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Perinatal fluoxetine prevents the effect of pre-gestational maternal stress on 5-HT in the PFC, but maternal stress has enduring effects on mPFC synaptic structure in offspring.

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ABSTRACT

Maternal affective disorders are frequently treated with selective serotonin reuptake inhibitor medications (SSRIs); with up to 10% of women being prescribed these medications during pregnancy. Infant development depends on the early serotonergic environment, which is altered by perinatal SSRIs, raising concern about how these medications affect neural outcomes. While clinical and preclinical research suggests an impact of SSRIs on the developing brain, more research is needed to determine the effects on neuroplasticity, the serotonergic system, and the hypothalamic-pituitary-adrenal axis in neural regions mediating behavior. The current work investigated the effects of the SSRI, fluoxetine, on the serotonergic system in the prefrontal cortex (PFC) during pre-adolescence, and changes to synaptic markers and glucocorticoid receptor density in the cingulate cortex (medial PFC) of pre-adolescent and adult Sprague-Dawley male and female rats. To model aspects of Perinatal Depression and maternal anxiety, pre-gestational maternal stress was used resulting in male and female offspring from 4 groups: 1) control, 2) perinatal fluoxetine exposed, 3) pre-gestational maternal stress exposed, and 4) pre-gestational maternal stress + fluoxetine. Perinatal fluoxetine prevented the effects of maternal stress on 5-HT levels and 5-HT turnover ratio in the PFC of pre-adolescent offspring, particularly in females. However, pre-gestational stress reduced synaptophysin and PSD-95 densities in the cingulate cortex, effects that were more pronounced in males. Interestingly, perinatal fluoxetine exposure reduced GR density in adult males in this same brain area. Together, results show differential effects of perinatal SSRIs and pre-gestational maternal stress on neurodevelopment in the PFC of males and females.

KEYWORDS: Synaptophysin; PSD-95; glucocorticoid receptor; Sex Differences; neuroplasticity; HPA axis.

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1. INTRODUCTION

Selective serotonin reuptake inhibitor medications (SSRIs) are the most frequently prescribed medication for perinatal depression and anxiety (Cooper et al., 2007; Fleschler and Peskin, 2008), with 2-10% of all pregnant women being prescribed these medications (Charlton et al., 2015; Cooper et al., 2007; Hayes et al., 2012; Lupattelli et al., 2014; Oberlander et al., 2006; Zoega et al., 2015). SSRIs cross the placental barrier and are present in breast milk (Baumann and Rochat, 1995; Kristensen et al., 1999), posing a risk for an altered serotonergic environment for the developing infant. While recent research shows that perinatal SSRI exposure is linked to poor neurobehavioral outcomes, such as increased risk of affective disorders and autism spectrum disorders (Glover and Clinton, 2016; Man et al., 2015; Oberlander et al., 2007; Oberlander et al., 2006), minimal work has investigated neurobiological consequences of such exposure. In addition, clinical work has demonstrated that maternal depression and health prior to conception can serve as a confounding factor (Brown et al., 2017; Mezzacappa et al., 2017; Oberlander and Zwaigenbaum, 2017), and therefore such interaction effects need to be further investigated. Indeed, women are known to be increasingly susceptible to perinatal depression if they suffer from a history of depressive-episodes, anxiety, or prolonged stress (Lancaster et al., 2010; Pawluski et al., 2017; Stewart, 2011). Therefore, this population of women are likely to remain on treatment, throughout the perinatal period.

Clinical research, using peripheral biomarkers and imaging data shows that prenatal SSRIs affect fetal and infant neurodevelopment. Prenatal SSRIs decrease S100B, an astroglial-specific Ca2+-binding protein critical for cell growth and differentiation, and reelin, a glycoprotein critical for neuronal migration and positioning, in human neonates at birth (Brummelte et al., 2013; Pawluski et al., 2009). Recent imaging data has confirmed that prenatal SSRIs alter the developing brain, with prenatal SSRI exposure decreasing activity in the basal ganglia and thalamus of preterm neonates (Podrebarac et al., 2016). However, further work is needed to assess the effects of perinatal SSRIs on central markers of neurodevelopment.

Research in animal models has provided a growing body of literature on the effects of perinatal SSRIs on development on many brain areas, such as the barrel cortex, the amygdala, the hippocampus, and the hypothalamus (Gemmel et al., 2017a; Glover and Clinton, 2016; Homberg et al., 2010; Oberlander et al., 2009; Olivier et al., 2013; Pawluski, 2012; Rayen et al., 2013, 2014). However, little is known about how perinatal SSRIs in combination with maternal stress, as a model of aspects of maternal depression, can affect offspring outcomes. Work in the hippocampus has shown that perinatal SSRIs can be protective against the effects of maternal stress on neurogenesis in offspring (Ishiwata et al., 2005; Lee et al., 2001; Rayen et al., 2015; Rayen et al., 2011), particularly in adolescence. However, whether these 'protective' effects extend to other brain areas remains to be determined.

The hippocampus, itself, is part of the prefrontal-limbic circuitry which is responsible for stress and emotional response as well as integration of limbic regions (Cardinal et al., 2003). The prefrontal cortex (PFC), in addition to playing a vital role in cognition, complex thought, decision making, and neuronal integration, plays a significant role in regulating behavior and emotion (Jin and Maren, 2015; Shiba et al., 2016). An important part of the PFC, known as the cingulate cortex, is a critical component of the prefrontal-limbic circuitry (Cardinal et al., 2003). The cross-talk between the hippocampus and PFC contributes to the etiology of multiple psychiatric disorders such as schizophrenia, depression, and phobias (Godsil et al., 2013; Kafetzopoulos et al., 2017; Maren et

al., 2013; Sigurdsson et al., 2010). However, little is known about how perinatal SSRIs affect the developing PFC and even less is known regarding how these effects differ in pre-adolescent and adult male and female offspring. Previous work has shown that early postnatal SSRI treatment can decrease serotonin levels (5-HT) and serotonin transporter (SERT) expression in the PFC of male and female offspring (Maciag et al., 2006; Xu et al., 2004; Zhou et al., 2015). Reductions in SERT expression in the cortical barrel of rat offspring following postnatal exposure to paroxetine is also associated with reduced cortical area (Xu et al., 2004), suggesting an impact on additional brain regions which rely on the cortex for afferent and efferent information. Postnatal SSRIs alter the trajectory of cortical development by reducing the density of dendritic spines (Lee and Lee, 2012), spiny stellate cells and thalamocortical afferents (Lee, 2009), and the length of subplate neuron dendrites (Liao and Lee, 2011). Interestingly, we have recently shown that early–life exposure to SSRIs has little effect on synaptophysin density in the medial PFC (cingulate cortex) of weanling offspring, particularly when using a model of maternal stress (Gemmel et al 2016). Thus, more work is needed to determine the effects of *perinatal* SSRIs on monoaminergic functioning and structural plasticity in combination with a model of maternal depression.

In addition, more work is needed to determine how early life changes to monoaminergic functioning, as a result of perinatal SSRI exposure, may impact additional neuro-regulatory systems, such as the Hypothalamic-Pituitary Adrenal (HPA) axis. The HPA axis, which receives serotonergic input, is a critical regulator of behavior and emotion (Andrews and Matthews, 2004; Gemmel et al., 2017a). In human infants, prenatal SSRIs alter the HPA axis via changes in cortisol levels and increases corticosteroid binding globulin (CBG) levels (Brennan et al., 2008; Davidson et al., 2006; Davidson et al., 2009; Oberlander et al., 2008; Pawluski et al., 2012a), work which has been replicated and studied further in animal models (Avitsur, 2017; Avitsur et al., 2016; Ishiwata et al., 2005; Pawluski et al., 2012c). While previous work has shown that early-life exposure to SSRIs decrease hippocampal glucocorticoid receptor (GR) expression (Pawluski et al., 2012c), additional work is needed to determine how serotonergic-dependent and behavior-mediating brain regions, such as the PFC, may be affected by perinatal SSRIs.

The current work aimed to determine the effects perinatal exposure to the SSRI fluoxetine on the developing PFC. We first investigated the effects of perinatal SSRIs on PFC monoaminergic systems as well as synaptic proteins and GR expression in the cingulate cortex of pre-adolescent male and female rat offspring. We then went on to investigate any enduring effect of perinatal SSRIs on synaptic proteins and GR expression in the cingulate cortex of adult male and female offspring. All work was done using a pre-gestational stress paradigm, which has been shown to serve as a model of aspects of Perinatal Depression (Huang et al., 2012), and allowed for a better analysis of the complex relationship between perinatal depression and SSRIs, and their impact on offspring neurobiology.

2. METHODS

2.1 Animals. Thirty-four Sprague-Dawley adult female rats (175-199 g, approximately 60 days of age) and 9 Sprague-Dawley adult male rats (275-299 g), from Harlan Laboratories Inc. (Indianapolis, Indiana), were kept in a 12:12-h light/dark schedule under standard laboratory conditions. Lights were on at 7:30h. Rats were housed in pairs in clear polyurethane bins with basic enrichment and ad libitum access to rat chow and tap water. The Institutional

Animal Care and Use Committee at Ohio University approved all experiments (IACUC, 12-H-053, 14-H-011), and every effort was made to minimize the number of animals used, as well as the pain and stress experienced by the animals.

Adult females were randomly assigned to stress or control groups prior to breeding (16 control, 18 stress); stressed females were housed individually and subject to chronic unpredictable stress (CUS) consisting of 0-2 stressors per day for 3 weeks. Stressors included 1 hour restraint under bright light, overcrowding, overnight exposure to damp bedding, food deprivation (12 h), forced swimming (5min), and cage rotation (12h). Following CUS, one male and one female were housed together for breeding. Gestation day (GD) 1, was identified following vaginal smear with evidence of sperm in the vagina, dams were individually housed. During breeding, each male was paired individually with up to 5 females.

2.2 Fluoxetine administration to dams. From GD10 until weaning (postnatal day (PD) 21), fluoxetine or vehicle (saline) was administered orally via a wafer biscuit twice a day to the dams. Each wafer was filled with 5mg/kg of fluoxetine or vehicle as described (Knaepen et al., 2013; Pawluski et al., 2014) for a total of 10mg/kg/day of fluoxetine. Fluoxetine and its active metabolite, norfluoxetine, can cross the placental barrier and can be passed to offspring via lactation, resulting in detectable levels of fluoxetine and norfluoxetine in the serum of mother and pups (Knaepen et al., 2013). Due to breeding difficulties, primarily as a result of the CUS, 6 vehicle and 8 fluoxetine non-stressed dams remained pregnant, and 5 vehicle and 5 fluoxetine stressed dams remained pregnant in the present study.

2.3 Offspring. On PD1, litters were culled to 4 males and 4 females. From birth until weaning (PD 21) offspring were housed with their mothers and maternal caregiving behaviors were observed two times per day (between 0900-1030 and 1400-1530) for 5 min from PD1 to PD6 as previously described (Gemmel et al., 2017b; Pawluski et al., 2012b; Rayen et al., 2011). The duration of the following maternal behaviors were assessed to understand the role of maternal care on offspring outcome: licking (licking/grooming), and nursing (arched-back nursing, blanket nursing or passive nursing). At weaning, offspring were housed with same-sex littermates. This paradigm yielded four groups of female and male offspring which were used at 2 ages (pre-adolescent and adult). Pre-adolescent offspring were used (PD27-30) to assess the effects of perinatal SSRI exposure on serotonergic levels in the PFC as well as markers of synaptic structure and the glucocorticoid receptor (GR) densities in the cingulated cortex in the medial PFC (mPFC). Pre-adolescent male and female offspring from the following 4 groups were used: 1) Control+Vehicle (CV; 16 animals, 8 male and 8 female), 2) Control+Fluoxetine (CF; 14 animals, 8 male and 6 female), 3) Pre-gestational Stress+Vehicle (PGSV; 16 animals, 8 male and 8 female), and 4) Pre-gestational Stress+Fluoxetine (PGSF; 12 animals, 6 male and 6 female). On PD 26 offspring were weighed and on PD 27-30, between 11:00 and 15:00, offspring were deeply anesthetized with isofluorane, decapitated, and brains were rapidly dissected. The right hemisphere was flash-frozen for high-performance liquid chromatography with electrochemical detection (HPLC-ED) techniques, and the left hemisphere immersion fixed in 4% paraformaldehyde for 48 hrs followed by saturation in 30% sucrose solution for approximately a week.

Adult offspring (PD62-65) were used to determine if there were any enduring effects of these early life exposures on synaptic structure and GR densities in the cingulate cortex. Adult male and female offspring from the

same 4 groups were used: 1) Control+Vehicle (CV; 12 animals, 6 male and 6 female), 2) Control+Fluoxetine (CF; 12 animals, 6 male and 6 female), 3) Pre-gestational Stress+Vehicle (PGSV; 12 animals, 6 male and 6 female), and 4) Pre-gestational Stress+Fluoxetine (PGSF; 12 animals, 6 male and 6 female). On PD 60, adult offspring were weighed and on PD 62-65, between 11:00 and 15:00, adult offspring were deeply anesthetized with isofluorane, decapitated, and brains rapidly dissected followed by immersion fixation of whole brains as described above.

All offspring were previously used in a social interaction test with a novel same-sex partner which involved habituation for 15 minute to the testing chamber and then testing for 10min with a novel same-sex partner as part of a larger study on behavioral outcomes (Gemmel et al., 2017b). No more than 2 males or 2 females were used per litter at each age. Behavioral assessment was completed 1-2 days prior to sacrifice.

To ensure the estradiol levels did not play a role in plasticity measures of adult female offspring, trunk blood was collected at sacrifice. Blood samples were centrifuged at 10,000×g for 10 min. Serum was collected and stored at −80 °C. All samples were run in duplicate using a commercially available ELISA kit (ALPCO Estradiol Rat ELISA 55-ESTRT-E01, ALPCO Diagnostics, Salem, NH, USA). The average coefficient of variation was less than 6%.

2.4 Monoamine Measurements. Flash frozen right brain hemispheres were assessed for levels of serotonin (5-HT), serotonin's metabolite, 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), dopamine's metabolite dihyroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the PFC using high performance liquid chromatography with electrochemical detection (HPLC-ED). Samples were weighed, homogenized, and deproteinized in 0.1 N perchloric acid solution (Applichem, Darmstadt, Germany) containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na_2EDTA (Riedel-de Haën AG, Seelze, Germany). Following deproteinization, samples were centrifuged at 4°C for 45 min at 20000 g and the supernatant was stored at - 80°C until analysis. The analysis utilized an LKB-2248 HPLC pump (Pharmacia Biotechnology AB, Bromma, Sweden) and BAS-LC-4C electrochemical detector (Bioanalytical Systems Inc., USA), as previously described (Kyratsas et al., 2013; Novais et al., 2013). The working electrode of the electrochemical detector was set at +800 mV. Reverse phase ion pairing chromatography was used to assess monoamines and metabolites. Mobile phase consisted of a 50 mM phosphate buffer at pH 3.0, with 300 mg/L 5-octylsulfate sodium salt as the ion pairing reagent and 20 mg/L Na_2EDTA (Riedel-de Haën AG); acetonitrile (Merck, Darmstadt, Germany) at 8-12% concentration. Reference standards were prepared in 0.1 N perchloric acid solution containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na_2EDTA. The column used was an Aquasil C18 HPLC Column, 100 mm X 1 mm, 5 µm Particle Size (Thermo Electron, Chesire, UK). Quantification was completed using a PC compatible HPLC software package (Clarity v.7 DataApex, Prague, Czech Republic) and by comparing the area under the curve against known external reference standards and expressed as ug/g of wet tissue. Following monoamine quantification, ratios (5-HIAA/5-HT, DOPAC/DA, HVA/DA) were calculated as a measure of turnover rates. Monoaminergic activities are better estimated when comparing individual neurotransmitter and metabolite levels as they reflect 5-HT and DA release and/or metabolic activity (Bessinis et al., 2013; Dalla et al., 2008; Kokras et al., 2009; Mikail et al., 2012). One control fluoxetine female was not included in analysis for having a 5-HT level 7 standard deviations greater than the mean, and some data were not available due to levels below the detection limit (10pg/20ul).

2.5 Immunohistochemistry. Immersion fixed brains were stored at -80°C until coronally sliced in 40 micron sections by cryostat (Leica Biosystems, Wetzlar, Germany) in series of 12. Tissue sections were then transferred to a glycol based antifreeze solution and stored at -20°C. During immunohistochemical staining, sections were rinsed in TBS and TBS plus 0.01% Triton X-100 (TBST) for synaptophysin and PSD-95 or PBS and PBST for glucocorticoid receptor (GR) between steps. Following initial rinses, tissue was incubated in 0.6% H_2O_2 for 30 minutes at room temperature and blocked in 5% Normal Goat Serum (NGS) (Lampire Biological Laboratories, Pipersville PA, USA) for 30 minutes. For PSD-95 staining, tissue underwent an additional antigen unmasking step, consisting of incubation in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes at 80°C. Tissue was then incubated in primary mouse anti-synaptophysin antibody (1:500, Sigma Aldrich, St. Louis MO, USA: NGS+TBST) and primary rabbit anti-PSD-95 antibody (1:1000, Abcam, Cambridge, MA, USA: NGS+TBST) overnight or in rabbit anti-GR antibody (1:500, Abcam, Cambridge, MA, USA: NGS+TBST) for two nights at 4°C. Following primary incubation, sections were incubated at room temperature for 2h in secondary antibodies (1:200, Vector laboratories, Burlingame, CA, USA) followed by additional processing using the avidine-biotine complex (ABC Elite kit; 1:1000; Vector laboratories, Burlingame, CA, USA) and DAB (3,3-diaminobenzidine; Vector laboratories, Burlingame, CA, USA). Sections were mounted on Superfrost Plus slides (Fischer Scientific, Pittsburgh, PA), dried, dehydrated, and cover-slipped with Permount (Fischer Scientific, Pittsburgh, PA). See Figure 1 for a representative photomicrograph of synaptophysin at 4x (Figure 1a) and 40x (Figure 1b) objective as well as PSD-95 (Figure 1c) and GR (Figure 1d) densities at 40x objective.

2.5.1 Quantification. For optical densities of synaptophysin, PDS-95, and GR-ir cells, two sections of the medial PFC (mPFC located between stereotaxic coordinates +3 mm to +2.5 bregma) were analyzed (Gemmel et al., 2015; Gemmel et al., 2016). Photomicrographs were taken for four areas within the cingulate cortex (Cg), two areas each from the Cg1 and Cg3 (mPFC) region. Immunoreactivity for all sections was examined under 40x objective using a Nikon Microphot SA and Nikon DS-Qi1MC camera with Nikon NIS Elements F4.00 software. The software ImageJ64 (Wayne Rasband, NIH, Bethesda MD, USA) was used for quantification of optical densities for all immunoreactive cells. The relative optical density was defined as the difference in optical density (grey level) after calibration between the area of interest and the background, which was an equivalent area adjacent to the area of interest with minimal staining.

2.6 Statistical Analysis. Data were analyzed using the software Statistica (Dell Inc.). Three-way Analysis of Variance Tests (ANOVA) were conducted on pre-adolescent offspring weight and levels of 5-HT, DA, relevant metabolites, and turnover ratios, with condition (maternal pre-gestational stress/control), treatment (fluoxetine/vehicle), and sex (female/male) as independent factors. ANOVAs were also conducted separately on optical densities of synaptophysin, PSD-95, and GR in the Cg of pre-adolescent and adult offspring with condition, treatment, and sex as independent factors. Analyses of covariance tests were run on all measures with maternal licking or nursing as covariates, and in adult females with estradiol level at sacrifice as a covariate. As previous findings show numerous sex differences on neuroplasticity and additional neurobiological outcomes (Pawluski et al., 2012c; Rayen et al., 2015; Rayen et al., 2013, 2014; Weinstock, 2001, 2011), separate two-way analysis of variance tests were computed for each sex with condition and treatment as independent factors. Separate analysis by sex

allows for the investigated of the sex specific effects of these early life exposures. Significant interaction effects were analyzed in more detail by a Fisher LSD *post hoc* tests. Pearson correlations were analyzed between maternal caregiving behaviors and offspring neurobiological measures. Significance was set at $p<0.05$.

3. RESULTS

3.1 Weight. Perinatal SSRI exposed offspring weighed significantly less than vehicle exposed offspring (main effect of treatment: $F(1,86)=5.21$, $p=0.02$, Table 1), regardless of maternal stress exposure. Separate analysis by sex resulted in a significant main effect of treatment on pre-adolescent female offspring ($F(1, 42)=4.67$, $p=0.04$, Table 1) with SSRI exposed pre-adolescent females weighing less than non-exposed pre-adolescent females. There were no effects of perinatal fluoxetine or pre-gestational stress on adult weight (Table 1). As expected, males weighed significantly more than females at both ages (pre-adolescent offspring main effect of sex: $F(1, 86)=53.89$, $p<0.0001$; adult offspring main effect of sex: $F(1,79)=646.72$, $p<0.0001$, Table 1).

3.2 5-HT system in pre-adolescent offspring. There were significant condition by treatment interaction effects on 5-HT levels ($F(1,51)=6.48$, $p=0.01$, Figure 2a,c) and 5-HIAA/5-HT ($F(1,51)=7.82$, $p=0.007$, Figure 2e,f) with PGSV offspring having less 5-HT and greater turnover ratios in the PFC when compared to all other offspring groups (p's < 0.02). There were additional treatment by sex interaction effects on 5-HT ($F(1,51)=6.16$, $p=0.02$) and 5-HIAA/5-HT ($F(1,51)=4.23$, $p=0.04$), with fluoxetine exposed females having significantly higher levels of 5-HT and lower turnover ratios compared to vehicle exposed females (p's<0.003) and higher levels of 5-HT compared to all fluoxetine and vehicle exposed male offspring groups ($p<0.02$). There was a significant condition by sex interaction effect on 5-HT ($F(1,51)=5.79$, $p=0.02$), with control females having greater 5-HT levels than control males ($p=0.03$) and pre-gestationally stress exposed females ($p=0.002$). Perinatal SSRI exposure also increased 5-HT and decreased 5-HIAA/5-HT in the PFC (main effect of treatment for 5-HT: $F(1,51)=15.94$, $p=0.0002$, Figure 2a; and 5HIAA/5HT: $F(1,51)=6.03$, $p=0.02$, Figure 2e). Pre-gestational stress also reduced 5-HIAA in the PFC (main effect of condition ($F(1,51)= 6.35$, $p=0.01$, Figure 2c).

When looking separately by sex, there were significant condition by treatment interaction effects on 5-HT ($F(1,25)= 4.29$, $p=0.049$, Figure 2b) and 5-HIAA/5-HT ($F(1,25)=6.89$, $p=0.01$, Figure 2f) in female offspring, with PGSV females having less 5-HT ($0.00004<p<0.002$) and greater turnover ($0.0005<p<0.002$) than all other groups. There was a significant main effect of maternal SSRI treatment on 5-HT($F(1,25)=17.63$, $p=0.0003$, Figure 2b), 5-HIAA ($F(1,25)=5.03$, $p=0.03$, Figure 2d), and 5-HIAA/5-HT ($F(1,25)=9.57$, $p=0.005$, Figure 2e), with perinatal SSRI exposure increasing 5-HT and 5-HIAA, and reducing turnover in the PFC in female offspring. There was also a significant main effects of condition on 5-HT ($F(1,25)=7.01$, $p=0.01$, Figure 2b) and 5-HIAA ($F(1,25)=4.33$, $p=0.048$, Figure 2d) with pre-gestational stress reducing 5-HT and 5-HIAA levels in female offspring. There were no significant differences in these measures in the PFC of male offspring (p's >0.05).

3.3 Dopamine system in pre-adolescent offspring. There was a significant condition by treatment interaction effect on dopamine turnover in the PFC of female offspring ($F(1,24)=5.08$, $p=0.03$, Table 1) with PGSV females having significantly more DOPAC/DA compared to PGSF females ($p=0.02$). Pre-gestational maternal stress decreased such

HVA levels in the PFC of females (main effect of condition ($F(1,25)=4.85$, $p=0.04$, Table 1). Males also had significantly higher HVA levels compared to female offspring (main effect of sex; $F(1,51)=4.93$, $p=0.03$, Table 1).

3.4 Synaptic proteins and GR densities. In pre-adolescent offspring, pre-gestational maternal stress significantly reduced pre- and post-synaptic protein densities in the Cg (synaptophysin-ir: ($F(1,40)=7.41$, $p=.009$, Figure 3a; PSD-95-ir: ($F(1,40)=5.83$, $p=.02$, Figure 4a). There were further sexually differentiated effects in pre-adolescent offspring, with pre-gestational maternal stress reducing post-synaptic density in the Cg of males (PSD-95-ir: $F(1,20)=5.02$, $p=.04$, Figure 4b), but not females.

In adult offspring, pre-gestational maternal stress significantly reduced PSD-95 density in the Cg (main effect of condition; $F(1,40)=10.03$, $p=0.003$, Figure 4c). This effect was most pronounced in adult males (main effect of condition in adult males; ($F(1,20)=7.45$, $p=0.01$, Figure 4d) and did not exist in adult females ($p>0.05$). In addition, perinatal SSRI exposure significantly reduced GR density in adult offspring (main effect of treatment: ($F(1,40)=6.16$, $p=0.02$, Figure 5c). Further analysis by sex revealed a significant main effect of treatment on adult GR density in males ($F(1,20)=5.10$, $p=0.04$, Figure 5d), but not females. There were no other significant main effects or interaction effects on synaptophysin densities, PSD-95 densities, or GR densities in male or female offspring (p's > 0.05). Estradiol levels at sacrifice did not significantly co-vary with any measures in adult females ($0.14<p<0.95$).

3.5. Maternal care-giving effects. ANCOVAs testing showed that maternal time licking/grooming significantly co-varied with synaptophysin density in the Cg ($p=0.001$) in pre-adolescent offspring only and resulted in a diminishing of these effects (main effect of maternal stress: $p=0.08$). Maternal licking/grooming also significantly co-varied with DOPAC/DA levels in the PFC of preadolescent female offspring ($p=0.03$) diminishing the effect (condition by treatment interaction effect: $p=0.06$). Overall, there were no significant differences in percent of time spent licking/grooming or nursing offspring during the first week postpartum (p's>0.2).

To assess the relationship between maternal behaviors and PFC measurements, Pearson correlations were completed on dopamine turnover and all synaptic outcomes. There was a significant positive correlation between maternal licking/grooming and pre-synaptic protein density in the Cg of pre-adolescent offspring ($r= 0.46$, $p=0.0009$, Figure 6a) and a significant negative correlation between maternal nursing and pre-synaptic density in the Cg of pre-adolescent offspring ($r= -0.44$, $p=0.002$, Figure 6b). There were no other significant correlations between maternal behavior and pre-adolescent or adult PFC measures.

4. DISCUSSION

Findings from the current study show that perinatal SSRI exposure may protect against some effects of maternal stress in the developing PFC. We also show that perinatal fluoxetine and pre-gestational maternal stress can have enduring effects on this same brain area, but these effects are sexually differentiated. More specifically, we found that perinatal fluoxetine prevents the effect of pre-gestational maternal stress on 5-HT and 5-HT turnover ratios in the PFC of pre-adolescent offspring, particularly in females. However, pre-gestational maternal stress significantly reduced the presence of synaptic proteins (synaptophysin and PSD-95) in the mPFC, effects that were

more pronounced in males. Interestingly, perinatal fluoxetine exposure, and not maternal stress, significantly reduced GR densities in the mPFC of adult males. Together, these results show differential effects of perinatal SSRIs and maternal stress on key physiological systems and neuroplasticity in the PFC of males and females.

4.1 Perinatal fluoxetine prevents the effect of maternal stress on the 5-HT system in the PFC. In the present study, pre-gestational maternal stress significantly reduced 5-HT and increased 5-HT turnover in the PFC, suggesting a change in the synthesis and/or metabolism of 5-HT and or the transportation of 5-HT by SERT. Previous work in rat fetuses has shown that pre-gestational stress increases 5-HT in the hippocampus and hypothalamus, decreases 5-HIAA levels in the hippocampus, and decreases 5-HT turnover (Huang et al., 2012). However, to our knowledge, this is the first work to show that pre-gestational maternal stress has enduring effects on the serotonergic system in the PFC by decreasing 5-HT and increasing its turnover. Interestingly, *prenatal* stress increases 5-HT and 5-HIAA levels in whole rat brains up to postnatal day 10 (Peters, 1990), yet has less marked effects on these same monoamines in rat offspring at weaning (Gemmel et al., 2015). Others have shown that maternal *prenatal* stress can decrease 5HT1AR on GABAergic cells in the mPFC in adult male, but not female, offspring (Zohar et al., 2014). Together this work shows pre-gestational, as well as prenatal, maternal stress can significantly affect the developing central 5-HT system but more work is needed to determine the time course of these effects in males and females.

Interestingly, perinatal fluoxetine exposure prevented the effect of pre-gestational maternal stress on 5-HT and 5-HT turnover ratios in the PFC of pre-adolescent offspring. This reversal, or prevention, of maternal stress effects on offspring outcomes by perinatal SSRI exposure has previously been shown, particularly in juvenile and adolescent rats: Perinatal fluoxetine can reverse the effects of maternal stress on immobility in the forced swim test, hippocampal neurogenesis, and 5-HIAA levels in the hippocampus of juvenile or adolescent offspring (Gemmel et al, 2016; Rayen et al 2011). This preventative effect of early-life exposure to SSRIs on maternal stress effects does not consistently extend into adulthood and, in fact, during adulthood different effects of perinatal exposure to SSRIs can emerge. For example, adult females, but not males, exposed early in life to fluoxetine show increased immobility in the forced swim test, and decreased synaptophysin density in the dentate gyrus, with no enduring effects of maternal stress exposure (Boulle et al., 2016a, b; Rayen et al., 2015). Such findings show that while perinatal SSRIs may prevent effects of maternal stress on neurobehavioral outcomes in juvenile offspring, these effects may significantly differ in adulthood.

It is perhaps not surprising that perinatal fluoxetine exposure would increase serotonin and metabolite levels in pre-adolescent offspring as fluoxetine and norfluoxetine can cross the placental barrier and can pass to the pups via lactation (Knaepen et al., 2013; Oberlander et al., 2009). Prenatal exposure to SSRIs (GD13-20) reduces 5-HT levels in the frontal cortex of prepubescent male rats, with no effect on 5-HIAA or 5-HIAA/5-HT levels (Cabrera-Vera et al., 1997), while postnatal exposure (PD1-21) has no effect on serotonin or serotonergic functioning in the PFC of male and female rats at weaning (Gemmel et al., 2015). Differences between the current work and previous work are likely due to differences in the timing of SSRI exposure – perinatal versus early postnatal. Serotonin signaling is critical for the development of neuronal circuits (Ansorge et al., 2008; Ansorge et

al., 2004; Fernandez et al., 2016), with serotonergic cells being among the earliest appearing cells in the 5-12th week after fertilization in humans (Herlenius and Lagercrantz, 2004; Sundstrom et al., 1993; Whitaker-Azmitia, 2001) and at gestation day 10-12 in rodents (Herlenius and Lagercrantz, 2004). Therefore, exposure to fluoxetine, beginning when serotonergic circuitry comes online (GD 10) and ending in the postnatal period may serve as a critical period of exposure, and may have more pronounced effects on serotonergic functioning as seen in the current study.

Changes in serotonin levels in the PFC after perinatal exposure to SSRIs may be due to changes in the plasma membrane serotonin transporter (SERT), a critical regulator of serotonin homeostasis (Kim et al., 2005). In humans, a peak of SERT is seen prenatally between weeks 16 and 22, when cortical sensory maps develop (Herlenius and Lagercrantz, 2004; Verney et al., 2002), thereby suggesting an effect of perinatal fluoxetine exposure on PFC development. Indeed, early postnatal SSRI exposure reduces SERT-ir density in the mPFC (Zhou et al., 2015) and cortical barrel of male and female rat offspring (Xu et al., 2004). Therefore, any changes to serotonin levels and/or serotonergic system functioning during the perinatal period as a result of perinatal SSRIs suggest an impact on the PFC and additional brain regions which rely on the cortex for afferent and efferent information. Previous work has shown that 5-HT levels in the PFC correlate to immobility behavior in the forced swim test (Mikail et al., 2012). Therefore, changes in serotonin as a result of perinatal SSRI exposure may indeed have additional effects on behavior- mediating bran regions.

It is particularly interesting to note that the effects of perinatal fluoxetine exposure on the 5-HT system in the PFC were more prevalent in pre-adolescent females. Indeed females are often more sensitive to perinatal SSRI exposure, particularly in the hippocampus, and during adulthood (Gemmel et al., 2017a; Pawluski et al., 2012c; Rayen et al., 2015; Rayen et al., 2013, 2014). Sexual differentiation of the brain is dependent on serotonin due to its critical role in hypothalamic–pituitary–gonadal (HPG) axis development (Dohler et al., 1991; Pawluski et al., 2015; Rayen et al., 2013, 2014), and thus, it may be that perinatal exposure to SSRIs alters sexual differentiation of the brain, and subsequently changes in the monoaminergic system, in a sexually differentiated manner which enhances effects in females, and not males.

4.2 Pre-gestational maternal stress, but not perinatal fluoxetine, affects synaptic structure in the mPFC. Pre-gestational maternal stress significantly reduced both pre- and post-synaptic density in the mPFC in male offspring. Previous work investigating the effects of *prenatal* stress on neuroplasticity and synaptic structure has reported a negative effect of maternal stress on spine type via reductions in the ratio of mPFC mushroom spines in adult male rat offspring (Michelsen et al., 2007)(females not studied). Prenatal stress also significantly reduces apical dendrite spine density, dendritic length, and dendritic complexity in the dorsal anterior cingulate and orbitofrontal cortex of male rat pups, while reducing both apical and basal dendritic spine density in the same regions, with no effect on dendritic length or complexity in female rat pups (Murmu et al., 2006). Others have shown that prenatal stress increases overall spine density in the mPFC of adult males and females, while increasing excitatory synapse number in the same region of male rats only (Mychasiuk et al., 2012). Taken together, this work shows that pre-gestational, and prenatal, maternal stress can have long-term effects on synaptic structure. In addition, the current work adds to a growing body of work showing that maternal stress results in sexually differentiated effects on neurodevelopment,

with males being particularly sensitive, and females being more resilient, to the effects of maternal stress. However, these effects may be age dependent in females with prenatal stress affecting plasticity after senescence (Koehl et al., 2009).

It is particularly interesting to note that while pre-gestational stress exposed differences in pre-synaptic density did not persist in the adult mPFC in the present study, there were persisting effects of post-synaptic density, via a reduction in PSD-95 expression. This suggests that pre-synaptic changes resulting from pre-gestational stress exposure may be more transient, while post-synaptic changes persist for a longer period of time. It is also important to consider that differences in such plasticity markers may be a result of localization differences. The pre-synaptic marker synaptophysin is present in the majority of synapses as an integral vesicular membrane protein, particularly in neuroendocrine cells (Glantz et al., 2007; Navone et al., 1986; Wiedenmann and Franke, 1985), while the scaffolding post-synaptic marker PSD-95 is concentrated at excitatory synapses (Sheng and Hoogenraad, 2007). Persistent effects of pre-gestational maternal stress exposure on PSD-95 suggests that such exposure may have a more substantial effect on non-neuroendocrine cells. This is, in part, supported by the lack of maternal stress effects on glucocorticoid receptor expression seen in the current study (discussed below).

Perinatal fluoxetine exposure did not affect markers of synaptic structure in the current study. Previous work shows that prenatal SSRIs reduce dendritic branching in cortical layer pyramidal neurons of adult mice (Smit-Rigter et al., 2012), and early postnatal SSRI exposure reduces dendritic spine density in the mPFC of adult male rats (Ko et al., 2014). Thus, perinatal SSRIs can affect other aspects of spines and synapse plasticity besides synaptic proteins.

4.3 Perinatal fluoxetine effects central HPA measures in the adult mPFC. Perinatal fluoxetine exposure significantly reduced GR density in the mPFC, particularly in male offspring. This extends previous work showing that early life exposure to fluoxetine significantly decreases GR density in the hippocampus of adolescent male, but not female, offspring (Pawluski et al., 2012c). Others have shown that perinatal exposure to SSRIs can affect the developing HPA axis via changes in serum corticosterone levels, stress responsivity and corticosteroid binding globulin capacity, particularly in male, and not female, offspring (Avitsur, 2017; Avitsur et al., 2016; Gobinath et al., 2016; Ishiwata et al., 2005; Pawluski et al., 2012a; Pawluski et al., 2012c). As previously mentioned, serotonin plays a critical role in and the developing HPA and HPG axes (Dohler et al., 1991; Erdeljan et al., 2001). In addition, in adulthood, HPA axis outcomes also correlate better with male behavior compared to female behavior (Kokras et al., 2012; Kokras et al., 2011). Therefore, the effects of perinatal SSRIs on GR-density in the mPFC, particularly in males, may be a result of changes in 5-HT via perinatal SSRI effects on gonadal steroids and the HPA system (Jarzab and Dohler, 1984; Viau, 2002; Viau and Meaney, 1991).

4.4. Maternal care-giving and synaptic proteins in the PFC. There is a wealth of literature showing the maternal care-giving behaviors can have long-term effects on many offspring outcomes (Caldji et al 2000; Kaffman and Meaney, 2007). Here we show that maternal licking/grooming duration in the first week postpartum is positively correlated with pre-synaptic protein density in the Cg of pre-adolescent offspring, with the duration of maternal nursing being negatively correlated with this same pre-synaptic protein in the Cg. While these effects do not persist

into adulthood, and were not evident with other neurobiological measures reported, the observed outcome does suggest that maternal caregiving may shape neurodevelopment in the early pre-adolescent period. This idea is also supported by our recent work showing an association between maternal care-giving and social behaviors in pre-adolescent rat offspring (Gemmel et al., 2017a). Therefore, maternal care may be a mediator of neurobehavioral outcomes in offspring earlier in life, with maternal caregiving effects being less evident in adult offspring.

Although we did not find evidence of a marked effect of pre-gestational stress or perinatal fluoxetine on maternal caregiving behaviors in the present study, maternal care can be modified by stress and fluoxetine treatment (Champagne and Meaney, 2006; Gemmel et al., 2017a; O'Donnell et al., 2014; Pawluski et al., 2012b; Smith et al., 2004; Weikum et al., 2013). Previous work we have done shows that postnatal SSRI treatment can significantly increase arched-back nursing (Pawluski et al., 2012b) and others have shown that gestational SSRI treatment increases the frequency of maternal licking of offspring, and in low doses, increases the duration of crouching over offspring (Johns et al., 2005). In addition alterations in maternal caregiving behaviors have been reported after repeated gestational stress (Belnoue et al., 2016; Brummelte et al., 2006; Champagne and Meaney, 2006; Haim et al., 2014; Hillerer et al., 2012). Thus, maternal caregiving behaviors can be mediated by maternal stress and SSRI treatment. Further work is needed to determine the extent to which the effects of these factors on offspring outcomes are due to changes in maternal caregiving behaviors.

5. Conclusions

This work adds to our understanding of the benefits and risks of perinatal SSRIs on the developing PFC. Here we show that perinatal SSRIs can protect against some effects of maternal stress, however both perinatal fluoxetine exposure and pre-gestational maternal stress can have additional, enduring, effects on the developing PFC; effects that are often sexually differentiated. With the increasing prescription rates for SSRIs during the perinatal period, further research in needed on how these medications affect different regions of the developing brain, particularly in the presence of maternal depression and anxiety, and behavioral outcomes. Understanding these effects will aid in improving therapeutic approaches for, and prevention of, maternal mental illnesses.

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Figure Legends

Figure 1 Representative photomicrographs of synaptophysin-ir cells in the Cg of the mPFC at a) 4x and b) 40 x objective, and c) PSD-95-ir and d) GR-ir cells in the Cg at 40x objective. (*Scale bar* is set at 25 µm)

Figure 2 Mean (±SEM) a,b) 5-HT levels (µg/g), c,d) 5-HIAA levels (µg/g wet tissue), and e,f) 5-HIAA/5-HT ratio in the PFC of pre-adolescent offspring. Figures indicate combined female and male outcomes (a,c,e) and sex-specific effects (b,d,f). ^ denotes significant main effect of treatment ($p<0.05$). *denotes significance ($p<0.05$). $^{@}$ denotes CV and CF males significantly differ from CV and CF females. CV = control + vehicle, CF= control + fluoxetine, PGSV= pre-gestational stress + vehicle, PGSF= pre-gestational stress + fluoxetine. (n=6-8/group/sex)

Figure 3 Mean (±SEM) synaptophysin-immunoreactivity (-ir) in the Cg of a,b) pre-adolescent and c,d) adult offspring. OD=optical density. Figures indicate combined female and male outcomes (a,c) and sex-specific effects (b,d). *denotes significance ($p<0.05$). CV = control + vehicle, CF= control + fluoxetine, PGSV= pre-gestational stress + vehicle, PGSF= pre-gestational stress + fluoxetine. (n=6/group/sex)

Figure 4 Mean (±SEM) PSD-95-ir in the in a,b) pre-adolescent and c,d) adult offspring. OD=optical density. Figures indicate combined female and male outcomes (a,b) and sex-specific effects (b,d). *denotes significance ($p<0.05$). CV = control + vehicle, CF= control + fluoxetine, PGSV= pre-gestational stress + vehicle, PGSF= pre-gestational stress + fluoxetine. (n=6/group/sex)

Figure 5 Mean (±SEM) glucocorticoid receptor (GR) –ir in the Cg in a,b) pre-adolescent and c,d) adult offspring. OD=optical density. Figures indicate combined female and male outcomes (a,b) and sex-specific effects (b,d). ^ denotes significant main effect of treatment ($p<0.05$). CV = control + vehicle, CF= control + fluoxetine, PGSV= pre-gestational stress + vehicle, PGSF= pre-gestational stress + fluoxetine. (n=6/group/sex)

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Table 1. Mean (±SEM) weight, dopamine levels (µg/g wet tissue), and dopamine turnover ratios in the prefrontal cortex (PFC) of pre-adolescent offspring. Fluoxetine reduced weight, particularly in female offspring. Preadolescent and adult males weighed significantly more than preadolescent and adult females. There was a main effect of sex on HVA levels in preadolescence, with males having higher levels than females, and a main effect of stress in females with maternal stress reducing HVA levels. There was a condition by treatment interaction effect on DOPAC/DA with PGSV female offspring having less turnover than PGSF female offspring in preadolescence. * denotes significance ($p<0.05$). (n=6-8/group/sex). CV = control + vehicle, CF= control + fluoxetine, PGSV= pre-gestational stress + vehicle, PGSF= pre-gestational stress + fluoxetine.

	CV	**CF**	**PGSV**	**PGSF**	**CV**	**CF**	**PGSV**	**PGSF**
	Female				**Male**			
Pre-adolescent Weight (g) *	70.7 ± 1.1	69.4 ± 0.8 *	71.0 ± 0.8	68.2 ± 1.0 *	77.5 ± 2.2	75.1 ± 1.1	79.2 ± 2.1	76.4 ± 1.3
Adult Weight (g) *	194.9 ± 4.4	192.5 ± 3.4	187.9 ± 3.0	192.3 ± 1.3	270.6 ± 5.3	276.7 ± 5.0	263.0 ± 4.0	275.0 ± 3.0
DA	0.01 ± 0.004	0.008 ± 0.001	0.006 ± 0.004	0.01 ± 0.003	0.01 ± 0.003	0.01 ± 0.005	0.01 ± 0.003	0.01 ± 0.003
HVA*	0.02 ± 0.005	0.02 ± 0.004	0.01 ± 0.003*	0.01 ± 0.002*	0.02 ± 0.002	0.02 ± 0.003	0.02 ± 0.003	0.02 ± 0.003
DOPAC	0.003 ± 0.001	0.004 ± 0.002	0.002 ± 0.0009*	0.0008 ± 0.0003*	0.003 ± 0.0005	0.004 ± 0.001	0.004 ± 0.002	0.005 ± 0.002
HVA/DA	3.6 ± 1.3	2.5 ± 0.67	6.3 ± 2.7	0.97 ± 0.20	10.2 ± 7.4	12.3 ± 9.3	7.3 ± 5.0	4.7 ± 2.2
DOPAC/DA	0.31 ± 0.09	0.56 ± 0.31	0.86 ± 0.32	0.06 ± 0.01	1.5 ± 0.94	1.4 ± 1.1	2.7 ± 2.2	0.80 ± 0.25

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Figure 1

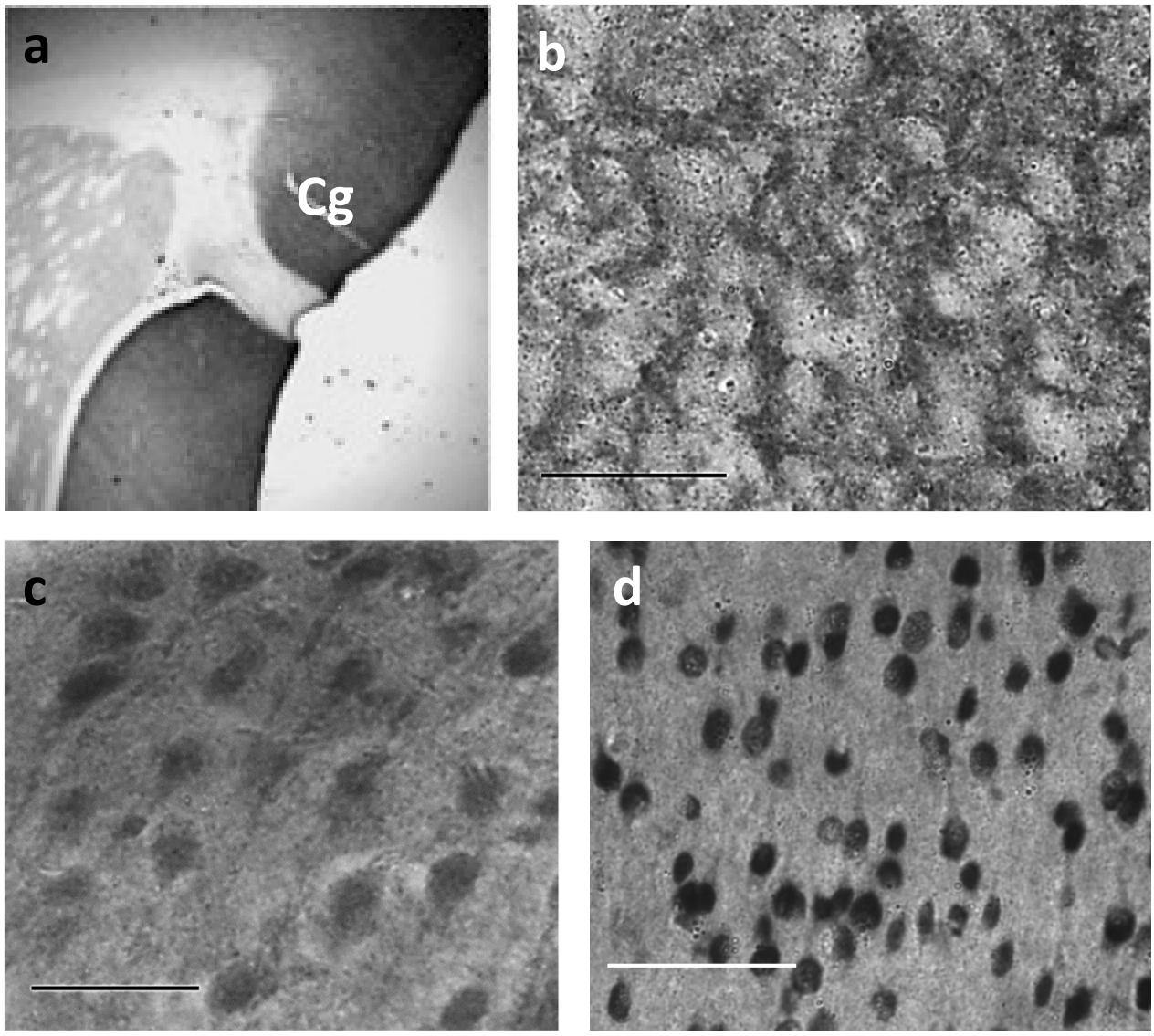


Figure 2

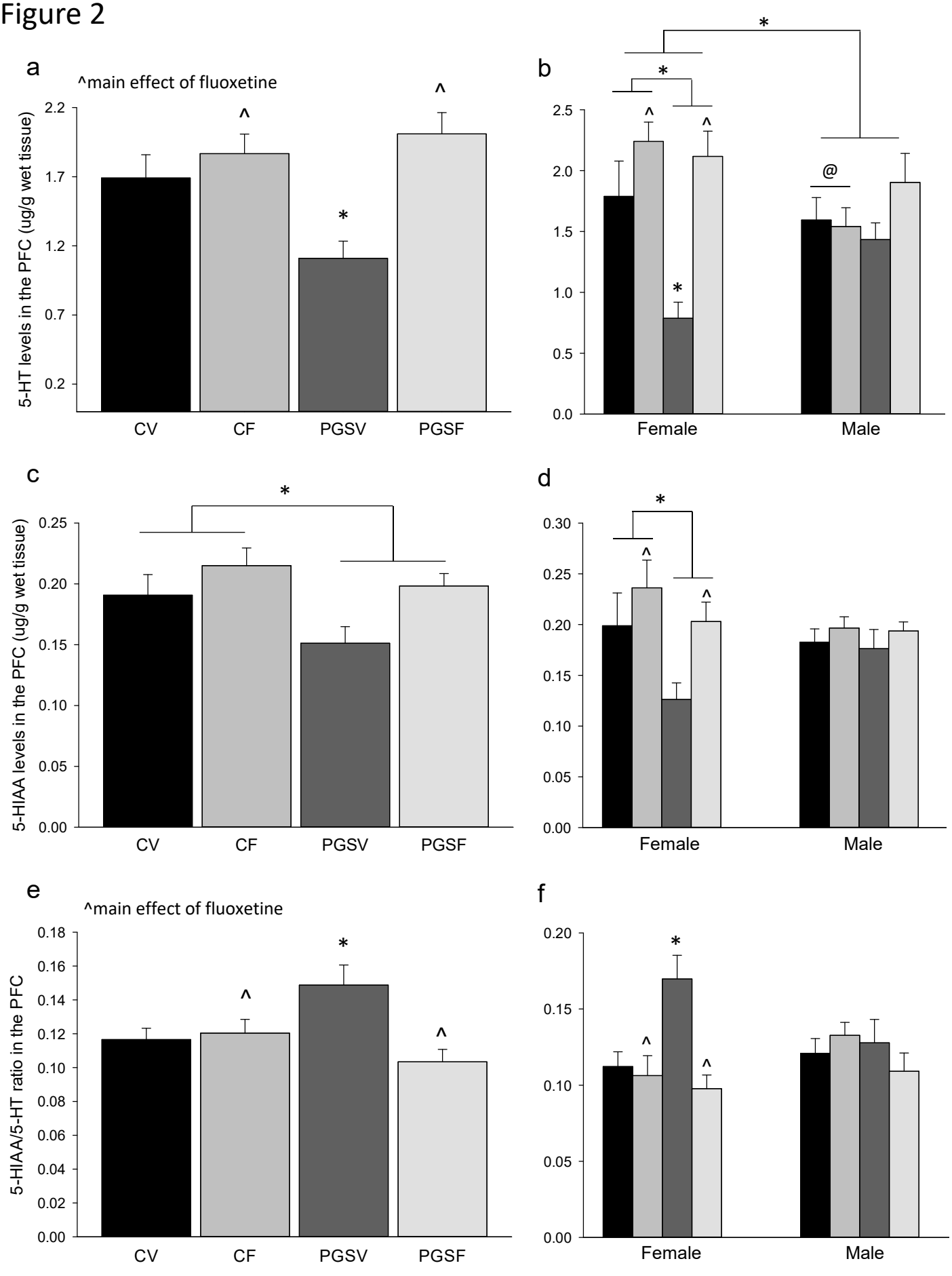

a
^main effect of fluoxetine
5-HT levels in the PFC (ug/g wet tissue)
2.2
1.7
1.2
0.7
0.2
CV
CF
PGSV
PGSF
b
2.5
2.0
1.5
1.0
0.5
0.0
Female
Male
c
5-HIAA levels in the PFC (ug/g wet tissue)
0.25
0.20
0.15
0.10
0.05
0.00
d
0.30
e
^main effect of fluoxetine
5-HIAA/5-HT ratio in the PFC
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
f
0.20

Figure 3

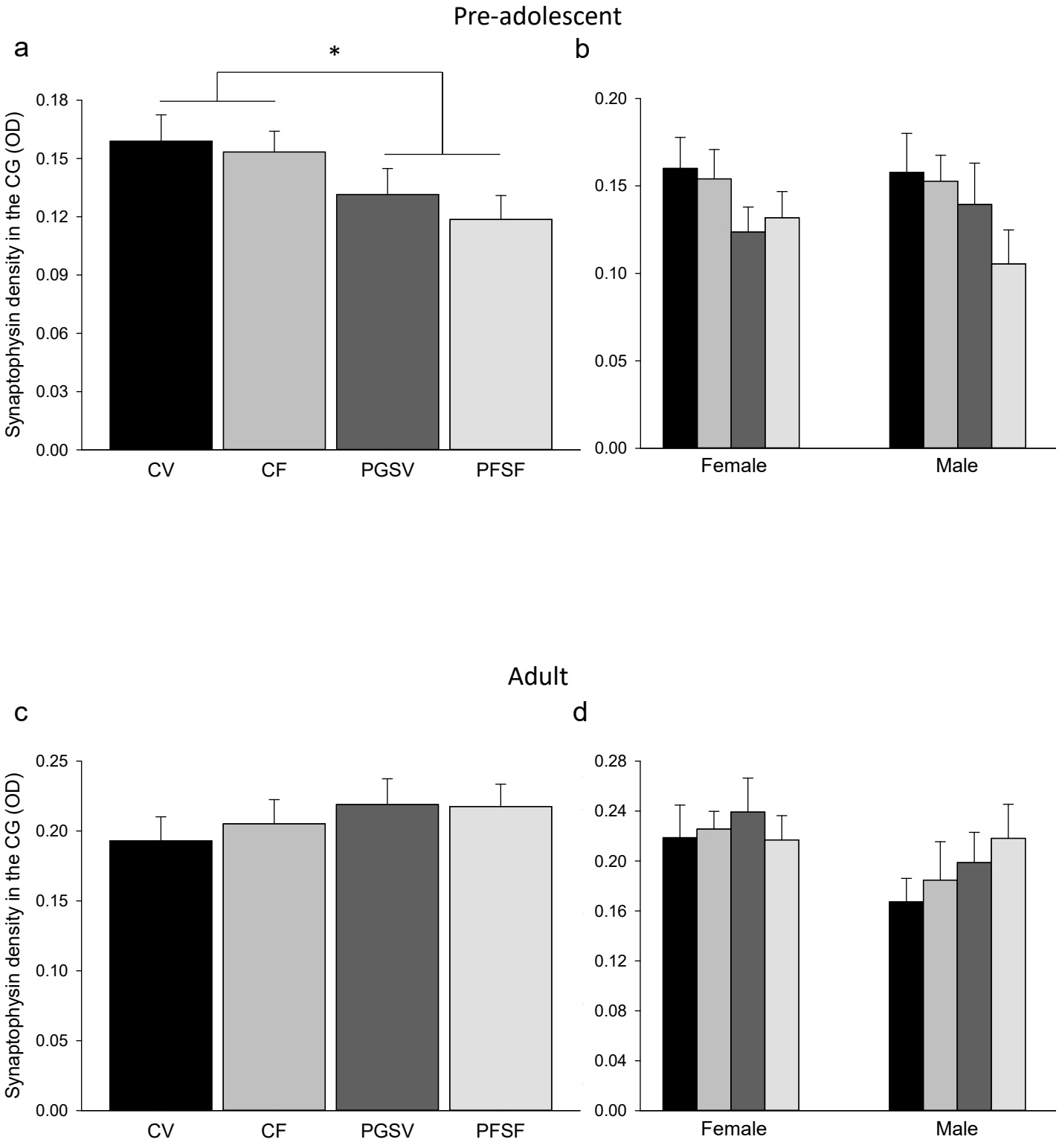

Pre-adolescent
a
b
*
Synaptophysin density in the CG (OD)
0.18
0.15
0.12
0.09
0.06
0.03
0.00
CV
CF
PGSV
PFSF
0.20
0.15
0.10
0.05
0.00
Female
Male
Adult
c
d
Synaptophysin density in the CG (OD)
0.25
0.20
0.15
0.10
0.05
0.00
CV
CF
PGSV
PFSF
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
Female
Male

Figure 4

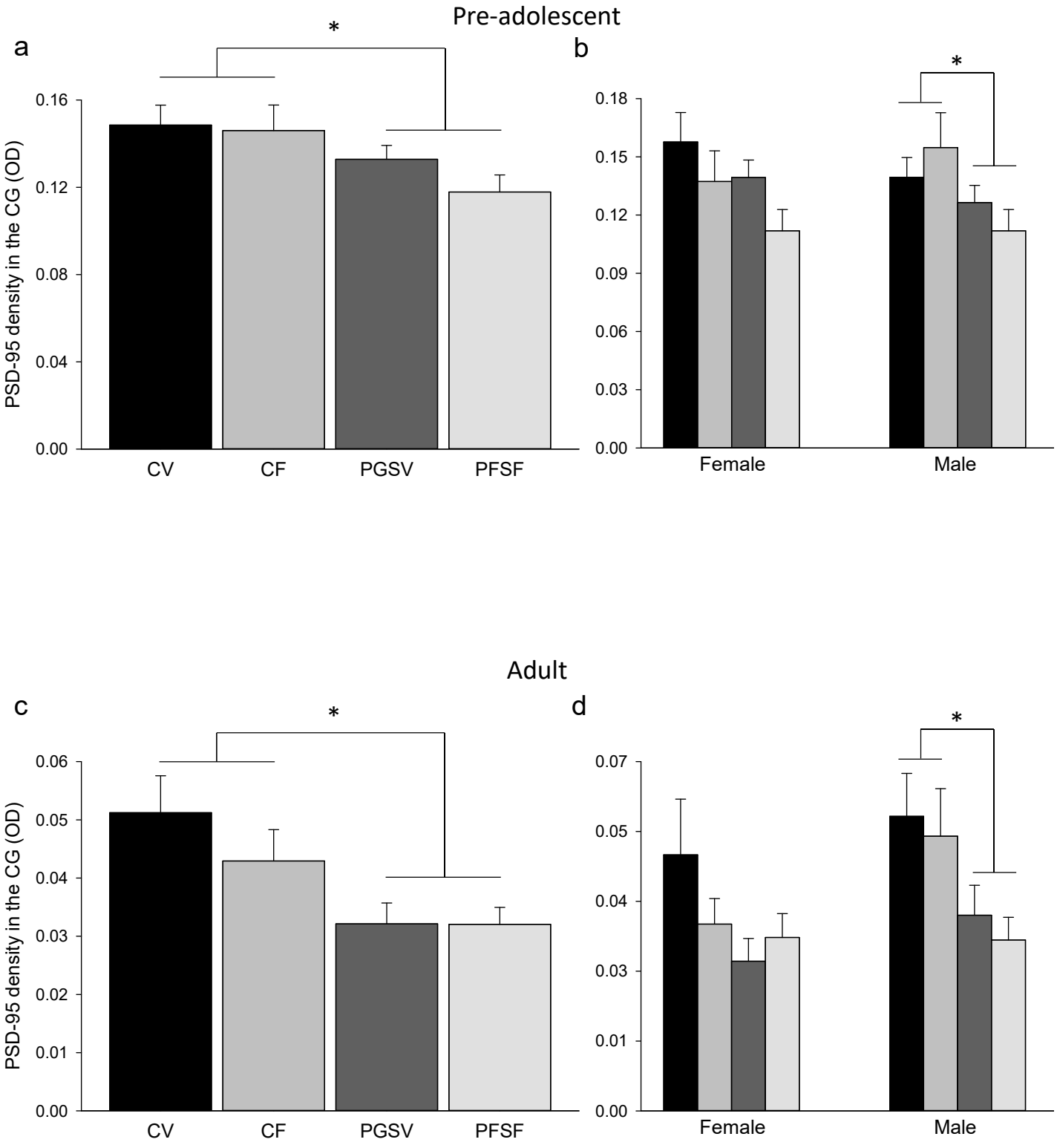

Pre-adolescent
a
b
*
*
PSD-95 density in the CG (OD)
0.16
0.12
0.08
0.04
0.00
0.18
0.15
0.12
0.09
0.06
0.03
0.00
CV
CF
PGSV
PFSF
Female
Male
Adult
c
d
*
*
PSD-95 density in the CG (OD)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.07
0.05
0.04
0.03
0.01
0.00
CV
CF
PGSV
PFSF
Female
Male

Figure 5

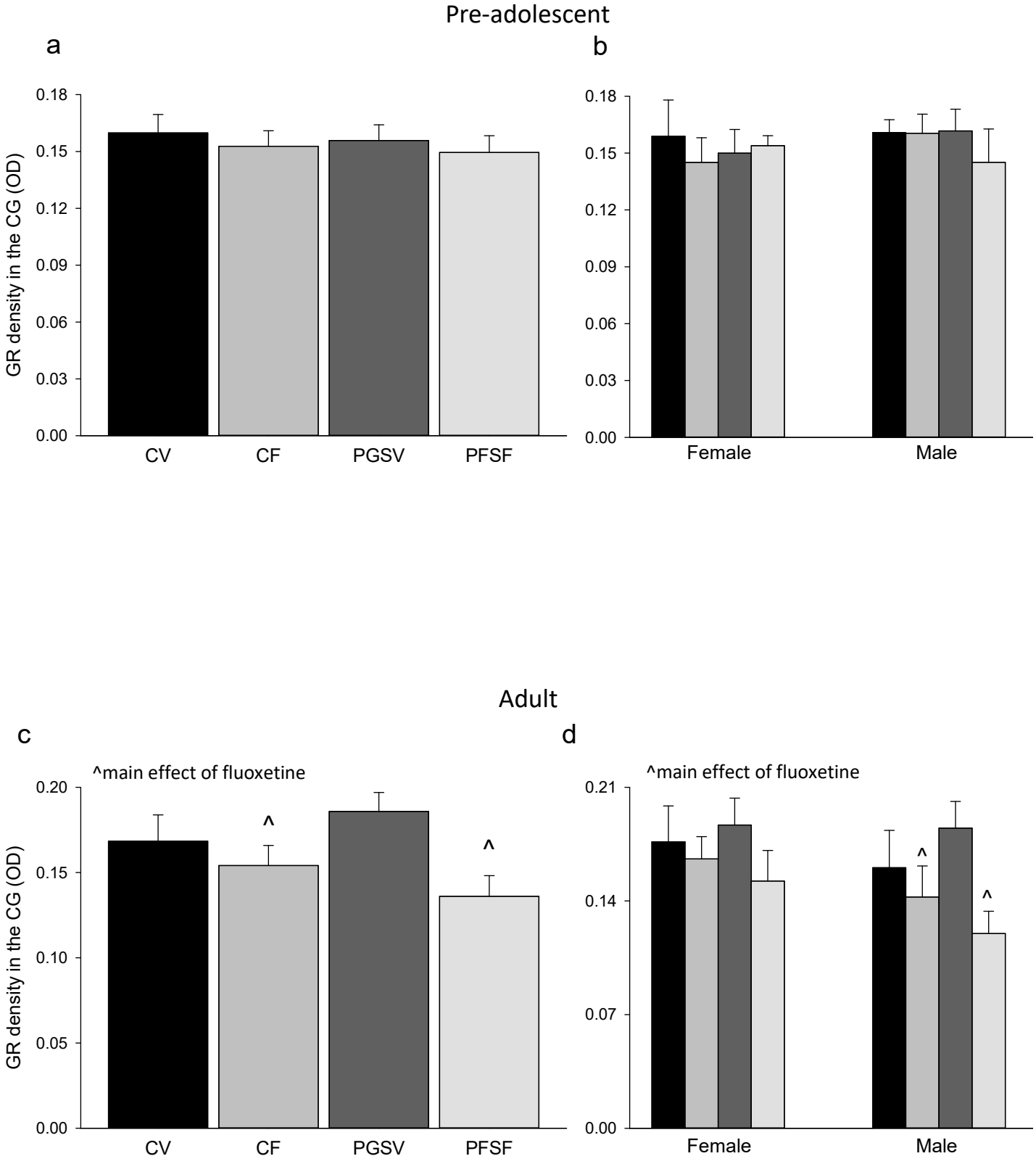
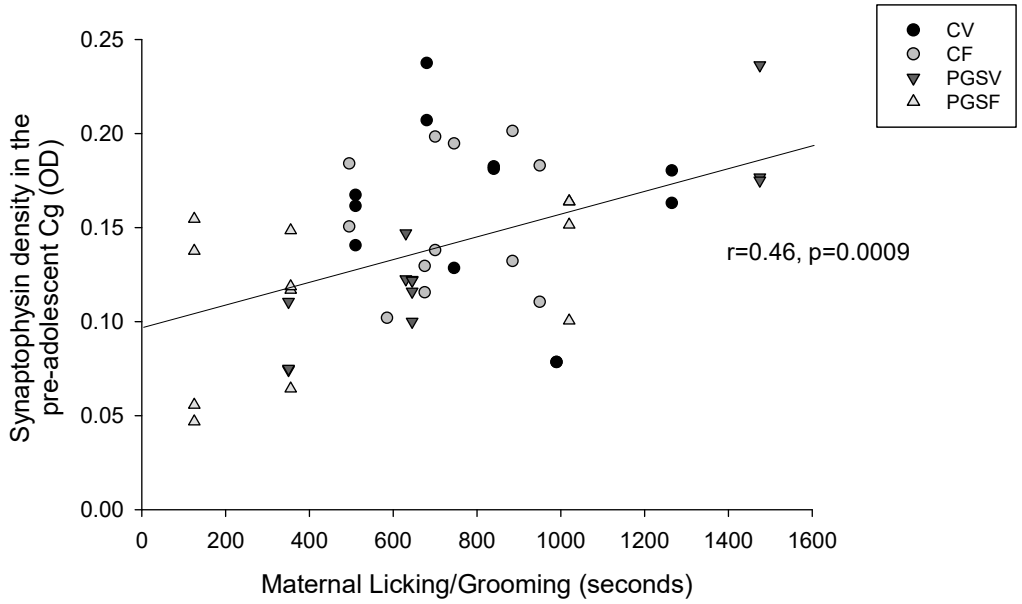
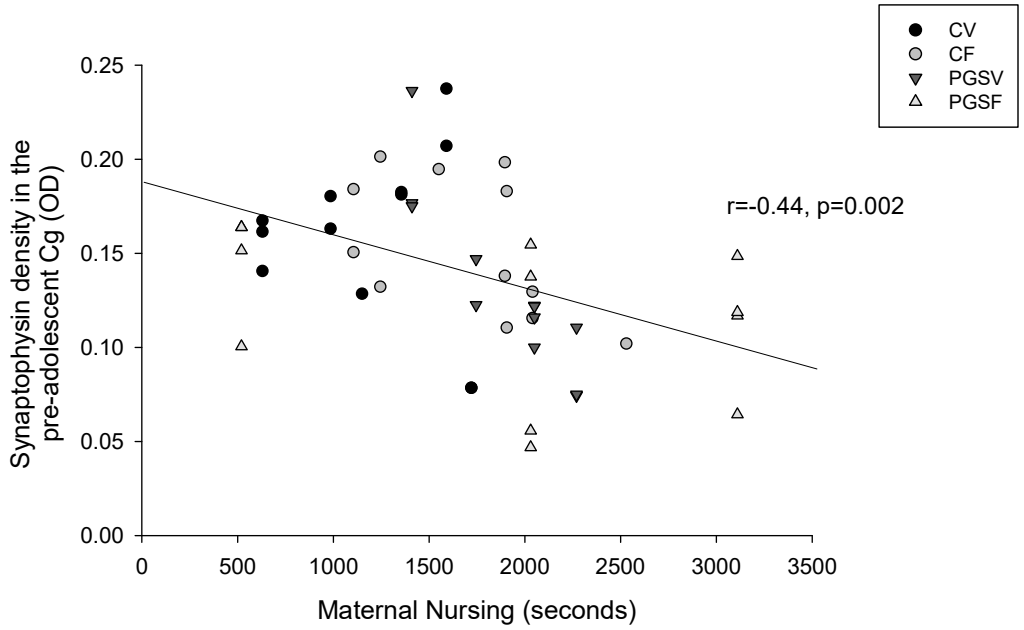

Pre-adolescent
a
b
GR density in the CG (OD)
0.18
0.15
0.12
0.09
0.06
0.03
0.00
CV
CF
PGSV
PFSF
Female
Male
Adult
c
d
^main effect of fluoxetine
^main effect of fluoxetine
0.20
0.15
0.10
0.05
0.00
0.21
0.14
0.07
^

Figure 6

a


CV
CF
PGSV
PGSF
r=0.46, p=0.0009
Synaptophysin density in the pre-adolescent Cg (OD)
Maternal Licking/Grooming (seconds)
0.00
0.05
0.10
0.15
0.20
0.25
0
200
400
600
800
1000
1200
1400
1600

b


CV
CF
PGSV
PGSF
r=-0.44, p=0.002
Synaptophysin density in the pre-adolescent Cg (OD)
Maternal Nursing (seconds)
0.00
0.05
0.10
0.15
0.20
0.25
0
500
1000
1500
2000
2500
3000
3500

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Highlights

- Perinatal SSRIs prevent the effects of maternal stress on preadolescent 5-HT in the PFC
- Pre-gestational stress, but not perinatal SSRIs, reduce synaptic markers in offspring cingulate cortex
- Perinatal SSRIs reduce glucocorticoid receptor density in the cingulate cortex, particularly in males
- Perinatal SSRIs effects are altered by pre-gestational maternal stress, offspring age and sex/gender

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