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Serotonergic treatment normalizes midbrain dopaminergic neuron increase after periaqueductal gray stimulation

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ABSTRACT

Electrical stimulation of the dorsolateral periaqueductal gray (dlPAG) in rats has been shown to elicit panic-like behaviour and can be a useful as an unconditioned stimulus for modelling anticipatory fear and agoraphobia in a contextual fear conditioning paradigm. In this study, we further analysed our previous data on the effects of escitalopram (a selective serotonin reuptake inhibitor, SSRI) and buspirone (a 5-HT1A receptor partial agonist) on dlPAG-induced anticipatory fear behaviour in a rat model using freezing as a measure. We then attempted to unravel some of the interactions with dopamine signalling using tyrosine hydroxylase (TH) immunohistochemistry to probe the effects on dopaminergic neurons. We showed that acute treatment of escitalopram, but not buspirone, was effective in reducing anticipatory freezing behaviour, while chronic administrations of both drugs were effective. We found that the dlPAG stimulation induced increase number of dopaminergic neurons in the ventral tegmental area (VTA) which was reversed in both chronic buspirone and escitalopram groups. We further found a strong positive correlation between the number of dopaminergic neurons and freezing in the VTA and showed positive correlations between dopaminergic neurons in the VTA and substantia nigra pars compacta (SNpc) in escitalopram and buspirone groups, respectively. Overall, we showed that chronic treatment with an SSRI and a 5-HT1A agonist reduced anticipatory freezing behaviour which seems to be associated, through correlative studies, with a reversal of dlPAG stimulation induced increase in number of dopaminergic neurons in the VTA and/or SNpc.

KEYWORDS: Deep brain stimulation; periaqueductal gray; fear-like behaviour; dopamine; serotonergic system

INTRODUCTION

The dorsolateral periaqueductal gray (dlPAG) plays a major role fear; it is thought to suppress the facilitatory effects of amygdala activation (Walker and Davis 1997), thereby inhibiting fear expression. Indeed, lesions of the dlPAG has been shown to enhance both unconditional freezing and cue conditioned freezing, and has been suggested to inhibit the ventral PAG and forebrain structures involved with defence (Oca et al. 1998). Electrical stimulation (which is thought to generate a temporary neural activity lesion (McIntyre and Anderson 2016)) of the dlPAG in rats has been shown to elicit panic-like behaviours such as vigorous running and jumping in an open-field arena (Lim et al. 2008, 2009). Memory of panic-like behaviour leads to contextual conditioning, where animals associate the context (e.g., the open-field) to fear, which models anticipatory anxiety and agoraphobia (Lim et al. 2008, 2010). Periaqueductal gray (PAG) serotonin transmission has been shown to play a crucial role in regulating the panic response; studies have shown that selective serotonin reuptake inhibitors (SSRIs) can attenuate or increase the threshold for escape behaviours in animals (Schenberg et al. 2002; Zanoveli et al. 2005; Hogg et al. 2006). In this study, we further analysed our previously published data (Lim et al. 2010), which studied the effects of escitalopram (an SSRI) and buspirone (a 5-HT1A receptor partial agonist) on dorsolateral PAG (dlPAG)-induced anticipatory anxiety). We used freezing, which is a species-specific defence response defined as the absence of all movement except that required for respiration (Blanchard and Blanchard 1969), as a measure of fear. It is known that both SSRIs and 5-HT1A receptor agonists exert effects on dopaminergic neurons, reducing TH cells and firing rate (Dremencov et al. 2009; Jahanshahi et al. 2010). Further, dopaminergic transmission has been shown to be important for processes and modulation of fear and memory (Abraham et al. 2014; Pignatelli and Bonci 2015; Luo et al. 2018; Tan et al. 2019b, a), and have been suggested to work as an opponent to serotonergic systems in conditioning tasks (Daw et al. 2002). Given that the central serotonergic systems is thought to regulate dopamine functions (Esposito et al. 2008), with this interaction playing a major role in learning and memory (Feria-velasco and Gonza 2008), we decided to study the effects of escitalopram (a selective serotonin reuptake inhibitor, SSRI) and buspirone (a 5-

HT1A receptor partial agonist) on dopaminergic neurons and their correlation to anticipatory fear. We further extended our previous data by examining dopaminergic neurons by tyrosine hydroxylase (TH)-immunohistochemistry of the ventral tegmental area (VTA), substantia nigra pars compacta (SNpc), dorsal raphe nucleus (DRN), and median raphe nucleus (MnR). Overall, we attempt to dissect dopaminergic mechanisms in which escitalopram and buspirone modulates defensive behaviour induced by dPAG stimulation.

MATERIALS AND METHODS

Animals

Animals used were previously reported (Lim et al. 2010), all data was based on new behavioural analysis on previously reported animals and molecular analysis of their tissue. Adult male Wistar rats (n=40, 12 weeks old) were individually housed in standard cages with food and water available *ad libitum*. The environmental conditions were maintained at a temperature of 21±1°C and 60%-65% humidity in a reverse 12 h/12 h light/dark cycle. This study was approved by the Animal Experiments and Ethics Committee of Maastricht University, as well as the Committee on the Use of Live Animals in Teaching and Research (CULATR) of The University of Hong Kong.

Experimental groups

Rats were randomly divided into six experimental groups: three groups receiving dlPAG deep brain stimulation (DBS) and three groups receiving sham stimulation and treated with either escitalopram (DBS-ESCIT, n=7; SHAM-ESCIT, n=7), buspirone (DBS-BUSP, n=7; SHAM-BUSP, n=6), or saline (DBS-SAL, n=7; SHAM-SAL, n=6), respectively. A schematic representation of the timeline of the dlPAG DBS and drug treatments is shown in Figure 1 (A).

Surgical procedure

The surgical procedures were performed as previously described (Temel et al. 2007; Lim et al. 2010). Rats were anaesthetized during the entire procedure using a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg) injected subcutaneously. Rats were placed in a stereotaxic frame (Stoeling, Wood Dale, USA) and electrodes were implanted at the level of the dlPAG (from Bregma: anterposterior, -7.6 mm; mediolateral, +0.7 mm; ventral, -4.8 mm; coronal approach angle of 10º)(Paxinos and Watson 2006) and secured to the skull using dental cement. The electrodes consisted of gold-plated needle combined with an inner wire of a platinum-iridium (Technomed, Beek, the Netherlands; IDEE Instruments, Maastricht University) with a tip and shaft diameter of 50 μm and 250 μm, respectively. All rats received a subcutaneous injection of Temgesic (0.1 mg/kg) for pain relief directly after the surgery and were allowed to recover for 2 weeks.

DBS procedures

The DBS procedures for inducing fear-like behaviour in rats were reported in our previous studies (Lim et al. 2009, 2010). To determine the level of the escape threshold, the dlPAG DBS groups had a preliminary session in their home cages. The stimulation amplitude was gradually increased until escape behaviour was observed. At each step, the stimulation period was 15 s followed by a stimulation-off period of 45 s. The stimulation frequency was set at 50 Hz and pulse width at 0.1 ms based on our previous studies (Lim et al. 2009, 2010). The effects of drugs on escape threshold was also done, and have been previously reported (Lim et al. 2010). The stimulation was delivered using a World Precision Instruments (WPI) digital stimulator (DS8000, WPI, Berlin, Germany) and a stimulus isolator (DLS100, WPI, Berlin, Germany). Real-time verification of the DBS stimulation parameters was monitored using a digital oscilloscope (Agilent 54622D oscilloscope, Agilent Technologies, Amstelveen). Animals requiring stimulation intensities above 100 μA were excluded from the analysis. After the threshold determination session, all rats were allowed a recovery period of 2 weeks. Sham animals were similarly connected to the stimulator, but no current was delivered.

Drug administration

The dosage and administration of BUSP and ESCIT in animals have been previously reported and shown to be effective in our previous study (Lim et al. 2010). A week prior to the actual experimental tests, all animals received 1 mL saline injections three times on alternating days to habituate them to the injection procedure. Animals were administered a single acute dose of drug before the first open-field behavioural test. Escitalopram oxalate (ESCIT; H. Lundbeck A/S, Copenhagen, Denmark) and Buspirone hydrochloride (BUSP; TOCRIS, Cookson Inc., Missouri, USA) were dissolved in saline (SAL; 0.9% NaCl). The animals received a subcutaneous injection (in the volume of 1 mL/kg) of ESCIT (10 mg/kg) 60 min before or BUSP (3 mg/kg) 120 min before the first open-field behavioural test, based on previous effective pharmacological profiles (Tunnicliff et al. 1992; Sánchez et al. 2003; Kakui et al. 2009; Lugenbiel et al. 2010). For testing the chronic effects of these drugs, animals received daily injections of ESCIT (10 mg/kg), BUSP (3 mg/kg) or SAL for 21 days. A final dose of SAL, ESCIT and BUSP was administered (at 60 min and 120 min, respectively) before the second open-field behavioural testing to investigate the chronic treatment effects.

Behavioural evaluation

Freezing behaviour of rats was evaluated in an open-field (square: 100 cm x 100 cm; height: 40 cm) made of clear plexiglass with an open top and a dark floor (Lim et al., 2010). 12h after dlPAG stimulation, rats were placed in the open-field arena for 10 min and their behaviour was recorded using a camera. Freezing is defined as the absence of all movement except that required for respiration (Blanchard and Blanchard 1969). The freezing response was manually scored by researchers who were blinded to the experimental design and animal groups.

Histological processing

At 2 h after the final behavioural test, rats were anaesthetized with Nembutal (75 mg/kg) and then perfused transcardially with Tyrode (0.1 M) and a fixative solution containing

paraformaldehyde, picric acid and glutaraldehyde in phosphate buffer (pH 7.6). Rats were post-fixed for 2h and incubated overnight in sucrose solution, and subsequently frozen rapidly with CO_2 and stored at -80 ºC. Brain tissues were sequentially cut (10 series) on a cryostat into 30 µm thick sections in the coronal plane and stored at -80 ºC. One series of the brain sections were processed for TH immunohistochemistry. Briefly, brain sections were incubated overnight with mouse anti-TH antibody (1:100, kindly provided by Dr C. Cuello, Canada) diluted in 0.1% bovine serum albumin (BSA) and Tris-Buffered Solution (TBS)-Triton (TBST) solution, followed by biotinylated donkey anti-mouse IgG (diluted 1:400, Jackson Immunoresearch Laboratories Inc, Westgove, USA). Sections were then incubated with avidin-biotin-peroxidase complex (diluted 1:800, Vectastain Elite ABC kit, Burlingame, USA) for 2 h. Between steps, sections were washed with TBS and TBST. Tissue sections were incubated with 3, 3'- diaminobenzidine tetrahydrochloride/nickel chloride solution to visualise the horseradish peroxidase (HRP) immune complex. The reaction was stopped after 10 min by washing the sections thoroughly with TBS. Subsequently, all sections were mounted on the Superfrost micro-slides (VWM, Illinois, USA) and cover-slipped with Permount mounting medium (Thermo Fisher Scientific, Waltham, USA). Another series of brain sections were stained with haematoxylin-eosin (Merck, Darmstadt, Germany) to evaluate the localisation of the electrode implantation sites (Fig 1B).

Evaluation of the TH-immunoreactive cells

Cell counts of TH-immunoreactive (TH-ir) cells were performed in the VTA, SNpc, DRN and MnR brain sections from the DBS and SHAM treatment groups. The procedure of cell counting was performed according to our previously established method (Liu et al. 2015). In brief, systematic quantification of the TH positive cells (3-4 sections per animal) was performed using an Axiophat 2 imaging microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany). The boundaries of the areas of interest (VTA, SNpc, DRN and MnR) were delineated according to the Paxinos and Watson rat brain atlas (Paxinos and Watson 2006). The TH positive cells were clearly defined within the areas of interest and quantified by an investigator who was blinded to the treatment

groups. Photomicrographs of TH-ir cells within the areas of interest were captured using an Olympus DP73 digital camera (Olympus, Hamburg, Germany) attached to a bright-field microscope. Brightness and contrast of the photomicrographs were adjusted in Adobe Photoshop (Adobe Systems, San Jose, USA).

Statistical Analysis

Statistical analysis was conducted in R (version 3.5.2) and visualizations were performed using the "ggplot2" package (Wickham 2016). As linearity of data cannot be assumed, outliers (n=2; 1 DBS-SAL, 1 DBS-BUSP in acute behaviour data) in the behaviour data were removed using the ROUT method (Motulsky and Brown 2006). As outliers were only found in acute and not chronic behavioural data in behavioural studies, no outliers were excluded from cell count and correlation studies. However, statistics of acute behaviour with outliers can be found in Supplementary Figure 2.

RESULTS

Acute administration of escitalopram, but not buspirone, reduced anticipatory freezing behaviour.

To test the efficacy of acute BUSP and ESCIT in reducing panic-like freezing behaviour, animals received electrical stimulation or sham stimulation of the dlPAG and were then tested in the open field. At 12 h post-stimulation, animals received either saline, BUSP, or ESCIT and were returned to the open field 120 min and 60 min later, respectively (Fig 1A). Two-way ANOVA revealed an effect of stimulation, drugs, and their interaction (lowest $F = 8.9$, all $ps < 0.05$) on the measured freezing behaviour. Tukey's post-hoc test revealed a significant difference between DBS-SAL and Sham-SAL groups ($p < 0.05$), indicating DBS induced increased freezing. Tukey's post-hoc test further revealed a significant difference between DBS-SAL and DBS-ESCIT ($p < 0.05$), but not between DBS-SAL and DBS-BUSP groups ($p = 0.999$), which indicated an acute effect of

escitalopram, but not buspirone, in reducing anticipatory freezing behaviour (Fig 1C). The heatmap and full table of p-values can be found in Supplementary Figure 1.

Chronic administration of buspirone and escitalopram reduced anticipatory freezing behaviour

To test the efficacy of chronic BUSP and ESCIT in reducing panic-like behaviour, animals received chronic administration of saline, BUSP, or ESCIT for 3 weeks. Animals then received electrical stimulation or sham stimulation of the dlPAG before testing in an open field. At 12 h after stimulation when animals were returned to the open field, two-way ANOVA revealed an effect of drug and DBS (lowest $F = 7.7$, all $ps < 0.05$) and a trending interaction effect ($F_{(2,32)}=3.2$, $p = 0.054$) on freezing behaviour. Tukey's post-hoc revealed a significant difference between DBS-SAL and Sham-SAL groups ($p < 0.05$), indicating DBS induced increased freezing. Tukey's post-hoc test further revealed a significant difference between DBS-SAL and both DBS-BUSP and DBS-ESCIT groups (all $ps < 0.05$), but no significant difference between DBS-BUSP and DBS-ESCIT groups ($p = 0.98$), indicating similar effects of chronic BUSP and ESCIT in reducing anticipatory freezing behaviour (Fig 1D). The heatmap and full table of p-values can be found in Supplementary Figure 1.

Chronic buspirone and escitalopram treatments and dlPAG DBS caused changes in the number of dopaminergic neurons.

To understand the effects of chronic treatment of BUSP and ESCIT and dlPAG DBS on dopaminergic neurons, TH immunohistochemistry and neuronal cell counting were performed on VTA, SNpc, DRN, and MnR brain sections (Fig 2A). Two-way ANOVA revealed an effect of dlPAG DBS on all structures (lowest $F = 4.7$, all $ps < 0.05$). There was an effect of drug in VTA (Fig 2B), DRN (Fig 2D), and MnR (Fig 2E) (lowest $F = 4.9$, all $ps < 0.05$), but not in SNpc (Fig 2C). There were no interaction effects, although it should be noted that VTA showed a trending interaction effect ($F_{(2,21)}= 3.4$, $p = 0.052$). Tukey's post-hoc test conducted on all structures revealed a significant difference between DBS-SAL and Sham-SAL groups in VTA ($p < 0.005$), but not in

SNpc, DRN, or MnR (all ps > 0.05), which suggests dlPAG DBS induced increased numbers of TH neurons in the VTA. There were a lower number of TH neurons in DBS-BUSP and DBS-ESCIT groups compared to the DBS-SAL group (all ps < 0.01), but no significant difference compared to their sham counterparts, which suggests chronic administration of both BUSP and ESCIT reversed the effects of dlPAG on TH neurons. DRN showed significantly lowered TH neuron counts between DBS-SAL and DBS-ESCIT groups ($p = 0.02$), and MnR showed a significantly lowered TH neuron count between Sham-SAL and Sham-ESCIT ($p = 0.04$) groups. The heatmap and full table of p-values can be found in Supplementary Figure 1.

TH neuron count correlated to anticipatory freezing behaviour

To understand the relationship between dopaminergic neurons, and anticipatory freezing behaviour, freezing and TH cell count in VTA, SNpc, DRN, and MnR of both dlPAG stimulated and sham stimulated were analysed together by Spearman's rank correlation (Fig 3A,C,E,G, respectively). Freezing and TH neuron counts were shown to be highly positively correlated in VTA ($R = 0.727$, $p < 0.001$) and moderately positively correlated in SNpc, DRN, and MnR (SNpc: $R = 0.513$, $p = 0.008$; DRN: $R = 0.464$, $p = 0.005$; MnR: $R = 0.387$, $p = 0.022$). Overall, dopaminergic neuron (in particular in the VTA) were positively correlated to anticipatory freezing behaviour.

Escitalopram treatment correlates to TH neuron count in VTA, whereas buspirone treatment correlates to TH neuron count in SNpc

To understand the relationship between chronic administration of BUSP or ESCIT and dopaminergic neurons, individual drug groups and TH cell counts in VTA, SNpc, DRN, and MnR were analysed by Spearman's rank correlation (Fig 3B, D, F, & H, respectively). ESCIT showed significant positive correlation with TH neuron count in the VTA ($R = 0.68$, $p = 0.06$), whereas BUSP showed significant positive correlation with TH neuron count in SNpc ($R = 0.95$, $p < 0.001$). No significance was seen in DRN or MnR for either drug. All R and p values are shown in the respective graphs (Fig 3B, D, F, & H).

DISCUSSION

In this study, we extended our previous findings to show that acute treatment of ESCIT, but not BUSP, was effective in reducing anticipatory freezing behaviour (mimicking agoraphobia). However, both drugs were comparably effective in reducing freezing behaviour after chronic administration. It should be noted that given both groups were returned to the same environment the second stimulation session to assess chronic effects. While this might mean that groups were not equivalent at the start of the second chronic test (ESCIT but not BUSP being effective in acute), both drugs still lowered freezing when administer chronically, and the comparison in efficacy in lowering freezing between drugs is unimportant to the main conclusion of the behavioural aspect of this paper. We further demonstrated that chronic BUSP or ESCIT reversed the dlPAG stimulation induced increase in number of dopaminergic neurons in the VTA, whereas only chronic ESCIT decreased dopaminergic neurons in the DRN. Lastly, we showed that there was a strong positive correlation between the number of dopaminergic neurons and freezing in the VTA, and positive correlations between dopaminergic neurons with ESCIT and BUSP treatments in the VTA and SNpc, respectively. This suggests that both drugs, while both reduce the number of dopaminergic neurons, exert their effects via different dopaminergic-related neuronal circuits.

The use of dlPAG stimulation is advantageous in that it directly induces the activation of the panic-circuit independent of the behavioural context and is highly reproducible (Jenck et al. 1995; Hogg et al. 2006; Lim et al. 2011). This feature is useful as an unconditioned stimulus in a contextual fear conditioning paradigm that effectively models agoraphobia. The use of freezing to measure fear response (rather than distance moved or corner time) is more relevant in cases where there is a lack of shelter or no close predators (Eilam 2005). Our behavioural data agree with previous studies showing anxiolytic drugs (including serotoninergic drugs) were effective in reducing panic-like responses in dlPAG stimulation models (Jenck et al. 1990, 1995, 1998; Hogg et al. 2006). Whether this effect is due to general anxiolytic effects, or effects on contextual fear memory, or a

combination of both, however, requires more work. Regardless, this study extended our previous findings (Lim et al. 2010) and showed that ESCIT and BUSP reduced freezing behaviour.

The mesolimbic dopamine pathway plays an important role in fear learning and memory, including encoding and consolidation (Pezze and Feldon 2004; Pignatelli and Bonci 2015). Therefore, it is not surprising that freezing activity was correlated to dopaminergic structures (namely VTA, SNpc, DRN, and MnR) in our model. Furthermore, both BUSP and ESCIT have been shown to affect dopaminergic transmission. BUSP was shown to antagonizes presynaptic inhibitory DA2 autoreceptors, increase circulating dopamine (Lechin et al. 1998), and occupy dopamine receptors (Ciano et al. 2017). Two major projection sites of the mesolimbic pathway are the amygdala and the medial prefrontal cortex (mPFC) (Pezze and Feldon 2004), structures heavily involved in fear conditioning (Herry et al. 2010). Indeed, higher stress and fear conditioning has been associated to higher dopamine transmission in both the mPFC and amygdala (Pezze and Feldon 2004), a potential mechanism of the effects seen in the current study. ESCIT was shown to increase the firing rate of VTA dopamine neurons (Ivanov and Konradsson-geuken 2011), although it should be noted that another study found an opposite effect (Dremencov et al. 2009). How serotonin modulators affect dopaminergic systems is still largely unknown and more studies are needed to fully understand their effects. Interestingly, the individual drug correlations appear to point towards ESCIT exerting its effects through/on VTA dopaminergic neurons, whereas BUSP exerts effects through/on SNpc dopaminergic neurons. This is in contrast to the TH cell count data, which showed both drugs exerted their effects mainly through/on the VTA. This data perhaps highlights the effects of both drugs on conditioned fear – restoration of dopamine synthesis in the VTA of dopamine deficient mice has been shown to reverse fear learning impairment (Fadok et al. 2009). Nevertheless, BUSP has been shown to have an anti-dyskinetic effect on a Parkinson's disease model (Azkona et al. 2014) and the SNpc is suggested to play a crucial role in this effect (Sharifi et al. 2012; Sagarduy et al. 2016), which implies that BUSP directly affects the SNpc, how much this affects anticipatory anxiety and/or learned fear requires further mechanistic work. Of note,

serotonin transporter inhibitors have been shown to reduce the amount of TH neurons in the SN (MacGillivray et al. 2011), and given the positive correlation of SN TH neurons and freezing, could explain in part the effects seen here. We should note that a limitation of this study is the correlative nature of this study, which makes it difficult to determine if the changes in TH neurons are due to BUSP/ESCIT, or if the changes in TH neurons are a consequence of changes in fear and memory caused by BUSP/ESCIT. The fact that a correlation was also present in Saline treated rats on TH count in the VTA seems to suggest that freezing (or fear/memory) is indeed driving the changes in TH neurons instead of drugs, however, a direct effect of drugs on TH count or a combination of both cannot be excluded. Another limitation is the relatively small sample size in terms of biological replicates (although multiple technical replicates for cell counts on each sample was performed, with averages of them being used for analysis), which might influence correlation studies. This has also limited our ability to obtain meaningful data from correlation studies breaking down dlPAG DBS and sham groups – though freezing as a measure would be sufficient given dlPAG DBS function was to induce fear which is measured by freezing. Overall, while the data suggest a complex interplay between VTA and SNpc dopaminergic neurons in the observed effects, more investigation is needed to fully understand this relationship.

Conclusions

In this study, we showed that both chronic administration of BUSP and ESCT were effective in reducing dlPAG stimulation induced anticipatory/contextual fear in our model. Our molecular work suggests that BUSP and ESCT are likely mediated via different structures or pathways in the mesolimbic dopaminergic system. A major limitation has been the lack of mechanistic studies on how the drugs affect dopaminergic systems, as well as the inconsistencies in the literature, making interpretation of the data difficult, especially in a correlative study such as this. More work is needed to fully understand the link between serotonergic drugs and their effects on dopaminergic neurons, and more causative studies are required to establish the effects of these serotonergic drugs on the various mesolimbic dopaminergic system and how they affect fear behaviour.

Ethical Statement

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485

486 **FIGURE LEGENDS**

487

488 **Figure 1. Chronic administrations of buspirone and escitalopram were effective in reducing**
489 **anticipatory fear freezing behaviours.** (A) A schematic representation of the timeline of the
490 experiments. (B) A 3-D reconstruction and lateral view and of the subdivisions of the

periaqueductal gray longitudinal columns. The dark dots represent the localization of electrode implantation sites. (C) & (D) show box-plots of the behavioural data from the acute and chronic treatments with saline, buspirone, and escitalopram in the dlPAG stimulation and sham groups, respectively. * represents $p < 0.05$ between bracketed groups, # represents $p < 0.05$ between corresponding dlPAG stimulation groups.

Figure 2. dlPAG DBS and chronic buspirone and escitalopram treatment caused changes in the number of dopaminergic neurons. (A) Representative low-power photomicrographs of VTA and SNpc sections from the brain of rats in dlPAG stimulation and sham groups treated with saline, buspirone, and escitalopram, respectively. Boxplot of TH cell counts for VTA (B), SNpc (C), DRN (D), and MnR (E) showing significantly lowered dopamine neurons counts with dlPAG DBS and chronic buspirone or escitalopram in VTA, and a significantly lowered dopamine neuron counts with escitalopram in DRN. * represents $p < 0.05$ between bracketed groups, # represents $p < 0.05$ between corresponding dlPAG stimulation groups.

Figure 3. Correlation plots of TH neuron cell count and freezing behaviour. Global correlation plots showed significant positive correlations between TH neuron count and anticipatory fear freezing behaviour in VTA (A), SNpc (C), DRN (E), and MnR (G). In individual drug groups, escitalopram showed a significant correlation in VTA (B), and a significant correlation in SNpc (D). No significant correlation of buspirone or escitalopram was seen in DRN (F) or MnR (H).

Supplementary Figure 1. Heat plot of p-values from Tukey's test. Numbers in the table are p-values from Tukey's post-hoc tests from the ANOVAs. The heatmap shows the levels of significance (red represents more significance and green represents less significance).