g., the context in which the fearful event happens) paired with the initial neutral stimuli or aversive stimuli—this is referred to as “conditioned fear”. Anxiety disorders based on conditioned fear are commonly treated using a form of cognitive behavioural therapy called exposure therapy, which involves new learning that attempts to inhibit or update the previous maladaptive learning but does not erase it, resulting in many patients unable to maintain the benefits and often leading to relapse (Baum, 1988; Bouton, 2002). Current attempts to improve cognitive behavioural therapy have met with several difficulties, including drug toxicity (when pharmacological treatments are used) and low treatment efficacy (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 an invasive technique that involves implanting electrodes in specific regions of the brain and using electrical stimulation to modulate the firing of neurons (Lim et al., 2015; Tan et al., 2010; Temel et al., 2009). It has been shown to be a promising treatment for depression and anxiety disorders (Khairuddin et al., 2020; Lim et al., 2015; Temel et al., 2012; Temel and Lim, 2013). However, few studies have systematically investigated the effects of DBS on fear memory. We previously

hypothesized that DBS would be able to disrupt memories through the disruption of the engram process (Tan et al., 2020; Tan et al., 2020; Tan et al., 2019). In this study, we examined the effects of DBS on the prelimbic cortex (PrL), which is a structure that is considered to be an ideal target (Lim et al., 2011; Tan et al., 2019) for the disruption of fear memories, as it is implicated in the expression of learned but not innate fear (Corcoran and Quirk, 2007).

Here we sought to investigate whether PrL DBS given to the animals during various stages of memory would disrupt fear memory. We applied acute PrL DBS during different stages of memory in order to study its effects. Conditioned fear was first investigated in rats using a standard tone-footshock fear conditioning paradigm, which is a highly robust and established method for testing anxiety (Ganella and Kim, 2014). With the regional specificity of DBS, we then probed the involvement of the hippocampus, a structure interconnected to the vmPFC that plays an important role in emotions and memory (Carreno et al., 2016; Jin and Maren, 2015; Liu et al., 2015; Phillips et al., 2019; Tan et al., 2020). Specifically, we found the involvement of the ventral hippocampus (vHPC), which plays a crucial role in anxiety-related behaviour and has monosynaptic projections connecting to the vmPFC (Adhikari et al., 2010; Padilla-Coreano et al., 2016).

Using qPCR, we found changes in the expression of various learning and memory-related receptors including dopamine D2 receptor (Drd2). We further conducted a reversal experiment using pharmacological methods, which revealed a partial causal role of Drd2. Lastly, using gas chromatography/mass spectrometry (GC/MS) analysis, we also identified changes in other neurotransmitter levels besides dopamine, which highlights the complex nature of the effects of DBS on memory. Overall, we showed the potential

application of DBS in modulating fear memories and unravelled some of the mechanisms behind its effects.

MATERIALS AND METHODS

Animals

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 open-top cages with food and water available *ad libitum*. The environmental conditions were maintained at 21±1°C and 60-65% humidity under a reversed 12/12 h light/dark cycle. All behavioural experiments were conducted during the dark phase. The total number of animals used in the study was estimated based on the recommendations from the CULATR. Power calculations predicted a significance effect of $\delta = 35\%$ with a standard deviation of $\sigma = 25\%$ for $p < 0.05$ and power of $\pi = 0.8$. In the behavioural studies, the number of animals in each group was determined by the following formula: $15.7 * (0.25/0.35)^2 = 8.01$ (n=8 animals). Taking into consideration the estimated loss and the overall statistical significance of the behavioural study, we estimated we would need 10 animals per group. Animals were assigned into groups by simple randomization. The timeline and the number of animals in the behavioural experiments are shown in the corresponding figures (Fig. 1-4).

Surgical and Deep Brain Stimulation Procedures

Surgery and DBS procedures were performed as previously described (Lim et al., 2015; Liu et al., 2015; Tan et al., 2020). The animals were initially anaesthetized with 5% isoflurane vapour mixed with oxygen until loss of righting reflex. Animals were mounted in a stereotaxic frame (Leica Biosystems, Nussloch, Germany) and maintained with 2.5%

isoflurane delivered through a nose cone. A midline incision was made to expose the skull and sagittal suture was used to align the skull along the anterior-posterior axis in the frame. Bilateral platinum-iridium electrodes (0.30 mm Diameter, 0.031 mm^2 exposed area) (Synergy Engineering Pte Ltd, Singapore) were implanted in the PrL (AP: +3.0 mm; ML: +/-0.6 mm; DV: -3.6 mm) based on the Paxinos & Watson Rat Brain Atlas (Paxinos and Watson, 2006). The electrode construct was anchored to the rat skull with stainless steel screws and dental acrylic (Paladur, Heraeus Kulzer GmbH, Hanau, Germany). 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) and similarly secured with dental acrylic. Rats were connected to the cables and stimulated using a digital stimulator (Model 3800 MultiStim: 8-Channel Stimulator; A-M Systems, Carlsborg, USA) with 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 (Liu et al., 2015; Tan et al., 2020). Sham animals were similarly implanted with electrodes and tested without stimulation. For verification of electrode/cannula localization, haematoxylin-eosin (Merck, Darmstadt, Germany) was performed to examine the implantation site.

Administration of Drugs

Rats were infused with either Quinpirole-HCl (10 μg of the salt per side, equivalent to 39.09 μmol per side; Sigma-Aldrich), Raclopride (1.67 μg of the salt per side, equivalent to 3.36 μmol per side; Sigma-Aldrich) 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 by two Hamilton syringes (10 μL) connected to the internal cannula via polyethylene tubing (Protech International, Texas, USA). The infusion volume (2 μL)

was delivered over approximately 3 min and the internal cannula was left in for an additional 3 min.

Fear Conditioning

Fear conditioning was performed using a startle and fear conditioning system (Panlab Harvard Apparatus, Massachusetts, USA). For the acquisition stage, the conditioned stimulus (CS) was a 10 s tone (Volume: 80 dB, Frequency: 5000 Hz), which was co-terminated with a 1 s footshock (0.6 mA) as the unconditioned stimulus (US). The protocol consisted of 2 min of adaptation, followed by three tone-footshock pairings with inter-trial intervals (ITIs) of 85 s and 135 s to prevent any association with time, and then 2 min of rest before removal from the chamber. To assess fear learning and memory, freezing was used as the dependent variable, which is a species-specific defence response defined as the absence of all movement except that required for respiration (Blanchard and Blanchard, 1969). For the context test at 24 h after conditioning, rats were placed in the chamber for 5 min and percentage freezing was reported during the test period. For the tone test at 24 h after the context test, rats were placed in the chamber and tested with a different context to the one received during conditioning. The tone tests consisted of 2 min of adaptation, followed by five tone presentations (10 s and 10 s ITI) in the absence of footshock, and then 2 min of rest before removal from the chamber. Percentage freezing was reported as the average of the five CS presentations. The chamber was washed with 70% ethanol and allowed to dry in between each animal testing. The movement of the animals in the fear conditioning chamber was assessed by a built-in pressure sensor. Blinding was not done, although the freezing values were calculated using a high sensitivity Weight Transducer System (StartFear System, Harvard Apparatus, Holliston, Massachusetts, USA) to avoid experimenter bias. Open Field Test was conducted 24 h after the tone test to control for locomotion differences. Animals were

allowed to explore the arena for 10 min. The behaviour of rats was recorded and analysed using a digital video camera with the Anymaze video tracking system 5.0 (Stoelting Co).

Modified Elevated Plus Maze

The elevated plus maze (EPM) used a four-arm maze made of black Plexiglass. The maze consisted of two opposing open arms (50x10 cm) and two opposing closed arms (50x10 cm) with 15 cm high walls that extended out from the central platform (10x10 cm). On day 1, a container with 5 mL of bobcat urine (aversive odour; PredatorPee, Maine, USA) was placed in one closed arm of the modified EPM and a container with 5 mL of rabbit urine (neutral odour) was placed in the opposite closed arm. Urine was changed every 4-5 animals tested. On day 2, empty containers without odour were placed in the two closed arms. Stimulation or sham stimulation was administered according to the protocol. For each trial, the animal was placed in the central platform and tested for 10 min. Discrimination Index (DI) was used to determine arm preference and was calculated from the time spent in each arm by the following equation: (aversive – non aversive)/(aversive + non aversive), which was used as a measure of avoidance fear memory. Their behaviour was also recorded using a digital video camera and analysed by Anymaze 5.0.

Real-time PCR

Immediately after the experiments, animals were sacrificed and their brains were removed. The dorsal hippocampus (dHPC) (Bregma -3.14 mm to -3.80 mm; 4 X 100 µm) and ventral hippocampus (vHPC) (Bregma -4.80 mm to -5.30 mm; 2 X 100 µm) were dissected out in a cryostat (Leica CM3050S, Nussloch GmbH, Germany) according to the anatomical regions based on the Paxinos & Watson Rat Brain Atlas. Sections were stored at -80°C until use. Total RNA was extracted using TRIzol reagent (Molecular Research

Center Inc., Ohio, USA) followed by reverse transcription using a PrimeScript™ RT reagent kit with gDNA eraser (Takara Bio USA, California, USA), and cDNA products were stored at -20°C until use. Real-time PCR was performed on a StepOne™ Real-Time PCR System (ThermoFisher Scientific, Massachusetts, USA). Reactions were performed in triplicate in MicroAmp 96-well plates under standard conditions (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 30 s) with SYBR Green fluorescence (Applied Biosystems, Life Technologies, Warrington, UK), and fluorescence was detected after each cycle. A melt curve from 60-95°C with a step increase of +5°C was plotted at the end of the cycling stage to evaluate the amplification products. Data were analysed using StepOne™ Real-Time PCR software. 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 described (Tan et al., 2020). 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 (Tan et al., 2020). A list of primer sequences can be found in Supp. Table 1.

Mass Spectrometry

The methodology of mass spectrometry experiment was performed as previously described (Tan et al., 2020). In brief, tissue homogenization and metabolite extraction were performed in 1.5 mL of methanol/MilliQ water (80%, v/v) with 0.1 mg norvaline as the internal standard. Tissue was homogenized on ice by 10 cycles of sonication at 10 microns for 20 s and 10 s pause time. Next, 750 µL of MilliQ water was added and the tube was vortexed for 30 s, and then 1200 µL of chloroform was added and the tube vortexed again. After agitation for 15 min, the sample was centrifuged for 5 min at 10000 g. The polar phase was isolated and the dried residue was redissolved and derivatized for 2 h at 37°C in 40 µL of methoxylamine hydrochloride (30 mg/mL in pyridine), followed

by trimethylsilylation for 1 h at 37°C in 70 μL MSTFA with 1% TMCS. A sample (0.2 μL) was analysed by GC-MS and the remaining sample was dried under vacuum.

The GC/MS spectra were acquired in SCAN and MRM mode on an Agilent 7890B GC and Agilent 7010 Triple Quadrupole Mass Spectrometer system (Agilent, CA, USA). The sample was separated in an Agilent DB-5MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness) with a constant flow rate of 1 mL/min. The GC oven program started at 60°C (holding time 1 min) and increased at 10°C/min to 120°C, then at 3°C/min to 150°C, followed by 10°C/min to 200°C, and finally 30°C/min to 280°C (holding time 5 min). Inlet temperature and transfer line temperature were 250°C and 280°C, respectively. Characteristic quantifier and qualifier transitions were monitored in MRM mode during the run. Mass spectra from m/z 50-500 were acquired in SCAN mode.

Data analysis was performed using the Agilent MassHunter Workstation Quantitative Analysis Software. Linear calibration curves for each analyte were generated by plotting the peak area ratio of external/internal standard against the standard concentration at different concentration levels. Analytes were confirmed by comparing the retention time and ratio of characteristic transitions between the sample and standard.

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.

Statistics

All statistical analyses were performed using GraphPad Prism 7.00. The statistical models are detailed in the various results sections. As linearity of data cannot be assumed,

outliers were removed using the ROUT method (Motulsky and Brown, 2006) (shown as red points on the figures). Results were considered significant for $p<0.05$. All data were presented as the Mean ± SEM of the individual data points (with the exception of Fig 6, as replicates were technical replicates). D'agostino & Pearson normality test was done on all datasets, and non-parametric data was analysed using Mann-Whitney test, which showed significant differences from the parametric tests. For GC/MS experiment, t-test with Holm-Sidak corrections was used to adjust the statistical power of multiple t-test comparisons.

RESULTS

PrL stimulation during retrieval specifically disrupts contextual fear memory

To investigate the effects of PrL DBS on conditioned fear memory, rats implanted with bilateral electrodes in the PrL were subjected to a standard tone-footshock fear conditioning paradigm (Fig. 1A, B). We first confirmed that our conditioned fear training paradigm was sufficient to generate robust fear responses from the animals, as indicated by the high freezing percentage during acquisition. For testing normal acquisition, two-way repeated measures ANOVA of the percentage of freezing in the conditioning trial with ITIs revealed an effect for the number of trials (Context: $F_{(2,28)}=57.05$, $p<0.001$; Tone: $F_{(3,42)}=99.52$, $p<0.001$), but not treatment (Context: $F_{(1,14)}=0.76$, $p=0.40$; Tone: $F_{(1,14)}=0.93$, $p=0.35$) or interaction (Context: $F_{(2,28)}=0.82$, $p=0.45$; Tone: $F_{(3,42)}=0.68$, $p=0.44$) (Fig. 1B), indicating no difference between groups prior to DBS in terms of basal behavioral responses to footshock.

We next probed the effect of PrL DBS when administered during memory acquisition. Rats (DBS n=8, Sham n=8) were stimulated in the home cage before continuous stimulation during the context acquisition for a total of 15 min (Fig. 1C-E). Unpaired t-test

of the context test and tone test showed no significant differences between sham and DBS groups (context test: $t_{(14)}$= 0.26, p=0.79; tone test: $t_{(14)}$=1.66, p=0.12), indicating no effect of PrL DBS on acquisition (Fig. 1D, E). We next asked whether fear memory was affected by the administration of PrL DBS during memory retrieval. To this end, rats were stimulated in the home cage, followed by continuous stimulation during the context test and tone test for a total of 15 min on each of the testing days (Fig. 1F-H). In animals that had been fear-conditioned to contextual and tone cues (DBS n=9, Sham n=7), PrL DBS delivered during memory retrieval specifically disrupted contextual fear memory ($t_{(14)}$=2.29, p=0.04) but not tone fear memory ($t_{(14)}$=1.06, p=0.31) (Fig. 1G, H). These results indicated that PrL DBS during retrieval selectively affects contextual fear recall and does not influence tone-fear recall.

Single PrL stimulation during consolidation disrupts both tone and contextual conditioned fear memory

To systematically characterize the effect of PrL DBS on fear memory disruption, we next asked whether contextual and tone fear memory were influenced by PrL DBS delivered post-acquisition, but prior to retrieval. When PrL DBS was applied for 15 min at 15 min after the acquisition task to ensure DBS only affected memory consolidation (DBS n=7, Sham=9) (Fig. 2A-E), DBS animals showed reduced freezing responses compared to sham animals on the following day during contextual fear memory recall ($t_{(14)}$=2.43, p=0.03) (Fig. 2B). A similar reduction was observed in the tone test, where the average percentage of freezing for all five tones showed a significant difference between sham and DBS groups ($t_{(12)}$=2.77, p=0.02) (Fig. 2C). To confirm that the observed difference was not due to the fear response induced by the novel context, percentage of freezing during the exploration period in the tone test was assessed. Unpaired t-test showed no significant differences between groups ($t_{(14)}$=0.22, p=0.83), indicating similar baseline

levels for the new context (Fig. 2D). To test whether the effects of PrL DBS are specific to fear memory retrieval, animals were placed in the open field 24 h after the tone test. Unpaired t-test showed no significant differences in distance travelled between sham and DBS groups ($t_{(14)}$=1.42, p=0.18), indicating no effect on locomotion (Fig. 2E). To establish the temporal specificity of PrL DBS in fear memory disruption, we also evaluated the effect of PrL DBS in rats stimulated for 15 min at 6 h after the acquisition task (DBS n=8, Sham n=10) (Fig. 2F-H). PrL DBS administered 6 h after acquisition did not influence contextual fear ($t_{(16)}$=0.32, p=0.75) and tone fear memory ($t_{(16)}$=1.42, p=0.17) (Fig. 2G, H). Altogether, the results in these experiments indicate that the reduced fear response observed in animals received PrL DBS during consolidation is contributed by disrupted fear memory and not unconditioned anxiety.

PrL DBS disrupts consolidation of avoidance fear memory

To investigate if the previous results of the disruption of the conditioned fear memory consolidation can be applied to avoidance fear memory, behaviourally naïve rats (DBS n=9, Sham n=9) implanted with bilateral electrodes in the PrL were tested in a modified EPM with aversive odour in one closed arm, followed by a retrieval task 24 h later without the odour. At 15 min after the acquisition task, rats were stimulated (or sham stimulated) in the home cage for 15 min. After 24 h, animals were placed back in the EPM without the aversive odour to test the retrieval of avoidance fear memory (Fig. 3A, B). Mann-Whitney test of the DI in the acquisition task showed no significant differences (p=0.61). Mann-Whitney test of the DI on day 2 in the retrieval task showed a significant difference (p=0.003), suggesting that PrL DBS was able to disrupt consolidation of fear memory (Fig. 3C). There were no significant differences in the time spent in the open arms on either day (D1: $t_{(16)}$=0.03, p=0.98, D2: Mann-Whitney p=0.06) (Fig. 3D), suggesting the differences seen were not due to differences in innate fear. Unpaired t-test of the distance

travelled on day 1 showed no significant differences ($t_{(14)}$=0.45, p=0.66) (Supp. Fig. 1A), whereas the distance travelled on day 2 showed a significant difference ($t_{(16)}$=2.17, p=0.045) (Supp. Fig. 1A). Despite the seemingly increased locomotor activity of PrL DBS animals on day 2, comparing the data in acquisition and retrieval shows that PrL DBS animals exhibited similar exploratory drive on both days. Moreover, we showed that PrL DBS was able to disrupt consolidation of memory in a fear conditioning test (Fig. 2), suggesting an effect on the memory itself. Also, we found no significant differences in distance travelled in stimulated animals when subjected to EPM testing (DBS n=18, Sham n=14) ($t_{(29)}$=1.10, p=0.28), indicating no alteration of exploratory drive in the PrL DBS animals (Supple. Fig. 1B), which is consistent with our previous results which showed that acute DBS (1 h prior to EPM) did not affect exploratory drive in naïve animals (Bhaskar et al., 2018). Hence the difference in DI is unlikely to be due to the change in exploratory drive, but rather a change in avoidance learning. Overall, the effects of DBS on the consolidation of memory provide a better and more encompassing explanation than its effects on exploratory drive.

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

To understand the molecular mechanisms of PrL DBS on the hippocampus, rats were sacrificed immediately on day 2 after the trials in the modified EPM. Real-time qPCR was performed in dHPC and vHPC sections to detect genes related to learning and memory (Handford et al., 2014; Lyon et al., 2011; Milton et al., 2013; Tan et al., 2015; Wilkerson and Levin, 1999). 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. 1C, D), indicating 15 min of PrL DBS did not induce long-term changes in the gene expressions.

To examine immediate changes in the receptor expressions in the hippocampus after PrL DBS, rats (DBS n=9, Sham n=11) were subjected to a similar trial in the modified EPM as in the consolidation DBS group, but were immediately sacrificed (Fig. 4A). Real-time PCR was performed in dHPC and vHPC sections to detect mRNA changes (Fig. 4A). The t-test showed no significant fold changes in the expressions of genes in the dHPC (t<1.65, all p>0.05) (Fig. 4B). However, t-tests showed significant fold changes in the genes expressions in the vHPC for 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 for Drd1, Grm2, Grm3, or GluN2B (t<0.17, all p>0.05) (Fig. 4C). Specifically, the changes in Drd2 expression along with the observed disrupted memory is in line with previous results that demonstrated the involvement of vHPC Drd2 in spatial working memory, which was found to be dose-dependently improved or inhibited by Drd2 agonist Quinpirole or Drd2 antagonist Raclopride, respectively (Wilkerson and Levin, 1999).

To detect changes in neuronal activity in the hippocampus upon PrL DBS, RT-qPCR was performed on mPFC, dHPC, and vHPC sections to examine the expression of immediate early gene c-Fos, a marker of neuronal activity. The t-test of the fold change 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. 4D), indicating reduced activation of the vPHC upon PrL DBS.

Uncorrected t-test was used in the analyses to increase the power to extract the potential mechanisms. Given the role of Drd2 in memory function and our previous proposal of dopamine as a potential target/mechanism of DBS in disrupting memory (Tan et al., 2020), the decrease in Drd2 gene expression in the vHPC observed in the current study may be the molecular mechanism underlying the deficit in memory. Hence, follow-up

experiments on the Drd2 were performed (next section) to validate the necessity of the receptor in fear memory disruption by PrL DBS.

vHPC dopamine D2 receptors are involved in the effects of PrL DBS on consolidation of memory

Based on our gene expression results, we further studied the role of vHPC Drd2 on the effects of DBS. To establish a causal role of vHPC Drd2 in the effects of PrL DBS on consolidation, rats (n=67: sham-aCSF n=13, DBS-aCSF n=9, sham-Quinpirole n=13, DBS-Quinpirole n=10, sham-Raclopride n=12, and DBS-Raclopride n=10; 3 animals were removed from each group on day 2 due to issues with the drug infusion) were implanted with electrodes in the PrL and guide cannulas in the vHPC. Rats were immediately administered aCSF, Quinpirole (a Drd2 agonist), or Raclopride (a Drd2 antagonist) via the guide cannula in the vHPC after subjected to the modified EPM. At 15 min after the acquisition task, rats were stimulated (or sham stimulated) in the home cage for 15 min. Rats underwent the same EPM without odour 24 h later to test retention of fear memory (Fig. 5A).

Two-way ANOVA of the DI in the acquisition task showed no significant differences (Interaction: $F_{(2,61)}$=0.02, p=0.98; Stimulation: $F_{(1,61)}$=0.09, p=0.76; Drug: $F_{(2,61)}$=1.85, p=0.17), indicating the baseline fear between groups were similar. Two-way ANOVA of the DI in the retrieval task showed an effect for interaction ($F_{(2,58)}$=3.95, p=0.03), stimulation ($F_{(1,58)}$=21.6, p<0.001), and drugs ($F_{(2,58)}$=7.84, p=0.001) (Fig. 5B). The disruption of fear memory by PrL DBS was verified by the significant difference in DI comparing aCSF sham group with aCSF PrL DBS group (p=0.01). As expected, PrL DBS animals infused with Raclopride also showed disorupted fear memory comparable to aCSF DBS animals (aCSF sham vs Raclopride DBS: p=0.026; aCSF DBS vs Raclopride

436 DBS: p=0.91), further validating the partially causal effect of dopamine receptor
437 inactivation on fear memory disruption. Within the DBS group, Tukey post-hoc test
438 revealed the infusion of Quinpirole effectively retained the preference towards the neutral
439 arm during recall (aCSF DBS vs Quinpirole DBS: p=0.001; aCSF DBS vs Raclopride
440 DBS: p=0.016), suggesting that activation of dopamine receptor blocked the disruption of
441 fear memory mediated by PrL DBS during consolidation (Fig. 5B). Sham groups showed
442 no significant differences with each other (lowest p=0.93), indicating that dopamine
443 modulation alone is not sufficient to disrupt fear memory.
444
445 Two-way ANOVA of time spent in the open arms in the acquisition task showed an effect
446 for drugs ($F_{(2,61)}$=5.07, p=0.01), but not interaction ($F_{(2,61)}$=1.23, p=0.30) or stimulation
447 ($F_{(1,61)}$=1.26, p=0.27). Tukey post-hoc test revealed a significant difference in only the
448 aCSF sham group compared with the Raclopride sham group (p=0.01) (Fig. 5C).
449 However, this effect disappeared in the retrieval task, with two-way ANOVA of time spent
450 in the open arms showing no significant effects (Interaction: $F_{(2,58)}$=1.52, p=0.23;
451 Stimulation: $F_{(1,58)}$=0.08, p=0.78; Drug: $F_{(2,58)}$=1.11, p=0.34) (Fig. 5C). The differences
452 seen in the acquisition task could be attributed to either batch or random effects, although
453 given the small differences in the actual mean time (around 40 s) and no differences in
454 the retrieval task, we believe the results are still valid. Lastly, no significant differences
455 were seen in the distance travelled in both the acquisition and retrieval tasks (F<2.50, all
456 p>0.05) (Fig. 5D). Overall, the data suggested that PrL DBS-induced disruption of
457 consolidation could be reversed by the D2/D3 receptor agonist Quinpirole, whereas
458 Raclopride alone was not able to disrupt memory consolidation. Together, our findings
459 support vHPC Drd2 plays a key role in PrL DBS on memory consolidation, although it
460 was not sufficient to disrupt consolidation on its own or to fully explain the action of PrL
461 DBS.

462

463 **PrL DBS modulates neurotransmitters in the vHPC**

464 To understand the effects of PrL DBS on neurotransmission, GC/MS was performed on
465 mPFC, dHPC, and vHPC slices for neurotransmitters/metabolites related to learning and
466 memory including Glu, GABA, HVA, DOPAC, DA, 5-HIAA, and 5-HT (Gottfries, 1990;
467 Johansen et al., 2011; Pananceau and Gustafsson, 1997; Peters, 2006; Riedel and
468 Reymann, 1996; Tan et al., 2015; Tan et al., 2020). All targets were within the linear
469 ranges of the standard curves, except for DA, which was excluded from the analysis as it
470 was only detected in the vHPC of the PrL DBS group. The t-test with Holm-Sidak
471 corrections for the relative fold changes in the mPFC revealed no significant differences
472 in neurotransmitter and their metabolite content ($t < 1.55$, $p > 0.05$) (Fig. 6A). The t-test
473 with Holm-Sidak corrections for the relative fold changes in the dHPC revealed a
474 significant increase in 5-HIAA ($t_{(4)}=9.50$, $p<0.001$) (Fig. 6B). The t-test with Holm-Sidak
475 corrections for the relative fold changes (target/average sham) in the vHPC revealed
476 significant differences in all targets ($t>3.49$, $p<0.05$), with decreases in GABA, Glu, and
477 5-HIAA, and increases in HVA, DOPAC, and 5-HT (Fig. 6C). Chromatographs are shown
478 in the supplementary materials (mPFC: Supp. Fig. 2; dHPC: Supp. Fig. 3; vHPC: Supp.
479 Fig. 4).

480

481 **DISCUSSION**

482 In this study, we systematically investigated the effects of PrL DBS on fear memory. The
483 use of a tone and context-footshock conditioning paradigm enabled a robust investigation
484 of the effect of PrL DBS on various stages of memory. We showed that PrL DBS during
485 consolidation was able to disrupt both contextual and tone-footshock conditioned fear
486 memory. We further extended our results with the use of a conditioned avoidance task
487 using a modified EPM, which allowed us to simultaneously control for locomotion and

innate fear differences during the tasks, both of which showed no significant differences. For molecular changes in the brain, we found the expression of dopaminergic and glutamatergic receptors were altered in the vHPC, and established a partial causal role of dopamine D2 receptors in these changes. Lastly, besides dopamine, we also found changes in other neurotransmitters in the vHPC.

Although recent work has implicated the efficacy of neuromodulation techniques in manipulating fear memory, few studies have systematically studied its effects on individual stages of the memory process, and most studies have focused on enhancing memory extinction (Do-Monte et al., 2013; Milad and Quirk, 2002; Milad et al., 2004; Poon et al., 2020; Rodriguez-Romaguera et al., 2012). Here we employed a systematic approach to examine the temporal specificity of PrL DBS administration and isolate the effects of DBS on the consolidation of memory by stimulating post acquisition. We also showed consistent results across multiple models of conditioned fear and in repeat experiments, which is important given the paradoxical ability of DBS to both enhance and disrupt memories (Tan et al., 2020; Tan et al., 2020; Tan et al., 2020). Despite the limitations of using conditioned fear to model anxiety disorders (LeDoux, 2015), it is one of the most well-established and translatable models currently available (Ganella and Kim, 2014).

We targeted the PrL as it has connections to both the hippocampus and the amygdala (Jin and Maren, 2015; Marek et al., 2013), which are structures heavily implicated in fear memory (Kim et al., 1993; LeDoux, 1995; Maren, 2001). In particular, the PrL has crucial roles in learned fear (Corcoran and Quirk, 2007). We found downstream effects of PrL DBS in the vHPC, but not the dHPC. This is consistent with previous findings demonstrating vHPC plays a crucial role in anxiety and has direct monosynaptic

projections to the PrL (Padilla-Coreano et al., 2016), suggesting a possible mechanism of the backpropagation of the signal. In contrast to previous findings that showed the respective involvement of dHPC in spatial encoding and vHPC in innate fear (Kheirbek et al., 2013), our results indicated effects on learned rather than innate fear. Additionally, vHPC slices were richer in CA3 areas compared to dHPC slices (Fig. 4A), and the obliteration of dopaminergic systems in CA3 was shown to affect memory consolidation, but not acquisition (Wen et al., 2015). However, effects on the amygdala cannot be excluded as indicated by Klavir et al. [32], who found that high-frequency optogenetic stimulation (similar in concept to axonal activation in DBS (Abulseoud et al., 2012)) of amygdala inputs to the PFC disrupted memory consolidation, but not acquisition of fear memory. We also found that retrieval PrL DBS specifically disrupted contextual fear memory but not tone fear memory. This might be explained by the effect of PrL DBS on vHPC inhibition, as the vHPC projection to amygdala was shown to be necessary for contextual fear memory (Jimenez et al., 2020), although further work is needed to verify this hypothesis. Interestingly, the site of DBS seemed to show no changes in cFos expression. However, it should be noted that the whole of the mPFC was microdissected out but only the PrL was stimulated, hence, changes specific to the PrL cannot be ruled out.

One possible mechanism of how DBS exerts its action is through the modulation of neurotransmitters such as monoamines (Hamani et al., 2010; Lim et al., 2015; van Dijk et al., 2012) and glutamate (Agnesi et al., 2010; Jimenez-Sanchez et al., 2016; Tawfik et al., 2010). Indeed, consistent with our qPCR results, the GC/MS results showed changes in all the tested neurotransmitters in the vHPC, whereas the dHPC only showed changes in the serotonin system and the mPFC showed no significant changes. Importantly, we found significant changes in dopaminergic metabolites in only the vHPC. Unfortunately,

dopamine levels were too low for reliable detection, making it difficult to accurately measure the dopamine turnover. Interestingly, we found increased dopamine metabolites in the vHPC of PrL DBS groups, whereas dopamine was detected in the PrL DBS groups, but not in the sham group. This initially seems to contradict our results (that dopamine agonist reversed the effects of PrL 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 PrL DBS. Further studies are needed to fully understand how PrL DBS affects the complex interplay of dopamine in modulating memory processes. Besides modulating dopamine, we showed that PrL DBS increased 5-HT levels in the vHPC, which was similar to the study by Volle et al. (Volle et al., 2018). Contrary to another study (Hamani et al., 2010), we showed that 5-HT was lower in the dHPC, but this might be due to differences in the length of stimulation (15 min in our study compared to 4 h in the other study). The increase in 5-HT in the vHPC was accompanied by lower 5-HIAA, which is similar to the actions of an MAO-inhibitor (Kaehler et al., 1999) and hints at increased 5-HT availability, although this requires further study. Besides monoamines, glutamate has also been shown to be modulated by DBS (Agnesi et al., 2010; Jimenez-Sanchez et al., 2016; Tawfik et al., 2010). In our study, we found both glutamate and GABA levels were lowered in the vHPC, which might follow the “disruption hypothesis”, whereby information flow is disrupted (Chiken and Nambu, 2016). It may be interesting for future studies to examine the cellular modifications, including DNA methylation and histone modifications, considering the large-scale neurotransmitter changes observed in this study, as such modifications are crucial to the development of anxiety disorders (Hutchinson et al., 2012; Kwapis and Wood, 2014; Poon et al., 2020; Poon et al., 2020). Overall, the findings suggest the modulation of dopamine transmission plays a major role in the effects of PrL DBS, which may also involve multiple neurotransmitters, although future studies are needed to further explore their contributions (Tan et al., 2020). This

study provides strong evidence for targeting the PrL with neuromodulation techniques to disrupt fear memory processes as a possible strategy for anxiety disorders.

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Conflict of Interest

All authors declared no competing financial interests or potential conflicts of interest.

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Figure Legends

Figure 1. PrL DBS during retrieval specifically disrupts contextual fear memories. Rats were fear conditioned in a tone-footshock paradigm. PrL DBS was performed during acquisition and retrieval. Rats were then tested for contextual fear memory and tone-footshock memory and their freezing behaviors were analysed. Histological micrograph of an example electrode implantation site, black arrows point to tips of the electrode **(A)**. Effective learning of both CS-US and context-US fear memory were observed. There were no differences between groups at baseline. Sound notation and black bolt indicates the tone and footshock administered to the animal during training respectively **(B)**. Experimental scheme of the tone-footshock fear conditioning paradigm. Rats were stimulated during acquisition (DBS n=8, Sham n=8) or retrieval (DBS n=9, Sham n=7), and then tested for contextual fear memory and tone-footshock memory. Light orange shading indicates electrical stimulation administered during the corresponding period in the scheme **(C, F)**. No significant differences in freezing were observed in both context test **(D)** and tone test **(E)** in the acquisition stimulation. A significant reduction of freezing behaviour was observed in context test of retrieval-stimulated rats **(G)**, but not in the tone test **(H)**. * $p<0.05$.

Figure 2. PrL DBS during consolidation disrupts both contextual and tone fear memories. Rats (DBS n=7, Sham n=9) were fear conditioned in a tone-footshock paradigm. PrL DBS was carried out 15 min after acquisition. Rats were then tested for contextual fear memory and tone-footshock memory **(A)**. With consolidation stimulation, PrL DBS rats exhibited significantly less freezing behaviour in both the context test **(B)** and tone test **(C)**. Analysis of freezing behaviour during the tone test exploration period showed no significant differences between sham and DBS animals, indicating the freezing was not generalized **(D)**. Analysis of the distance travelled in the open field test

showed no significant difference between sham and DBS animals, indicating no locomotion changes **(E)**. For DBS performed 6 h after acquisition (DBS n=8, Sham n=10) **(F)**, no significant differences in freezing were observed in both context test **(G)** and tone test **(H)**. * $p<0.05$.

Figure 3. PrL DBS disrupts consolidation of avoidance fear memory

Schematic figure of the modified elevated plus maze. Rats were trained in the modified elevated plus maze with aversive odour (bobcat urine) in one closed arm and neutral odour (rabbit urine) in the opposite closed arm **(A)**. Experimental scheme of the modified elevated plus maze experiment. Rats were tested for arm preference 24 h after acquisition. Rats were stimulated during consolidation (DBS n=9, Sham n=9). Light orange shading indicates electrical stimulation administered during the corresponding period in the scheme **(B)**. With consolidation stimulation, **C** shows there were no significant differences in the discrimination index during acquisition, whereas significant differences were observed in the retrieval test, indicating disruption of the consolidation of memory. No significant differences were seen in the time spent in the open arms during acquisition and retrieval **(D)**. ** p<0.01.

Figure 4. qPCR of various memory- and neuronal activity-related genes immediately after stimulation. Rats (DBS n=9, Sham n=11) were trained in a modified elevated plus maze with aversive odour in one closed arm and neutral odour in the opposite closed arm. PrL DBS was carried out 15 min after acquisition and rats were immediately sacrificed. mPFC, dHPC, and vHPC sections were micro-dissected for qPCR **(A)**. **B** shows there were no significant differences in the dHPC. **C** shows downregulation of Drd2, Grm5, and Grin2A in the vHPC. C-Fos gene expressions were not significantly

changed in the mPFC or dHPC, but was significantly downregulated in the vHPC **(D)**. * $p<0.05$.

Figure 5. vHPC Dopamine D2 receptor is involved in the effects of DBS on consolidation of memory. Rats (n=68; sham-aCSF n=13, DBS-aCSF n=9, sham-Quinpirole n=13, DBS-Quinpirole n=10, sham-Raclopride n=12, DBS-Raclopride n=10) were trained in a modified elevated plus maze with aversive odour in one closed arm and neutral odour in the opposite closed arm. aCSF, Quinpirole, or Raclopride were immediately infused into the vHPC, indicated by the light blue shading. PrL DBS was carried out 15 min after acquisition **(A)**. There was no significant difference in the discrimination index during acquisition, whereas significant differences were observed between DBS and sham groups for aCSF and Raclopride, but not for Quinpirole during retrieval. None of the sham groups showed significant differences with each other. Quinpirole DBS group was significantly different from the aCSF and Raclopride groups **(B)**. For the time spent in the open arms, there was a significant difference between the aCSF and Raclopride sham pre-treatment groups, but no significant difference was observed in the retrieval task, which suggests minor batch differences, but overall no effect on innate fear **(C)**. No significant difference was seen in the distance travelled, indicating no effects on locomotion **(D)**.

Figure 6. Mass spectrometry analysis of various neurotransmitters and metabolites. mPFC, dHPC, and vHPC slices were analysed by GC/MS for Glutamate, GABA, HVA, DOPAC, 5-HIAA, and 5-HT. No significant differences were observed in the mPFC **(A)**, whereas there were significant differences in the dHPC with an increase in 5-HIAA **(B)**. There were also significant differences in the vHPC with decreases in

Glutamate, GABA, and 5-HIAA, and increases in HVA, DOPAC, and 5-HT **(C)**. * $p<0.05$; *** $p<0.001$

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 Table 2. Statistical values of behavioral tests

Supplementary Figure 1. PrL DBS does not affect locomotion, and qPCR of various synaptic plasticity- and neuronal activity-related genes 24 h after stimulation. For consolidation stimulation in the avoidance EPM experiment, there was no significant difference in the distance travelled during the acquisition task, whereas there was a significant difference in distance travelled in the retrieval task, suggesting higher exploratory drive rather than a difference in locomotion **(A)**. No significant differences were seen in the distance travelled in the consolidation DBS group during EPM acquisition, suggesting PrL DBS during the EPM task does not affect exploratory drive **(B)**. Rats from the consolidation experiment (Fig. **2B**) were sacrificed and dHPC and vHPC sections were micro-dissected for qPCR. No significant differences were seen in the identified gene expressions in both dHPC and vHPC (**C** & **D**). * $p<0.05$.

Supplementary Figure 2. Mass spectrometry chromatographs of various neurotransmitters and metabolites in the mPFC. mPFC slices were analysed by GC/MS for Glutamate, GABA, HVA, DOPAC, 5-HIAA, and 5-HT. **A** shows chromatographs of the Sham group, and **B** shows chromatographs of the PrL DBS group.

Supplementary Figure 3. Mass spectrometry chromatographs of various neurotransmitters and metabolites in the dHPC. dHPC slices were analysed by GC/MS for Glutamate, GABA, HVA, DOPAC, 5-HIAA, and 5-HT. **A** shows chromatographs of the Sham group, and **B** shows chromatographs of the PrL DBS group.

Supplementary Figure 4. Mass spectrometry chromatographs of various neurotransmitters and metabolites in the vHPC. vHPC slices were analysed by GC/MS for Glutamate, GABA, HVA, DOPAC, 5-HIAA, and 5-HT. **A** shows chromatographs of the Sham group, and **B** shows chromatographs of the PrL DBS group.

Fig 1

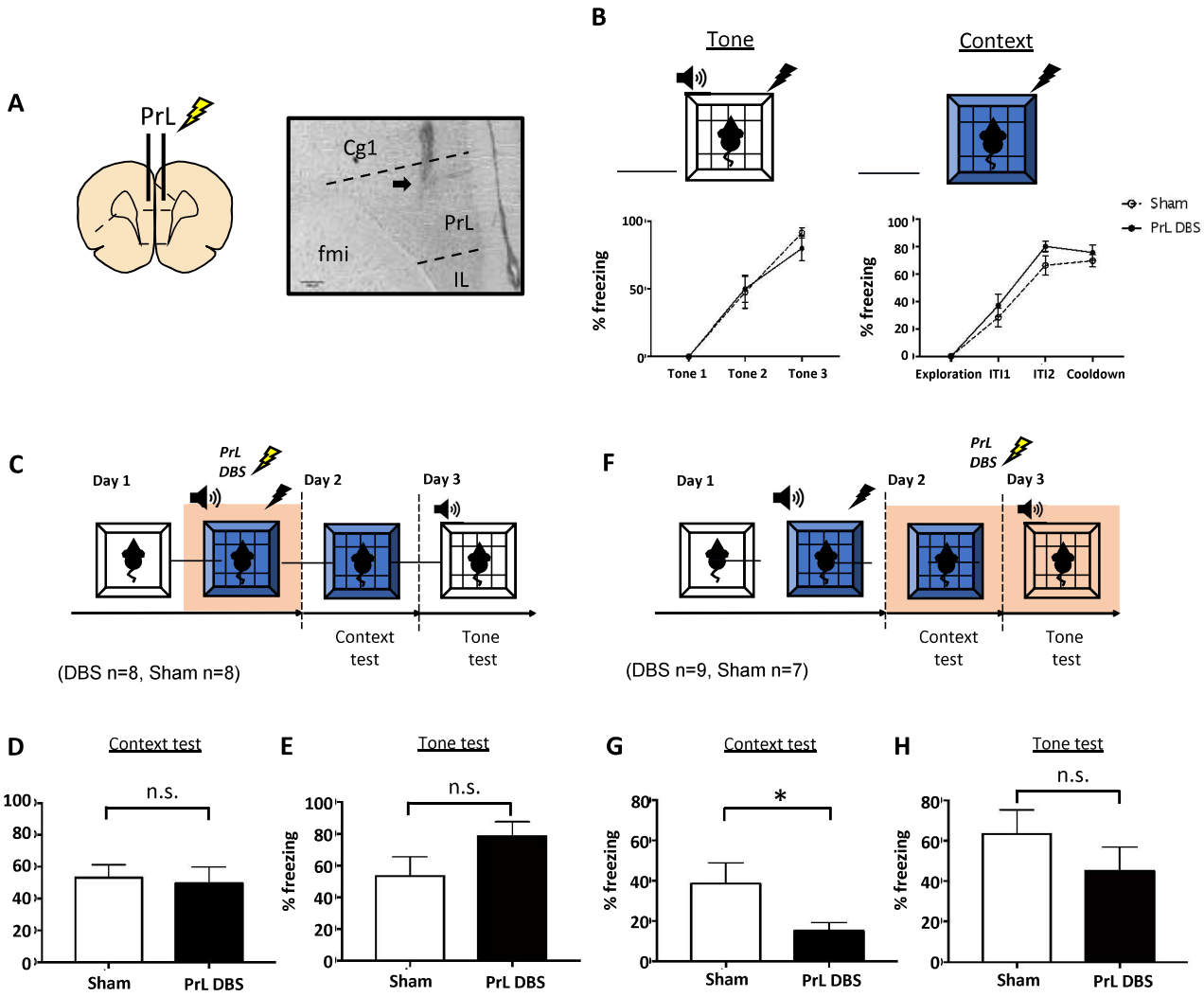

A
PrL
Cg1
PrL
fmi
IL
B
Tone
Context
Sham
PrL DBS
% freezing
Tone 1
Tone 2
Tone 3
Exploration
ITI1
ITI2
Cooldown
C
PrL DBS
Day 1
Day 2
Day 3
Context test
Tone test
(DBS n=8, Sham n=8)
F
PrL DBS
Day 1
Day 2
Day 3
Context test
Tone test
(DBS n=9, Sham n=7)
D
Context test
n.s.
% freezing
Sham
PrL DBS
E
Tone test
n.s.
% freezing
Sham
PrL DBS
G
Context test
*
% freezing
Sham
PrL DBS
H
Tone test
n.s.
% freezing
Sham
PrL DBS

Fig 2

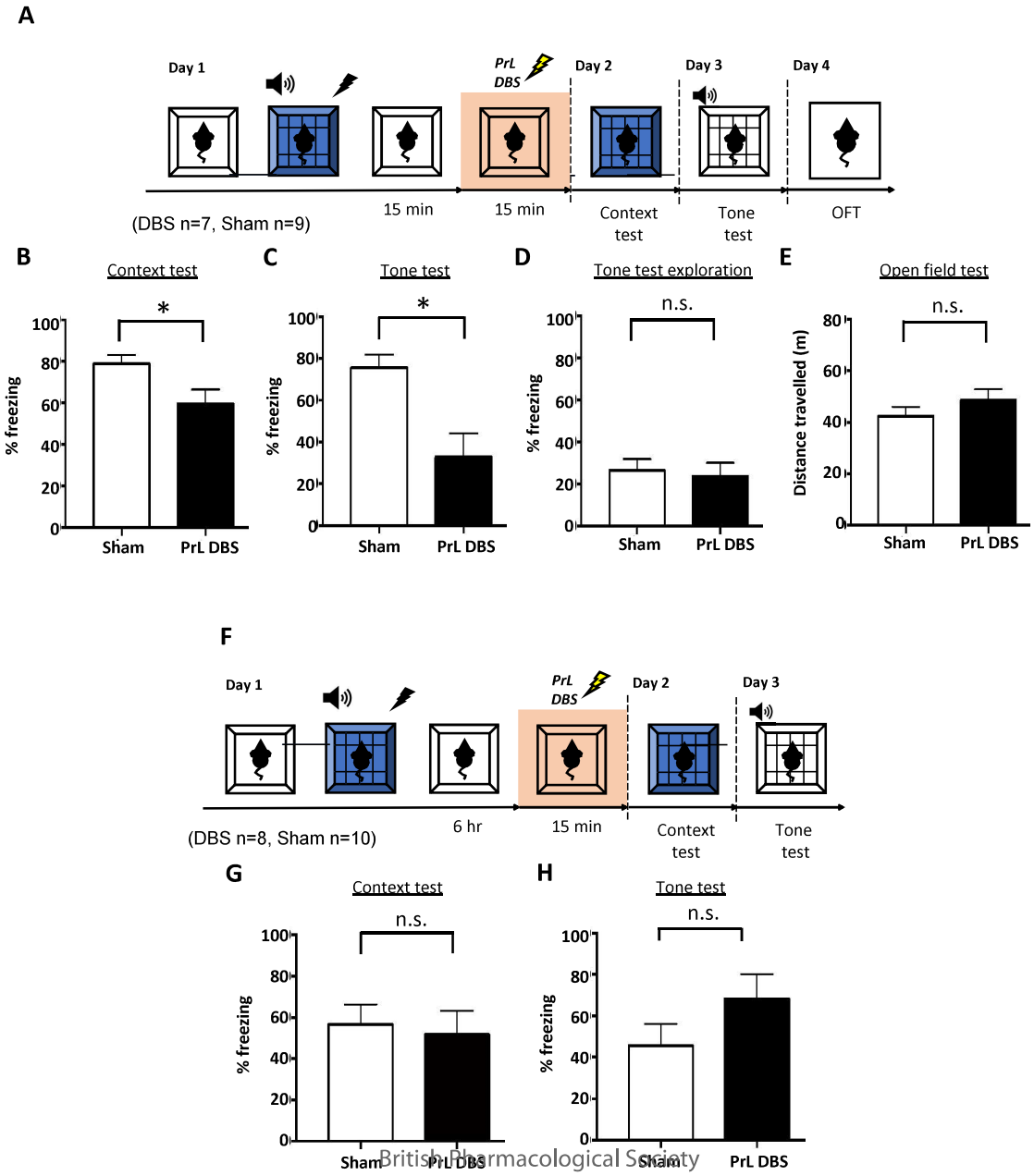


Fig 3

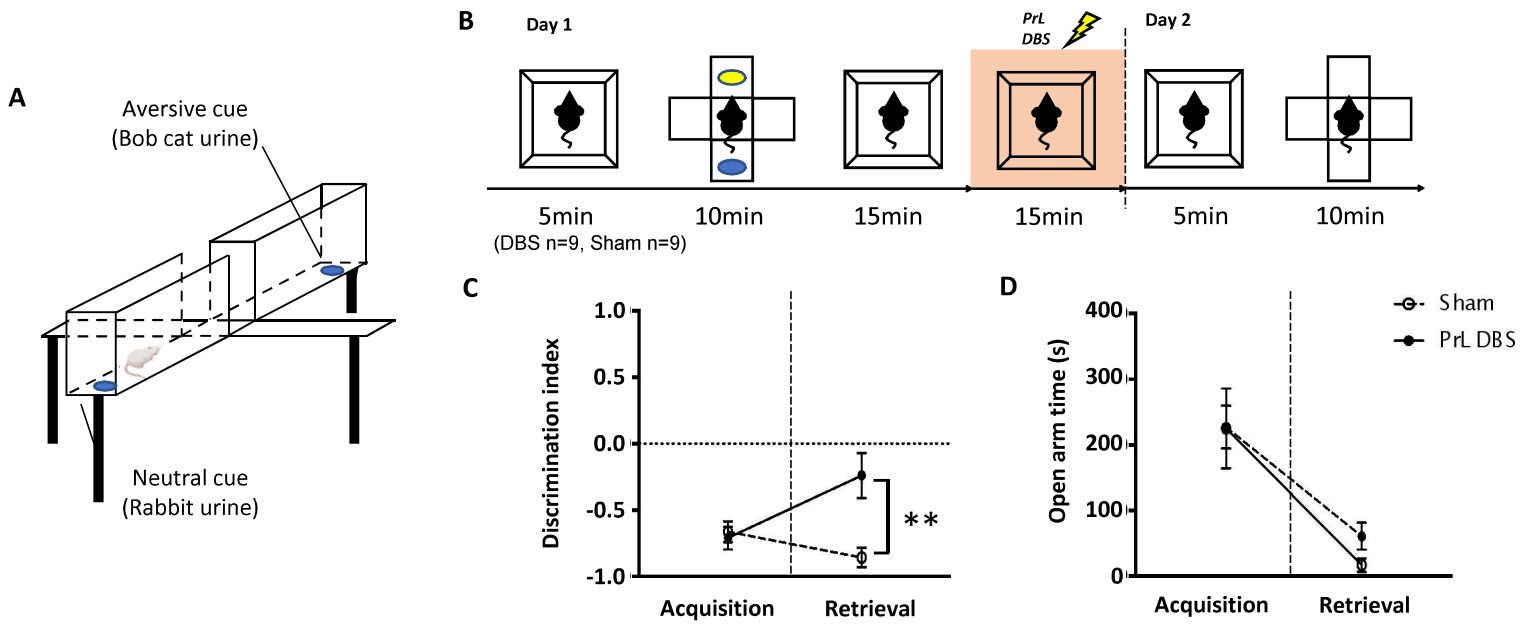

A
Aversive cue
(Bob cat urine)
Neutral cue
(Rabbit urine)
B
Day 1
PrL
DBS
Day 2
5min
10min
15min
15min
5min
10min
(DBS n=9, Sham n=9)
C
Discrimination index
1.0
0.5
0.0
-0.5
-1.0
**
Acquisition
Retrieval
D
Open arm time (s)
400
300
200
100
0
Sham
PrL DBS
Acquisition
Retrieval

Fig 4

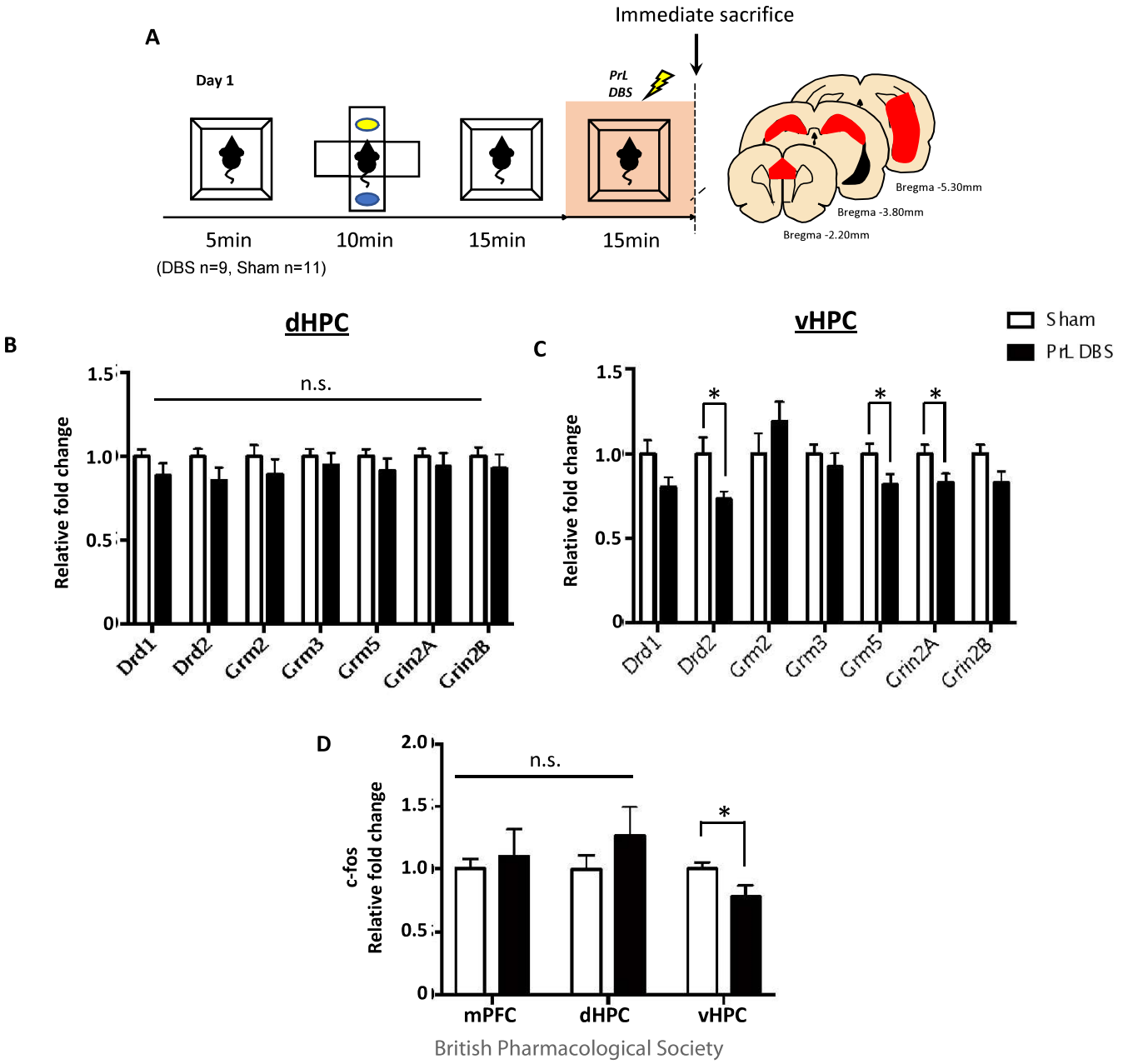

A
Immediate sacrifice
Day 1
PrL DBS
5min
10min
15min
15min
(DBS n=9, Sham n=11)
Bregma -5.30mm
Bregma -3.80mm
Bregma -2.20mm
B
dHPC
n.s.
Relative fold change
1.5
1.0
0.5
0
Drd1
Drd2
Grm2
Grm3
Grm5
Grin2A
Grin2B
C
vHPC
Sham
PrL DBS
*
D
c-fos
Relative fold change
2.0
mPFC
dHPC
vHPC

Fig 5

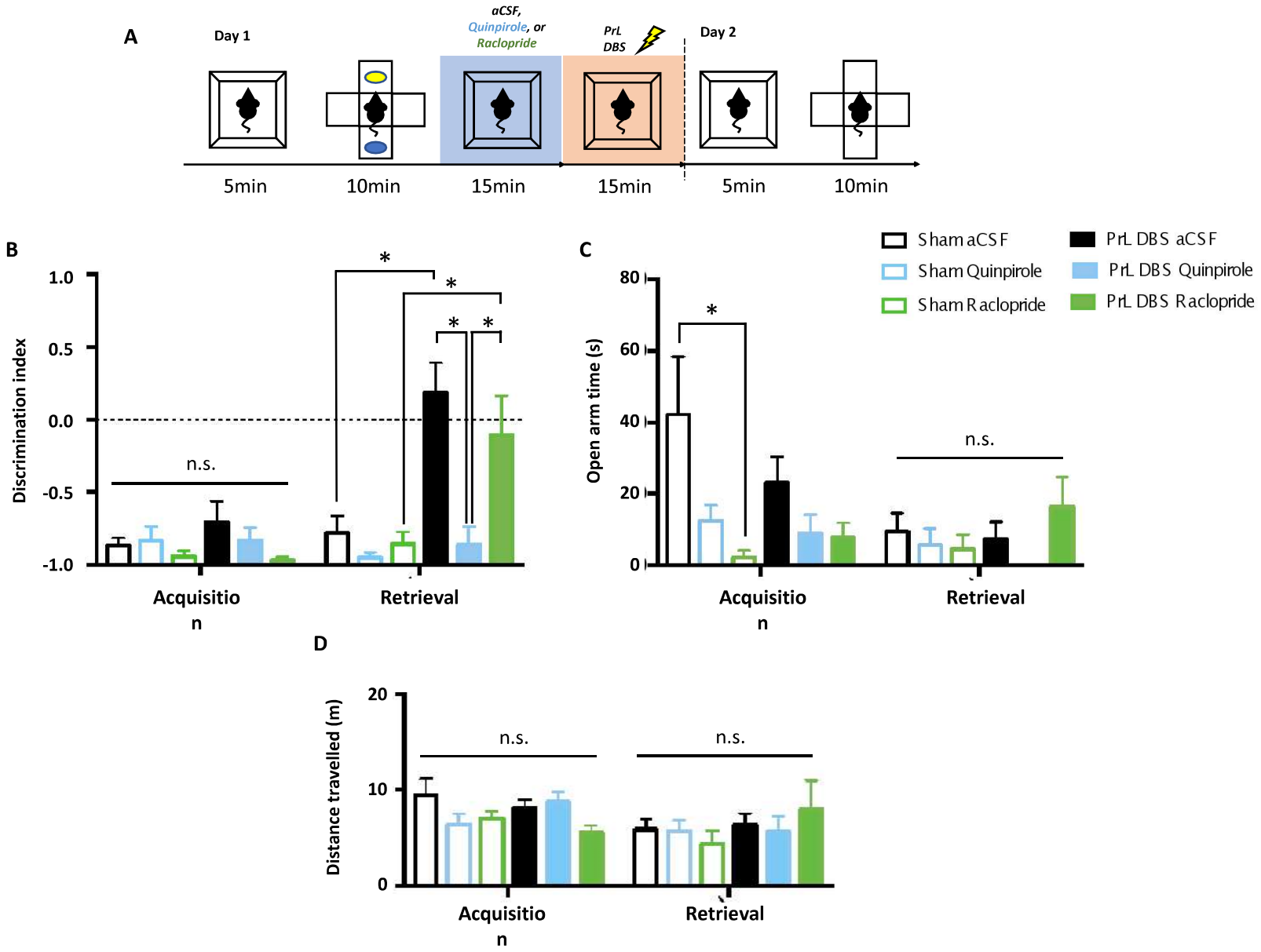
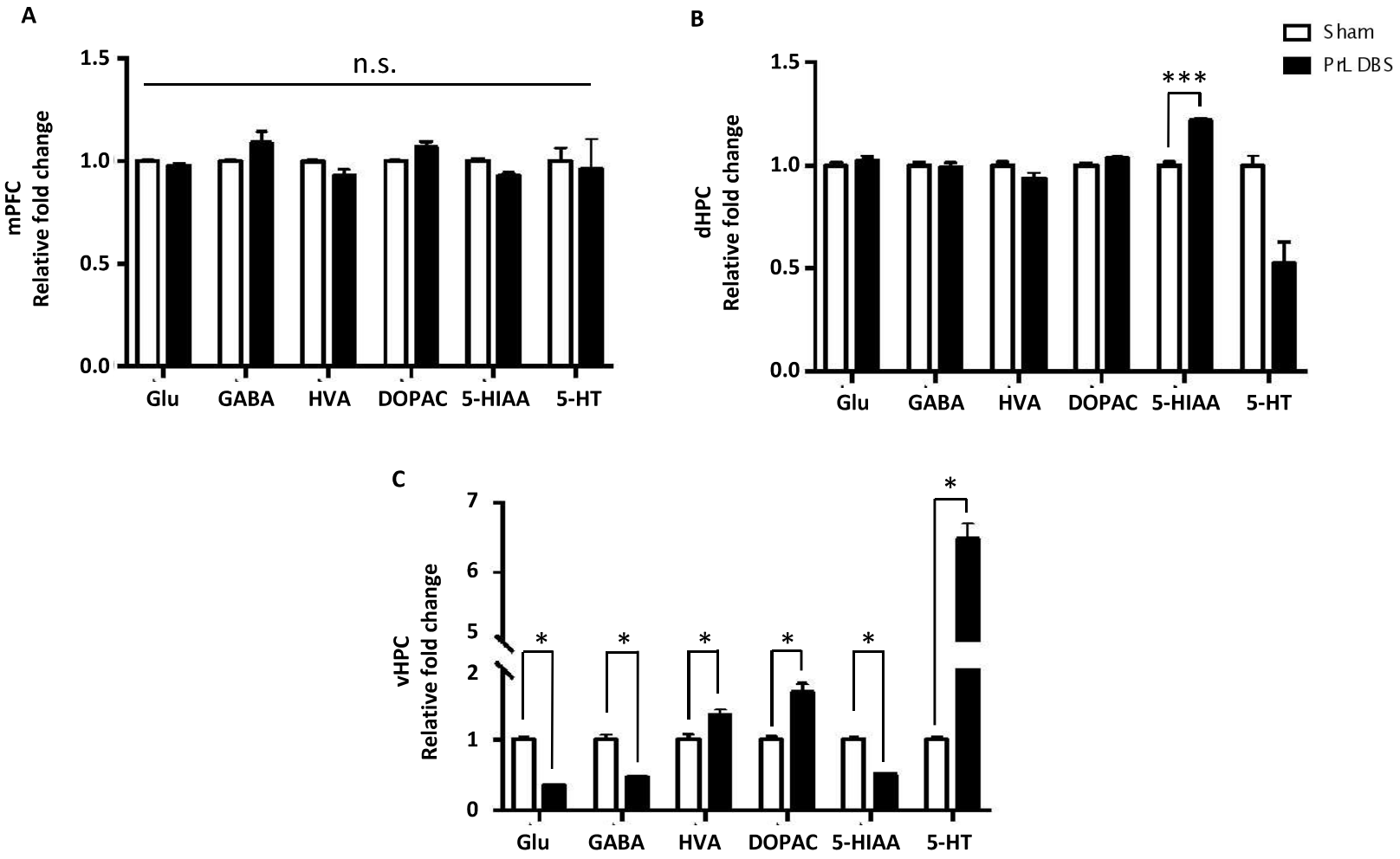

A
Day 1
aCSF,
Quinpirole, or
Raclopride
PrL
DBS
Day 2
5min
10min
15min
15min
5min
10min
B
Discrimination index
1.0
0.5
0.0
-0.5
-1.0
n.s.
*
*
*
*
Acquisitio
n
Retrieval
C
Sham aCSF
Sham Quinpirole
Sham Raclopride
PrL DBS aCSF
PrL DBS Quinpirole
PrL DBS Raclopride
Open arm time (s)
80
60
40
20
0
*
n.s.
Acquisitio
n
Retrieval
D
Distance travelled (m)
20
10
0
n.s.
n.s.
Acquisitio
n
Retrieval

Fig 6


A
n.s.
mPFC
Relative fold change
1.5
1.0
0.5
0.0
Glu
GABA
HVA
DOPAC
5-HIAA
5-HT
B
Sham
PrL DBS
***
dHPC
Relative fold change
C
vHPC
Relative fold change
7
6
5
2
1
0
*

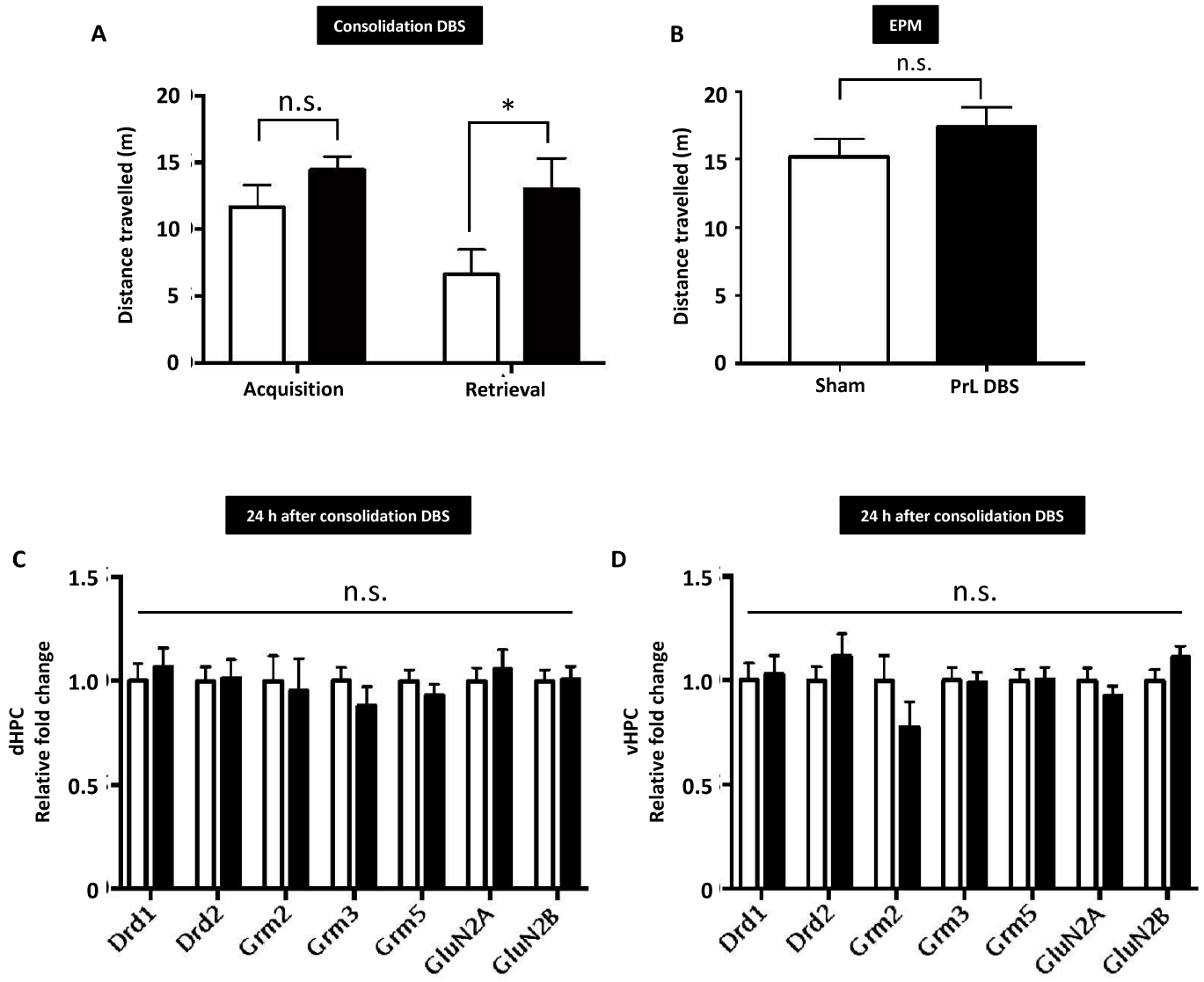
Supp Table 1

Target Gene	Sense	Anti-Sense	References
Drd1	5'-CCTTCGATGTGTTTGTGTGG-3'	5'-GGGCAGAGTCTGTAGCATCC-3'	(Dick et., 2015)(67)
Drd2	5'-TTCTGTCCTTCACCATCTCC-3'	5'-GACCAGCAGAGTGACGATGA-3'	(Dick et al., 2015)(67)
Grm2	5'-AGTCCTTAGCTGGGGAGCCT-3'	5'-AACCATCCTCTCTATCCCAGAGTAAC-3'	(Ermolinsky et al., 2008)(68)
Grm3	5'-TAGGCTGTTAGACAAAGTGCTCA-3'	5'-GAAGGGGCTGTTAATTAGGGCA-3'	(Ermolinsky et al., 2008)(68)
Grm5	5'-ACCAAGACCAACCGTATTGC-3'	5'-AGACTTCTCGGATGCTTGGA-3'	(Tan et al., 2015)(69)
Grin2a	5'-GCACCAGTACATGACCAGATTC-3'	5'-ACCAGTTTACAGCCTTCATCC-3'	(Calabrese et al., 2012)(70)
Grin2b	5'-TTCATGGGTGTCTGTTCTGG-3'	5'-GGATGTTGGAGTGGGTGTTG-3'	(Calabrese et al., 2012)(70)
c-Fos	5'-CCGACTCCTTCTCCAGCAT-3'	5'-TCACCGTGGGGATAAAGTTG-3'	(Rogers et al., 2004)(71)
HPRT	5'-CTCATCGGACTGATTATGGACAGGAC-3'	5'-GCAGGTCAGCAAAGAACTTATAGCC-3'	(Covacu et al., 2009)(72)

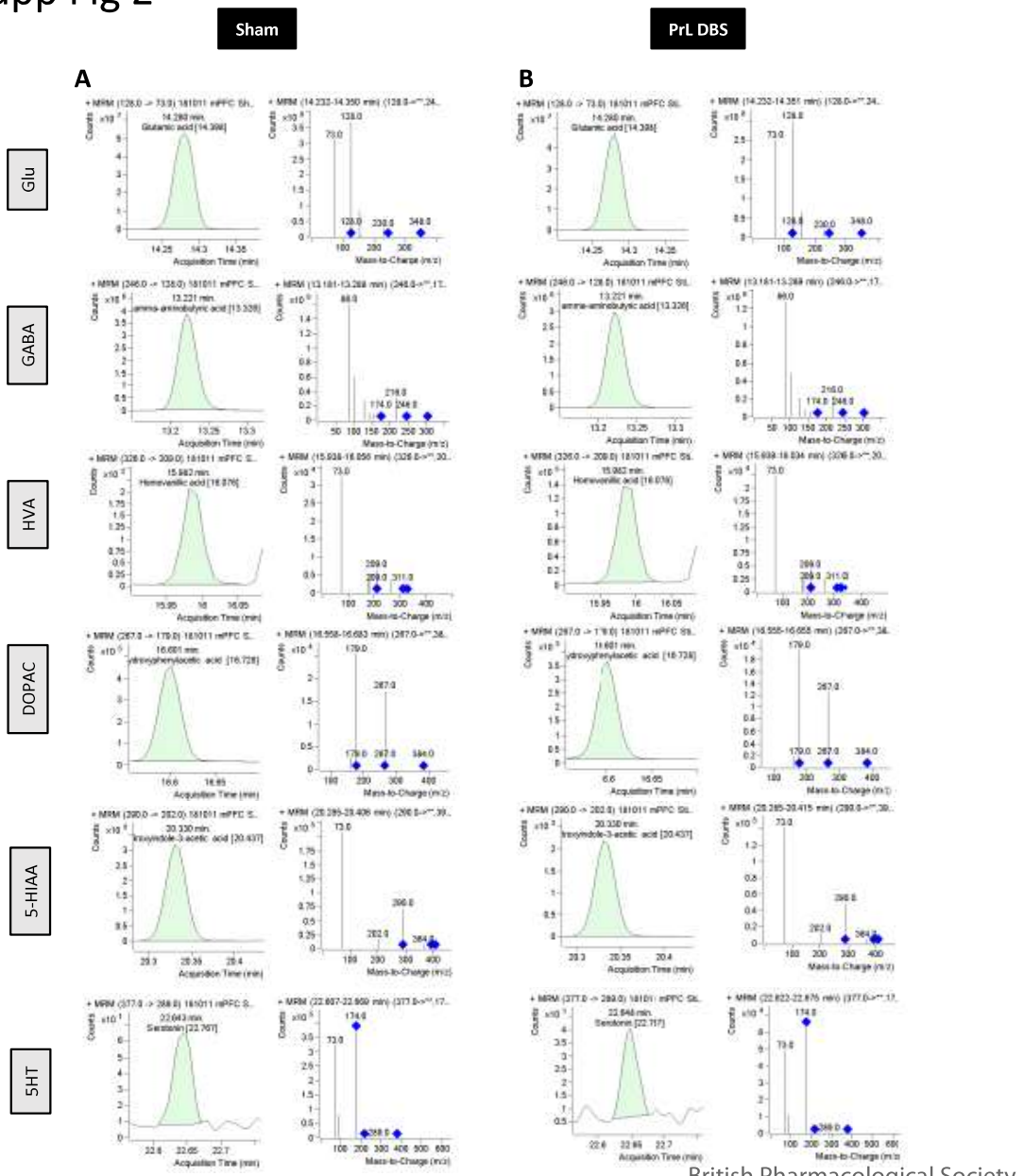
Supp Table 2

Figure		t/f score	p-value
1B (Context)	Interaction	f(2,28) = 0.82	0.45
	Time	f(2,28) = 57.05	<0.01
	Stimulation	f(1,14) = 0.76	0.40
1B (Tone)	Interaction	f(3,42) = 0.68	0.44
	Time	f(3,42) = 99.52	<0.01
	Stimulation	f(1,14) = 0.93	0.35
1D		t(14) = 0.22	0.79
1E		t(14) = 1.66	0.12
1G		t(14) = 2.43	0.04
1H		t(14) = 1.06	0.31
2B		t(14) = 2.43	0.03
2C		t(12) = 2.77	0.02
2D		t(14) = 0.22	0.83
2E		t(14) = 1.42	0.18
2G		t(16) = 0.32	0.75
2H		t(16) = 1.42	0.17
3C	Acquisition	Mann-Whitney	0.61
	Retrieval	Mann-Whitney	0.003
3D	Acquisition	t(16) = 0.03	0.98
	Retrieval	Mann-Whitney	0.06
5B (Acquisition)	Interaction	f(2,61) = 0.02	0.98
	Drug	f(2,61) = 1.85	0.17
	Stimulation	f(1,61) = 0.09	0.76
5B (retrieval)	Interaction	f(2,58) = 3.95	0.03
	Drug	f(2,58) = 7.84	<0.01
	Stimulation	f(1,58) = 21.6	<0.01
5C (Acquisition)	Interaction	f(2,61) = 1.23	0.03
	Drug	f(2,61) = 5.07	0.01
	Stimulation	f(1,61) = 1.26	0.27
5C (Retrieval)	Interaction	f(2,58) = 1.52	0.23
	Drug	f(2,58) = 1.11	0.34
	Stimulation	f(1,58) = 0.08	0.78
5D (Acquisition)	Interaction	f(2,61) = 1.20	0.31
	Drug	f(2,61) = 2.50	0.90
	Stimulation	f(1,61) = 0.57	0.45
5G (Retrieval)	Interaction	f(2,58) = 0.10	0.90
	Drug	f(2,58) = 0.32	0.72
	Stimulation	f(1,58) = 0.02	0.88

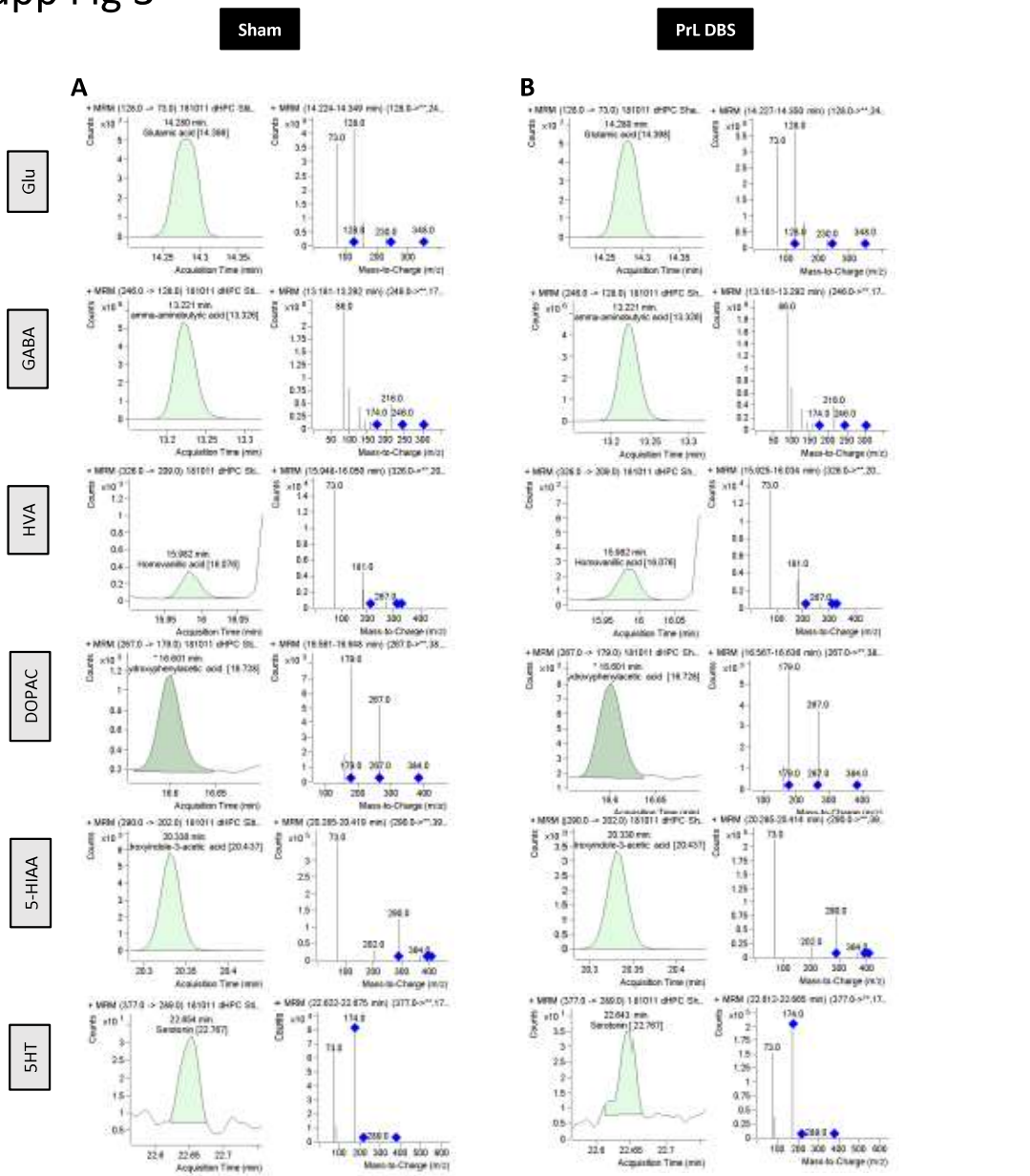
Supp Figure 1



Supp Fig 2



Supp Fig 3



Supp Fig 4