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Effects of amphetamine exposure during adolescence on behavior and prelimbic cortex neuron activity in adulthood

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Abstract

Repeated exposure to psychostimulants during adolescence produces long-lasting changes in behavior that may be mediated by disrupted development of the mesocorticolimbic dopamine system. Here, we tested this hypothesis by assessing the effects of amphetamine (AMPH) and dopamine receptor-selective drugs on behavior and neuron activity in the prelimbic region of the medial prefrontal cortex (PFC). Adolescent male, Sprague-Dawley rats were given saline or 3 mg/kg AMPH between postnatal day (P) 27 and P45. In Experiment 1, locomotor behavior was assessed during adulthood following challenges with a dopamine D₁ (SKF 82958) or D₂ (quinpirole) receptor-selective agonist. In Experiment 2, pre-exposed rats were challenged during adulthood with AMPH and a D₁ (SKF 83566) or D₂ (eticlopride) receptor-selective antagonist. In Experiment 3, the activity of putative pyramidal cells in the prelimbic cortex was recorded as rats behaved in an open-field arena before and after challenge injections with AMPH and one of the antagonists. We found that compared to controls, adolescent pre-exposed rats were more sensitive to the stimulant effects of AMPH and the dopamine receptor agonists, as well as to the ability of the antagonists to reverse AMPH-induced stereotypy. Prelimbic neurons from AMPH pre-exposed rats were also more likely to respond to an AMPH challenge in adulthood, primarily by reducing their activity, and the antagonists reversed these effects. Our results suggest that exposure to AMPH during adolescence leads to enduring adaptations in the mesocorticolimbic dopamine system that likely mediate heightened response to the drug during adulthood.

Keywords

adolescent; development; D₁ receptor; D₂ receptor; locomotor activity; sensitization

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1. Introduction

Individuals with a long history of AMPH* misuse often exhibit impairments in tasks assessing executive cognitive functions such as impulse control, attention, working memory, and decision-making (McKetin and Mattick, 1998; Ornstein et al., 2000; Monterosso et al., 2005; Woods et al., 2005; Casaletto et al., 2015). Notably, however, cognitive impairment is not an inevitable consequence of repeated AMPH exposure (Scott et al., 2007; Hart et al., 2012) and the development of drug-induced cognitive dysfunction may depend on a number of ancillary factors. One such potential factor is the initial age of drug exposure, with those beginning drug use during adolescence being most at risk (Vonmoos et al., 2013). This hypothesis is supported indirectly by data showing that brain regions known to be important for cognition, including those in the corticolimbic circuitry such as the PFC, amygdala, and hippocampus, are among the last to develop adult-like structure and function, and appear highly susceptible to environmental influences such as drug use (Paus et al., 2008; Gulley and Juraska, 2013). In addition, cross-sectional analyses have suggested that those with the longest duration of psychostimulant abuse, which are individuals who started drug use in early adolescence, are the most susceptible to deficits in decision making (Rogers et al., 1999) and have a greater likelihood of developing a substance use disorder (Wu and Schlenger, 2003; Lopez-Quintero et al., 2011; Gilder et al., 2014).

The mechanisms that underlie this enhanced vulnerability of adolescents to the adverse consequences of repeated AMPH exposure are uncertain, but a leading candidate is drug-induced changes in the normal development of the mesocorticolimbic dopamine system (Gulley and Juraska, 2013). Studies in rodents have demonstrated that during adolescent development, there are significant changes in the density of monoamine transporters and dopaminergic fibers (Kalsbeek et al., 1988; Benes et al., 2000; Moll et al., 2000; Willing et al., 2017). Dopamine neurons in the VTA, which project to multiple areas including the PFC and NAc, have been shown to be more active during the adolescent period compared to adulthood and this appears to be due to a relatively reduced GABAergic tone in the adolescent VTA (McCutcheon et al., 2012). This finding is not unequivocal, however, as a recent study reported no age-dependent differences in baseline firing rates of VTA dopamine neurons (Kim et al., 2016). This study also showed neurons recorded from adolescents were less responsive to reward anticipation and delivery compared to those recorded from adults. In terms of receptors for dopamine, there is an overproduction and subsequent decline of dopamine D₁ and D₂ receptor expression in the PFC and NAc as rats age from pre-adolescence into young adulthood (Andersen et al., 2000; Tarazi and Baldessarini, 2000; Brenhouse et al., 2008). It has also been suggested that signaling via D₁-D₂ heteromers, particularly in the striatum and NAc, is also unique in adolescents compared to adults and this may contribute to enhanced vulnerability in adolescents to the effects of abused drugs (Perreault et al., 2014).

In the current study, we tested the hypothesis that repeated exposure to AMPH during periadolescence (P27-P45) leads to long-lasting changes in the function of D₁ and D₂ receptors in adulthood. In Experiment 1, we investigated if adult rats pre-exposed to AMPH

* AMPH – amphetamine; PFC – prefrontal cortex; VTA – ventral tegmental area; NAc – nucleus accumbens; P – postnatal day

in periadolescence were sensitized to the motor-activating effects of the D₁-selective agonist SKF 82958 or the D₂-selective agonist quinpirole compared to saline-treated controls. In a second experiment, we tested if the D₁-selective antagonist SKF 83566 or the D₂-selective antagonist eticlopride would differentially influence AMPH-induced stereotypy in periadolescent pre-exposed rats compared to controls. Lastly, in Experiment 3 we used *in vivo* electrophysiology in periadolescent pre-exposed rats and controls to investigate functional changes in putative pyramidal cells of the prelimbic region of the medial PFC. Previous studies have revealed an important role of the medial PFC in AMPH-induced motor activation and sensitization (Steketee, 2003; Hall et al., 2009; Gulley and Stanis, 2010; Mathews and McCormick, 2012; Aguilar-Rivera et al., 2015). Moreover, the activity of pyramidal output cells is tightly regulated by D₁ and D₂ receptors (Seamans and Yang, 2004) and we recently found that periadolescent AMPH exposure alters D₁ receptor-mediated inhibition in these cells *in vitro* (Kang et al., 2016a; Paul et al., 2016).

2. Results

2.1. Experiment 1 – Effects of AMPH pre-exposure on D₁ and D₂ agonist challenge

Relative to baseline, there was an increase in motor activity after injection with the D₁ agonist SKF 82958 in both controls and AMPH pre-exposed rats (Fig. 1). For ambulation, we found a significant main effect of time bin ($F_{8,168} = 16.7$, $p < 0.001$) and a significant group x time bin interaction ($F_{8,168} = 6.30$, $p < 0.001$). As shown in Fig. 1a, rats pre-exposed to AMPH had a significantly greater maximal response that persisted for the entire 90-min post-drug interval. The D₁ agonist also increased stereotypy (Fig. 1b) and rearing (Fig. 1c) behavior. Separate ANOVAs indicated significant main effects of time bin for stereotypy ($F_{8,168} = 5.79$, $p < 0.001$) and rearing ($F_{8,168} = 4.54$, $p < 0.001$). However, in contrast to ambulation, there were no significant main effects of group or group x time bin interactions ($ps > 0.05$).

During challenge sessions with the D₂ agonist quinpirole, rats pre-exposed to AMPH were more sensitive to drug-induced increases in ambulation (Fig. 2a). Two-way repeated measures ANOVA of these data revealed significant main effects of group ($F_{1,21} = 6.76$, $p < 0.05$) and time bin ($F_{8,168} = 97.5$, $p < 0.001$), as well as a significant group x time bin interaction ($F_{8,168} = 4.97$, $p < 0.001$). Pre-exposed rats showed a significant increase in agonist-induced ambulatory activity beginning 30 min post-injection, while the activity of control animals did not significantly change across the 90 min post-injection period. Similarly, analysis of stereotypy (Fig. 2b) indicated a significant main effect of time bin ($F_{8,168} = 22.3$, $p < 0.001$) and a significant group x time bin interaction ($F_{8,168} = 6.88$, $p < 0.001$). For rearing (Fig. 2c), there was a significant main effect of time bin ($F_{8,168} = 10.1$, $p < 0.001$) and a group x time bin interaction that was at the threshold for being considered statistically significant ($F_{8,168} = 1.20$, $p = 0.053$). Thus, for all three measures of motor activity, AMPH pre-exposed rats exhibited greater sensitivity to the effects of quinpirole.

2.2. Experiment 2 – Effects of AMPH pre-exposure on AMPH and D₁ or D₂ antagonist challenge

In a separate group of rats, we assessed the effects of AMPH pre-exposure on the response to a challenge injection of AMPH and to subsequent injection with a D₁ or D₂ antagonist. As shown in Fig. 3, a challenge injection of 3 mg/kg AMPH significantly increased motor activity regardless of pre-exposure; however, AMPH pre-exposed rats exhibited significantly less AMPH-induced ambulation than controls (Fig. 3a). A two-way repeated measures ANOVA on these data indicated significant main effects of group ($F_{1,100} = 10.7$, $p < 0.01$) and time bin ($F_{5,500} = 179.9$, $p < 0.001$), as well as a significant group x time bin interaction ($F_{5,500} = 15.4$, $p < 0.001$). This relative reduction in ambulation was likely a result of response competition, as AMPH-induced stereotypy was significantly greater in pre-exposed rats relative to controls (Fig. 3b). Two-way repeated measures ANOVA of stereotypy indicated significant main effects of group ($F_{1,100} = 33.6$, $p < 0.001$) and time bin ($F_{5,50} = 60.3$, $p < 0.001$), as well as a significant group x time bin interaction ($F_{5,500} = 10.6$, $p < 0.001$). Rearing behavior followed a similar trend to ambulation, with pre-exposed rats rearing less than controls after AMPH (Fig. 3c). Two-way repeated measures ANOVA on these data revealed significant main effects of group ($F_{1,100} = 14.9$, $p < 0.001$) and time bin ($F_{5,500} = 88.9$, $p < 0.001$), and a significant group x time bin interaction ($F_{5,500} = 20.4$, $p < 0.001$).

The effects of the dopamine receptor antagonists on AMPH-induced stereotypy were assessed after challenge injections with either saline, SKF 83566 (D₁ antagonist), or eticlopride (D₂ antagonist). As shown in Fig. 4, the cumulative stereotypy response was greater in pre-exposed rats relative to controls, and continued to increase across the session in pre-exposed rats given saline at challenge. In rats given SKF 83566 (Fig. 4a) or eticlopride (Fig. 4b) at this challenge injection, however, this continued increase in stereotypy across the session was blocked. A three-way repeated measures ANOVA on these data indicated significant main effects of group ($F_{1,96} = 8.09$, $p < 0.01$) and challenge ($F_{8,768} = 136.0$, $p < 0.001$), and a significant group x challenge interaction ($F_{16,768} = 1.71$, $p < 0.05$). There was no effect of challenge on stereotypy in controls that received saline during adolescence (p -values > 0.05).

2.3. Experiment 3 – Effects of AMPH pre-exposure on medial PFC neuron activity

A total of 417 neurons were recorded from the medial PFC of control and AMPH pre-exposed rats ($n = 5/\text{group}$) during two open-field challenge sessions. All electrodes were localized to the prelimbic region (Fig. 5a), with recordings from individual neurons isolated and analyzed with NeuroExplorer (Fig. 5b). During baseline (15 min prior to AMPH injection), we found no significant differences in mean firing rate across the two recording sessions so these data were collapsed. Moreover, the baseline activity of the 202 units recorded from controls (2.98 ± 0.18 spikes/sec) was not significantly different from that recorded in the 215 units from pre-exposed rats (3.17 ± 0.17 spikes/sec).

Cells were classified as increased, decreased, or not changed based on modulations in firing rate after AMPH challenge (Fig. 6a). In both control and AMPH pre-exposed rats, the majority of recorded prelimbic neurons ($> 78\%$) were responsive to 3 mg/kg AMPH. Of

these AMPH-responsive units, the most frequent response-type was a decrease in firing rate post-injection (Fig. 6b). We found a significant difference between groups in the population distribution of unit responses ($\chi^2 = 13.56$, $p < 0.01$). In pre-exposed animals relative to controls, there was a greater proportion of units that decreased firing rate after AMPH injection and a smaller proportion showing no change. The percentage of cells excited by AMPH was similar in the two groups.

Separate analyses of decreased and increased units revealed that the magnitude of change was greatest in the AMPH pre-exposed group (Fig. 7). For decreased units, there were main effects of group ($F_{1,226} = 8.12$, $p < 0.01$) and time ($F_{11,2486} = 158.9$, $p < 0.001$), as well as a group x time interaction ($F_{11,2486} = 22.6$, $p < 0.001$). For increased units, there was a main effect of time ($F_{11,1353} = 18.0$, $p < 0.001$) and a group x time interaction ($F_{11,1353} = 2.12$, $p < 0.05$). Between-group differences in response magnitude were significant by 20 min after AMPH injection and persisted for the duration of the recording period in units that had drug-induced decreases in firing rate (Fig. 7a). For those with drug-induced increases (Fig. 7b), where responses in pre-exposed rats were more variable, between-group differences were statistically significant by 25 min after injection.

At 45 min after they were administered AMPH, rats were given an injection of the D₁-selective antagonist SKF 83566 (0.03 mg/kg) or the D₂-selective antagonist eticlopride (0.03 mg/kg) to assess the ability of these drugs to alter AMPH-induced changes in medial PFC firing rate. As shown in Fig. 8, data were analyzed by comparing the mean normalized firing rate during the last 15 min of the 45-min AMPH response interval to the same measure during the last 15 min of the 45-min antagonist response interval. For the D₁ antagonist, we found a significant main effect of injection ($F_{1,99} = 10.9$, $p = 0.001$) and a near-significant main effect of group ($F_{1,99} = 3.43$, $p = 0.067$) in decreased units (Fig. 8a); there was only a significant main effect of injection ($F_{1,62} = 4.33$, $p < 0.05$) for increased units (Fig. 8b). For the D₂ antagonist, we found a significant main effect of injection ($F_{1,124} = 24.8$, $p < 0.001$) and a near-significant main effect of group ($F_{1,124} = 3.03$, $p = 0.084$) in decreased units (Fig. 8a); none of the main effects or the interaction was significant for increased units (Fig. 8b). Thus, both antagonists tended to reverse AMPH-induced changes in firing rate, but this effect was most robust for the D₁-selective compound SKF 83566 in units that decreased firing rate after AMPH. Moreover, the effect was similar in control and pre-exposed rats.

In addition to the analysis of firing rate, burst firing activity of prefrontal neurons was also assessed during each recording session. Separate two-way repeated measures ANOVAs for bursting rate revealed significant main effects of injection during both sessions (SKF 83566: $F_{2,400} = 15.5$, $p < 0.001$; eticlopride: $F_{2,436} = 40.5$, $p < 0.001$) and a significant group x injection interaction ($F_{2,400} = 4.38$, $p < 0.05$) for the first session when the D₁ antagonist was administered. As shown in Fig. 9a, spontaneous bursting after saline injections in the first test session was blunted in pre-exposed animals relative to controls. This effect of pre-exposure was evident in the second test session (Fig. 9b), but the group difference was reduced and not statistically significant. In both sessions, bursting rate tended to decrease after injections of 3 mg/kg AMPH, particularly in controls. AMPH-induced reductions in bursting rate were relatively unaffected by challenges with either antagonist.

Analysis of the percentage of spikes occurring in bursts also revealed effects of AMPH and antagonist challenges, but these varied depending on the recording session. In the session where the D₁ antagonist was tested, we found a significant group x injection interaction ($F_{2,400} = 4.00$, $p < 0.05$). As shown in Fig. 9c, prelimbic units recorded from pre-exposed rats had an increase in the percentage of spikes occurring in bursts after AMPH injection and this was reversed after injection with the SKF 83566. In the session where the D₂ antagonist was tested (Fig. 9d), we found a significant main effect of time ($F_{2,436} = 14.0$, $p < 0.001$), and a near significant group x injection interaction ($F_{2,436} = 2.97$, $p = 0.052$). Thus, in both groups there was a significant increase in the percentage of spikes occurring in bursts and this effect was reversed to a statistically significant extent in the pre-exposed group of rats.

3. Discussion

The developing dopamine system of the adolescent brain may be an especially vulnerable target of the plasticity induced by repeated drug exposure. In the current study, we tested the hypothesis that AMPH exposure during periadolescence induces changes in dopamine receptors that would persist into adulthood, lead pre-exposed rats to be more sensitive to the behavioral effects of AMPH and dopamine receptor-selective drugs, and alter the function of putative pyramidal cells in the prelimbic region of the medial PFC. In Experiment 1, we found that AMPH pre-exposed rats, compared to controls, were more sensitive to the motor activating effects of the D₁ agonist SKF 82958 and the D₂ agonist quinpirole. With the D₁ agonist, this sensitization was isolated to ambulation, whereas the D₂ agonist induced a relatively greater effect on both ambulation and stereotypy. In Experiment 2, we found that pre-exposed rats were more sensitive to the stereotypy-inducing effects of AMPH as well as to the ability of a D₁ or D₂ antagonist to attenuate AMPH-induced stereotypy. Lastly, our electrophysiological recordings of prelimbic neurons (Experiment 3) revealed that cells recorded from pre-exposed rats, compared to those from controls, were less likely to be firing in bursts under baseline conditions. Following an AMPH challenge, these neurons were also more likely to be inhibited by AMPH, they exhibited a greater magnitude of change from baseline firing, and they were more sensitive to D₁ and D₂ antagonist-induced reversal of AMPH's effects on burst firing. Together, these results support the hypothesis that AMPH exposure during adolescence alters the development of the dopaminergic system, and in particular D₁ and D₂ receptor function, such that in adulthood animals are more sensitive to the neural and behavioral effects of drugs that influence dopamine receptor function.

It has been known for some time that rats pre-exposed to AMPHs in adulthood will exhibit behavioral sensitization to D₂, but not D₁, receptor-selective agonists after long withdrawal periods (Levy et al., 1988; Ujike et al., 1990; Vanderschuren et al., 1999). Here, we show that AMPH exposure during adolescence leads to a sensitized response to both D₁ and D₂ receptor-selective agonists when challenges are given in adulthood. This suggests that drug exposure during adolescence, which is a developmental stage when these receptors are overproduced and subsequently pruned (Andersen et al., 2000; Tarazi and Baldessarini, 2000; Brenhouse et al., 2008), may lead to unique changes in dopamine receptor expression and/or function compared to drug exposure during adulthood. Supporting this hypothesis, we recently showed that the same AMPH exposure protocol we used here induced a

reduction of D₁ expression in the medial PFC (Kang et al., 2016b) and inhibited D₁-mediated inhibition of pyramidal cell output in the medial PFC (Kang et al., 2016a) when assessed in adulthood. Also consistent is our current finding that adult rats exposed to AMPH during adolescence were more sensitive to the effects of dopamine receptor-selective antagonists on AMPH-induced stereotypy.

A comparison of the effects of the different receptor-selective drugs on ambulation and stereotypy in pre-exposed rats suggests that adolescent AMPH exposure may have a differential impact on D₁ and D₂ receptors. We found the D₁ agonist SKF 82958 tended to induce a greater potentiation of ambulation, whereas the D₂ agonist quinpirole had a greater effect on stereotypy. Previous work in adult male rats demonstrated that the 0.5 mg/kg dose of quinpirole used here tends to suppress locomotor activity in lieu of more repetitive behaviors such as sniffing (Horvitz et al., 2001), so the relatively reduced effect on ambulation may not be surprising. However, adolescent rats are reported to be more sensitive to the effects of quinpirole compared to adults (Frantz and Van Hartesveldt, 1999). A recent study comparing D₁ and D₂ agonist responses in adolescent and adult male rats (Dwyer and Leslie, 2016) showed adolescents are more sensitive to both the ambulation- and stereotypy-inducing effects of quinpirole, but not to the effects of D₁ agonists. Thus, it might be the case that exposure to AMPH during development induces a functional state of relative D₂ receptor supersensitivity. This would be consistent with our previous findings of reduced D₁ receptor expression in the mPFC (Kang et al., 2016b) and a reduced ability of the D₁ agonist SKF 38393 to increase spontaneous inhibitory postsynaptic currents in the mPFC (Kang et al., 2016a) following exposure to AMPH during adolescence. However, this hypothesis requires future study, especially since the current experiments did not investigate a full dose-response curve for the agonist drugs that were used. Moreover, automated methods of assessing stereotypy have limitations with regard to the amount of information that can be obtained about the unique components of repetitive behaviors (Rebec and Bashore, 1984).

Previous work revealed that when AMPH-exposed adolescents were given an AMPH challenge in adulthood, they exhibit sensitized locomotor responses (McPherson and Lawrence, 2006; Mathews et al., 2011; Labonte et al., 2012; Richetto et al., 2013; Shanks et al., 2015) and stereotypy (Hankosky et al., 2013; Sherrill et al., 2013). However, it was not previously demonstrated that dopamine receptor antagonists could partially reverse this sensitized response in adolescent-exposed rats or that D₁ and D₂ receptor-selective antagonists were similarly efficacious. Activation of D₁, and to a lesser extent D₂ receptors, is known to be important for the induction of AMPH sensitization (Ujike et al., 1989; Vezina, 1996; Meng et al., 1998; Karper et al., 2002; Tanabe et al., 2004; Tournier et al., 2013; Kai et al., 2015), but their relative roles in its expression have been less well characterized. The fewer number of studies investigating expression of sensitization have analyzed adult-exposed rats after relatively short withdrawal periods (14 days) and suggest a more prominent role for D₁ receptors (Shuto et al., 2006; Shi and McGinty, 2011) or a D₁-D₂ heteromer (Shen et al., 2015). The current findings are suggestive of an enhanced sensitivity of the developing adolescent brain to the ability of AMPH to induce enduring changes in the function of D₁ and D₂ receptors in the PFC, though this hypothesis will require further investigation with comparison groups of subjects that are outside the window of adolescent development when they are exposed to the drug (e.g., juveniles and adults).

Our electrophysiology results suggest that AMPH-induced adaptations in the prelimbic cortex play a role in the behavioral adaptations we observed. Previously, neurons in the prelimbic and infralimbic regions of the medial PFC from AMPH-exposed adult rats were shown to be more responsive to AMPH compared to those recorded from controls when a challenge injection was given following a short withdrawal (Homayoun and Moghaddam, 2006; Gulley and Stanis, 2010). In these studies, which like the current experiments were done in freely behaving rats, the effects of AMPH pre-treatment differed depending on dose and withdrawal duration. In the earlier study (Homayoun and Moghaddam, 2006), adult rats were treated for 5 days with 2 mg/kg AMPH or saline and were subsequently given a 2 mg/kg AMPH challenge following a 10-day withdrawal period. The authors reported that compared to controls, neurons from AMPH pre-exposed rats were more responsive to the challenge injection and the most frequent response observed was an inhibition in firing. In experiments from our laboratory (Gulley and Stanis, 2010), adult rats were treated with 1 mg/kg AMPH for 5 days and challenged with the same dose following a 4-day withdrawal. Like the earlier study, AMPH pre-exposed rats were more responsive to AMPH and the predominant effect was a decrease in firing rate and bursting. Here, when exposure to a higher dose of AMPH (3 mg/kg) occurred during peri-adolescent development and the AMPH challenge was given ~30 days later in adulthood, we observed a similar predominance of AMPH-induced decreases in firing rate. Additionally, we found that prelimbic neurons from AMPH pre-exposed rats were more sensitive to D₁ and D₂ antagonist-induced reversal of AMPH's effects on burst firing, though the ability of these antagonists to reverse changes in firing rate were similar in pre-exposed and control rats. A similar reversal of AMPH's effects on neural activity in behaving rats was previously reported in recordings from dorsal striatum (Rosa-Kenig et al., 1993). Together, the previous and current findings suggest that the longer-term adaptations in medial PFC circuitry that AMPH induces likely involve changes in the sensitivity of D₁ and D₂ receptors and they may be more pronounced following adolescent exposure or a more protracted withdrawal period. Differentiating between these hypotheses of age or withdrawal duration dependency requires future studies, but our previous *in vitro* electrophysiology findings support the important role of exposure age for determining the effects of AMPH exposure on PFC neuronal physiology (Kang et al., 2016a; Paul et al., 2016).

The specific mechanisms through which AMPH alters the anatomical and functional development of the PFC during adolescence remains to be elucidated, but several lines of evidence point to an important role for drug-induced changes in the ontogeny of GABAergic signaling. Studies in non-human primates and rodents have revealed that normal PFC development involves significant increases in GABAergic function, including an increase in the expression and excitability of fast-spiking, parvalbumin-positive GABAergic interneurons (Caballero et al., 2014), an increase in spontaneous inhibitory postsynaptic currents in the pyramidal cells on which these interneurons synapse (Hashimoto et al., 2009; Cass et al., 2014), and changes in the subunit composition of GABA_A receptors that would facilitate inhibitory transmission (Datta et al., 2015). In addition, dopaminergic control of inhibitory signaling in the PFC changes significantly during adolescence. For example, D₁- and D₂-mediated increases in GABAergic interneuron excitability occurs sometime between P36 and P50 in male rats (Tseng and O'Donnell, 2007). Our current finding of increased

burst firing in prelimbic neurons from AMPH pre-exposed rats is consistent with a drug-induced change in the adolescent development of GABAergic signaling and/or its control by dopamine receptors, but we did not directly test that hypothesis here. However, we have shown previously that adolescent AMPH exposure impairs D₁-mediated inhibition of pyramidal neurons in the PFC (Kang et al., 2016a) and leads to disinhibition in these cells following AMPH challenge in adulthood (Paul et al., 2016). Moreover, comparable effects on GABA and dopamine signaling in the PFC have been reported following adolescent exposure to the dopamine transporter blockers cocaine (Cass et al., 2013) and GBR 12909 (Khan et al., 2017), the substituted amphetamine MDMA (3,4-methylenedioxymethamphetamine; Cadoni et al., 2017) or the cannabinoid receptor agonist WIN 55,212-2 (Cass et al., 2014).

4. Conclusion

The results of the current study and others (Laviola et al., 2001; McPherson and Lawrence, 2006; Labonte et al., 2012; Reynolds et al., 2015; Kang et al., 2016a; Tendilla-Beltrán et al., 2016) point to a key role for drug-induced changes in the structural and functional development of the mesocorticolimbic dopamine system in the neural and behavioral changes induced by adolescent AMPH exposure. One intriguing possibility, which awaits testing, is that the phasic dopamine changes caused by an injection of AMPH have the unique ability to reorganize the structural and functional connections between the VTA and the PFC in adolescents compared to adults. A recent optogenetic study revealed this potential mechanism for heightened mesofrontal circuit plasticity in adolescents compared to adults and further demonstrated that D₂ receptors play an important role in regulating this plasticity (Mastwal et al., 2014). Dopamine systems are undoubtedly not alone in being affected by AMPH, however, and long-lasting changes in GABAergic (Cass et al., 2013), glutamatergic (Counotte et al., 2011) and cholinergic (O'Dell, 2009) functioning have also been reported following exposure to AMPHs and other psychostimulants during adolescence.

Because AMPH is used in the treatment of attention deficit hyperactivity disorder (ADHD) in children and adolescents, there is obvious concern about the potential for early-life drug exposure to induce long lasting changes in brain function and behavior. The current study does not address this issue, however, as the exposure dose used here (3 mg/kg/day injected i.p.) is likely to be beyond what would be used therapeutically. Future studies that use more clinically relevant dosing of AMPH are clearly warranted. Another important goal of future studies will be to more specifically link drug-induced disruptions with the consequences they induce, especially those related to cognitive dysfunction and other behaviors known to be critically involved in addiction. It will also be important to provide more detailed analysis of the developmental timing of drug exposure as multiple recent studies have revealed that drug exposure during specific times during peri-adolescence can influence later neurophysiological and behavioral outcomes (Adriani et al., 2004; Kang et al., 2016b; Tendilla-Beltrán et al., 2016) and that puberty may influence drug effects on neuronal development differently in males and females (Dagher et al., 2001; Juraska and Willing, 2017).

5. Experimental Procedure

5.1. Subjects

The male subjects ($n = 96$ for all experiments) were offspring of male and female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) bred in our animal facility. After weaning at P22, rats were housed 2–3 per cage with food and water available *ad libitum*. Rats were maintained on a 12:12 hr light/dark cycle (lights on at 0800) and experimental procedures were conducted during the light phase. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois, Urbana-Champaign, and were in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

5.2 Open-field Apparatus

Locomotor activity was monitored in open-field arenas ($41 \times 41 \times 41$ cm) constructed of transparent acrylic walls and surrounded by photobeam frames (Coulbourn Instruments; Allentown, PA, USA) that recorded horizontal (lower frame; 2.5 cm above the arena floor) and vertical activity (upper frame; 15 cm above the arena floor). Each open-field arena was housed in a sound-attenuating cubicle ($76 \times 80 \times 63$ cm) that contained a 76 mm speaker fixed to one side wall to provide white noise (70 dB), two ceiling mounted white lights (4 W each), and a centrally mounted overhead camera (not used in this study). Computer software (TruScan v 2.01, Coulbourn Instruments) was used to record photobeam breaks and to calculate ambulation (m), rearing (number), and stereotypy. Stereotypy was measured as total number of repetitive movements, as defined by the recording software.

5.3. Drugs

D-amphetamine sulfate and SKF 82958 hydrobromide (D_1 agonist) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SKF 83566 hydrobromide (D_1 antagonist), eticlopride hydrochloride (D_2 antagonist), and (–) quinpirole hydrochloride (D_2 agonist) were purchased from Tocris (Minneapolis, MN, USA). All drugs were dissolved in sterile saline (0.9% NaCl). Dosages were calculated based on the weight of the salt and given via intraperitoneal (i.p.) injection at a volume of 1 ml/kg.

5.4. Pre-treatment

At weaning, rats were assigned to exposure groups so that animals from 12 litters were represented similarly across groups. Cagemates (2–3 rats/cage) were assigned to the same treatment groups; controls were given 0.9% saline (1 ml/kg) and rats in the pre-exposed group were administered AMPH (3 mg/kg). Beginning on P27, one injection was given every other day for a total of 10 injections. For each injection, rats were transferred from the colony to a testing room, and placed individually into acrylic tubs ($46 \times 25 \times 22$ cm) lined with hardwood bedding where they remained undisturbed for 60 min post-injection.

5.5. Experiment 1

Approximately 30 days after their last pre-exposure injection (P70–P80), rats ($n = 23$) were transferred from the colony to a separate testing room where they remained in their

homecages for a 30-min acclimation period. Next, they were placed individually in an open-field arena for 15 min, then removed and injected with 1 ml/kg saline. They were immediately returned to the open-field and 30 min later challenged with either a dopamine D₁ (SKF 82958, 1.0 mg/kg) or a D₂ (quinpirole, 0.5 mg/kg) receptor agonist. Assignment of the specific agonist was made randomly. Doses were chosen based on previous studies showing they were near or above threshold for affecting behavior in rats pre-exposed to AMPH in adulthood (Vanderschuren et al., 1999).

Following agonist injections, rats were allowed to behave undisturbed for 90 min and then were returned to their homecage. Three days later, the drug challenge procedure was repeated, but rats received the dopamine receptor agonist they did not get during the first drug challenge. The order of drug challenges was counterbalanced within each group (i.e. control and pre-exposed).

5.6. Experiment 2

Approximately 30 days after the last pre-exposure (P70-P80), rats (n = 51) were given two challenge sessions that were separated by three days. During the first session, rats were placed individually in an open-field arena for 15 min, then removed and injected with 1 ml/kg saline (Injection 1). Thirty min later, they were administered 3 mg/kg AMPH (Injection 2) and allowed to freely move about the chamber for another 45 min. Lastly, rats were challenged (Injection 3) with saline, a dopamine D₁ antagonist (SKF 83566, 0.03 mg/kg), or a D₂ antagonist (eticlopride, 0.03mg/kg) and monitored for an additional 45 min. These doses were chosen based on previous studies showing they would decrease AMPH-induced stereotypy (Garrett and Holtzman, 1994; Fritts et al., 1997). This procedure was repeated three days later during a second session. However, rats in the antagonist challenge groups were given the antagonist for Injection 3 that they did not receive during the first challenge session; the order of injections was assigned randomly. Rats previously challenged with saline on session one received saline again for Injection 3.

5.7. Data analysis for Experiments 1 and 2

In Experiment 1, ambulation, stereotypy, and rearing measures were summed in 15-min bins and analyzed using separate two-way ANOVAs, with time bin as the within-subjects factor and group (control, pre-exposed) as the between-subjects factor. Similarly, in Experiment 2, ambulation, stereotypy, and rearing measures were summed and analyzed using separate two-way ANOVAs with time bin as the within-subjects factor and between-subjects factors of group (control, pre-exposed). Cumulative stereotypy, which was obtained for each rat by summing this measure for the 45-min period after injections with AMPH and challenge drugs (i.e., saline or the dopamine receptor antagonists), was analyzed with three-way mixed factor ANOVAs with injection as the within-subjects factor and between-subjects factors of group (control, pre-exposed) and challenge drug (saline, SKF 83566, eticlopride). Main effects and interactions were further analyzed using Holm-Sidak multiple comparison procedures. Statistical analyses were conducted using R Statistical Computing Software (R: a Foundation for Statistical Computing; Vienna, Austria) or Systat 11 (Systat Software Inc.; San Jose, CA, USA).

5.8. Experiment 3

Rats ($n = 5/\text{group}$) underwent surgical procedures for implantation of microwire electrode arrays between P65 and P70 using methods we described previously (Gulley and Stanis, 2010). The array, which was implanted using stereotaxic coordinates for the prelimbic region of the medial PFC (3.0 mm AP, 0.7 mm ML, and 3.5mm DV to bregma; Paxinos and Watson, 2007) consisted of eight Teflon-insulated stainless steel wires (50- μm diameter each) arranged in a 2×4 pattern (NB Labs; Denison, TX, USA). Rats were allowed to recover from surgery for 5 days.

Between P70 and P80, which was approximately 30 days after the last pre-exposure injection, rats were transferred from the colony to a separate testing room, where they remained in their homecages for a 30-min acclimation period. During this time, voltage signals from each microwire were amplified with a unity gain field effect transistor (FET) headstage and transmitted via a shielded lightweight cable connected to a multi-channel commutator, which allowed the animal to freely move about the testing environment during recording sessions. Extracellular signals were amplified, band-pass filtered (250 Hz to 8 kHz), and digitally captured with a 40 kHz sampling rate using a Multichannel Acquisition Processor (Plexon Inc.; Dallas, TX, USA). Single-unit activity originating from putative pyramidal cells was identified based on waveform (i.e. spike) characteristics using well established criteria (Jung et al., 1998; Barthó et al., 2004; Homayoun et al., 2005; Homayoun and Moghaddam, 2006; Gulley and Stanis, 2010). Spike sorting was also performed both online and offline from continuous data using both manual and automated procedures (Sort Client and Offline Sorter; Plexon Inc.). Single-units were discriminated if the absolute refractory period of the target signal was > 1.1 ms and the waveform amplitude was at least 2.5:1 above background. Units with an average firing rate > 10 spikes/s, which were putative fast spiking interneurons, made up a small percentage of the total units recorded in this study ($\sim 7\%$) were thus excluded from analysis.

Rats were subsequently placed into an open-field arena for 15 min, then removed and injected i.p. with 1 ml/kg saline. Thirty minutes later, they were administered 3 mg/kg AMPH and allowed to freely move about the chamber for another 45 min. Lastly, they were injected with the D_1 antagonist SKF 83566 (0.03 mg/kg, i.p.) and monitored for an additional 45 min. During a second recording session, which took place two days after the first, the same procedure was used except the last injection given to rats was the D_2 antagonist eticlopride (0.03mg/kg, i.p.).

5.9. Experiment 3 data analysis

Electrophysiological data were imported into NeuroExplorer (NEX Technologies, Madison, AL, USA) and analyzed with custom scripts and methods similar to those used previously in our lab and others (Homayoun and Moghaddam, 2006; Gulley and Stanis, 2010). Neurons recorded during sessions 1 and 2 were treated as independent units, although the anchoring of the electrode connectors to the skull leaves the possibility that the same or similar population of cells were sampled across sessions. Baseline firing rates, which were designated as the mean activity (in Hz) during the saline phase of each recording session (15-min period after saline injection and before AMPH injection), were analyzed with two-

way repeated measures ANOVA (group x session). The 99% confidence interval for this baseline period was then used to classify each unit's response to AMPH. A unit was categorized as "increased" or "decreased" after AMPH if its activity post-injection was above or below, respectively, the 99% confidence interval for (1) five of the nine 5-min recording bins, or (2) at least four consecutive 5-min bins post-injection (Gulley and Stanis, 2010). Those units not meeting one of these criteria were classified as "not changed". The distribution of response types was compared between the control and pre-exposed groups using chi-square (χ^2).

To compare the magnitude change after AMPH challenge during sessions 1 and 2 combined, mean firing rate was calculated for each unit in 5-min bins and activity was normalized to each unit's baseline (mean firing during the 15-min saline injection period). This was done by dividing the unit's mean firing rate by its mean baseline firing rate and then expressing the value as a percentage. These data were subsequently analyzed with separate two-way repeated measures ANOVAs (group x time) for increased and decreased response types. The ability of D₁ and D₂ antagonists to alter AMPH-induced changes in firing rate were analyzed by comparing the mean normalized firing rate (% baseline) for the last 15 min of the AMPH injection interval to the last 15 min of the antagonist response interval. These data were then analyzed with separate two-way mixed factor repeated measures ANOVAs (group x injection) for each response type (increased or decreased) and antagonist (SKF 83566 or eticlopride). For all ANOVA tests, main effects and interactions were further analyzed using Holm-Sidak multiple comparison procedures where appropriate.

To analyze burst firing, spike train data were analyzed with the NeuroExplorer using the Poisson surprise method, which is robust against irregular patterns of activity and changes in mean firing rate that may obscure burst detection (Legédy and Salcman, 1985; Homayoun et al., 2005; Homayoun and Moghaddam, 2006). A minimum surprise value of 5 was set as the confidence level for detection. For each unit, bursting rate (bursts/min) and the percent of spikes in bursts were compared between groups using separate two-way repeated measures ANOVAs (group x injection). Significant differences were followed-up with Holm post-hoc comparisons where appropriate. Statistical analyses for data obtained in Experiment 3 were conducted using SigmaPlot 12.5 (Systat Software Inc.; San Jose, CA, USA).

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Highlights

- Rats exposed to amphetamine in adolescence are hypersensitive to D₁ and D₂ agonists
- Adolescent amphetamine exposure enhances antagonist-induced reversal of stereotypy
- Amphetamine decreases prelimbic neuron activity more in adolescent-exposed rats

Fig. 1.

Ambulation (a), stereotypy (b) and rearing (c) in an open-field arena following challenge with 1.0 mg/kg SKF 82958 ($n = 11-12/\text{group}$). Arrows indicate time-bins when rats were removed from the open-field and injected (i.p.) with saline and the D_1 agonist. * $p < 0.05$, ** $p < 0.01$ vs baseline (45-min bin) within group; # $p < 0.05$ vs control within time bin; @ $p < 0.05$ vs baseline collapsed across group

Fig. 2.

Ambulation (**a**), stereotypy (**b**) and rearing (**c**) in an open-field arena following challenge with 0.5 mg/kg quinpirole ($n = 11-12/\text{group}$). Arrows indicate time-bins when rats were removed from the open-field and injected (i.p.) with saline and the D_2 agonist. $*p < 0.05$, $**p < 0.01$ vs baseline (45-min bin) within group; $\#p < 0.05$ vs control within time bin; $@p < 0.05$ vs baseline collapsed across group

Fig. 3.

Ambulation (**a**), stereotypy (**b**) and rearing (**c**) in an open-field arena following challenge with 3.0 mg/kg AMPH. Arrows indicate time-bins when rats were removed from the open-field and injected (i.p.) with saline and AMPH. Data are presented collapsed across antagonist treatment group for pre-exposed rats ($n = 25$) and controls ($n = 26$). # $p < 0.001$ vs control within time bin, ** $p < 0.001$ vs baseline (45-min bin) within group

Fig. 4. Stereotypy following 3.0 mg/kg AMPH and challenge injections of saline, 0.03 mg/kg SKF 83566 (a) or 0.03 mg/kg eticlopride (b). Data are presented as the cumulative response for the entire 45-min post-injection interval ($n = 8-9/\text{group}$). *** $p < 0.001$ vs AMPH period within group; # $p < 0.05$ vs pre-exposed rats given a saline challenge

Fig. 5. Schematic of recording locations in the prelimbic cortex and example sorting of recordings from two putative pyramidal cells. **(a)** A total of 417 recordings were made from electrodes implanted in the prelimbic region of the medial PFC. Closed circles represent approximate electrode tip locations for AMPH pre-exposed rats, whereas open circles represent locations for controls ($n = 5$ rats/group). Numbers indicate the distance anterior to bregma (images adapted from Paxinos and Watson 2007). **(b)** Images taken from the NeuroExplorer data analysis package that show the clustering of two distinct units (indicated in grey and black) based on principle component (PC) analysis of waveform characteristics. PC1 and PC2 are plotted against time and represented in 3-D space. Multiple tracings for waveforms from the two discriminated units are also shown.

Fig. 6.

Putative pyramidal neurons from the prelimbic cortex that responded to a 3.0 mg/kg AMPH injection with an increase (+), decrease (–) or no change (NC) in firing rate. **(a)** Representative examples of each type of unit response (see Methods for classification procedure). Data are mean firing rate in 5-min bins during a 15-min saline injection baseline and 45 min after AMPH injection ($t = 0$ min). **(b)** The population distribution of categorized units recorded from control and pre-exposed rats. The number of cells in each category is given in parentheses. Because there were no significant differences in the distribution of response types between recording sessions, data are presented collapsed across sessions. $**p < 0.01$, χ^2 statistic comparing proportion of responses in control and pre-exposed groups

Fig. 7. Magnitude of firing rate changes in prelimbic cortex cells classified as decreasing (**a**) or increasing (**b**) following a challenge injection of 3.0 mg/kg AMPH (designated by the arrow). Data are normalized to the pre-injection baseline for each unit. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, vs. the last time bin before AMPH injection ($t = 45$ min) within group; # $p < 0.05$ vs. pre-exposed group within time bin

Fig. 8. Magnitude of firing rate changes in decreased (a) and increased (b) cells during the last 15-min of the 45-min recording period following 3.0 mg/kg AMPH injection and the last 15 min of the 45-min recording period following the D₁ antagonist SKF 83566 (0.03 mg/kg) or the D₂ antagonist eticlopride (0.03 mg/kg). Data are normalized to the pre-injection baseline for each unit.

Fig. 9.

Burst firing during saline baseline (SAL), following 3.0 mg/kg AMPH, and after challenge with the D₁ antagonist (SKF 83566; panels **a**, **c**) or the D₂ antagonist (eticlopride; panels **b**, **d**). Matching letters indicate significant between or within-group differences ($p < 0.05$).

*** $p < 0.001$ vs SAL, collapsed across group