HHS Public Access

Author manuscript

Eur Neuropsychopharmacol. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

Eur Neuropsychopharmacol. 2016 September ; 26(9): 1496–1506. doi:10.1016/j.euroneuro.2016.06.003.

Preventative treatment in an animal model of ADHD: Behavioral and biochemical effects of methylphenidate and its interactions with ovarian hormones in female rats

Jodi L. Lukkes[a,b], **Nadja Freund**[a,c], **Britta S. Thompson**[a], **Shirisha Meda**[a], and **Susan L. Andersen**[a,b,*]

[a]Laboratory for Developmental Neuropharmacology, McLean Hospital, USA

[b]Harvard Medical School, USA

[c]Department of Psychiatry and Psychotherapy, University of Tübingen, Tübingen, Germany

Abstract

Clinical and preclinical studies on attention deficit hyperactivity disorder (ADHD) show that juvenile males that are exposed to methylphenidate (MPH) show reduced risk for substance use later in life. In contrast, little is known about whether females have the same enduring treatment response to stimulants and how gonadal hormones influence their behavior later in life. Females received either a sham or 6-hydroxydopamine (6-OHDA) microinjection in the prefrontal cortex (PFC) at postnatal day (P)10. Subjects were then treated with Vehicle or MPH (2 mg/kg, p.o.) between P20–35 and tested during late adolescence/young adulthood (P60); half of these subjects underwent ovariectomy at P55 to determine hormonal influences. Females with 6-OHDA were depleted of PFC dopamine by 61% and demonstrated increased impulsive choice (delayed discounting) and preferences for cocaine-associated environments relative to control females. Both MPH and ovariectomy reduced impulsive choice and cocaine preferences in 6-OHDA females, but had no enduring effect in Sham females. Ovariectomy itself did not significantly affect impulsivity. Juvenile MPH interacted strongly with 6-OHDA to increase D4, D5, Alpha-1A, Alpha-2A, and 5-HT-1A mRNA receptor expression in the PFC. MPH alone effected D1 mRNA, while 6-OHDA increased BDNF; all markers were decreased by ovariectomy. Together, these data suggest that 6-OHDA changes in dopamine are not only relevant for ADHD-like behaviors, but their long-term modulation by treatment and the influence of cyclical differences in menstrual cycle.

*Corresponding author at: Laboratory for Developmental Neuropharmacology, McLean Hospital, 115 Mill Street, Belmont, MA 02478, USA. Tel.: 1-(617)-855-3211; fax: +1 617 855 3479. sandersen@mclean.harvard.edu (S.L. Andersen).

Contributors

Authors Lukkes and Andersen designed the study, wrote the protocol, and managed the literature searches and analyses. Author Andersen undertook the statistical analysis, and author Lukkes wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

Keywords

BDNF; Discounting; Dopamine; Estrogen; Impulsive choice; Norepinephrine

1. Introduction

The childhood disorder of attention deficit hyperactivity disorder (ADHD) has an average onset of seven years of age (Merikangas et al., 2010). Symptoms of ADHD include increased impulsivity, elevated novelty seeking, and insensitivity to changes in reward contingencies (Sonuga-Barke, 2003). These behaviors are risk factors for substance use disorder (SUD), which is elevated in individuals with ADHD (Kollins, 2003). The risk for SUD is two-fold higher in females with ADHD compared with males with ADHD or typical females (Biederman et al., 2006). Pre-pubertal treatment with stimulants, including methylphenidate (MPH), does not increase and may actually reduce the risk of developing SUD in some ADHD cases (Mannuzza et al., 2008; Wilens et al., 2003). Preclinical studies parallel this clinical observation and show that exposure to MPH in male juvenile rats (P20-P35) reduces drug seeking (Andersen et al., 2002b; Warren et al., 2011).

Females are rarely examined in research on ADHD in humans (Nussbaum, 2012) or in animal models, including the spontaneously hypertensive rat (SHR (Somkuwar et al., 2013)), dopamine transporter knockouts (Gainetdinov et al., 1999), and rats with dopamine depletions using 6-hydroxydopamine (6-OHDA; (Davids et al., 2002; Freund et al., 2014). 6-hydroxydopamine depletions in immature, but not mature (Breese et al., 2005), animals increase ADHD-like behaviors of hyperactivity and impair working memory (Davids et al., 2002), but see (Boyce and Finlay, 2005). Localized prefrontal (PFC) 6-OHDA depletions during development (Freund et al., 2014) may better recapitulate observations of young adults with ADHD who have decreased DOPA decarboxylase in this region (Ernst et al., 1998). Early postnatal 6-OHDA depletions in juvenile female rats increased novelty seeking and impulsivity and D1 receptors on glutamatergic cells in the PFC (Freund et al., 2014), which has been associated with elevated cocaine self-administration.

The ADHD-like risk behaviors of impulsivity and increased sensitivity to cocaine-associated environments found following PFC 6-OHDA depletions (Freund et al., 2014) can aid in determining whether early MPH treatment increases (e.g., as we found in typically developing females (Brenhouse et al., 2009)) or decreases risk behaviors in older females. Ovarian hormones may also influence the expression of lasting treatment effects on behavior and on underlying mechanisms. Similar to females with ADHD (Van Voorhees et al., 2012), estrogen facilitates drug-seeking behavior in adult rats possibly by increasing dopamine levels in the brain (Becker, 1990; Russo et al., 2003).

The biochemical underpinnings of 6-OHDA, MPH, and the effects of ovariectomy (OVX) within a *developmental* context have received even less attention than typical females. This study focused on dopamine, noradrenergic, and 5-HT1A receptors given their role in ADHD-like behaviors and treatment approaches. Impulsive choice is modulated by dopaminergic (Fernando et al., 2012), serotonergic (Pattij and Vanderschuren, 2008; Winstanley et al., 2006), and noradrenergic (Chamberlain et al., 2007) signaling in the PFC.

MPH targets both dopaminergic and noradrenergic systems (Berridge et al., 2006) within the PFC to reduce impulsivity. Prior studies in non-lesioned male rats exposed to MPH as juveniles show an enduring decrease in D3 mRNA at P60 with no significant change in the other dopamine receptors (Andersen et al., 2008). An increase in brain-derived growth factor (BDNF) has also been observed in juvenile MPH-exposed males (Andersen and Sonntag, 2014; Simchon Tenenbaum et al., 2015). In contrast, BDNF decreased in adolescent SHR rats treated with MPH (Fumagalli et al., 2010); whether these differences are attributable to the model or age of exposure is not known. Regardless, little is known about females. Together, the current study may help fill the gap between clinical and preclinical knowledge about whether early medication exposure affects behavior and biochemistry under differing levels of gonadal hormones and PFC dopamine levels.

2. Experimental procedures

2.1. Subjects

Lactating female Sprague-Dawley rats and their litters obtained from Charles River (Worcester, MA) were housed on a 12:12 h light: dark cycle, with lights on at 06.00 h and food and water provided *ad libitum*. Litters with an n=10 pups and a 5/5 male/female ratio arrived on postnatal day 6 (P6) and were weaned and group-housed (4/cage) on P21. Only females were used (*N*=103), with one subject/litter in any individual condition. A total of 14 different litters were used, with extra females and males distributed to different studies. The timeline of the study is shown in Figure 1A. All animals were treated in accordance with the policies established by NIH and the McLean Hospital Institutional Animal Care and Use Committee.

2.2. Surgeries

2.2.1. 6-OHDA lesions—On P10, female rats were pretreated with desipramine (20 mg/ml in a 25 μl injection) to protect noradrenergic terminals. Subjects were anesthetized by hypothermia and bilaterally injected with 0.5 μg in 0.5 μl of either 6-hydroxydopamine (6-OHDA) or 0.9% saline into the plPFC (AP: +2.8, ML: ±0.5, DV: 2.6; following our previous methods (Freund et al., 2014)).

2.2.2. Ovariectomy (OVX)—On P55, all females that were involved in behavioral studies were injected with a ketamine/xylazine mixture (80/12 mg/kg) and underwent either sham or OVX surgery (Andersen et al., 2002a). Briefly, subjects were anesthetized with ketamine/xylazine anesthesia, and under aseptic conditions, an incision was made either at the midline. The ovaries were removed and oviduct tied off. The main incision was sutured with continuous absorbable sutures.

2.2.3. Drugs—d,l-Methylphenidate HCl (MPH) and cocaine HCl were obtained from Sigma (St Louis, MO). Each drug was dissolved in 0.9% saline (Vehicle, Veh) in a volume of 1 ml/kg.

Experiment 1. Enduring effects of MPH and ovarian hormones on delay discounting and place conditioning

Females ingested MPH (2 mg/kg) or Veh via Frootloop (General Mills) twice daily between P20–35 (Brenhouse et al., 2009). This MPH dose was based on previous preclinical studies and represents a clinically relevant dose for humans (Andersen et al., 2002b; Brenhouse et al., 2009).

2.2.4. Delay discounting—At P57, females (n=8–9/group) were food-deprived to 90% of free-feeding weight and began training at P60 to measure changes in impulsive choice in an operant chamber with a Bussey-Saksida Touch Screen (Lafayette Instruments, Lafayette, IN). Subjects underwent four phases of training to assure that each phase was learned sufficiently before the next phase following our previous protocols (Lukkes et al., 2015). Phase 1 trained subjects to initiate each session with a nose poke in the food hopper, which produced a square on one side of the touch screen. Poking the square produced one food pellet (45 mg, Dustless Precision Pellets, Bio-Serv, French-town, NJ) and subjects needed to reach a criterion of 60 rewards in 90 trials to move to Phase 2. Phase 2, initiated by a magazine nose poke, now produced two symbols on the screen that were counterbalanced across, but not within, subjects. One symbol was associated with the delivery of an immediate reward and the other with a delayed reward that was used throughout the rest of the experiment. Criterion was responding 60 of 90 trials to the delay square for two consecutive days to index learning. Phase 3 trained subjects to discriminate between larger (4 pellets) vs. smaller (1 pellet) rewards. Criterion was set at 45 responses out of 50 for the square that was the assigned delay square for two consecutive days. Phase 4 presented a block of delays comprised of 12 trials of 0,10, 20, 40 and 60 seconds in succession. The presentation of one square at the beginning of a block signaled a change in delay condition, and was followed by two forced choice trials. After a nose poke and the delivery of the reinforcer, the magazine light extinguished for 100 seconds and the next trial began with the presentation of the symbols. Data from the last 10 trials at each delay were averaged across three consecutive days or until the within-subject factor of “day” was not significant. Most subjects stabilized within 3–4 days on Phase 4.

2.2.5. Place conditioning—Following delay discounting, the same subjects in the four groups (n=8–9/group; Veh/intact; Veh/OVX; MPH/intact; MPH/OVX) were tested at P82 for unbiased place conditioning to cocaine (10 mg/kg) according to the methods of (Andersen et al., 2002b). Ten mg/kg is a threshold dose of cocaine that we have repeatedly used for determining enduring MPH effects (Andersen et al., 2002b; Brenhouse et al., 2009; Carlezon et al., 2003). The place conditioning chambers had compartments that differed in color, floor texture, and lighting and were separated by a middle compartment (Med Associates, St. Albans, VT). Screening occurred for 30 min on Day 1 and subjects that showed a bias (4 1080 seconds spent on one side) were eliminated from the experiment. During two 60 min conditioning sessions on Days 2 and 3, rats received a 1 ml/kg i.p. injection of Veh in the morning (09.00 h) and were confined to one side and four hours later, received 10 mg/kg i.p. cocaine and confined to the other side in the afternoon to avoid any possible carryover effects of cocaine. On Day 4, rats freely explored the entire apparatus for 30 min in a drug-free state. Place conditioning scores were determined as the difference of the time spent on the drug side - time spent on the saline side.

2.2.6. Dopamine and Norepinephrine enzyme-linked immunoassay (ELISA)— Ninety minutes following the onset of day 4 of CPP, subjects were rapidly decaped, brains collected, and the plPFC microdissected and then stored at −80 °C until assayed for DA and NE levels. Tissue was homogenized in 0.01 N HCl in the presence of EDTA and sodium metabisulfite. Measurement of plPFC, ilPFC, accumbens, and striatal DA and NE levels were performed using a dopamine and norepiphrine enzyme linked immunoassay kit (2-CAT [N-D] Research ELISA; Rocky Mountain Diagnostics, CO). Samples were run in duplicate and 100 µl of the extracted homogenate was used for each sample. All samples were processed according to the manufacturer's directions.

Experiment 2. Quantitative, real time-polymerase chain reaction (qRT-PCR)

A separate set of Sham/6-OHDA females exposed to Veh or MPH were used and staged into high or low estrous states based on vaginal cytology and sacrificed. Monoamine receptor and BDNF mRNA levels were measured using qRT-PCR as previously described (Andersen et al., 2008).

2.2.7. qRT-PCR—For qRT-PCR analyses, subjects were dichotomized into high and low hormonal states based on vaginal cytology at the time of sacrifice. Based on (Emanuele et al., 2003) low levels of estrogen are found during meta- and di-estrous phases of the cycle and high estrogen levels are found during pro- and estrous phases of the cycle. Briefly, the plPFC was the main target of dissection for this analysis, and HPLC analysis confirmed that the ilPFC demonstrated significant depletion effects as well (the implications are discussed below). The total mRNA from the plPFC (n=6/group) was prepared, processed to cDNA (50 ng/ µl mRNA total mRNA equivalent), and analyzed with qRT-PCR using the IQ SYBR Green SuperMix (BioRad, Hercules, CA). Primer sequences were based on published methods and are given in Table 1 for GAPDH, $BDNF_{total}$, D1 to D5 (Andersen et al., 2008), Alpha-1A, Alpha-2A (Sun et al., 2012), and 5-HT1A ((Perez-Garcia et al., 2006). Gene expressions were normalized to the housekeeping gene product glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and plotted as a fold change relative to Sham intact Veh females.

2.3. Statistical analysis

Between-subject ANOVAs (SPSS v. 22) were used to analyze the delay discounting data from Phase 4 with depletion (2: Sham/6-OHDA), treatment (2: Veh/MPH), and hormones (2: intact/OVX) were used as between-subjects measures and delay (5) and day (3) as within-subject variables. The criterion for sensitivity to delay was when delay was significant or significantly interacted with the other between-subjects variables (Mar and Robbins, 2007). All subjects displayed such a sensitivity to delay and no effect of day was present in any group. An alternative way of capturing impulsivity is to determine the average of the number of large reinforcers received across three days of testing. While similar to "K" the indifference point (Mazur, 2007), the DDT_{50} is the delay period when 50% of the large reinforcers are received (Lukkes et al., 2015). Place conditioning data was analyzed similarly: depletion (2: Sham/ 6-OHDA), treatment (2: Veh/MPH), and hormones (2: intact/ OVX) were used as between-subjects measures and pre- and post-conditioning as a within-

subject variable. Post-hoc analyses were corrected with Bonferroni and qRT-PCR. Significance was set at P<0.05. Correlational analyses with a two-tailed Pearson's r test were conducted to determine the relationship between discounting and place conditioning scores.

3. Results

Experiment 1. Enduring effects of MPH and ovarian hormones on delay discounting and place conditioning

3.1. Depletion confirmation

Dopamine and noradrenergic levels were characterized by ELISA and revealed that dopamine in Veh subjects was: 6-OHDA: 0.12 ± 0.02 vs Sham: 0.21 ± 0.07 ng/mg wet plPFC tissue weight, similar to other reports (Boyce and Finlay 2005); depletion was also evident in the ilPFC: 6-OHDA: 0.09 ± 0.02 vs VEH: 0.38 ± 0.14 ng/mg wet tissue weight. Norepinephrine levels remained stable following 6-OHDA lesions in the plPFC (Figure 1B). The specificity of 6-OHDA to the plPFC is evidenced by the absence of effects in downstream regions of the striatum and nucleus accumbens (Figure 1B).

3.2. Delay discounting and place conditioning in the model

To establish whether the behavioral effects of neonatal PFC 6-OHDA depletions endure into adulthood (Freund et al. [2014] tested juveniles), comparisons were made between Sham Veh and 6-OHDA Veh rats for delay discounting and place conditioning behavior. Learning to discriminate larger versus smaller reinforcers in Phase 3 of the discounting task was faster in *6–4* OHDA Veh females (3.9 ± 0.9 days to reach criterion) compared to Sham Veh females (8.2 ± 0.9 days; $P<0.005$), suggesting that 6-OHDA did not produce learning or motivational deficits. In testing Phase 4, developmental 6-OHDA depletions increased impulsive choice compared to Sham control subjects (Figure 1C). Main effects of depletion ($F1,11 = 8.28$, $P<0.05$) and [delay] ($F4,44=12.82$ $P<0.001$) indicated that 6-OHDA increased impulsive choice; [day] was not significant. Only 6-OHDA subjects demonstrated a significant place preference for cocaine-associated environments and not Sham subjects (main effect of depletion: $F1,12=4.79$, $P<0.05$) that was driven by the post-conditioning phase (Bonferroni post-hoc comparison, $P<0.05$; Figure 1D).

3.3. Effects of MPH and OVX on delay discounting

The interactive effects of juvenile MPH exposure and hormones on delay discounting were tested with a five-way mixed ANOVA of 2 (hormones) × 2 (treatment) × 2 (depletion) × [delay] × [day], which was not significant. A depletion × treatment interaction: ($F1,47=4.94$, $P<0.05$) and main effects of hormones ($F1,47=5.34$, $P<0.05$), depletion ($F1,47=6.1$, $P<0.05$), and [delay] ($F4,188=38.8$, $P<0.001$) were observed. Both 6-OHDA and OVX increased discounting overall, although treatment decreased discounting in the 6-OHDA animals and not the Sham controls. A main effect of [delay] indicated that subjects decreased responding to the large reinforcer as the duration of the delay grew. To better understand whether the enduring effects of juvenile MPH treatment and hormones on impulsive choice depended on

the animal model, subsequent analyses were divided into 6-OHDA (Figure 2A and C) and Sham (Figure 2B and D) groups.

Within 6-OHDA subjects, a significant main effect of treatment was observed ($F_{1,28}=6.93$, $P<0.05$). MPH increased responding for the large reinforcer in both intact and OVX 6-OHDA rats overall (Figure 2A and C). A significant main effect of [delay]: ($F_{4,112}=24.7$, $P<0.001$) indicated that subjects selected fewer large reinforcers as the delay interval increased. Unlike 6-OHDA females, neither juvenile MPH exposure (treatment: $P=0.51$) nor OVX ($P=0.08$) effected discounting in Sham females (Figure 2B, D). However, a main effect of [delay]: ($F_{4,72}=15.87$, $P<0.001$) was observed in Sham females similar to 6-OHDA females.

3.4. Effects of MPH and OVX on place conditioning

A four-way mixed ANOVA of 2 (hormones) × 2 (treatment) × 2 (depletion) × [conditioning] was significant ($F_{1,49}=4.58$, $P<0.05$). The interaction between treatment × depletion × [conditioning] was also significant ($F_{1,49}=4.53$, $P<0.05$). Conditioning overall exerted a significant main effect ($F_{1,49}=13.41$, $P<0.001$), and pre-conditioning did not interact with any of these factors as this was a non-biased paradigm (all P's>0.4). To better understand how 6-OHDA influenced place conditioning, analyses were divided into 6-OHDA and Sham groups.

In the 6-OHDA depletion group, a three-way significant interaction (hormones × treatment × [conditioning]: $F_{1,29} = 6.18$, $P<0.05$) was observed, demonstrating that MPH reduced place preferences in intact 6-OHDA subjects (Figure 3A,B). OVX reduced place preferences in both Veh and MPH treated 6-OHDA subjects to similar levels as those found in intact, MPH treated 6-OHDA females. In contrast, Sham subjects treated with MPH demonstrated significant place preferences ([conditioning]: $F_{1,20} = 5.84$, $P<0.05$) with no other significant interactions or main effects (Figure 3B).

3.5. Inter-relationship between delay discounting and place conditioning

We investigated the enduring effects of juvenile MPH exposure by examining the delay discounting point where the subject selected 50% of the large reinforcers (DDT_{50}). This DDT_{50} was based on the non-linear fit of the relationship between delay and the number of large reinforcers received for each subject (Lukkes et al., 2015). A hormones × treatment × depletion ANOVA revealed a main effect of MPH treatment ($F_{1,47}=4.62$, $P<0.05$) on the DDT_{50}, independent of hormones or depletion (Figure 3C), suggesting that MPH decreased impulsivity (an elevated DDT_{50}).

Correlations between the amount of impulsive choice (represented by DDT_{50}) and place conditioning scores indicated a significant negative correlation in the intact 6-OHDAVehrats ($r=-0.847$, $P<0.05$; Figure 3D); these data suggest greater impulsivity (lower DDT_{50}) was associated with greater cocaine preferences in the 6-OHDA model. MPH disrupted this relationship in intact 6-OHDA rats. Correlations in the Sham females were not significant (both P's>0.25).

Experiment 2. Quantitative, real time-polymerase chain reaction (qRT-PCR)

An overall hormones × treatment × depletion interaction was not observed for any of the receptors studied (P's 0.3–0.8). However, hormones × depletion interactions were observed for all receptors studied (all P's ≤ 0.03, even following Bonferroni correction for multiple comparisons). With minor exception, 6-OHDA reduced mRNA levels for all receptors. Within the 6-OHDA group, treatment with MPH increased mRNA levels for D4, D5, Adra-1A, Adra-2A, 5-HT-1A, and BDNF. Treatment alone had no significant effect unless dopamine levels were altered either by 6-OHDA or by hormones. Higher levels of hormones increased mRNA levels in 6-OHDA females, but decreased mRNA levels in Sham females. Fold-change in mRNA expression relative to Sham intact rats and P values for all statistical analyses are presented in Table 2.

4. Discussion

Early developmental reductions of PFC dopamine by 6-OHDA produced enduring increases in impulsivity and place preferences for cocaine-associated environments, and altered PFC mRNA receptor expression relative to Sham rats. Such behavioral increases are consistent with behavioral symptoms associated with ADHD, which include impaired discounting and increased risk for SUD that are related to hypofunction in PFC regions (Sonuga-Barke, 2003). Moreover, our study is the first to show that juvenile MPH treatment permanently reduced these behaviors in adult 6-OHDA females, in line with clinical findings of reduced substance use following treatment (Klassen et al., 2012). Reduced place preferences in MPH treated 6-OHDA rats are opposite to our previous findings in MPH treated typical adolescent rats (Brenhouse et al., 2009) or Sham rats in the current study, suggesting that treatment studies (at least in females) maybe more appropriate in subjects demonstrating an ADHD-like phenotype. The observation that MPH effectiveness depends on baseline dopamine levels is consistent with clinical observations (Volkow et al., 2005). Low PFC dopamine also led to an increase in the saliency of cocaine-associated environments. The more impulsive a female was, the more preference she had for cocaine-associated cues, but only in the 6-OHDA subjects. Juvenile MPH exposure disrupted the relationship between impulsive choice and cocaine preferences, consistent with clinical findings of reduced SUD in individual treated before puberty (Mannuzza et al., 2008). This increased sensitivity to the rewarding effects of cocaine in 6-OHDA rats did not manifest until later in life (this study) as our juvenile characterizations of 6-OHDA lesions produced no increase in cocaine place conditioning (Freund et al., 2014). SUD and its modulation by MPH does not manifest until adolescence, in concert with typical rising rates of drug use by teenagers (Wilens et al., 2003).

Notwithstanding, another major contribution of this study is the demonstration that the enduring effects of MPH interact with gonadal hormones to alter behavior and receptors. Ovarian hormones, especially estrogen, have pro-dopaminergic effects (Becker et al., 1984) that could reduce impulsivity much like MPH if the conditions were right. Hypotheses about how MPH reduces ADHD symptoms involve both modulation of autoreceptor activity to modulate dopamine overflow and a change in the signal-to-noise ratio of corticostriatal afferents (Volkow et al., 2005). Methylphenidate increases tonic dopamine levels that dampen noise while amplifying the phasic signal of corticostriatal afferents (Grace, 2000). When dopamine levels are low in 6-OHDA females, OVX further increased impulsivity

(P=0.08) and attenuated place preferences for cocaine. Under these lower dopamine conditions, the enduring effects of MPH are even more effective (e.g., increasing the DDT_{50}; reducing place preferences). Together, developmental 6-OHDA depletions in females increase ADHD-like behaviors into adulthood that are sensitive to early intervention with MPH and manipulation of ovarian hormones levels.

Previous studies show that developmental 6-OHDA manipulations often produce downstream effects that are generally the opposite of adult 6-OHDA depletions. For example, global 6-OHDA depletions in adulthood result in motor impairment (Breese et al., 1987). In contrast, global 6-OHDA dopamine depletions in juveniles increase other ADHD-like behaviors of hyperactivity and working memory deficits (Davids et al., 2002). More localized developmental 6-OHDA depletions in the PFC increase activity in males (but see (Boyce and Finlay, 2005)), but does not alter activity in females, and reduces drug nicotine self-administration later in life in females relative to controls (Rezvani et al., 2008). The current study shows that PFC 6-OHDA permanently increases impulsive choice that was initially observed in juvenile females (Freund et al., 2014). These results are similar to direct 6-OHDA lesions into the plPFC in adulthood, which reduce outcome evaluation in goal-directed responding (Balleine and Dickinson, 1998; Lex and Hauber, 2010). While direct depletion of the region of interest in adulthood is well-suited for testing the role of dopamine, develop-mentally, lesions of one region are likely to have subsequent effects on other brain regions (Pycock et al., 1980). The apparent lack of specificity of 6-OHDA that also reduced dopamine in the ilPFC may have to do with the spreading of developmental lesions (Teicher et al., 1998). Our previous study targeted and ‘hit’ the plPFC with 6-OHDA selectively (Freund et al., 2014). However, lesions with 6-OHDA are not always straight-forward. Rather, depletions that were performed under the identical situations can range from no depletion to a significant loss of dopamine. We have previously observed that an initial 6-OHDA lesion in the striatum progressively spread into a loss of dopamine in the nucleus accumbens with further maturation (Teicher et al., 1998). The same phenomenon may have occurred here with spreading from the plPFC to the ilPFC. Whether both plPFC and ilPFC are involved in these behaviors cannot be ruled out, although evidence shows plPFC involvement in delay discounting (Loos et al., 2009; Sonntag et al., 2014). However, all of the regional dissections for mRNA analysis were from plPFC. Other research models of ADHD-like behaviors have a delayed onset of behavioral impairment during adolescence (e.g., (Somkuwar et al., 2013)), whereas ADHD typically appears during childhood.

Neonatal 6-OHDA dopamine depletions affect more than just dopamine (Broaddus and Bennett, 1990), and changes have been found in glutamate (de Azeredo et al., 2010) and GABA receptors (Podkletnova et al., 2000). Our findings significantly expand this literature by further showing how PFC 6-OHDA depletions strongly interact with early treatment and later hormone levels to modulate receptor mRNA. Depletion with 6-OHDA significantly affected 5-HT-1A receptors, which also interacted with both treatment and hormone levels. The role of 5-HT-1A is consistent with previous findings of their role in impulsivity (Winstanley et al., 2003). The interaction of 6-OHDA with hormonal status increased D3 mRNA at a trend level in the high ovarian hormone state, whereas juvenile MPH exposure in typical, non-lesioned male rats reduced PFC D3 mRNA (Andersen and Sonntag, 2014). These findings indicate that dopamine levels are integral for varying levels of expression. 6-

OHDA depletions also interacted with juvenile MPH to increase D4, D5, Alpha-1A, Alpha-2A, 5-HT-1A, and BDNF mRNA relative to Sham controls also exposed to MPH. These increases following MPH in 6-OHDA females under high hormone conditions implicate these receptors in decreased impulsivity and place conditioning in 6-OHDA females. MPH-induced increases in D4, D5, and Alpha1A in 6-OHDA subjects may work by enhancing GABAergic activity. The D4 polymorphism reduces the inhibitory function and has been associated with increased risk taking behavior (LaHoste et al., 1996). An increase in D4 following MPH could facilitate GABA functions in the PFC (Zhong and Yan, 2014), similar to D5, which is localized on PFC pyramidal neurons, but is also found on ~75% of parvalbumin-immunopositive GABA inter-neurons (Oda et al., 2010). Increased Alpha-2A may increase cortical excitability by disinhibition (Andrews and Lavin, 2006). Changes in Alpha-1A could reduce place preferences, although previous research in adult animals shows no effect of Alpha-1A formation of a place preference (reviewed in (Schmidt and Weinshenker, 2014)).

Juvenile versus adult MPH exposure produces opposite effects in typical animals in BDNF (Andersen, 2005), although 6-OHDA and hormonal state alters that relationship. Reduced PFC BDNF is associated with greater cocaine-seeking and reinstatement (reviewed in (Barker et al., 2015)), which is consistent with increased place preferences in our 6-OHDA females. Similarly, increased BDNF in the plPFC decreases drug-taking behavior in adult male rats (Berglind et al., 2007); juvenile MPH in 6-OHDA females increased BDNF and reduced cocaine place preferences. Overall, little work has examined the three way interaction of MPH (or cocaine) × BDNF × estrogen. Treatment with 17β-estradiol in intact adult females facilitates extinction of cocaine taking behavior (Larson and Carroll, 2007). Ours is the first study to show that elevated ovarian hormones may work through increased BDNF to reduce drug seeking. Indeed, the gene for BDNF contains an estrogen response element providing a mechanism where estrogen can easily increase BDNF (Harte-Hargrove et al., 2013). Here, both MPH treatment and 6-OHDA interacted with elevated ovarian hormones to increase BDNF levels that reduced impulsivity and cocaine preferences.

Changes in D1 mRNA were evident in response to MPH, regardless of depletion state. High delay discounting is found in mice with elevated PFC D1/D5 receptors (Loos et al., 2009) or following viral-mediated overexpression of D1 on plPFC glutamatergic neurons (Sonntag et al., 2014). Prefrontal cortex 6-OHDA depletions reduced D1 mRNA and increased discounting relative to Shams. Our previous study in early 6-OHDA depleted *juvenile* females localized an increase in D1 to plPFC projection neurons to the accumbens (Freund et al., 2014). However, D1 mRNA from tissue cannot specifically localize these D1 receptors to projection neurons. The observation that MPH increased D1 and reduced discounting raises the possibility that these D1 receptors may be on non-projection neurons.

Finally, assessment of ovarian hormones by estrous cycle staging fully illustrates how different hormone states not only influence behavior as discussed above, but also receptor mRNA and the expression of treatment effect. Developmental reductions in PFC dopamine levels minimized the effect of MPH under low ovarian hormone conditions overall, consistent with place preferences following OVX in females (Russo et al., 2003). In the current study, we used 10 mg/kg dose of cocaine as a threshold dose and did not observe a

significant place preference in our Sham-operated females. In non-lesioned females (with no PFC manipulation), others have found significant, but rather modest, place preferences to 10 mg/kg cocaine (Crawford et al., 2011) or less. For example, pre/post conditioning difference of ~250 sec was reported in one study (Bobzean et al., 2014) and a 37 s difference from a hypothesized difference of 0 at baseline reported in another study (Grotewold et al., 2014). However, this dose highlighted the increased sensitivity to cocaine-associations in the 6-OHDA females.

Depletion with 6-OHDA facilitated MPH-induced receptor mRNA expression changes when ovarian hormone levels were high. Previous studies found that estrogen increases dopamine release in the striatum of adult females by decreasing the affinity of the dopamine transporter (Becker, 1990) and dopamine receptor expression *in vitro*. Estrogen has neuroprotective effects also against dopamine cell loss (Baquet et al., 2005). An additional possible mechanism could involve estrogenic effects on GABA neurons. Increases in estrogen produce a decrease in GABA transmission (Almey et al., 2016), which could also explain the findings hormones × treatment interaction in the Sham females. In the intact females, estrogen would decrease GABA, and treatment reverses the effects via the receptors as discussed above. Estrogen manipulations have no apparent effect on PFC 5-HT-1A mRNA in the PFC (Landry and Di Paolo, 2003), although 6-OHDA may have unmasked a subtle influence. D1 and D5 dopamine receptors were also elevated in high ovarian hormone states, as shown previously (Frye and Vongher, 1999). Juvenile MPH in 6-OHDA females increased D1, D5, and Alpha-2A receptor mRNA expression during high ovarian hormone levels. While estrogen has received the most attention, progesterone reduces drug craving (Fox et al., 2013) and may also be involved. As the menstrual cycle likely influences acute treatment response, our data suggest the stage of cycle will also influence enduring treatment effects.

The results of this study demonstrate the importance of dopamine levels for the effectiveness of MPH on behavior in female rats. Early postnatal 6-OHDA lesions localized to the PFC resulted in low dopamine levels later in life. Depleted females demonstrated increased delay discounting and a greater sensitivity to cocaine-associated environments relative to Sham control females. These effects were reduced by juvenile MPH exposure, but not in Sham females. Relevant changes in dopamine, serotonin, and noradrenergic receptors also reflect both MPH and ovarian hormone levels. Future studies from our lab will determine the location of which cortical neuronal phenotype these receptor changes occur to better understand these ADHD-relevant findings in females.

Acknowledgments

The authors acknowledge the expert pipetting skills of Dr. Kai Sonntag.

Role of the funding source

Funding for this study was provided by NIDA Grant DA-015403 and MH-091114; Neither NIDA no NIMH had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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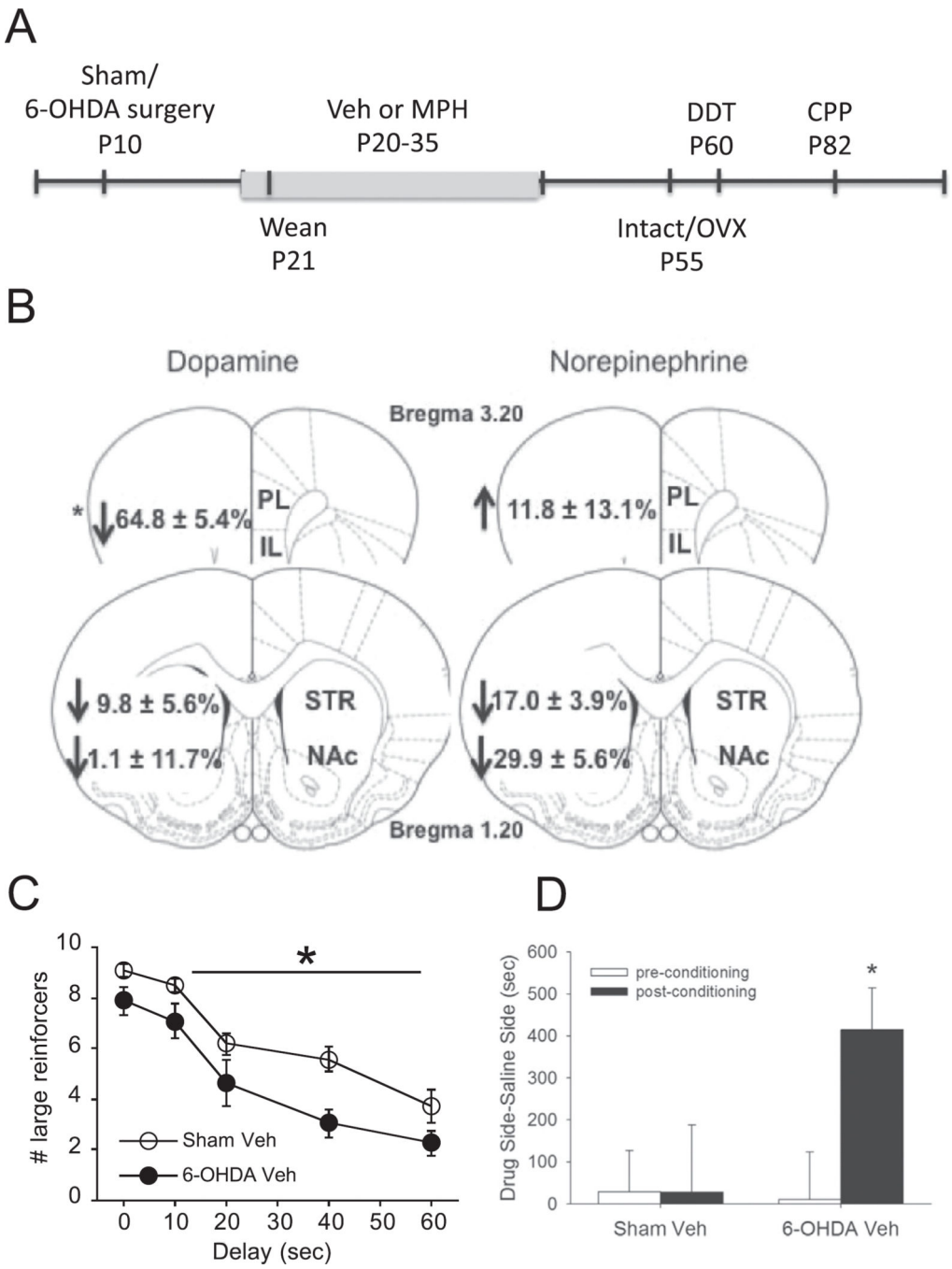

Figure 1.

(A) Timeline illustrating experimental design. (B) Percent change in DA and NE levels of 6-OHDA females relative to Shams in the prelimbic (PL) and infralimbic (IL) PFC (combined), nucleus accumbens (NAc), and striatum (STR). C left) Enduring effects of 6-OHDA plPFC lesions in females on impulsive choice; * and the line indicate the delay periods when significant differences were observed between groups; and right) place conditioning on 6-OHDA and Sham females. Means ± SE presented. *P<0.05.

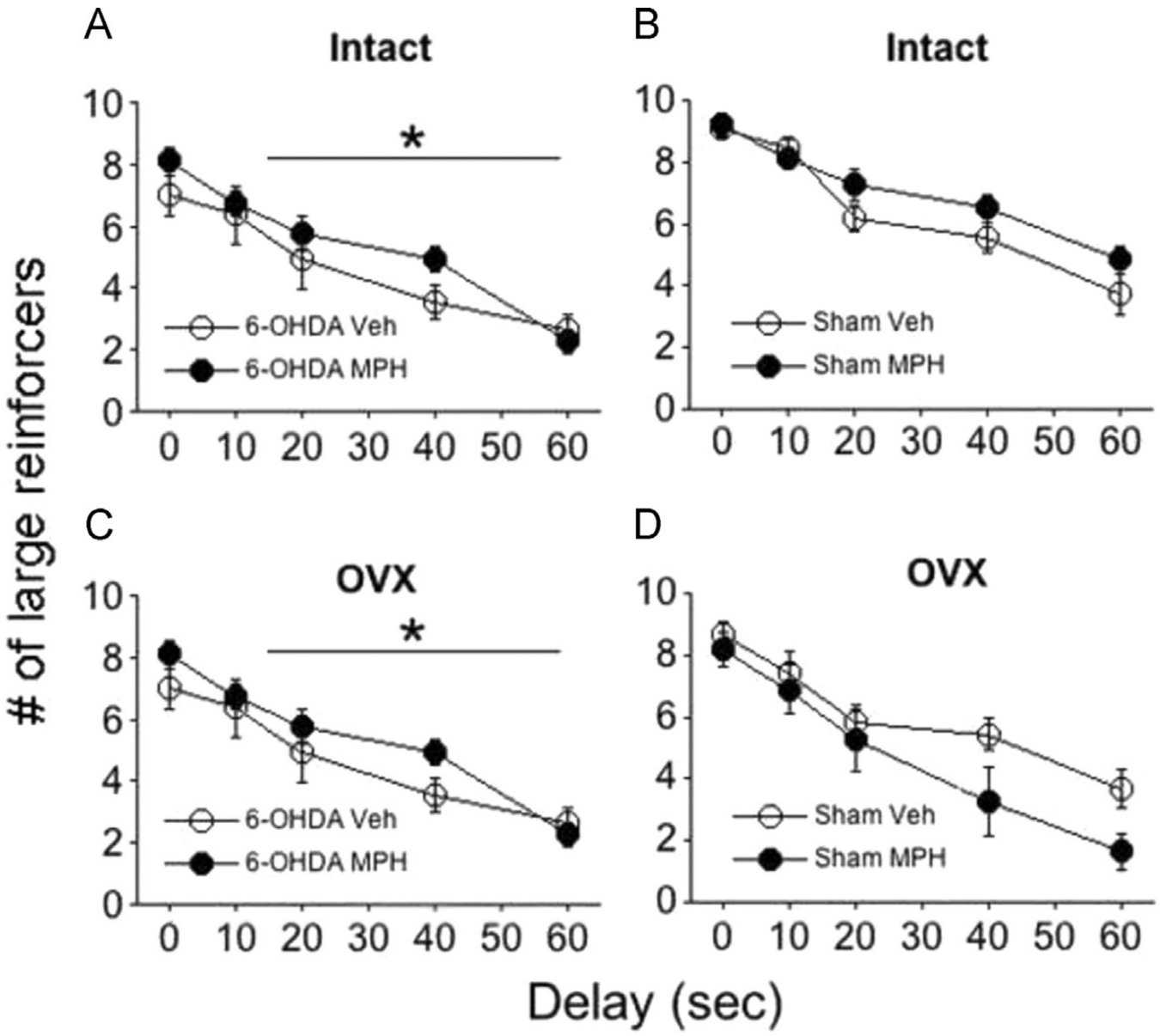

Figure 2.

Enduring effects of juvenile MPH on the number of large reinforcers during delay discounting in (A, C) intact or ovariectomized (OVX) 6-OHDA or (B, D) Sham females. The *line indicates an overall significant main effect of treatment between groups; Means ± SE presented. *P<0.05.

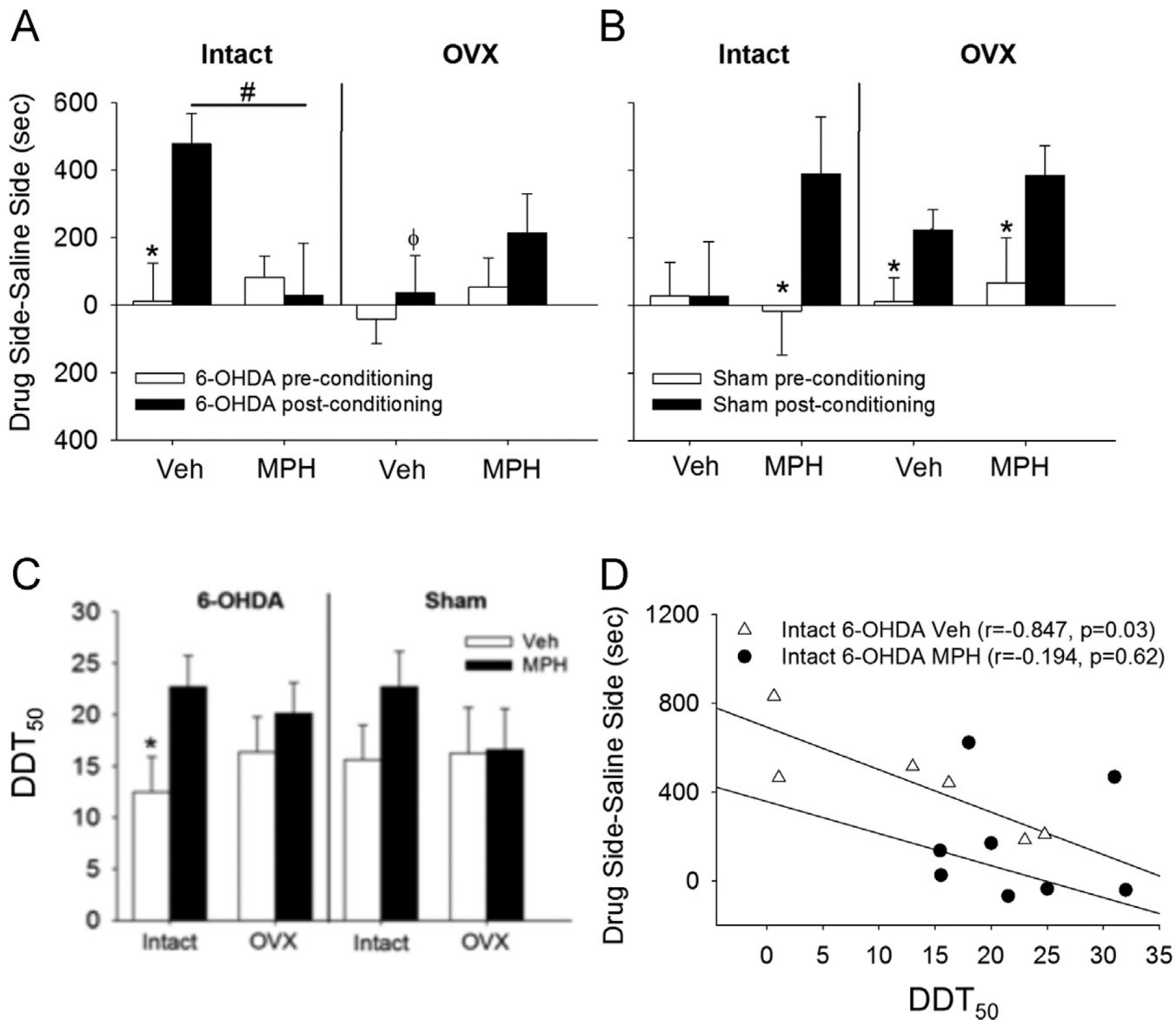

Figure 3.

Enduring effects of juvenile MPH on preferences for cocaine-associated environments in A) intact or OVX 6-OHDA or B) intact or OVX Sham females. Means ± SE presented. **P*<0.05 comparison between pre- and post-conditioning; #P<0.05 comparison of post-conditioning effects of Vehvs MPH in the intact condition; and ϕ*P*<0.05 comparison between intact and OVX inVeh6-OHDA for post-conditioning effects. C) The enduring effects of juvenile MPH on the indifference point for selecting large reinforcers in delay discounting in 6-OHDA and Sham females. *P<0.05 comparison between Vehicle and MPH of the intact 6-OHDA group; D) A negative correlation is found between the DDT_{50} of discounting and place preference in intact 6-OHDAVeh females (r= −0.847, P=0.03; open triangles). This correlation is absent when 6-OHDA females are exposed to MPH as juveniles (r= −0.194, P=0.62; closed circles).

Table 1

Primers used to characterize monoamine receptors, BDNF, and GAPDH.

D1-F:	5′-AGATGACCCCCAAAGCAG-3′	R:5’-ACGTCCTGCTCAACCTTG-3′
D2-F:	5′-CAGACCATGCCCAATGGC-3′	R:5’-CACACCGAGAACAATGGC-3′
D3-F:	5′-AAGCGCTACTACAGCATCTGC-3′	R:5’-GGATAACCTGCCGTTGCTGAG-3′
D4-F:	5′-CCTGATGTGTTGGGACGCCTTTC-3′	R:5’-TGGTGTAGATGATGGGGTTGAGGG-3′
D5-F:	5′-AAAGACTGGCTTCCCTTGTGTC-3′	R:5’-CTGATGTTTACCGTCTGCACTG-3′
Alpha1A-F:	5′-GCGAATCCAGTGTCTTCGCAG-3′	R:5’-ACCATGTCTCTGTGCTGTCCC-3′
Alpha2A-F:	5′- CTGTTCACCGTGTTTGGCAAC-3′	R:5’-AAAGGGAATGACCAGCGTGG-3′
5-HT1A-F:	5′- CCAAAGAGCACCTTCCTCTG-3′	R:5’-CTTGCGCTTTGCTTCAGC-3′
BDNF $_{total}$-F:	5′- ACTCTGGAGAGCGTGAATGG- 3′	R: 5’- TACTGTCACACACGCTCAGC- 3′
GAPDH-F:	5′-AACTCCCATTCTTCCACCTTTG-3′	R: 5’-CCCTGTTGCTGTAGCCATATTC-3′

Table 2

Fold changes in plPFC mRNA expression in Sham and 6-OHDA female rats treated with VEH or MPH.

	Low E2 (Meta- and Di-Estrous)		High E2 (Pro- and Estrous)		*P* values		
	VEH	**MPH**	**VEH**	**MPH**	**OH × Tmt**	**Deplete × Tmt**	**Deplete × OH**
6-OHDA							
D1	0.26±0.05	0.31±0.06	0.40±0.03	1.43±0.22	0.05	0.154	0.006
D2	0.18±0.04	0.24±0.05	0.3±0.04	0.66±0.15	0.01	0.110	0.001
D3	0.19±0.05	0.14±0.03	0.41±0.14	0.65±0.16	0.03	0.100	0.001
D4	0.10±0.03	0.15±0.04	0.41±0.12	0.78±0.14	0.03	0.008	0.001
D5	0.15±0.04	0.23±0.06	0.39±0.08	1.02±0.24	0.02	0.040	0.001
BDNF total	0.53±0.09	0.45±0.06	0.62±0.05	0.91±0.16	0.002	0.800	0.02
Alpha-1A	0.04±0.01	0.15±0.04	0.22±0.06	0.70±0.24	0.004	0.001	0.001
Alpha-2A	0.09±0.02	0.14±0.05	0.29±0.07	0.70±0.10	0.006	0.003	0.001
5-HT-1A	0.08±0.03	0.14±0.03	0.30±0.02	0.52±0.16	0.13	0.040	0.002
Sham					**Tmt**	**Deplete**	**OH**
D1	1.0±0.21	0.73±0.25	0.37±0.12	0.59±0.17	0.06	0.88	0.29
D2	1.0±0.16	0.54±0.19	0.11±0.04	0.36±0.13	0.1	0.1	0.18
D3	1.0±0.18	0.51±0.16	0.25±0.10	0.31±0.11	0.5	0.08	0.57
D4	1.0±0.12	0.48±0.16	0.27±0.17	0.23±0.10	0.7	0.9	0.9
D5	1.0±0.17	0.60±0.20	0.24±0.10	0.39±0.15	0.3	0.3	0.91
BDNF total	1.0±0.18	0.56±0.17	0.28±0.06	0.83 ± 0.21	0.4	0.7	0.82
Alpha-1A	1.0±0.19	0.31±0.10	0.13±0.05	0.16 ± 0.05	0.8	0.18	0.42
Alpha-2A	1.0±0.26	0.36±0.08	0.19±0.05	0.18 ± 0.07	0.57	0.14	0.49
5-HT-1A	1.0±0.29	0.46±0.16	0.34±0.10	0.28 ± 0.09	0.46	0.02	0.58

OH=ovarian hormones; MPH=methylphenidate; Tmt=treatment; VEH=vehicle.