AGE-DEPENDENT EFFECTS OF CHRONIC AMPHETAMINE ON PRELIMBIC-NUCLEUS ACCUMBENS CIRCUIT FUNCTION AND GOAL-DIRECTED BEHAVIOR

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

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Abstract

Non-medial amphetamine (AMPH) use is prevalent in the adolescent population. Empirical evidence suggests that individuals starting using drugs during adolescence have a higher chance of developing drug-related problems. It is hypothesized that adolescents may have a heightened vulnerability to drug induced plasticity such as cognitive deficits and neuroadaptations. My pilot studies suggested that the high frequency stimulation (HFS)-induced long-term depression (LTD) in the prelimbic cortex (PL) is likely to be a target influenced by repeated AMPH in an age-of-exposure dependent manner, which results in abnormalities in the reward circuit function and goal-directed behavior. Using a rat model, this hypothesis was tested with three specific aims: (1) to investigate the ontogeny of this LTD function (Experiment 1), (2) to assess the effect of adolescent and adult amphetamine on HFS-induced LTD in the PL and the potential mechanisms underlying AMPH-induced changes (Experiment 2) and (3) to determine whether AMPH-induced changes in the PL and in the reward circuit are associated with impaired cognitive flexibility (Experiment 3). In Experiment 1, field potentials in brain slices from male naive rats were assessed to test the effect of age on the expression of this LTD. The role of GABA and dopamine receptors in the HFS-induced LTD was investigated using respective agonist or antagonist. In Experiment 2, brain slices from male rats pre-exposed to repeated 3.0 mg/kg AMPH i.p injections were used in field potential and patch-clamp recordings to assess AMPH's effect on the expression of LTD and dopamine's modulation of inhibition. In Experiment 3, male and female rats were treated as in Experiment 2 and subjected to a strategy set-shifting task to assess AMPH's effect on cognitive flexibility. Upon finishing the behavioral testing, each rat was sacrificed for slice recordings to assess AMPH's effect on HFS-induced plasticity in the PL-nucleus accumbens circuit. These studies revealed unique behavioral changes

and neuroadaptations following adolescent AMPH exposure. Our results are consistent with the hypothesis that adolescent brain is more vulnerable to the detrimental effect of drugs.

Acknowledgments

I would like to thank my advisor Dr. Josh Gulley for his guidance and tremendous support throughout my Ph.D. study and research, for his patience when helping me preparing every talk and manuscript, for believing in me and encouraging me all the time.

I would like to thank my committee members: Dr. Janice Juraska, Dr. Hee Jung Chung, Dr. Catherine Christian and Dr. Nu-Chu Liang. Thank you for insightful comments and encouragement.

My sincere thanks also goes to Dr. “Lee” Cox, Dr, Kush Paul and Dr. Roberto Galvez who provided me an opportunity to work them, who trained me on doing electrophysiology, and who gave access to the laboratory and research facilities. Without they precious support it would not be possible to conduct this research.

I would like to thank my fellow labmates Dr. Luke Sherrill, Dr. Emily Hankosky, Dr. Lindsey Hammerslag and Sara Westbrook. Thanks for your support all the years.

Last but not the least, I would like to thank all my family for their love and unconditional support.

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Chapter 1: Dissertation objectives

Adolescent-, compared to adult-onset, drug users have a greater propensity to develop substance use disorder (SUD; Chen et al., 2009; Grant & Dawson, 1997). This heightened vulnerability to addiction could be explained by the characteristics of adolescent behavior such as increased impulsivity and risk-taking (Burnett et al., 2010; Spear, 2008; Hammerslag et al., 2017). Yet another contributing factor is that the adolescent brain may be more susceptible to drug-induced cognitive deficits (Gulley and Juraska, 2013) that bias individuals towards actions contributing to addiction and related disorders (Gould, 2010). Consistent with this hypothesis, previous studies from our lab found evidence for more pronounced impairment in working memory (Sherrill et al., 2013) and impulse control (Hankosky and Gulley, 2013; Hammerslag et al., 2014) following repeated exposure to amphetamine (AMPH) in adolescence compared to adulthood. Notably, the medial prefrontal cortex (mPFC), a brain region that critically regulates these behaviors, undergoes significant structural and functional remolding during adolescence. Thus, it is hypothesized that exposure to AMPH during adolescence leads to unique adaptations in the mPFC that underlie more severe cognitive deficits (Gulley and Juraska, 2013).

Empirical evidence from human (Fung et al., 2010) and animal studies (Erickson et al., 2002; Caballero et al., 2014a; Caballero and Tseng, 2016a) suggests a significant increase in the functionality of the GABA system in the mPFC throughout adolescent development. For example, high frequency stimulation (HFS; 50 Hz) delivered *in vivo* is found to produce a GABA-mediated long-term depression (LTD) in the mPFC only in rats that are older than postnatal day (P) 45-50 (mid- to late-adolescence, Caballero et al., 2014b). The protracteddevelopment of GABA signaling is suggested to be a critical step for the mPFC to mature functionally that also serves as a labile period that introduces heightened susceptibility to

environmental adversities including exposure to drugs of abuse (Caballero et al., 2016a, b). Indeed, *in vivo* studies have found that exposure to cocaine, a CB_1 receptor agonist or an NMDA receptor antagonist during peri-adolescence abolishes the expression of GABA-dependent LTD in adulthood (Cass et al., 2014a; Cass et al., 2014b; Thomases et al., 2014). However, the mechanisms underlying this HFS-induced LTD and its functional significance is unknown.

In my initial studies, I found a similar age- and GABA-dependent LTD in the prelimbic cortex (PL) using slice recording. Interestingly, AMPH exposure during adolescence, but not adulthood, led to a greater impairment in this GABA function (see Chapter 4). These results suggest that the deep-layer output cells are more disinhibited and therefore likely to generate aberrant activity in the brain circuits to which they project. Notably, the nucleus accumbens (NAC) is a major output site of the PFC and plays a key role in integrating relevant information from corticolimbic regions to program goal-directed behavior (Goto and Grace, 2008). Currently, there is a gap in our understanding about the impact of AMPH on the functional connectivity between PFC and NAC as most literature has studied the two regions individually. The studies proposed in this dissertation will follow up on my preliminary findings with the objectives of 1) revealing the potential mechanisms underlying the HFS-induced LTD in the PL and AMPH-induced changes in its expression and 2) determining the consequences of AMPH-induced alteration in this LTD function on the level of circuit and behavior. My central hypotheses are that: (a) HFS induces a GABA-dependent LTD only in the adult PL, which is partially due to increased dopamine receptor function; (b) AMPH exposure during adolescence will produce a long-lasting disruptive effect on HFS-induced LTD in the PL, which is more pronounced compared to controls or rats given AMPH in adulthood and is partially attributable to reduced inhibitory tone in the deep layer of the PL; and (c) NAC cells in the adolescent-exposed group

are expected to exhibit the greatest increase in their responsiveness to PL stimulation compared to other groups and this will be associated with more evident abnormalities in goal-directed behavior. I will test my hypotheses by pursuing the following specific aims:

Aim 1: Investigate the ontogeny of HFS-induced LTD in the PL

In Experiment 1, I will use field potential recordings in brain slice from naive rats to assess the effect of age on HFS-induced plasticity in the PL and confirm the involvement of $GABA_A$ and dopamine receptors in the expression of this LTD. My working hypotheses are: 1) HFS delivered in the superficial layer of adult PL induces an LTD in the deep layer and such LTD is reduced or absent in peri-adolescent rats; 2) intact function of $GABA_A$ and dopamine receptors is required for HFS to induce LTD in the adult PL; 3) Reduction or absence of HFS-induced LTD in peri-adolescent rats is due to a decreased dopamine receptor function.

Aim 2: Investigate the mechanisms underlying age-dependent differences in AMPH's effects on HFS-induced LTD in the PL

In Experiment 2.1 and 2.2, I will use field potential or patch-clamp recordings in brain slice from rats pre-exposed to chronic AMPH in either adolescence or adulthood to determine age-of-exposure dependence differences in AMPH's effect on HFS-induced plasticity and dopamine's modulation of spontaneous inhibitory currents in the pyramidal cells in the PL. My working hypotheses are: 1) repeated AMPH exposure will have a long-lasting disruptive effect on the expression of HFS-induced LTD in the PL; 2) Repeated AMPH exposure impairs dopaminergic modulation of inhibition in the deep layer of PL; 3) The effects of AMPH on LTD and inhibitory function in the PL are more pronounced following adolescent drug exposure.

Aim 3: Investigate age-dependent differences in AMPH's effects on HFS-induced plasticity in the PL-NAC circuit and goal-directed behavior.

In Experiment 3.1 and 3.2, I will use a rat model of strategy set-shifting to assess the effect of repeated AMPH exposure in either adolescence or adulthood on cognitive flexibility. Upon finishing Experiment 3.1 and 3.2, rats will be subjected to field potential recordings for assessing AMPH's effect on HFS-induced plasticity in the PL-NAC circuit. My working hypotheses are that rats exposed to AMPH during adolescence, compared to adulthood, 1) will have more impaired flexibility in executing goal-directed behavior such that they will persist in a previously learned response that is no longer appropriate; 2) they will have a higher propensity of expressing HFS-induced LTP in the PL-NAC circuit.

Chapter 2: Background and significance

Non-medical use of AMPHs among adolescents

In the US, the percentage of adolescents who have ever used AMPHs [mainly AMPH and methamphetamine (mAMPH)] without medical supervision by the 12th grade peaked to 26% in 1981 and declined in recent years to approximately 10%, which is just behind alcohol, nicotine and marijuana (Johnston et al, 2016). The annual prevalence of non-medical AMPH use in 8-12th graders, however, has been increasing since 2009 and reached 7.7% in 2016 (NIDA). Such pervasive use may be related to the perception of AMPH's harmful effects as data from the Monitoring the Future study suggested that in the past decade only about 60% of 8-12th graders considered regular AMPH use a significant health risk. This percentage is lower than that of most other drugs including even alcohol (~65%). In contrast, the annual prevalence of mAMPH use in 8-12th graders stabilized around 1% in the past decade, perhaps because the drug has been perceived to be more harmful such that over 75% of the 8-12th graders considered using mAMPH once or twice of as a great heath risk (Johnston et al, 2016). This number is very close to that of heroin and cocaine. But the fact is, mAMPH is rapidly metabolized to p-OH-AMPH, norephedrine and AMPH, with the first two metabolites having no psychoactive effects (Gygi et al., 1996). In other words, AMPH is the active form of mAMPH, the drug that is perceived as harmful as cocaine. The misuse of AMPH in the relatively large percentage of young people raises an alarming concern as it is known that repeated exposure to the drug is associated with cognitive abnormalities that are contributing factors for the development of addiction (McKetin and Mattick, 1997; Ornstein et al., 2000; Gould, 2010). This is supported by studies that showed an increased rate of drug dependence and related mental disorders in adolescent-onset AMPH users (Degenhardt et al., 2007; Lakhan and Kirchgessner, 2012).

Most adults who develop a substance use disorder report having started drug use in adolescence or young adulthood (Johnston et al, 2016). Adolescence is defined as the transitional period between childhood and adulthood. For humans, an important marker for the onset of adolescence is puberty (Casey et al., 2008), which typically starts around 10 to 11 years old in females and around 12 years old for males (Kali and Cavanaugh, 2010). In laboratory rat models, it has been argued that adolescence starts around postnatal day (P) 28, which is before the onset of puberty (Spear, 2000). This is because certain neural and behavioral changes that are noted as early as P28 are similar to those seen in human adolescents (Tirelli et al., 2003; Sisk and Foster, 2004; Spear, 2011). An important aspect of adolescent development is that higher cognitive functions emerge and improve substantially, which would require neuroanatomical and functional remodeling in multiple brain regions (Casey et al., 2008). Recent studies suggest that significant anatomical changes, especially in the mPFC, occur closely around puberty onset and these changes may be related to the refinement of higher cognitive functions such as working memory and cognitive flexibility during adolescence (Drzewiecki et al., 2016; Willing et al., 2016; Juraska and Willing, 2016). Light has also been shed on the mesocortical pathway as it is suggested that the developmental changes in the related circuits are critically involved in the maturation of reward-related behavior, impulse control, decision-making other higher cognitive function (Chamber et al, 2003; Naneix et al., 2012). In this context, it has been hypothesized that drug exposure during this period may influence the normal ontogeny of multiple neurotransmitter systems, producing long-lasting, if not permanent, adaptations in brain and behavior (Gulley and Juraska, 2013). However, laboratory animal studies of the neurobiological and cognitive changes induced by AMPH have typically used rats and mice that were either adults or their age was unspecified. There has been a considerable gap in our knowledge about

the impact of AMPH on the adolescent brain even though it is suggested to be particularly susceptible to environmental insults (Gulley and Juraska, 2013).

Studies from our lab and others have found disruptive effects of exposure to moderate or abuse-relevant doses of AMPH during adolescence on associative learning (Richetto et al., 2012), cognitive flexibility (Hankosky et al., 2013), impulse control (Counotte et al., 2011; Hankosky and Gulley, 2013; Hammerslag et al., 2014, Hammerslag et al., 2017) and working memory (Sherrill et al., 2013). Most of the studies from our lab used a chronic treatment regimen where drug was administered to Sprague-Dawley rats from P27-P45, which is an early span of peri-adolescence that includes time before and after puberty onset in females and a primarily pre-pubertal time period for males. We also included a separate group of rats that received the same dose of AMPH in adulthood (P85-P103), which allowed us to demonstrate that some of the disruptive effects of AMPH are specific to periadolescent exposure. The mechanisms underlying these age-dependent effects of AMPH have become of great interest as they are important for understanding why drug use during adolescence is more detrimental compared to exposure during adulthood.

The cognitive functions we examined previously are sensitive to lesion or disruption of the PFC, one of the latest-maturing brain regions that undergoes significant neuroanatomical and functional remodeling during adolescence (Casey et al., 2008). While the full picture of PFC maturation is still not clear, relatively more light has been shed on the ontogeny of the prefrontal dopamine system, a primary target of psychostimulant drugs like AMPH (Lüscher and Malenka, 2011). For example, rodent studies have shown that throughout adolescence there are progressive increases in dopamine fiber density (Kalsbeek et al., 1988; Benes et al., 2000; Willing et al., 2017), changes in dopamine receptor levels that follow an inverted “U-shaped” trajectory

(Anderson et al., 2000; Tarazi and Baldessarini, 2000) and cell type-dependent functional alterations (Tseng and O'Donnell, 2007; Caballero et al., 2016). In this regard, it is not surprising that the neurobiological or behavioral outcomes resulting from drug exposure depend on when drugs are introduced to the brain, even within the span of adolescence (Spear, 2015). In a previous study, we compared the effect of repeated AMPH exposure from P27 to P45 (same one as used in other studies from our lab) and from P37 to P55 on dopamine receptor level in adulthood. Only the early onset exposure led to a significant reduction in D_1 receptor expression in the mPFC, suggesting decreased dopamine function (Kang et al., 2016a). In keeping with this, my initial study revealed a persistent reduction in dopaminergic modulation of GABA function in the PL following the same early onset AMPH exposure and this effect was dependent on age of exposure (Paul et al., 2016). The studies proposed in this dissertation will follow on these preliminary findings to investigate age-dependent effects of AMPH on dopamine- and GABA-related functions in the PFC.

Adolescent development of prefrontal GABA function and its susceptibility to drugs

It is known that the GABA system in the PFC has a protracted ontogeny throughout adolescence. GABAergic cells take up 15-20% of the total cell population in the PFC (Santana et al., 2009) and they have a distinct morphology and physiology. The number of GABAergic cells in the PFC does not seem to change significantly from peri-adolescence to adulthood (Willing and Juraska, 2016), but a notable change in these cells during adolescence is the up- or down-regulation of calcium binding protein that is cell-type specific (Caballero et al., 2014; Erikson and Lewis, 2002; Fung et al., 2010). In rats, for example, the level of parvalbumin (PV), which is the hallmark protein expressed in a subtype of fast-spiking interneurons (FSI), is found to be lowest in juveniles (P25–P35) but increases during adolescence (P45–P55) to adult-like levels. In

contrast, the level of calretinin (CR) expressed in non-fast-spiking interneurons (non-FSI) is reduced in adults compared to juvenile and adolescent animals (Caballero et al., 2014b). Such changes indicate significant functional remodeling since the electrophysiological properties (i.e. firing frequency) of FSIs and non-FSIs largely depend on the level of corresponding calcium binding protein (Caillard et al., 2000). PV expression is known to strongly rely on glutamatergic signaling (Behrens et al., 2007). Hence the developmental change of PV level is also suggested to reflect increased excitatory influence selectively occurring in PV-positive cells during adolescence (Caballero et al., 2014b). This is hypothesis is supported by the finding of elevated frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in FSIs from adults compared to juveniles. Accordingly, it was also demonstrated that the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded in glutamatergic pyramidal cells in the adult mPFC was increased compared to those in adolescents (Caballero et al., 2014a). Taken together, these findings suggest that prefrontal GABAergic interneurons may increase their synaptic contact with pyramidal cells and are thereby becoming integrated into the mPFC neural network where they provide critically important inhibitory regulation (Caballero and Tseng, 2016). With the addition of the maturing interneurons, it is hypothesized that the PFC may tune up its information processing capacity to "match the computational demands posed by parallel developing circuits, ultimately altering PFC output and control over subcortical structures" (Caballero and Tseng, 2016).

In keeping with this notion, a series of recent *in vivo* studies demonstrated GABAergic modulation of neuronal activity in response to high frequency stimulation (HFS) in the mPFC emerges only after mid- to late-adolescence. Specifically, male rats that were $\geqslant$ 45 days old exhibited an LTD-like change in the evoked field potential in the deep layer of the mPFC

following four trains of 50-Hz stimulation delivered in the ventral hippocampus (vHC; Caballero et al. 2014b). This LTD was absent when the $GABA_A$ receptor antagonist picrotoxin was infused into the mPFC before delivering HFS in the vHC (Caballero et al., 2014b). Importantly, exposure to cocaine (Cass et al., 2013) or the NMDA receptor antagonist PCP (Thomases et al., 2014) during early-adolescence (P35-P40) has been shown to abolish this LTD function in adulthood, suggesting a susceptibility to drugs during the development of this function.

It is speculated that the HFS-induced LTD may serve as an "anti-plasticity" mechanism for the PFC (Laroche et al., 2000; Caballero et al., 2014b). Specifically, high frequency neuronal spiking (30 to >100 Hz) in the mesocorticalimic pathway is often found to be associated with the occurrence of environmental salience (Benchenane et al., 2011). Also, cortical-cortical communication may rely on high frequency oscillation. For example, in primates, gamma oscillations (30~120 Hz) are hypothesized to carry the dialog between sensory cortex and frontal areas that determines what is relevant for goal-directed behavior (Rich and Shapiro, 2009). Previous studies found that HFS delivered in the superficial layer of PFC induces LTP in the deep layer cells when $GABA_A$ receptors are blocked (Otani et al., 2003; Matsuda et al., 2006; Kolomiets et al., 2009; Xu and Yao, 2010; Sheynikhovich et al., 2013). In this regard, an inhibitory mechanism, possibly this HFS-induced LTD, seems to be necessary to prevent the PFC network from being saturated with LTP and thereby losing its flexibility for processing and coding information (Laroche et al., 2000). Also, this GABA-dependent LTD may serve as a noise control mechanism such that high frequency activity in the superficial layer may be less likely to activate cells in the deep layer, thus avoiding unnecessary output. Theoretically, the lack of HFS-induced LTD following AMPH exposure may confer a noisy PLC network that allows

irrelevant high frequent neural activity to "leak out" and influence downstream targets such as the NAC.

Circuit-level implications of altered GABA-dependent LTD in the PLC

The NAC is a major target of the PLC and plays a critical role in coding the top-down control information from the PLC with other corticolimbic inputs and then transfer this into behavior output (Mink, 1996). Studies have shown that afferents from the PLC and other limbic structures converge onto single NAC projection neurons (O'Donnell and Grace, 1995; French and Totterdell, 2002) that are hyperpolarized (~-80mV) at resting state. It was found that excitatory drive from one afferent brain structure tends to shift the membrane potential of these cells to a depolarization plateau, referred as an "Up state", that is just below their typical threshold for firing action potentials (Goto and O'Donnell, 2001; Goto and Grace, 2008). Supra-threshold spike firing requires coincide inputs from more than one afferent brain regions (Mulder et al., 1998; Goto and Grace, 2008). Such electrophysiological properties are suggested to be fundamental for NAC cells to integrate and compute info for programing possible behavior response. As shown in Fig. 2.1A, glutamatergic inputs from PFC, AMG and HC elicit differential responses in the NAC as a pool of neural representations of possible outputs. Fig. 2.1B depicts the hypothesized influence of a disinhibited PFC onto NAC cells as found in

AMPH-exposed rats. Potentially increased neuronal drive into the NAC (bolded arrows) may bring more cells into the "Up state" (Gruber and O'Donnell, 2009) and coincide with other inputs, leading to discharges that may not represent the most appropriate output.

Another important property of the NAC output cells is that their synapses can undergo long-term plastic changes (Goto and Grace, 2008). Furthermore, the synaptic plasticity in the NAC cells has an interesting feature: HFS delivered in the deep layer of PFC triggers LTP in the NAC and simultaneously induces LTD at the synapses receiving projections from HC, and vice versa (Goto and Grace, 2005; Belujon and Grace, 2008). A similar "competition" has been observed between the HC to NAC and the AMG to NAC circuits (Goto and Grace, 2008). These between-circuit synaptic interactions may serve as a mechanism that increases the signal-to-noise ratio in a specific circuit when coding a behavioral output as the potential influences from other afferent inputs to the same cell are reduced. This could also be a mechanism for NAC cells to sustain flexibility when they need to update output patterns in response to changes in afferent

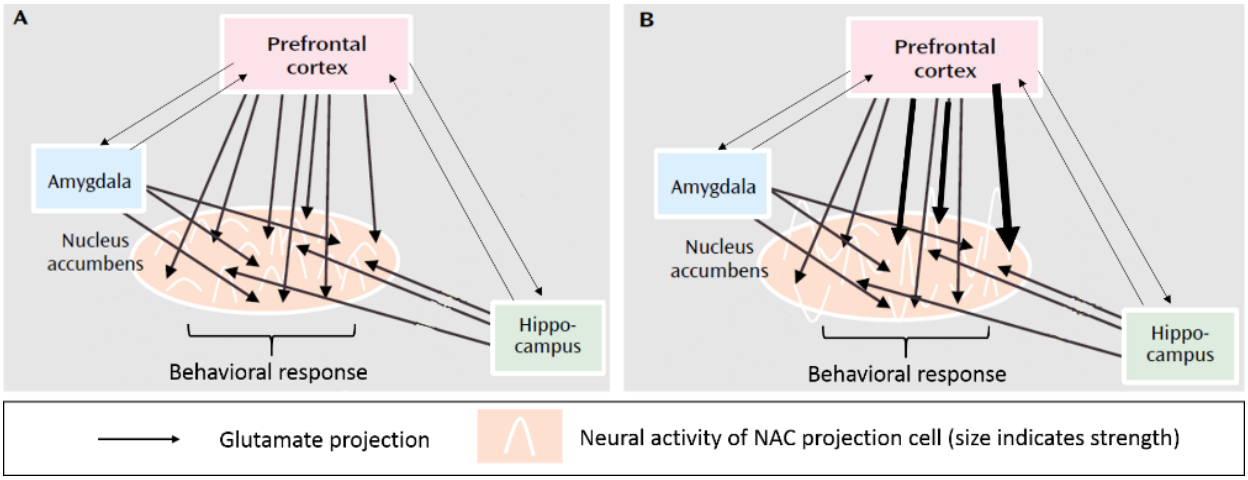

Figure 2.1 Schematic of NAC neuronal ensemble activity under the influence of corticolimbic inputs. **A**. Under normal conditions, inputs from corticolimbic regions (example in the figure: HC, AMG and PFC) generate a pool of neural response that represents potential behavioral output (Goto and Grace, 2008). **B**. With aberrant input from PL, more NAC cells may enter the "Up State" ready for output. When these cells receive additional influences from PFC or other region, they may resulting in abnormal output. The circuit diagrams are adopted and modified from Chambers et al., 2003.

inputs, especially when multiple corticolimbic structures are recruited in a cognitive behavior exerting influence over NAC simultaneously (Goto and Grace, 2008).

As discussed above, if there is a lack of HFS-induced LTD in the PL, high frequency activity that is irrelevant and should be retained in the PL may come to impact the NAC and produce within- and between-circuit plasticity. This would be expected to have a significant impact on how information is processed and stored in the NAC, and further on cognitive functions that critically rely on the intact PL-NAC communication. The research proposed here will test this hypothesis and determine if altered PL-NAC function predicts certain behavioral abnormalities, for example, deficits in cognitive flexibility.

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Chapter 3: Ontogeny of HFS-induced LTD in the PL*

Rationale

Converging evidence suggests that prefrontal GABAergic interneurons increase their functionality and synaptic influence on pyramidal cells throughout adolescence and in turn provide critically important inhibitory regulation of the PFC network (Caballero and Tseng, 2016). Consistent with this hypothesis, a series of recent *in vivo* studies demonstrated GABAergic modulation of neuronal activity in the mPFC in response to HFS emerges only after mid- to late-adolescence. Specifically, male rats that were $\geqslant$ 45 days old exhibited a LTD-like change in the evoked field potential in the deep layer of the mPFC following four trains of 50-Hz stimulation delivered in the ventral hippocampus (vHC; Caballero et al. 2014b). In contrast, identical HFS delivered in the basolateral amygdala (AMG) induced long-term potentiation (LTP) in the deep layer of mPFC in both adolescent and adult rats (Caballero et al., 2014b). The lack of LTD in the mPFC following HFS in the amygdala suggests that the ability of HFS in the ventral hippocampus to induce LTD in the mPFC may reflect a unique characteristic of the hippocampus-mPFC pathway. However, it also may indicate that this late-emerging GABAergic modulation is an intrinsic property of the local network within the mPFC. A primary goal of the current experiment is to investigate the latter possibility by determining if a GABA-sensitive LTD could be induced *in vitro* by HFS within the mPFC of rats scarified at pre- and post-adolescent stages of development.

This experiment also aims to reveal the potential neuronal mechanisms for HFS-induced LTD so we may gain more insight into AMPH's disruptive effect on this function. Early studies

*Kang S, Cox CL, Gulley JM (2018) High frequency stimulation-induced plasticity in the prelimbic cortex of rats emerges during adolescent development and is associated with an increase in dopamine receptor function. Neuropharmacology 141:158-166.

using juvenile rats showed that dopamine plays a prominent role in regulating inhibitory tone in the mPFC (Seamans et al 2001, 2004), partially through altering the excitability of interneurons (Gorelova et al., 2002). Later, it was found that there are significant changes in the expression of PFC dopamine receptors throughout adolescent development (Anderson et al., 2000; Brenhouse et al., 2008; Naneix et al., 2012) and accordingly, the dopaminergic regulation of inhibition has been shown to be influenced by age (Paul and Cox, 2013). The delayed maturation of dopamine's function in the mPFC is also consistent with the finding that prefrontal GABAergic cells increase their responsiveness to dopamine receptor stimulation during adolescent development. For example, after rats are 50 days old, non-FSIs become responsive to dopamine D_1 receptor stimulation, whereas FSIs become responsive to D_2 receptor-mediated increases in excitability (Tseng and O'Donnell, 2007). Notably, the timing of these functional changes in dopamine's effects on interneurons is similar to when HFS-induced LTD emerges in the mPFC (Caballero et al. 2014b). Thus, we hypothesized that the ontogeny of HFS-induced LTD involves functional changes in both D_1 and D_2 receptors from peri-adolescence to adulthood such that the absence of LTD in the peri-adolescent mPFC is associated with a relatively reduced function of dopamine receptors.

Methods

Subjects

A total of 48 male Sprague-Dawley rats, which were offspring of breeders maintained in our facility, were used in these experiments. Rats from individual litters were weaned on postnatal day (P) 22. They were housed with same-sex littermates in groups of 2-3 per cage. Approximately half of the subjects were housed individually for about 24 hours before being sacrificed for recordings because their cagemates had been removed for recordings on the

previous day. These cases were equally distributed across all experimental conditions. All rats were kept on a 12-h light/dark cycle (lights on at 0800 h) in a temperature-controlled room with food and water available *ad libitum.* All procedures were performed during the light period of the cycle, were consistent with the 'Principals of Laboratory Animal Care' (NIH Publication no. 85-23) and were approved by the IACUC at the University of Illinois, Urbana-Champaign, USA.

Brain slices preparation

Rats were assigned to be sacrificed for collection of brain slices when they were within the age ranges targeted for this study: P35-P45, P45-P55, P55-P65, P65-80, and P95-135. The mean (+ SEM) age at sacrifice for each of these groups, which were chosen based on previous studies of functional developmental of plasticity in the mPFC (Caballero et al., 2014), was 37.4 ± 1.09, 49.0 ± 0.91, 59.5 ± 1.76, 71.7 ± 1.2, and 117 ± 6.92 days, respectively. On the day they were sacrificed, rats were deeply anesthetized with 55 mg/kg of sodium pentobarbital and decapitated. The brain was removed quickly and chilled in ice-cold, oxygenated slicing medium containing (in mM): 2.50 KCl, 1.25 NaH_2PO_4, 10.0 $MgCl_2$, 0.50 $CaCl_2$, 26.0 $NaHCO_3$, 11.0 glucose, and 234 sucrose. Tissue slices (450 μm thickness) containing the prelimbic cortex (PLC) were then cut with a vibrating tissue slicer (Pelco EasiSlicer, Ted Pella INC.) in the coronal orientation and transferred to a holding chamber in artificial cerebrospinal fluid (aCSF) containing (in mM): 126 NaCl, 2.50 KCl, 26.0 $NaHCO_3$, 2.00 $CaCl_2$, 1.25 $MgCl_2$, 1.25 NaH_2PO_4, and 10.0 glucose). Slices were kept in the aCSF gassed with 95% O_2/5% CO_2 first at 32°C for 20 min and then room temperature for 1 h before recordings.

Field potential recording

Brain slices were transferred to a recording chamber (Harvard apparatus, Holliston, MA) and submerged under a thin film of aCSF (30-31°C) gassed with 95% O_2/5% CO_2. A dissecting stereoscope was used to identify specific regions of the slice and to aid in the placement of electrodes. Extracellular signals were obtained by using low resistance (300–600 kΩ insulated tungsten electrodes (FHC, Inc., Bowdoinham, ME) placed in the deep layer of PLC. Extracellular stimuli were delivered through a concentric bipolar tungsten electrode (FHC, Inc., Bowdoinham, ME) placed in either layer II/III or the deep layer of the PLC in line with the recording electrode as shown in the schematics (Fig. 3.1 and Fig. 3.3). Stimuli consisted of either single testing pulses (100 µs duration, 50-350 pA at 0.067 Hz) or 4 trains of HFS (100 pulses at 50 Hz per train, inter-train inverval = 10 s). The intensity of the test pulse was adjusted to 50% of the intensity required to produce the maximum synaptic response. HFS trains or ligands were applied after a stable baseline response was obtained for at least 10 min. Ligands, which included bicuculline methiodide (BMI), SCH23390, sulpiride, DNQX, SKF38393, quinpirole or tetrodotoxin (TTX; all obtained from Tocris, Minneapolis, MN), were diluted in dimethyl sulfoxide (DMSO) or deionized water as stock solutions (10-40 mM) and kept at -20 °C before use. For all the drug applications, 5-20 µl stock solution or vehicle (DMSO or deionized water) was diluted in 200 ml aCSF gassed with 95% O_2/5% CO_2 (30-31°C) to the desired concentration (indicated in the figures or legends) and bath applied to the slice throughout the recording or for 10 min (indicated in the figures or legends). Recorded signals were amplified (1,000X), filtered (low pass =10 kHz), sampled at 20kHz and digitally stored.

Data analysis

The slope of the first 1-2 ms of the field excitatory postsynaptic potential (fEPSP) was averaged in 1-min bins. For each slice, the averaged slope was then normalized to the mean slope of its 10-min baseline. All data are presented as mean ± SEM. Statistical tests consisted of one- and two-way ANOVA (drug × age) with Tukey post hoc tests performed where appropriate.

Results

Field potential recordings were obtained in a total of 125 slices obtained from 48 male rats. A schematic diagram showing the approximate location of stimulating and recording electrodes is shown in Fig. 3.1. Single stimuli delivered in the superficial layer of the PLC typically produced one deflection in cells recorded from the deep layer of the PLC (Fig. 3.1A). Bath application of the AMPA receptor antagonist DNQX completely blocked this response, suggesting that this component is primarily mediated by an AMPA receptor-dependent postsynaptic response. Occasionally, we observed an additional deflection following single stimuli (Fig. 3.1B), which was resistant to DNQX. Further application of 1μM TTX completely blocked this early component, suggesting that the DNQX-insensitive deflection is a presynaptic fiber volley (Wilson and Cox, 2007). The fiber volley was not included in the analysis because it did not appear in all the recordings and only the slope of the DNQX-sensitive component was used for analyses.

As shown in Fig. 3.2, the effect of HFS on the fEPSP slope in the deep layer of the PLC was age-dependent. Following four trains of HFS delivered in the superficial layer, we found a sustained decrease of fEPSP slope in rats from groups older than P55 (Fig 3.2D-F). This was confirmed by significant one-way ANOVAs for the P65-80 and P95-135 groups ($F_{49,235}$ = 10.8,

$p < 0.001$ and $F_{49,261} = 3.19$, $p < 0.001$, respectively). In contrast, this same stimulation in younger rats failed to significantly change the fEPSP slope compared to baseline (Fig 3.2A-C). These effects were compared between groups by obtaining the mean fEPSP slope from the 30 to 40 min period following HFS (Fig. 3.2G). One-way ANOVA revealed a significant main effect of group ($F_{4,22} = 6.81$, $p = 0.001$). Post-hoc analysis suggested that the mean fEPSP slope in young adult rats (≥ P65) was significantly lower than that in peri-adolescent rats (≤ P55). Slices obtained from rats between P55 and P65 also exhibited a mean reduction in fEPSP slope to about ~80% of baseline, but this was not statistically significant.

To test whether the LTD we observed in the deep layer PLC of rats ≥ P55 was the result of specifically stimulating deep layer afferents originating from the superficial layers of the PLC, we investigated the effects of applying HFS within the deep layer in slices from young adult rats (≥ P65). With electrodes positioned as schematically depicted in Fig. 3.3, we found that HFS failed to induce LTD and instead produced a transient increase in the fEPSP slope (Fig. 3.3A). However, one-way ANOVA of the time course data suggested no significant differences in the fEPSP slope across all time points ($F_{49,236} = 1.01$, $p > 0.05$).

To test if delayed development of GABAergic signaling was involved in age-dependent differences in HFS-induced plasticity in the PLC, we studied the effects of inhibiting GABA signaling during recordings. Following applications of vehicle, we observed similar age-dependent differences in HFS-induced plasticity as we had seen previously (Fig. 3.4). Specifically, there was a significant decrease in fEPSP slope in slices from young adults (P65-P80; Fig. 3.4C), but no significant change in those from peri-adolescent rats (P35-P45; Fig. 3.4B). However, HFS delivered in the presence of the $GABA_A$ receptor antagonist BMI (1μM) not only eliminated the LTD of the fEPSP, but led to a significant increase in the fEPSP slope in

rats that were ≥ P65. There continued to be no significant effect of HFS in slices from peri-adolescent rats. Analysis of the mean slope of the fEPSP from 30-40 min post-HFS (Fig. 3.4D) revealed a significant main effect of drug ($F_{1,20} = 20.4$, $p < 0.001$) and a drug by age interaction ($F_{1,20} = 8.60$, $p = 0.008$). Post-hoc analysis suggested a significant effect of BMI only in the young adult group.

To determine if dopamine receptor activation was required for the HFS-induced LTD observed in young adult rats, we applied dopamine receptor antagonists to slices for the duration of recordings (Fig. 3.5). As shown in Fig. 3.5B, we found that HFS failed to produce a lasting decrease in the fEPSP slope with the presence of either the D_1 (SCH 23390; 10 μM) or the D_2 receptor antagonist (sulpiride; 10 μM). Analysis of the mean slope of the fEPSP from 30-40 min post-HFS with one-way ANOVA revealed a significant effect of group ($F_{2, 13} = 18.61$, $p < 0.001$). Post-hoc comparisons suggested that both drug groups were significantly different from control (Fig. 3.5C).

We next determined if application of dopamine receptor agonists alone was sufficient to produce long-term changes in the fEPSP and if agonist-induced changes were age-dependent (Fig. 3.6A). As shown in Fig. 3.6B, fEPSP recordings from both periadolescent and young adult brain slices remained stable and relatively unchanged following vehicle exposure. Following application of a D_1 agonist (SKF38393; 10 μM), the fEPSP increased significantly in slices from young adult rats. There was also a small increase in the fEPSP from peri-adolescent rats, but this change was not significantly different from baseline (Fig. 3.6C). The D_2 agonist quinpirole (1 μM) also had a small, but non-significant effect on the fEPSP in peri-adolescent rats. In slices from young adult rats, quinpirole (1 μM) led to a significant decrease in the fEPSP (Fig. 3.6D) that similar to the decrease observed in this age group following HFS. Analysis of the mean

slope of the fEPSP from 30-40 min post-HFS (Fig. 3.6E) revealed a significant main effect of drug ($F_{2,23} = 50.1$, $p < 0.001$) and a drug by age interaction ($F_{2,23} = 13.7$, $p < 0.001$). Post-hoc tests suggested that within the young adult group, both the D_1 and D_2 agonist conditions were significantly different from vehicle. Within each drug condition, there was also a significant difference between the young adult and periadolescent groups.

To further elucidate the role of dopamine receptors in HFS-induced LTD, we next determined if their activation prior to delivery of the stimulating pulse would impact HFS-induced LTD. Vehicle exposure just prior to HFS delivery had no impact on the plasticity induced in slices from young adult rats (Fig. 3.7A). Stimulation of D_1 receptors increased the fEPSP in slices from young adult, but not peri-adolescent rats as we observed in our prior experiment (cf. Fig. 3.6B), but subsequent application of HFS reversed this effect and returned the fEPSP to just below 100% of its baseline prior to drug application (Fig. 3.7B). Stimulation of D_2 receptors (Fig. 3.7C) decreased the fEPSP in slices from both peri-adolescent and young adult rats to a similar extent that we had seen in our prior experiment (cf. Fig. 3.6C). Following HFS, the fEPSP recorded from young adult rats decreased further below baseline while the fEPSP recorded from peri-adolescents was relatively stable. Two-way ANOVA of the mean slope of the fEPSP 30-40 min post-HFS (Fig. 3.7D) revealed a significant main effect of drug ($F_{2,25} = 13.6$, $p < 0.001$) and age ($F_{1,25} = 25.0$, $p < 0.001$). Post-hoc tests showed that only the D_1 agonist significantly altered HFS-induced changes in the fEPSP and this was the case only in slices from young adult rats.

Discussion

Using *in vitro* extracellular recordings, the current study revealed that GABA-sensitive plasticity in the PLC emerged in the late peri-adolescent period in male rats. Specifically, HFS

delivered in the superficial layer of the PLC produced a persistent decrease in evoked field potential in the deep layer PFC in rats > P55 and this decrease was blocked by a $GABA_A$ receptor antagonist. We further demonstrated that dopamine signaling was involved in the expression and, potentially, in the ontogeny of HFS-induced LTD in the PLC. This is evidenced by the findings that antagonism of either D_1 or D_2 receptors prevented the expression of HFS-induced LTD in the adult PLC and that both D_1 and D_2 receptors in the peri-adolescent PLC were less responsive to their respective agonists when compared to the adult group. Together, these results indicate that the functional up-regulation in prefrontal dopamine receptors may be an important factor enabling a late-maturing GABA system to regulate the mPFC neural network.

As mentioned above, findings by Caballero and colleagues suggest a GABA-related developmental alteration in the deep layer cells that respond to hippocampal input (Caballero et al., 2014b). Besides afferent innervations from subcortical regions, deep layer cells in the mPFC also receive projections from the superficial layers (Yang et al., 1996). Several early investigations have demonstrated that under certain artificial conditions, HFS is able to produce long-term plastic change in the glutamtergic transmission between the superficial and the deep layer in the juvenile or adult mPFC (Otani et al., 2003; Huang et al., 2004; Kolomiets et al., 2009; Sheynikhovich et al., 2013). However, based on Caballero at al., GABA may play a prominent role in modulating HFS-related activity in the mPFC and significant developmental changes are likely to occur this modulation during adolescence (Caballero et al., 2014b; Caballero et al., 2016).

To test this hypothesis, we kept the GABA transmission undisturbed in our recordings and compared the effect of HFS on the field potential across age. We found that HFS delivered

within the mPFC indeed produced an LTD and this effect was apparent only in rats > P55. We further confirmed that the expression of LTD was sensitive to the antagonism of $GABA_A$ receptors, which is the same as the LTD observed in the hippocampus-mPFC circuit (Caballero et al., 2014b). In fact, with $GABA_A$ receptor blocked, HFS from the hippocampus (Caballero et al., 2014b) or delivered in the superficial layer produces an LTP in the deep layer cells in the adult mPFC. Collectively, these data suggest that as the mPFC matures, cells in the deep layer become more responsive to HFS-induced plasticity and the net result LTD may be due to a greater increase in inhibition relative to excitation. Such significant shift in the excitation-inhibition balance may alter how information is processed within the mPFC network and since it emerges when animals are approaching closely to adulthood, this GABA-related function may be one of the limiting factors for the mPFC to fully mature (Caballero et al., 2016).

The up-regulation in the GABA tone that may underlie the ontogeny of HFS-induced LTD could be explained by a series of pre- and post-synaptic changes in the interneurons during late-adolescence. GABAergic cells in the mPFC are generally categorized as FSIs and non-FSIs based on their electrophysiological properties. According to several studies, functional up-regulation after P45 in male rats seems to occur preferentially in FSIs that express parvalbumin (PV) protein (Caballero et al., 2013; Caballero et al., 2014b; Tseng and O'Donnell, 2007). The FSIs increase in number, their level of PV protein expression and their responsiveness to glutamate (Caballero et al., 2013) as discussed above. Post-synaptically, $GABA_A$ receptors containing α_1 subunits are suggested to selectively respond to the input from PV-expressing cells. It is found that the mRNA of the α_1 subunit continues to increase during the peri-adolescent development (Datta et al., 2015) and accordingly there are more inhibitory currents in the post-synaptic pyramidal cells that are mediated by α_1-containing $GABA_A$ receptors (Hashimoto et al.,

2010), suggesting that the pyramidal cells become more responsive to FSIs' input during adolescence. Since FSIs are believed to provide major inhibitory tone in the mPFC, especially in layer V (Naka and Adesnik, 2016), these changes are suggested to significantly increase GABA tone and render novel patterns of inhibitory modulation in the mPFC (Caballero and Tseng, 2016), which is consistent with the HFS-induced LTD found in the current study.

In addition to the developing GABA system, functional changes in dopamine receptors are also likely involved in the ontogeny of the HFS-induced LTD. As mentioned above, dopamine plays an important role in regulating inhibition in the mPFC (Seamans and Yang, 2004) and there are numerous developmental changes in the dopamine receptors that may significantly influence GABA functions (Paul and Cox, 2013; Tseng and O'Donnell, 2007). Furthermore, peri-adolescent disturbance in dopamine signaling by cocaine exposures abolishes the HFS-induced LTD in the ventral hippocampus-mPFC circuit in adulthood (Cass et al., 2013).

To investigate the relationship between dopamine signaling and the HFS-induced LTD, we first demonstrated that either D_1- or D_2-like receptor antagonist prevented the expression of LTD. Notably, the concentration of SCH23390 and sulpiride used in the current study has been commonly seen in other *in vitro* electrophysiological investigations (Kroner and Lavin, 2012; Paul et al., 2013; Xu and Yao, 2010; Banks et al., 2105) and would be considered moderate, not likely to compromise the selectivity of these antagonists. Thus, it is unlikely that the blockade of LTD was due to non-selective antagonism of dopamine receptors, suggesting that both D_1 and D_2 dopamine receptors are necessary for HFS to induce LTD.

Early investigations found that high concentration (>50 μM) of dopamine applied to the mPFC slice suppresses fEPSP and produces an LTD-like response (Law-Tho et al., 1995; Mair and Kauer, 2007). In this regard, the HFS-induced LTD may be explained by dopamine's effect

on the fEPSP and the absence of LTD in the adolescent mPFC may be due to a reduced dopamine receptor function. To test this hypothesis, we examined the effect of D_1 and D_2 agonist on the field potential in the adolescent and adult mPFC. In the adult rats, D_2 agonist produced a persistent decrease in the field potential that seemed to mimic an LTD while D_1 agonist led to a significant increase in the field potential. In contrast, neither of the D_1 or D_2 agonist produced significant changes in rats sacrificed between P35 and P45. These results support part of our hypothesis that there is a significant functional increase in dopamine receptor after P45 but at the same time they can not explain the expression of LTD and appear contradictory to our finding that both D_1 and D_2 antagonist blocked the expression of LTD, especially because D_1 agonist led to an increase in the field potential that is opposite to an LTD.

It is notable that approximately 80% of D_1 and D_2 receptors are located on the pyramidal cells in the mPFC (Santana et al., 2009). Thus it is likely that the effects of dopamine agonist we observed were primarily driven by their actions on the pyramidal cells. In fact, the effects of D_1 and D_2 agonist on the field potential and the age-related changes found in the current study are in keeping with dopamine's effect on excitatory currents or potentials in the pyramidal cells according to several previous investigations. For example, in rats older than at least P45, D_1 activation was found to increase NMDA receptor-mediated depolarization in the deep layer pyramidal cells in the mPFC (Tseng and O'Donnell, 2005; Flores-Barrera et al., 2014). D_2 activation was found to decrease the AMPA receptor-mediated EPSP in deep layer pyramidal cells and this effect was more pronounced in rats older than P50 (Tseng and O'Donnell, 2007). In this regard, the expression of HFS-induced LTD may not mainly rely on dopamine's action on the pyramidal cells. This notion is supported by our receptor occlusion results. In the adult mPFC, HFS was able to substantially decrease the field potential further after 10 min application

of either D_1 or D_2 agonist. Together, our results indicate that the HFS-induced LTD is unlikely due to global activation of dopamine receptors in the mPFC or in the pyramidal cells but may be more relying on dopamine-mediated changes in interneurons. This is consistent with the anatomical arrangement of dopamine release pools in the mPFC. It is known that VTA dopamine cells project directly into both superficial and deep layers and early studies found that these fibers prefer to make contact with GABAergic interneurons over pyramidal cells (Sesack at al., 1998). Interestingly, there is also an increase in the dopamine varicosities specifically around prefrontal GABA cells throughout the adolescent development (Sesack at al., 1998). Therefore, it is possible that the dopamine release following HFS may have a biased impact on the interneurons, resulting in an overall suppression in synaptic transmission.

Based on the available evidence from the literature, we speculate that the HFS-induced LTD is an augmented inhibition that masks potential changes in excitation rather than a mere reduction in excitation. The putative enhanced inhibition is due to, at least partially, D_1- and D_2-mediated facilitation in the GABAergic output (Tseng and O'Donnell, 2007). It is well established that dopamine elevates inhibitory tone in adult mPFC and the increase in inhibitory function could last over 30-40 min as shown in previous studies (Kroner and Lavin, 2012; Paul et al., 2013; Kang et al, 2016). This prolonged action of dopamine on inhibition seems consistent with the post-HFS changes found in the current study. Furthermore, non-FSIs and and FSIs start to respond to the excitatory effect of D_1 and D_2 agonist, respectively, after P50 (Tseng and O'Donnell, 2007). The timing when these additional dopamine-mediated inhibitory forces start to appear in the mPFC is in close neighborhood when the HFS becomes able to produce LTD. Third, the enhanced inhibition following HFS does not necessarily disagree with the potential increase in excitation that may likely co-exist at a limited set of synapses and can not be detected

in the populational responses in the current study (Laroche et al., 2000). The potentially increased glutamate transmission may provide further drive on nearby interneurons and the net effect is a reduction in the populational activcity. An alternative explanation is that the adult-level GABA inhibition limits the post-synaptic depolarization following HFS, resulting in a low Ca^{2+} rise that is known to lead to LTD (Hirsch and Crepel, 1992; Graham et al., 2010). The absence of LTD in peri-adolescent PLC could be due to a relatively weak and inmatured GABA tone that is unable to attenuate the HFS-induced depolarization. Future studies are required to directly test these hypotheses.

Another interesting finding in the current study is that the HFS delivered in the deep layer did not produce significant changes in the cells that are also in the deep layer. We did this experiment because the deep-layer pyramidal cells in the mPFC also receive intra-layer glutamate influence and we sought to determine if there is also a GABA-sensitive LTD to regulate intra-layer high frequency transmission. However, it seems that the GABA-sensitive LTD in the deep layer is elicited specifically by high frequency superficial layer input. In contrast to our results, a recent *in vitro* study showed that the HFS delivered in the superficial layer in the mPFC produced an LTP in the same layer (Konstantoudaki et al., 2018) and this LTP was insensitive to blockade of $GABA_A$ receptors. These differential expressions of synaptic plasticity could be explained by the cellular arrangement in the mPFC. For example, the intra-superficial layer LTP could be mediated by the horizontal "long range" glutamate projections between pyramidal cells specifically found in layer 2/3 (Kritzer and Goldman-Rakic, 1995). In contrast, layer V pyramidal cells and surrounding interneurons are hypothesized to be organized in aggregates that resemble the structure of columns (as in non-humam primates) or barrels (as in rodents) in other neocorteces (Naka and Adesnik, 2016). Activity in layer V pyramidal cells

readily recruits surrounding interneurons, which in turn elicits inhibition on nearby excitatory and inhibitory cells (Naka and Adesnik, 2016). This arrangement may be important for confining activity in a given neural aggregate or column to prevent unnecessary influences on neighboring columns. In this regard, the lack of significant changes following HFS delivered in the deep layer could be due to the inter-column inhibition that did not allow signal propagation between columns. However, it is not clear if we would have seen any significant changes in the field potential when the electrode placement had been further manipulated. Future investigations are required to determine the cellular and molecular mechanisms underlying this LTD.

Taken together, our results reveal a GABA-sensitive regulation in the deep layer in the mPFC emerging during late-adolescence. It is activated by HFS, which is in keeping with several *in vivo* findings (Caballero et al., 2014b; Cass et al., 2013). While the functional significance of this GABA-regulation requires further investigation, our results suggest that it is expressed when the HFS also becomes able to produce LTP in the mPFC. Perhaps, as suggested earlier, this inhibitory function may serve as an "anti-LTP" or a threshold control mechanism to prevent too much LTP saturating the mPFC network (Laroche et al., 2000). With a focus on the developmental changes in mPFC, the current study further demonstrates increases in dopamine receptors may play an important role in the maturation of HFS-induced LTD. Given the well-studied dopamine-GABA interplay, this HFS-induced LTD may also serve to regulate the signal-to-noise level in the deep layer output cells (Seamans et al., 2001; 2004; Caballero and Tseng, 2016).

Figures

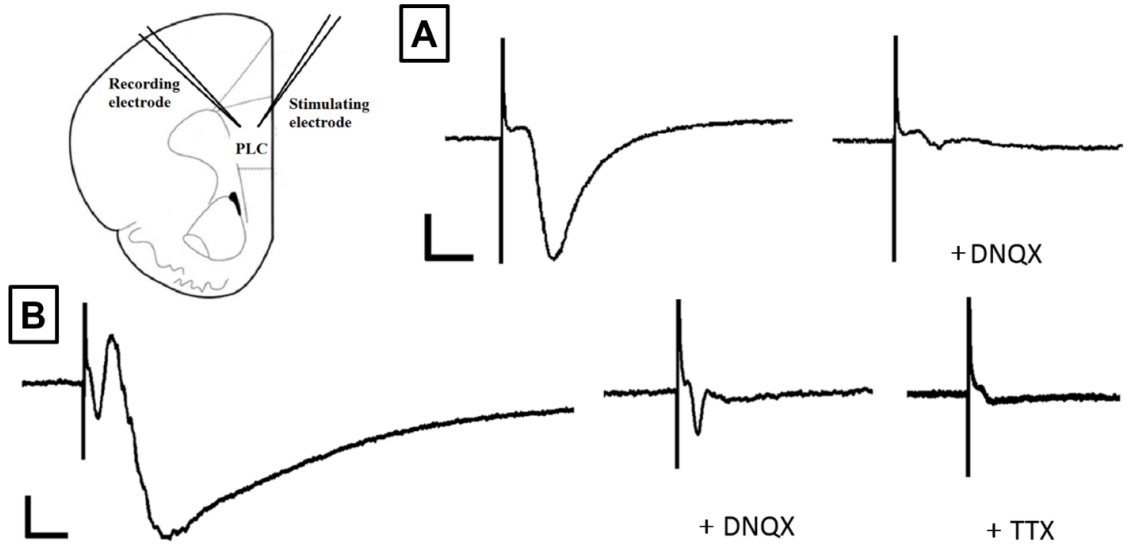

Figure 3.1 Schematic diagram of stimulating and recording electrode placements in the prelimbic cortex (PLC) and sample traces showing the components of the fEPSP that were analyzed. Single testing pulses **(**100 µs duration; 50-350 pA at 0.067 Hz**)** produced either single (**A**-**B**) or double (**C**-**E**) deflections that could be blocked by DNQX (10 µM) or DNQX (10 µM) + TTX (1µM)**.** Calibrattion axes: 50 µV for the ordinate; 50 ms for the abscissa in A, 20 ms for the abscissa in B.

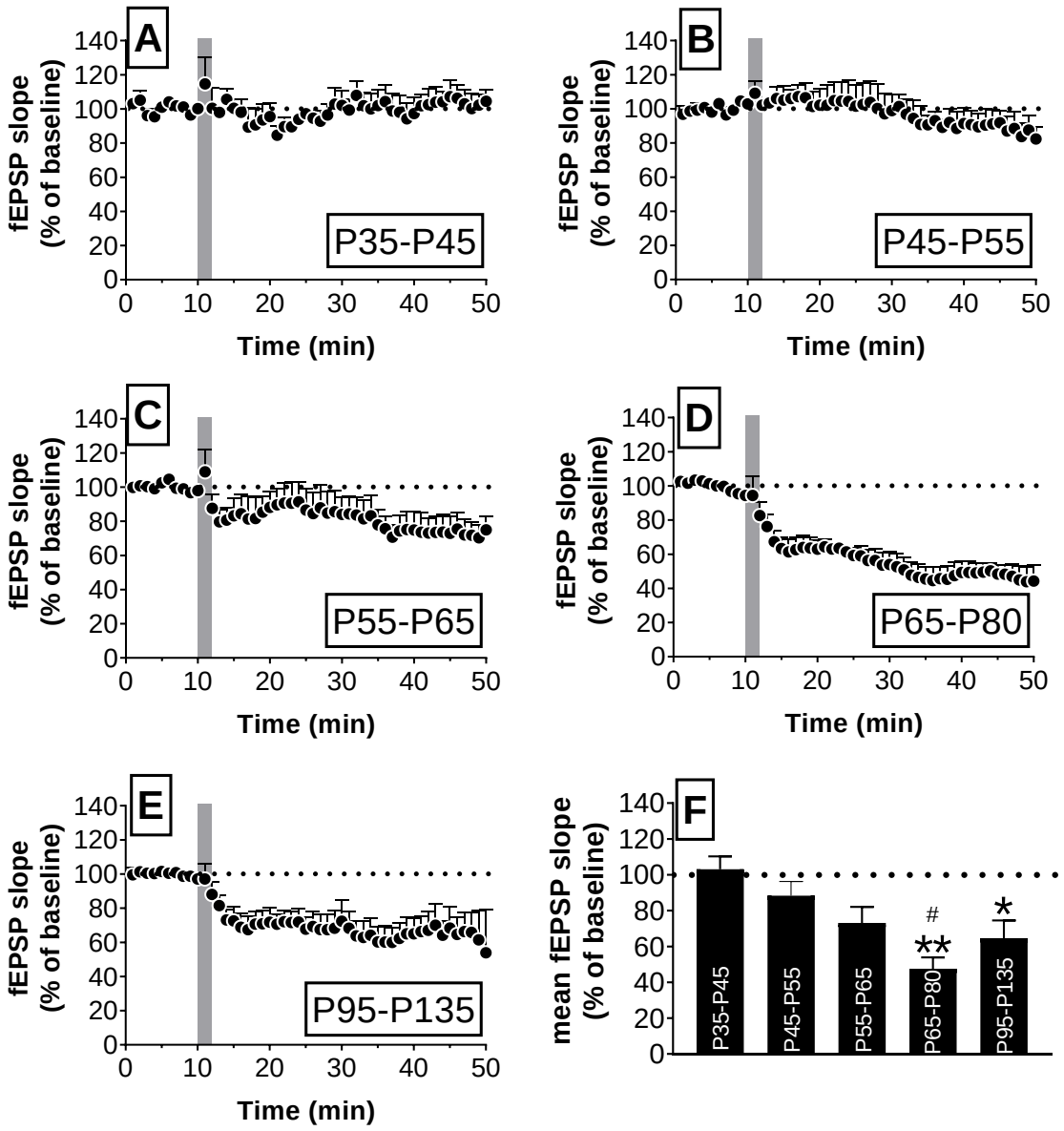

Figure 3.2 Age-dependent differences in deep layer PLC plasticity following HFS delivered in superficial layer PLC. (**A-E)** Effects of HFS delivered in layer 2/3 on the fEPSP slope recorded in layer 5/6 from rats in the indicated age range (n = 5-6 slices obtained from 4 rats/age group). The shaded vertical bar indicates the HFS period. **F.** Mean fEPSP response during the 30-40 min period following HFS for each age group. $^{**}p < 0.001$ vs. P35-P45; $^{*}p < 0.05$ vs. P35-P45; $^{\#}p < 0.05$ vs. P45-P55.

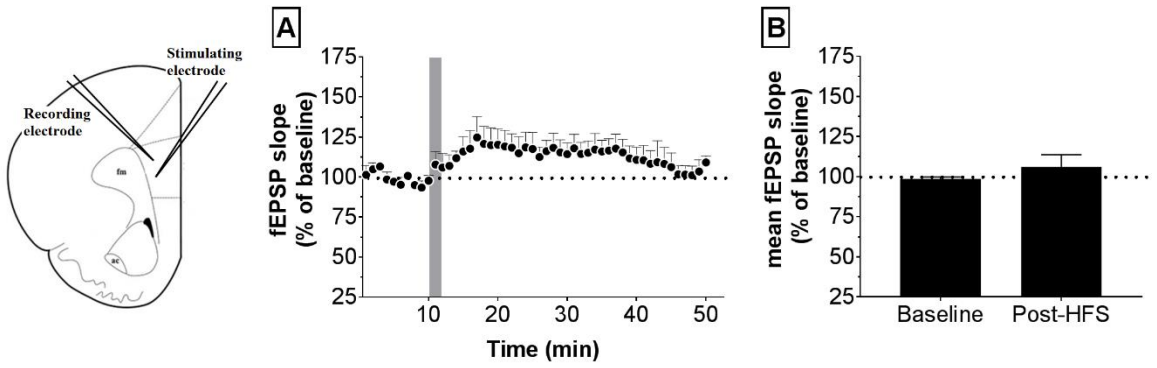

Figure 3.3 The effect of intra-laminar HFS on the evoked field potential in the deep layer PLC from young adult rats (P65-P80, n = 6 slices from 4 rats). Left panel, schematic diagram of the location for stimulating and recording electrodes, which were placed in layer 5/6 approximately 400-450 µm apart. **A**. Effects of HFS (period indicated by shaded vertical bar) on fEPSPs. **B.** Mean fEPSP response during the 10 min baseline period and the 30-40 min period following HFS.

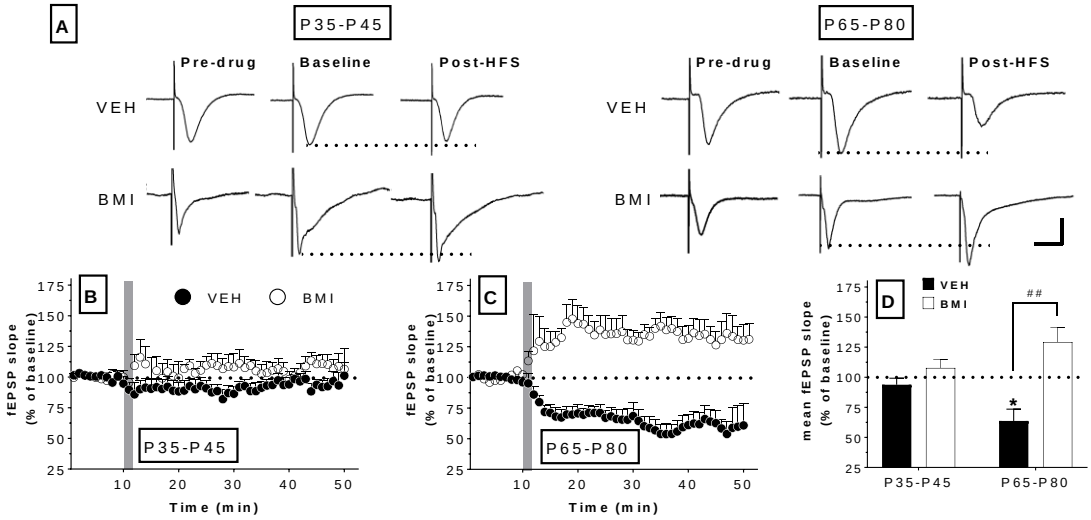

Figure 3.4 The effect of $GABA_A$ antagonist bicuculline (BMI) on HFS-induced plasticity in the PLC of peri-adolescent (P35-P45) and young adult rats (P65-P80). **A.** Representative traces sampled before (Pre-drug) and after (Baseline) application of 1µM BMI or vehicle (VEH) and following HFS (Post-HFS; calibrarion: 50 µV, 20 ms). **B-C.** Time course of the fEPSP slope before and after HFS (shaded vertical bar) in peri-adolescent and young adult rats (n=5-6 slices from 3 rats per age group). Slices were exposed to BMI or VEH for the duration of the recording shown. **D.** Mean fEPSP response from the 30-40 min period post-HFS**.** $^{*}p < 0.05$ vs. P35-P45 VEH; $^{\#\#}p < 0.001$ vs. P65-P80 VEH.

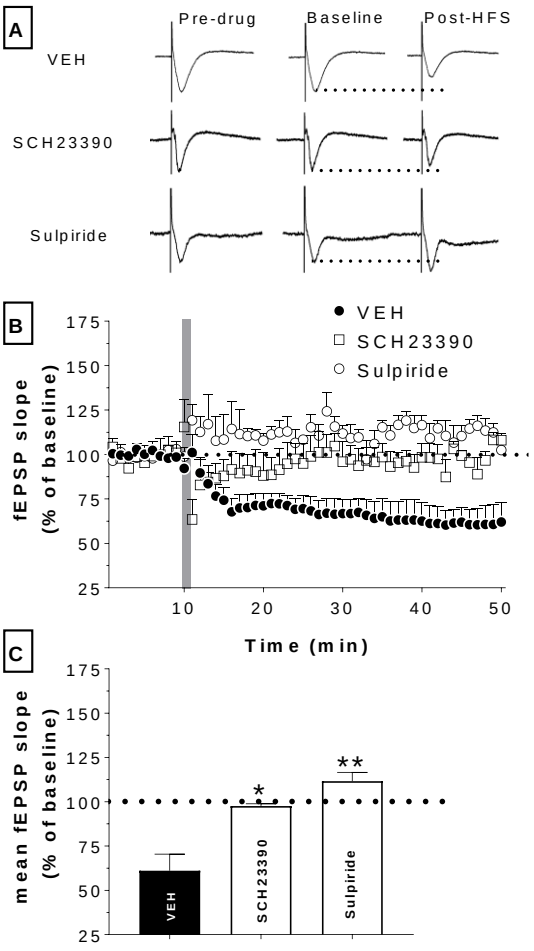

Figure 3.5 The role of dopamine D_1 and D_2 receptors in HFS-induced plasticity in the PLC of young adult rats (P65-P80). **A.** Representative traces sampled before application of vehicle (VEH), the D_1 anagonist SCH23390 (10 µM) or the D_2 antagonist sulpiride (10 µM), which are labeled Pre-drug, after their application (Baseline), and following HFS (Post-HFS; calibrarion: 50 µV, 20 ms). **B.** Time course of the fEPSP slope before and after HFS (shaded vertical bar) in young adult rats (n = 6 slices from 3 rats per age group. The time course shown includes the Baseline and Post-HFS periods (i.e., slices were exposed to VEH or a dopamine receptor antagonist for the duration of the recording shown). **C.** Summary of the mean fEPSP response from the 30-40 min period post-HFS**.** *$p < 0.05$ vs VEH; **$p < 0.001$ vs VEH.

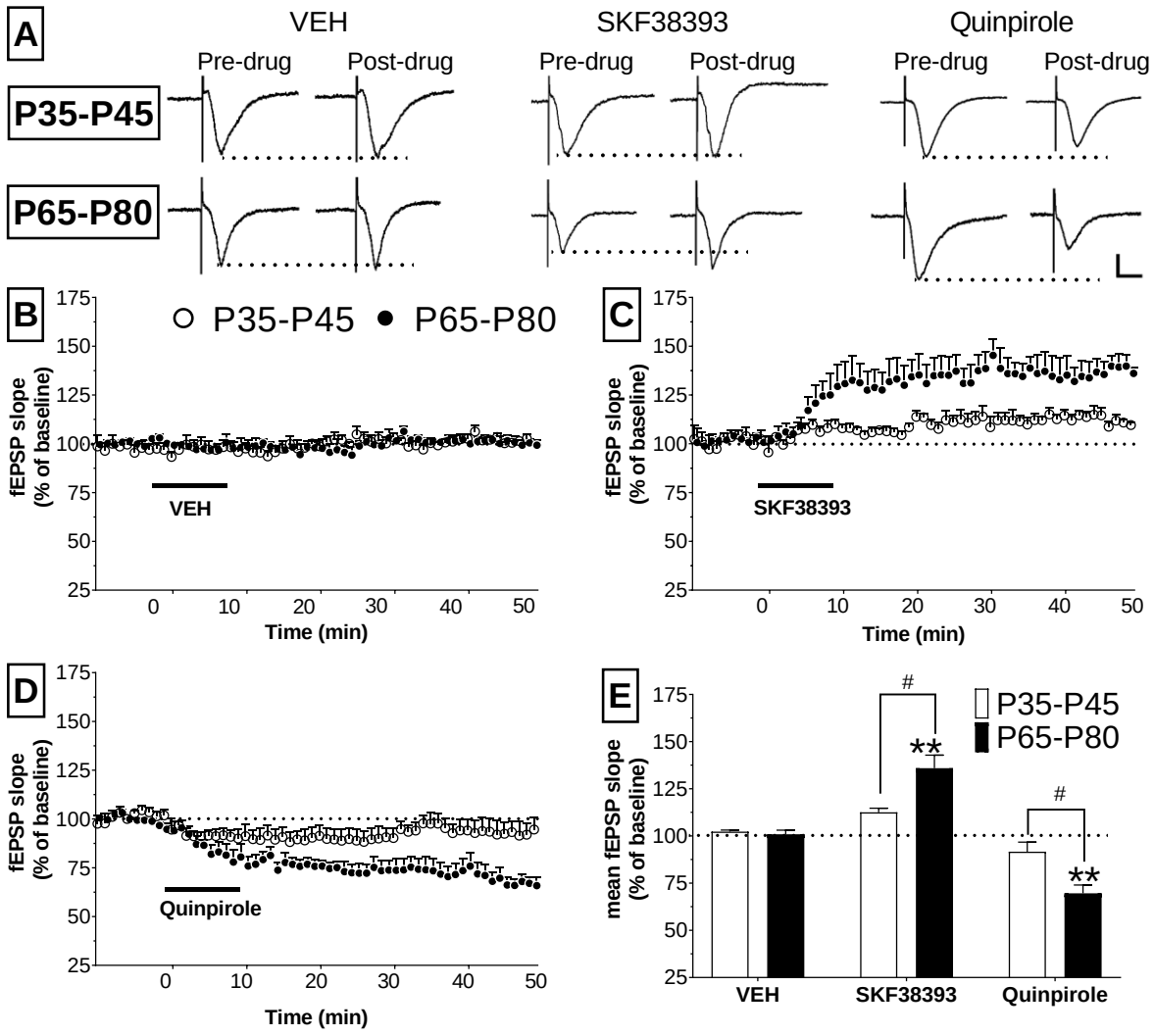

Figure 3.6 Age-dependent effect of dopamine D_1 and D_2 receptors agonists on evoked fEPSP in the PLC of peri-adolescent (P35-P45) and young adult (P65-P80) rats. A. Representative traces sampled before and after application of VEH or drug (Pre-drug and Post-drug, respectively; calibrarion: 50 µV, 20 ms). (**B-D**) Time course of the fEPSP slope before and after application of VEH, the D_1 agonist SKF38393 (10 µM) or the D_2 agonist quipirole (1 µM). The 10-min application period is indicated by the horizontal bar (n = 4-6 slices from 3 rats per group). **E.** Summary of the mean fEPSP response from the 30-40 min period after VEH or drug application**.** $^{**}p<0.001$ vs VEH within age group; $^{\#}p<0.05$ vs P35-P45 within drug condition.

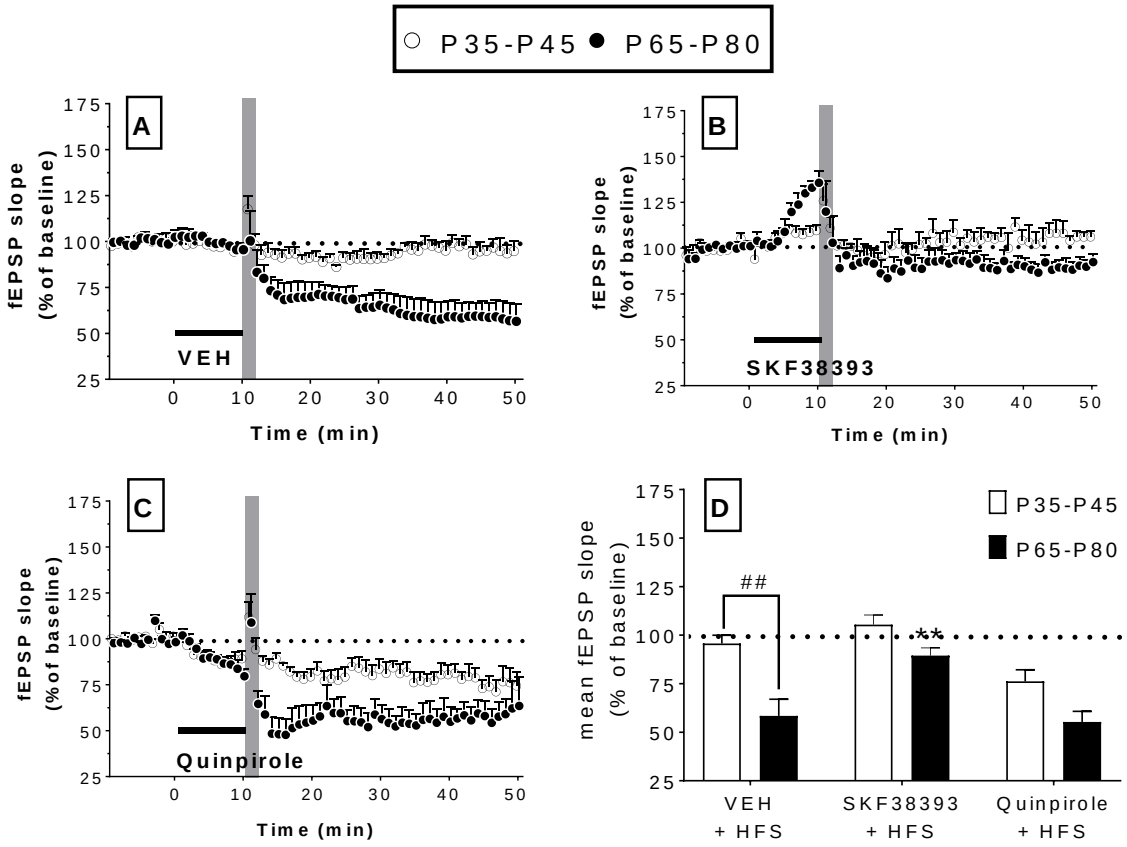

Figure 3.7 Age-dependent effect of dopamine D_1 and D_2 receptors agonist paried with HFS on evoked fEPSP in the PLC (n = 4-6 slices from 3 rats per group). (**A-C**). Time course of the fEPSP slope before and after application of VEH, the D_1 agonist SKF38393 (10 µM) or the D_2 agonist quipirole (1 µM). The 10-min application period is indicated by the horizontal bar. The shaded vertical bar indicates the HFS period. **D.** Summary of the mean fEPSP response from the 30-40 min period post-HFS**.** $^{**}p<0.01$ vs VEH within age group; $^{\#\#}p<0.01$ vs P35-45 within drug condition.

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Chapter 4: Mechanisms underlying age-dependent differences in AMPH's effects on HFS-induced LTD*

Rationale

The results from Experiment 1 suggest that HFS-induced LTD depends on intact $GABA_A$ and dopamine receptors and its protracted ontogeny may involve functional changes in dopamine-GABA signaling in the mPFC. Given their profound effects, it is likely that drugs that impact GABA or dopamine signaling may disturb the normal development of HFS-induced LTD (Cass et al., 2013; Thomases et al., 2014) and produce unique and long-lasting changes in its expression. The purpose of the experiments in this aim is to compare the effect of repeated AMPH exposure in adolescence or adulthood on HFS-induced LTD in the PL to determine if there are age of exposure-dependent effects of AMPH.

In regard to the potential mechanisms underlying AMPH-induced changes, this experiment will focus on dopamine's modulation of inhibition in the mPFC. it is known that dopamine increases the excitability of GABAergic interneurons by depolarizing their membrane closer to spike firing threshold via D_1-like but not D_2-like receptors (Gorelova et al., 2002). Accordingly, in peri-adolescent and adult rats, D_1 receptors stimulation leads to an increased number of evoked action potentials in FSIs and non-FSIs (Tseng and O'Donnell, 2007) and in turn, a higher frequency of sIPSCs in pyramidal neurons in the mPFC (Seamans et al., 2001; Kroener and Lavin, 2010; Paul and Cox, 2013). Stimulation of D_2 receptors decreases sIPSC frequency in pyramidal cells (Seamans et al., 2001; Kroener and Lavin, 2010; Paul and Cox, 2013) but paradoxically increases FSIs output only in adult rats (Tseng and O'Donnell, 2007).

* Kang S, Paul K, Hankosky ER, Cox CL, Gulley JM (2016a) D1 receptor-mediated inhibition of medial prefrontal cortex neurons is disrupted in adult rats exposed to amphetamine in adolescence. Neurosci. 324:40-49.

Notably, these effects of dopamine can last for over 30 min. Taken together, evidence suggests that dopamine has prolonged actions on inhibitory tone in pyramidal cells in the mPFC via activating both D_1 and D_2 receptor and the facilitating effects on inhibition seems to rely primarily on D_1 receptors (Seamans and Yang, 2004). In this regard, HFS-induced LTD may involve increased inhibition in pyramidal cells following D_1 receptor activation. Based on my previous finding (Kang et al., 2016b), I hypothesize that dopamine's modulation of inhibition in the PL is significantly impaired following AMPH exposure due to reduced D_1 function. Furthermore, I hypothesize a greater impairment will be associated with adolescent compared to adult AMPH exposure.

The current experiment first used field potential recordings in brain slices to determine if there were age-dependent differences in AMPH's effect on HFS-induced LTD. Then, patch-clamp was used to determine if inhibitory tone in the mPFC differentially responds to dopamine receptor activation in rats exposed to AMPH during adolescence compared to adulthood. In Experiment 2.1, recordings were obtained from rats in the adolescent- and adult-exposure groups following a 3-5 week withdrawal period. In Experiment 2.2, it was determined if the effects of adolescent exposure would persist into adulthood by repeating the Experiment 2.1 when rats were all approximately 4.5 months old. Thus, the withdrawal period from the last AMPH injection varied from 3-5 weeks and 11-14 weeks for adult- and adolescent-exposed rats, respectively. Non-selective activation of dopamine receptor was assessed following by exposing slices to dopamine, whereas selective activation of D_1 receptors was assessed with exposure to SKF38393.

Methods

Subjects

A total of 80 male Sprague-Dawley rats were used in these experiments. We chose only males because we haven't seen any significant sex difference in HFS-induced LTD in the PL in our pilot study. Animals were weaned on P22 and housed 2-3 per cage with ad libitum access to food and water. They were kept on a 12-h light/dark cycle (lights on at 0800) with experiments performed between 0830h and 1830h.

AMPH treatment.

Rats were assigned to one of three treatment groups – control, adolescent-exposed, or adult-exposed – such that all groups were represented in each litter. Injections (i.p.) were given every other day during adolescence and/or adulthood as dipicted in Figure 4.1. Those assigned to the control group were given 0.9% saline (1 ml/kg), whereas those in the treatment groups were given 3 mg/kg AMPH (d-amphetamine hemisulfate salt; Sigma-Aldrich, St. Louis, MO, USA). For the adult-exposed rats in Experiment 2.1 and both adolescent- and adult-exposed rats in Experiment 2.2, potential effects of injection experience were controlled by administering injections at both adolescent and adult time periods (Figure 3.1). For all injections, animals were transported to a testing room, given their injection, and placed individually in a clear plastic tub (46 x 25 x 22 cm) lined with hardwood bedding. After 60 min, rats were returned to their home cages in the colony room.

Electrophysiology

Rats were sacrificed between P66 and P77 (mean = P70) for those in the adolescent-exposed groups or between P125 and P136 (mean = P130) for those in the adult-exposed groups.

This kept the withdrawal period between groups at 3-5 weeks. Animals were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.), perfused with cold, oxygenated slicing medium containing (in mM): 2.5 KCl, 10.0 MgCl2, 0.5 CaCl2, 1.25 NaH2PO4, 26.0 NaHCO3, 11.0 glucose, and 234.0 sucrose, and then decapitated. Their brain was quickly removed, sliced into 450 µm or 350 µm coronal sections using a vibrating tissue slicer for field potential recording or patch-clamp, respectively. These sections were transferred to a holding chamber where they were incubated for at least 1h before recording. The procedure for field potential recordings is identical to that as described in Experiemnt 1. For patch-clamp recordings, individual slices were subsequently transferred to a submersion-type recording chamber on a modified microscope stage (Axioskop 2FS; Zeiss Instruments, Thornwood NY) and continuously superfused with oxygenated physiological saline at 32 ºC. This solution contained (in mM): 126.0 NaCl, 2.5 KCl, 1.25 MgCl2, 2.0 CaCl2, 1.25 NaH2PO4, 26.0 NaHCO3, and 10.0 glucose. It was gassed with 95% O2/5% CO2 to a final pH of 7.4. A 5x objective was used to identify layer V/VI of mPFC and a 63x water-immersion objective equipped with differential interference contrast optics was used to visualize individual neurons.

Using the whole-cell configuration, intracellularly recorded currents were amplified by a Multiclamp 700 amplifier (Molecular Devices, Foster City, CA) with voltage-clamp protocols generated using pClamp software. Recording pipettes (tip resistance = 3-6 MΩ) were filled with a solution containing (in mM): 117.0 Cs-gluconate, 13.0 CsCl, 1.0 MgCl2, 0.07 CaCl2, 0.1 EGTA, 10.0 HEPES, 2.0 Na2-ATP, 0.4 Na-GTP and 0.3% biocytin. The pH and osmolarity were adjusted to 7.3 and 290 ±1 mosM, respectively. Data were obtained from recordings with the access resistance stabilized between 10 and 20 MΩ. Layer V/VI pyramidal neurons in either the prelimbic or infralimbic regions of the mPFC were identified by their soma shape and apical

dendrite oriented towards layer I. A roughly equal number of cells from these subregions of the mPFC were recorded across the groups in Experiments 1 and 2. For recordings of sIPSCs, glutamate receptor antagonists, CPP (10 μM) and DNQX (20 μM) were present in the bath and currents were recorded at a 0 mV holding potential. Neurons were filled with biocytin and subsequent recovery was used to confirm they were pyramidal cells.

A motorized syringe pump was used to apply agonists by injecting a bolus into the input line of the recording chamber. All concentrations reported here are the final bath concentrations that were estimated as previously described (Cox et al., 1995). Dopamine was made with 0.08% ascorbic acid every 2-3 h and was kept on ice and away from light. To minimize light-induced oxidation, lights were off when dopamine was bath-applied. All chemicals were obtained from Tocris (St. Louis, MO) and doses were chosen based on our previous studies (Paul and Cox, 2013).

Data analysis

Data are presented as mean ± SEM unless noted otherwise. Analysis of field potential was the same as described in Experiment 1. Analysis of sIPSC time course was done with two-way, mixed factor ANOVA (time x group). Group differences in the peak response to bath-applied drugs were determined by using an adjacent-averaging data smoothing method (Seamans and Yang 2004, Trantham-Davidson et al., 2004; Paul and Cox, 2013). For each recording, the peak response area was determined as a 5-min window centered around the 1-min bin with the maximal deviation from baseline (the shaded areas in Figs. 4.2-4.6). This peak response was analyzed with two-way ANOVA (group x age) followed by Tukey's post-hoc analyses where appropriate.

Results

Experiment 2.1

We recorded fEPSP in the PL from control (n=7-8 slices from 4 rats/group) or AMPH-exposed rats (n=8-9 slices from 4 rats/group) 3 to 4 weeks after their last injection. As shown in Fig. 4.2, we found that HFS triggered LTD in controls but not in AMPH pre-exposed rats. Two-way ANOVA with repeated measures revealed a significant main effect of group ($F_{3, 28}$ = 11.33, p <0.001), time ($F_{38, 1064}$= 5.834, p <0.001) and time by group interaction ($F_{114, 1064}$= 4.148, p <0.001). Post hoc tests suggest significant group differences between AMPH-exposed group and their corresponding control, and between adolescent- and adult-AMPH group. Analysis of the mean slope of the fEPSP from 25-30 min post-HFS (Fig. 4.2, right panel) revealed a significant main effect of treatment ($F_{1, 27}$ = 21.23, p <0.001) and age ($F_{1, 27}$ = 8.538, p = 0.008) and a near-significant treatment by age interaction ($F_{1, 27}$ = 3.307, p = 0.08).

We next recorded sIPSCs in layer V/VI pyramidal neurons from control (n = 14 and 13 for adolescent and adult, respectively) or AMPH-exposed rats (n = 12 and 13 for adolescent and adult exposed, respectively). The averaged frequency and amplitude of baseline sIPSC are summarized in the Table 4.2. Although the adolescent control rats had the lowest values of the four groups, the frequency and amplitude were not significantly different.

In control rats, bath application of dopamine (50 μM, 4 min) produced a significant increase in the sIPSC frequency, but this effect of dopamine was absent in AMPH-exposed rats. Two-way ANOVA of frequency time course data revealed a significant main effect of time ($F_{34,476}$ = 5.58, p < 0.001) and a significant group by time interaction ($F_{102,476}$ = 1.32, p < 0.05). Two-way ANOVA of the peak response showed significant differences in sIPSC frequency between control and AMPH-exposed groups ($F_{3,15}$ = 3.90, p < 0.05), but no age of exposure-

dependent differences (Fig. 4.3). Dopamine application did not change the sIPSC amplitude for any groups.

In our previous studies, this DA-mediate increase in inhibitory activity has been linked to the selective activation of D1 receptors (Paul & Cox, 2013). Thus, to determine if D1 receptor function was altered by repeated AMPH exposure, we next tested the ability of the selective D1 agonist SKF38393 to alter sIPSC activity. SKF38393 (10 μM, 90 s) produced a large increase in sIPSC frequency in controls (Fig. 4.3), but this effect was reduced in both adolescent- and adult-exposed rats. A significant main effect of time ($F_{34,510} = 10.9$, $p < 0.001$), group ($F_{3,510} = 3.59$, $p < 0.05$) and time by group interaction ($F_{102,510} = 1.67$, $p < 0.001$) was observed. Two-way ANOVA of peak response indicated a significant main effect of group ($F3,15 = 3.87$, $p < 0.05$). Post-hoc comparisons revealed a significant group difference between controls and AMPH-exposed groups, but there were no significant age of exposure-dependent differences. Analysis of sIPSC amplitude revealed no statistically significant differences for either the time course or peak response data (Fig. 4.3).

Experiment 2.2

A different group of rats were injected with saline or 3mg/kg AMPH in their adolescence (P27-P45) or adulthood (P85-P103) and sacrificed at proximately same age for field potential and patch clamp recording (Fig. 4.1). The mean withdrawal period was nearly 13 weeks for rats exposed to AMPH during adolescence and nearly 5 weeks for those exposed during adulthood. We recorded fEPSP in the PL from control (n=7 slices from 4 rats) and AMPH-exposed rats (n=10-11 slices from 4 rats/group). As shown in Fig. 4.5, we found again that HFS-induced LTD is altered in AMPH pre-exposed rats. For the time course data, Two-way ANOVA revealed a significant main effect of group ($F_{2, 25} = 3.854$, $p = 0.035$) and interaction of time by group ($F_{76,}$

$_{950}$ = 2.797, $p < 0.001$). Post hoc tests suggest a significant difference between adolescent-exposed group and control. Analysis of the mean fEPSP slope 25-30 min post-HFS revealed a significant main effect of group ($F_{2, 26} = 7.178$, $p = 0.003$) and post hoc suggest that adolescent-exposed group is significantly different from adult-exposed group and control.

We then recorded sIPSCs in layer V/VI pyramidal neurons from control (n = 15) or AMPH-exposed rats (n = 8 and 12 for adolescent and adult groups, respectively). For this cohort of animals, sIPSC frequency during baseline tended to be relatively lower in controls compared to AMPH exposed groups. However, as with Exp. 2.1, there were no statistically significant group differences in this measure or in the amplitude of sIPSCs (Table 4.1).

We found an increase in sIPSC frequency, but no change in amplitude, following dopamine application (50 μM, 4 min) in pyramidal neurons from controls (Fig. 4.6). This dopamine-mediated facilitation was significant reduced or abolished in neurons from adult- and adolescent-exposed rats. Statistical analysis of sIPSC frequency revealed a significant main effect of group ($F_{2,641} = 3.88$, $p < 0.05$), time ($F_{34,641} = 2.53$, $p < 0.001$) and time by group interaction ($F_{68,641} = 3.17$, $p < 0.001$). Analysis of the peak response (Fig. 4.6) revealed significant differences in sIPSC frequency between control and AMPH-exposed groups, with adolescent- and adult-exposed rats exhibiting a similar lack of sensitivity to dopamine. When D_1 receptor function was assessed by bath application of SKF38393 (10 μM, 90 s), we observed an effect similar to what was seen in Experiment 2.1. Specifically, there was an increase in sIPSC frequency in slices from control rats, but this effect was attenuated in slices from AMPH-exposed rats (Fig. 4.7). Two-way ANOVA revealed a significant main effect of time ($F_{34,374} = 13.2$, $p < 0.001$) and a time by group interaction ($F_{68,374} = 2.12$, $p < 0.001$). Analysis of the peak response revealed that both adolescent and adult-exposed groups exhibited this reduced

sensitivity to the D_1 agonist (Fig. 4.7), but the effect was greatest for those exposed in adulthood. This was confirmed statistically through a significant main effect of group ($F_{2,11} = 4.17$, $p < 0.05$) followed by a significant post-hoc comparison between controls and the adult-exposed group. The amplitude of sIPSCs did not significantly change over time or between groups.

Taken together, we found that layer V/VI pyramidal cells recorded from rats exposed to AMPH during adolescence or adulthood were no longer sensitive to dopamine-induced increases in sIPSCs. This effect was at least partially mediated by a reduced sensitivity of D1 receptors in exposed rats. There was no apparent dependency of this effect on age of exposure.

Discussion

This experiment set out to determine if there are age-of-exposure dependent effect of repeated AMPH on HFS-induced LTD in the PL. We further tested if AMPH exposure would induce changes in dopamine-mediated modulation of inhibitory transmission in the mPFC and if the effect of AMPH depends on the timing of exposure. We controlled for withdrawal duration and testing age in experiment 2.1 and 2.2, respectively, and found persistent changes in the LTD function following AMPH exposure. Furthermore, layer V/VI pyramidal cells recorded from rats exposed to AMPH during adolescence or adulthood were no longer sensitive to dopamine-induced increases in IPSCs. This effect was at least partially mediated by a reduced sensitivity of D1 receptors in exposed rats. However, there was no apparent dependency of this effect on age of exposure.

Consistent with our hypothesis, we observed long-lasting disruptive effects of repeated AMPH on HFS-induced LTD in the PLC. Specifically, adult exposure seemed to result in an

impaired expression of LTD. In contrast, slices from adolescent-exposed group exhibited an LTP-like change following HFS, a phenomenon observed previously when we had $GABA_A$ antagonist present before delivering HFS (Fig. 3.4, Experiment 1). This result is consistent with a previous finding that HFS in the vHC led to an LTP in the mPFC rather than an LTD as in controls following daily injections of cocaine between P35 and P40 (Cass et al., 2014). These authors found such effect approximately 5 weeks after the cocaine injection while the current study demonstrated that altered LTD expression was still prominent 14 weeks after adolescent drug exposure. Based on our results in Experiment 1, we speculate that the altered LTD following AMPH exposure could be due to a reduction in inhibitory function in the mPFC.

It is known that dopamine plays a critical role regulating inhibitory transmission in the mPFC (Seamans et al., 2001; Trantham-Davidson et al., 2004). To investigate the potential mechanism underlying AMPH-induced changes in the LTD, we examined the baseline sIPSCs function in layer V/VI pyramidal cells as well as their response to dopamine receptor manipulations. Our data suggest that repeated AMPH exposure, regardless of the age when it occurs, results in long-lasting disruption of dopamine's modulatory influence on sIPSCs. Previous studies in juvenile (P14-P28) and young adult (P50-P100) rodents have shown that high concentrations of dopamine (>1 μM) applied *in vitro* increase sIPSC frequency in mPFC pyramidal cells primarily via D_1 activation (Seamans et al., 2001; Gonzalez-Islas and Hablitz, 2001; Gonzalez-Burgos et al., 2005; Kroener and Lavin, 2010; Paul and Cox, 2013). In line with this, we found a stable increase of sIPSC frequency in control rats following D1 activation by dopamine or SKF 38393. This effect was significantly attenuated in AMPH pre-exposed rats, suggesting a long-lasting impairment of D_1-mediated regulation of sIPSC activity. Others have reported that AMPH exposure during adolescence or young adulthood is associated with

adaptive changes in mesocortical dopamine circuits (Labonte et al., 2012; Reynolds et al., 2015) and D_1 receptor function (Fletcher et al., 2005; Peterson et al., 2006; Tse et al., 2011) following 3 days to 4 weeks of withdrawal.

In line with the persistent alteration in the LTD function, our results suggest the reduced responsiveness to dopamine in the mPFC can last at least 14 weeks following adolescent exposure. Interestingly, the D1 receptor insensitivity seemed to diminish after this long withdrawal period in adolescent-exposed animals, suggesting mechanisms other than D1 dysfunction contributing to the blunted response to dopamine. However, from the current study, it is not clear if D1 function is still impaired following a prolonged withdrawal in those exposed during adulthood. In addition, the detailed mechanisms underlying the attenuated inhibitory tone following chronic AMPH exposure will require additional study. Previous work suggests that the D1-dependent facilitation of sIPSC activity is due to increased presynaptic GABA release, as dopamine and selective D1 agonists have no significant effect on the frequency or amplitude of miniature IPSC recorded in mPFC pyramidal cells (Seamans et al., 2001; Gorelova et al., 2002; Kroener and Lavin, 2010). D1 activation increases the excitability of interneurons, which results in an enhancement of their output (Gorelova et al., 2002; Gonzalez-Burgos et al., 2005). Thus, it is possible that repeated AMPH exposure induces changes in interneuron physiology such that they become less sensitive to D1 stimulation and/or have a reduced output capacity (Morshedi and Meredith, 2007). However, the current study does not allow us to rule out the possibility that there are postsynaptic changes that may contribute to the reduced inhibition in the pyramidal cells. Future studies will be necessary to determine the detailed mechanisms for D1 function deficiency and if there are also long-lasting changes in D2 receptor function that might also contribute to the effects of AMPH exposure on inhibitory tone in the PFC (Paul et al., 2016).

Whereas AMPH-induced adaptations in dopamine receptor expression and/or signaling are a likely candidate mechanism for the effects we observed, the current studies do not allow us to rule out a potential involvement of 5-HT receptor changes. Previous work has shown that the D1 agonist we used in this study (SKF 38393) has moderate affinity for 5-HT receptors in vitro (Briggs et al., 1991). However, the effect of 5-HT receptor activation on sIPSC frequency is transient, lasting less than 15 min (Tan et al., 2004). In contrast, we found previously (Paul and Cox, 2013) and in the current study that dopamine and SKF 38393 had more enduring effects on sIPSC activity. Thus, AMPH-induced changes in serotonergic signaling are unlikely to play a major role in the loss of sensitivity of prefrontal neurons to D1-induced increases in sIPSCs that we observed in drug exposed rats.

The PFC's top-down control of executive functions relies on deep layer output neurons that are tightly controlled by interneurons (González-Burgos et al., 2002). Accordingly, disruption of GABAergic function in the mPFC results in a broad spectrum of cognitive impairments (Gonzalez-Burgos et al., 2011; Enomoto et al., 2011). The current study suggests that a deficit in D1-mediated inhibitory transmission in the mPFC is a candidate mechanism through which repeated AMPH exposure induces lasting deficits in cognition in both humans (Ornstein et al., 2000) and laboratory animals (Gulley and Juraska, 2013). Importantly, the drug exposure paradigm we used in Exp. 2.2 is identical to the one we used previously to demonstrate impairments in working memory (Sherrill et al., 2013), cognitive flexibility (Hankosky et al., 2013) and impulse control (Hammerslag et al., 2014) after a prolonged abstinence, where certain cognitive changes were specific to adolescent exposure. These diverging behavioral outcomes suggest that there are different neuroadaptations following exposures at the two developmental stages. In this initial study of seeking the drug-induced plasticity specific to adolescent exposure,

we found a unique change in the HFS-induced LTD function but not in dopamine-mediated inhibition in the mPFC. Future studies are required to elucidate the mechanisms underlying the plasticity induced specifically by adolescent exposure.

In summary, our findings demonstrate that chronic AMPH exposure leads to differential expression of HFS-induced LTD and reduced inhibitory transmission in the mPFC as a result of alterations in dopamine receptor function. Importantly, these effects persist after a protracted withdrawal period. The findings from adolescent-exposed rats reveal that drug-induced changes in mPFC function last throughout the adolescent period and linger well into adulthood, long after drug exposure has ceased. Adolescents may be especially vulnerable to certain aspects of drug-induced plasticity, which in turn may confer a greater risk for developing cognitive dysfunction, addiction and other psychological disorders (Gulley and Juraska, 2013). Previous work in adolescent rats exposed to cocaine (Cass et al., 2013) or cannabinoids (Cass et al., 2014) has also demonstrated lasting changes (up to 35 days) in inhibitory tone and HFS-induced LTD in the mPFC. Thus, the ability of drugs of abuse to alter the normal developmental trajectory of prefrontal circuitry may be a principal mechanism through which adolescent drug exposure can lead to longstanding, if not permanent, changes in prefrontal control over behavior. It will be important for future studies to determine the precise mechanisms that underlie drug-induced plasticity leading to vulnerability, as well as understand what factors might lead to resilience to the effects of drugs (Hammerslag and Gulley, 2016).

Table and Figures

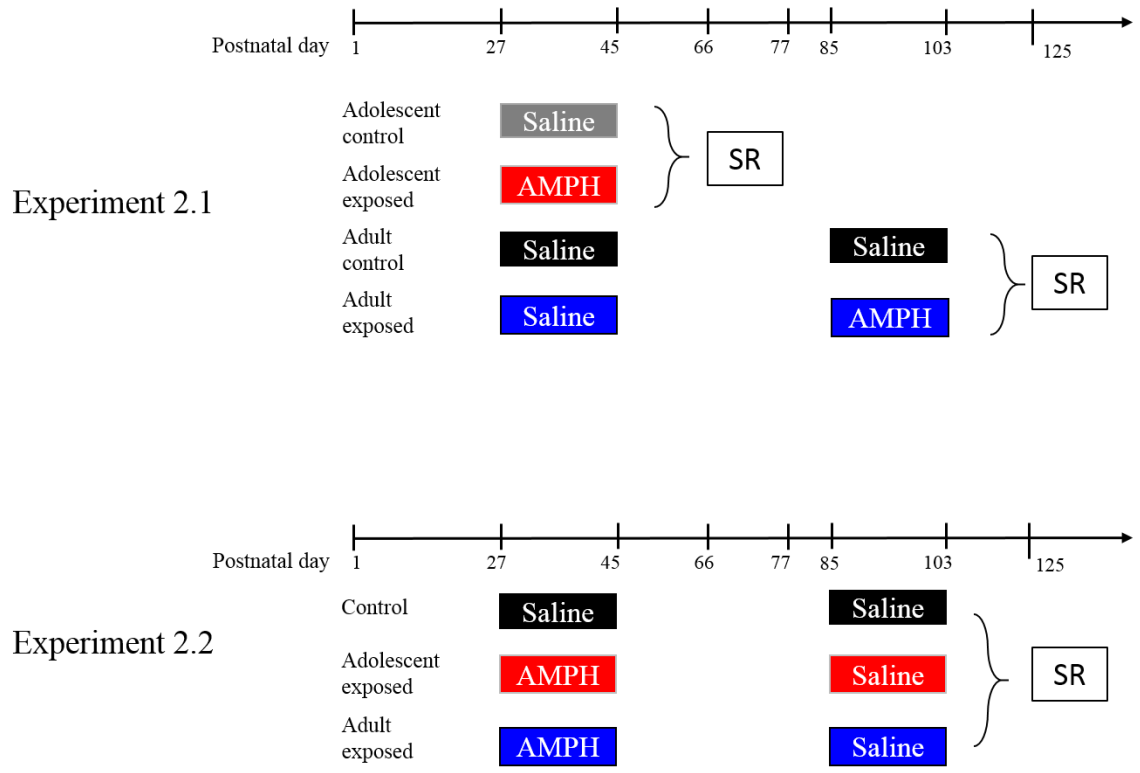

Figure 4.1 Summary of groups injected (i.p.) with either saline or 3 mg/kg AMPH a total of 10 times (once every other day) during the ages noted. For Experiment 2.1, rats were sacrificed for slice electrophysiology 3-5 weeks following their last injection. In Experiment 2.2, rats were sacrificed at about the same age in adulthood such that withdrawal periods were 3-5 weeks and 11-14 weeks for adult and adolescent groups, respectively. SR=slice recording.

	Age at recording (P)	sIPSC frequency (Hz)	sIPSC amplitude (pA)
Experiment 2.1			
Control			
Adolescent	70 ± 2.2	8.9 ± 1.1	27.3 ± 1.1
Adult	133 ± 2.8	10.6 ± 1.5	29.1 ± 1.3
Exposed (withdrawal duration)			
Adolescent (26 ± 1.2 days)	71 ± 1.2	10.0 ± 1.4	30.1 ± 2.1
Adult (25 ± 0.8 days)	128 ± 0.9	9.4 ± 1.3	31.0 ± 1.1
Experiment. 2.2			
Control	134 ± 1.5	7.4 ± 0.9	22.6 ± 0.6
Exposed (withdrawal duration)			
Adolescent (88 ± 2.1 days)	132 ± 2.1	9.5 ± 1.1	23.5 ± 0.9
Adult (33 ± 2.0 days)	135 ± 2.0	9.1 ± 1.2	22.2 ± 0.8

Table 4.1. Summary of postnatal age (P) at sacrifice for slice electrophysiology experiments and sIPSC characteristics during baseline recording. Also shown for AMPH-exposed rats is the mean number of withdrawal days between the last drug injection and sacrifice.

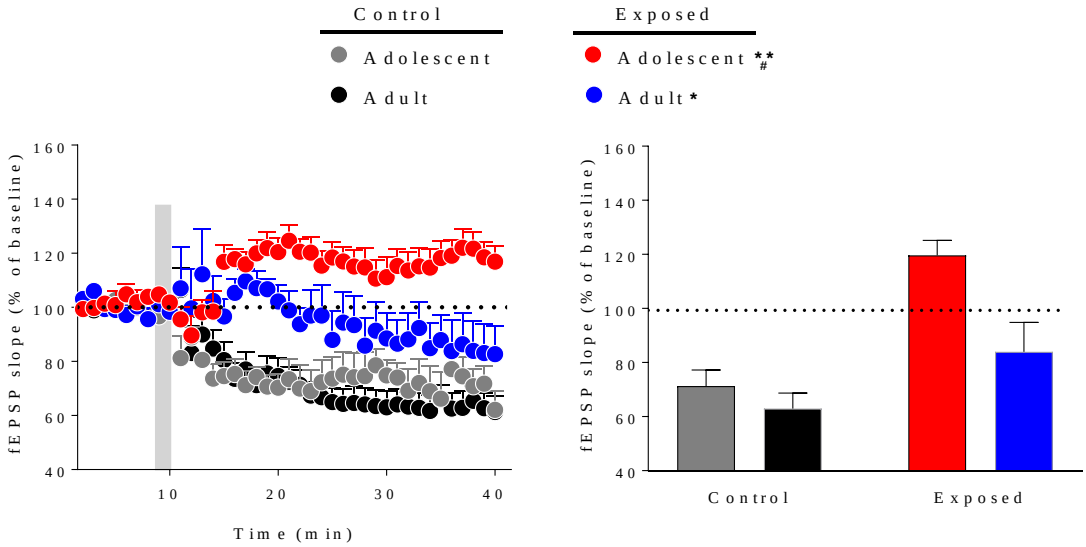

Figure 4.2 HFS-induced LTD is impaired in AMPH-exposed rats in Experiment 2.1. Left: time course of the fEPSP slope before and after HFS (shaded vertical bar) in control and AMPH-exposed group (n = 7-9 slices from 4 rats per group), $^{**}p < 0.01$, $^{*}p < 0.05$ vs. age-matched control; $\#p < 0.05$ vs. adult-exposed group. Right: Mean fEPSP response during the 25-30 min period following HFS for each age group.

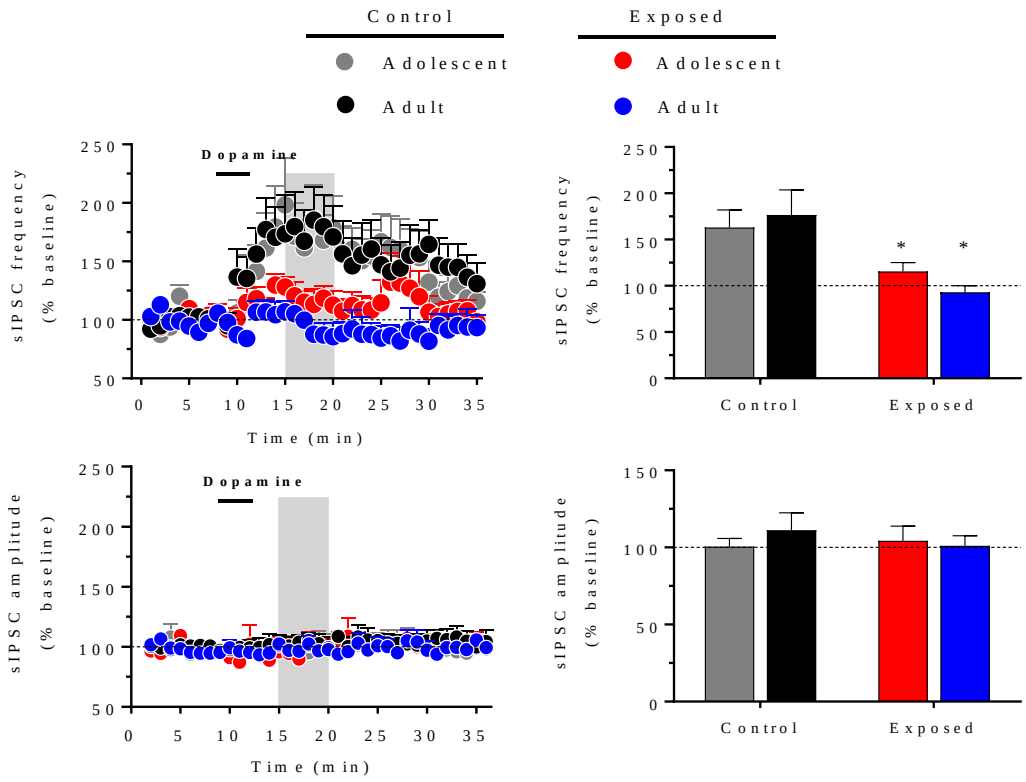

Figure 4.3. Time course and peak response for sIPSC amplitude and frequency in slices exposed to dopamine (DA; 50 μM for 4 min, as indicated by horizontal bar) in Experiment 2.1. Recordings were obtained from 4-6 cells/group in slices from 3-4 rats/group. Shaded regions in the time series indicate the areas of peak response (see Methods). The mean responses during these periods are summarized in the bar graphs. *$p < 0.05$, *vs.* age-matched control.

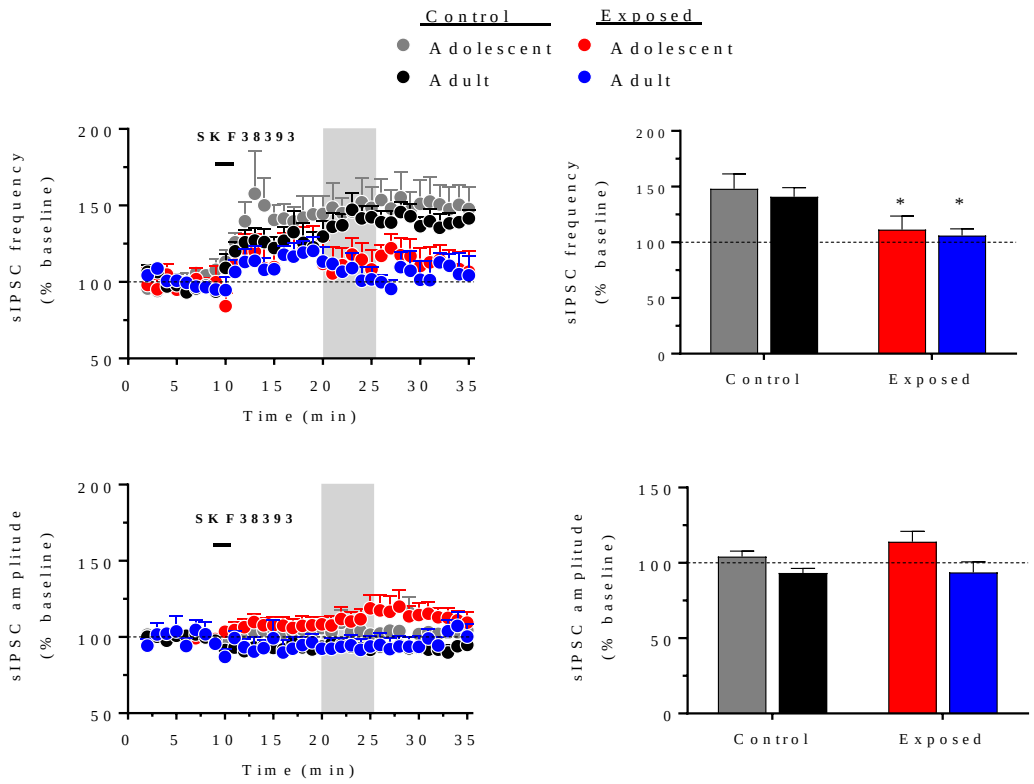

Figure 4.4 Time course and peak response for sIPSC amplitude and frequency in slices exposed to SKF38393 (10 μM for 90 s, as indicated by horizontal bar) in Experiment 2.1. Recordings were obtained from 4-7 cells/group in slices from 3-4 rats/group. Data are presented as described for Fig. 2. *$p < 0.05$, vs. control.

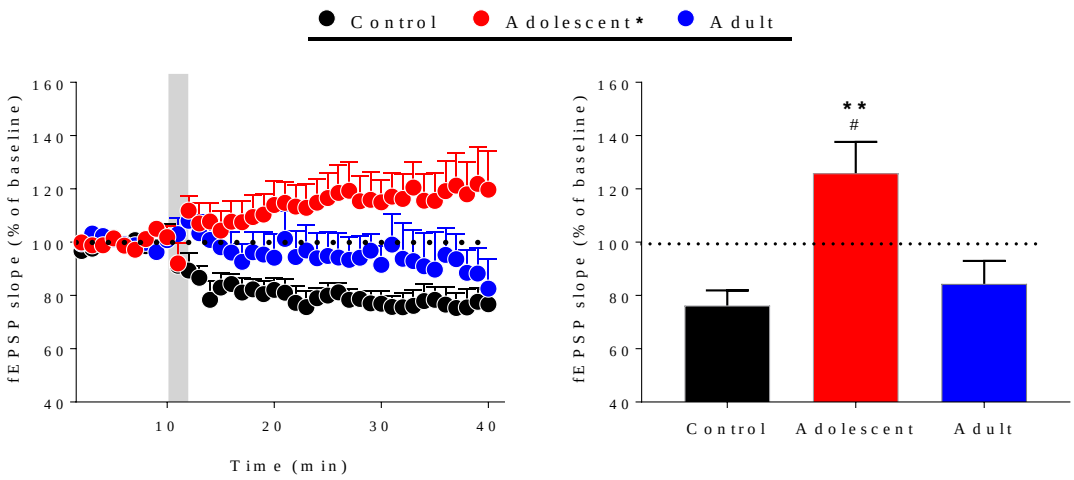

Figure 4.5 HFS-induced LTD is impaired in AMPH-exposed rats in Experiment 2.2. Left: time course of the fEPSP slope before and after HFS (shaded vertical bar) in control and AMPH-exposed group (n = 7-11 slices from 4 rats per group), *$p < 0.05$ vs. control. Right: Mean fEPSP response during the 25-30 min period following HFS for each age group. **$p < 0.01$ vs. control; #$p < 0.05$ vs. adult.

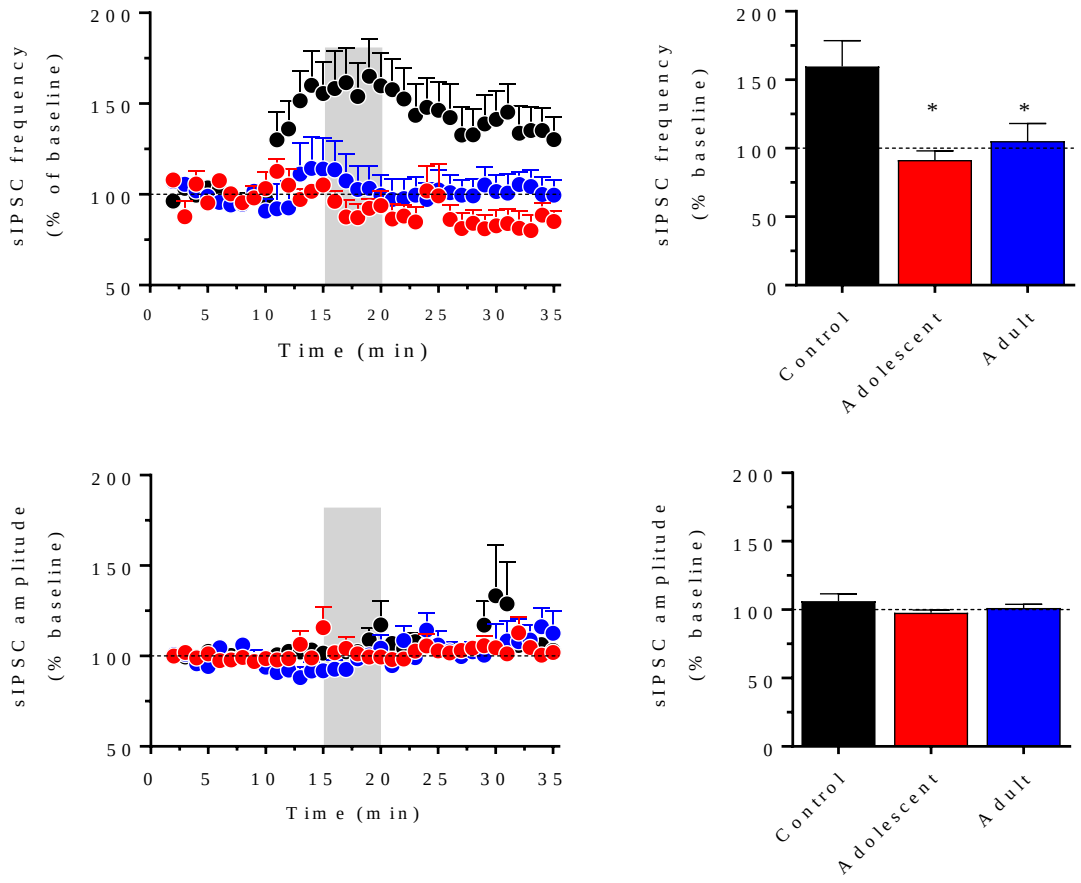

Figure 4.6 Time course and peak response for sIPSC amplitude and frequency in slices exposed to dopamine (DA; 50 μM for 4 min, as indicated by horizontal bar) in Experiment 2.2. Recordings were obtained from 4-10 cells/group in slices from 4-5 rats/group. Data are presented as described for Fig. 4.3. *$p< 0.05$, *vs.* control.

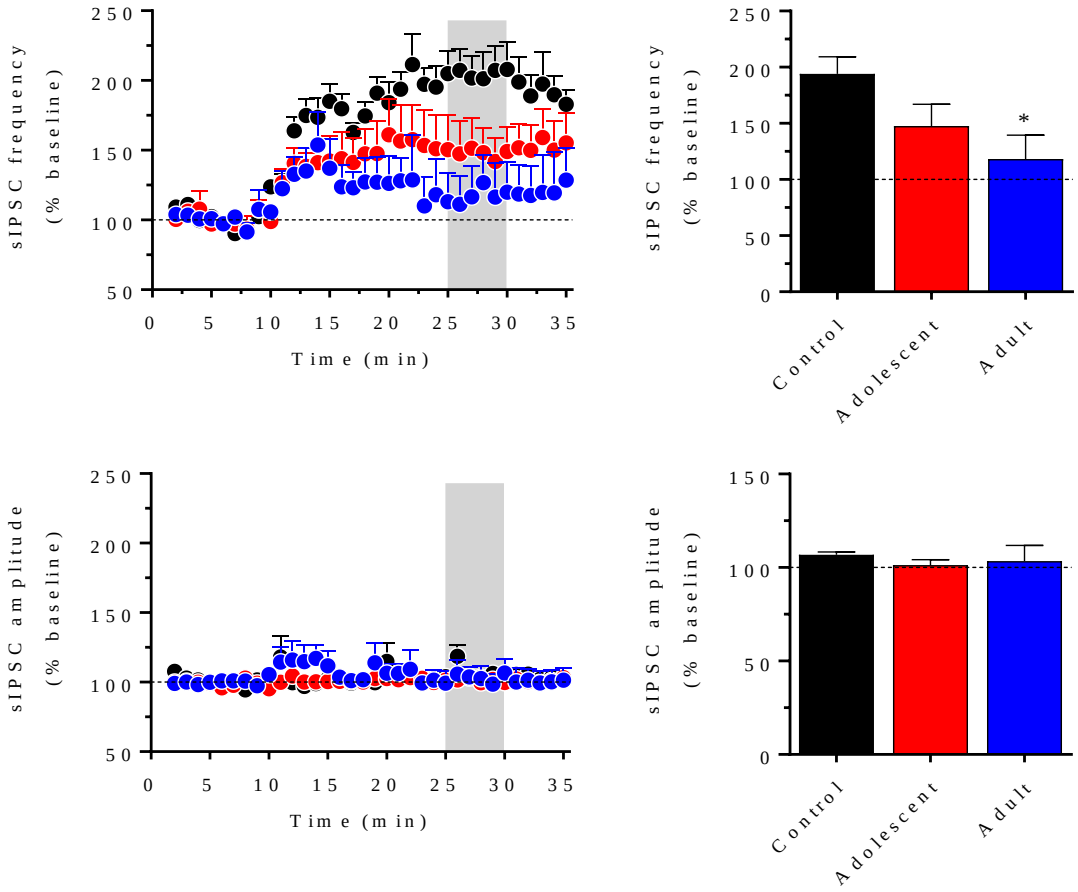

Figure 4.7 Time course and peak response for sIPSC amplitude and frequency in slices exposed to SKF38393 (10 μM for 90 s, as indicated by horizontal bar) in Experiment 2.2. Recordings were obtained from 4-5 cells/group in slices from 4-5 rats/group. Data are presented as described for Fig. 4.4. *$p < 0.05$, vs. control.

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Chapter 5: Age-dependent differences in AMPH's effects on HFS-induced plasticity in the PL-NAC circuit and goal-directed behavior

Rationale

It has been shown that repeated AMPH exposure leads to lasting structural and functional changes in the PFC and NAC (Robinson and Kolb, 1997; Robinson and Kolb, 1999; Piazza et al., 1991; Lu and Wolf, 1999) but there have been relatively few studies examining the long-term impact of AMPH on neuronal physiology, as well as on the two regions functioning in an intact circuit (Onn and Grace, 2000). Results from the previous chapter indicate that the deep layer output cells in the PL of rats pre-exposed to AMPH may become disinhibited following activation by high frequency stimulation, especially when drug exposure occurs during adolescence. This could result in an abnormal increase in the PL's influence on cells in the NAC. For example, it is found that HFS delivered in the deep layer of the mPFC produces LTP in the NAC both *in vivo* and *in vitro* (Goto and Grace, 2005; Shen and Kalivas, 2013). Based on our findings in Experiment 2, repeated AMPH exposure may alter the HFS-induced plasticity in the PL-NAC circuit in an age-dependent manner. In addition, anatomical evidence suggests that besides the deep layer cells, prefrontal pyramidal cells in the superficial layer also project to the NAC (Pinto and Seasack, 2000; Vertes, 2004). However, it is not clear if high frequency activity in the superficial layer would also induce long-term plasticity in NAC cells, which is also potentially sensitive to adaptations following repeated AMPH exposure. In this experiment, I will test the hypothesis that following repeated AMPH exposure, HFS delivered in the superficial and deep layer is more likely to produce LTP in the NAC and this effect is more pronounced following adolescent AMPH exposure.

As discussed in the background, the cells in the NAC have intriguing electrophysiological properties that are considered critical for incorporating information from limbic structures and the executive control from the mPFC (Goto and Grace, 2008). Specifically, it has been shown that individual NAC cells form synaptic contact with afferent input from PFC, hippocampus (HC) and amygdala (AMG) (Goto and Grace, 2005) and potentially other inputs. HFS-induced LTP formed in PFC-NAC projection simultaneously decreases the efficacy of synapses between that NAC cell and afferent input from HC and AMG (Goto and Grace, 2005), and vice versa, as if these afferent inputs are competing for a predominant control over that cell (Goto and Grace, 2005, 2008). This synaptic competition between circuits is suggested to be a potential mechanism that enables NAC cells to flexibly adapt their activity or output in response to the changes in afferent input (Goto and Grace, 2008). In this regard, possible aberrant influences from a disinhibited PL that may occur in AMPH-exposed rats are likely to disrupt this mechanism. In line with the framework proposed by Grace and Goto (2008), this adaptation may lead to an impaired ability of an individual to select the appropriate behavioral response to obtain a specific goal in the face of changing contingencies. In other words, AMPH exposure is hypothesized to induce a reduced flexibility in behavior. Indeed, it has been observed in humans and lab rodents that there is an impaired cognitive flexibility following a history of AMPH exposure (van der Plas et al., 2009; Fletcher et al., 2005; Hankosky et al., 2013; Kantak et al., 2014). However, it is still not clear whether adolescent drug exposure leads to a greater impairment in behavioral flexibility (Hankosky et al., 2013; Kantak et al., 2014).

In this experiment, I used a strategy set-shifting task to investigate the effect of AMPH on cognitive flexibility and HFS-induced plasticity in the PL-NAC circuit of rats exposed to the drug during adolescence or adulthood. Male and female rats were treated with either saline or 3.0

mg/kg AMPH as in experiment 2.1 and underwent behavioral testing 3-4 weeks after their last injection. Subsequently, they were sacrificed for brain slice recordings to determine AMPH's effect on plasticity function in the PL-NAC circuit.

Methods

Subject and treatment

A total of 168 male and female Sprague-Dawley rats were used in this experiment. Rats from individual litters were weaned on P22, housed in groups of 2 per cage and randomly assigned to groups using a sex (male or female) by treatment (saline or AMPH) by age (adolescence or adulthood) design with 8-12 rats per group. All groups were represented approximately equally in each litter. Rats were kept on a 12-h light/dark cycle (lights on at 0800 h) in a temperature-controlled room with food and water available *ad libitum* except where noted below. Rats were injected i.p. with either saline or 3 mg/kg AMPH, every other day from P27 to 45 (adolescent exposure) and P85 to 103 (adult exposure) as described in Experiment 2.

Apparatus

A wooden, four-arm plus maze painted black was used for behavioral training and testing. Four legs attached to the ends of each arm elevated the maze 100 cm above the floor. Each arm was 40 cm long and 10 cm wide and had 20-cm high walls. Cylindrical food wells (2 cm wide x 0.5 cm deep), which recessed in the floor and were located 2 cm from the end wall, were used for placement of food rewards.

Experiment 3.1 - Egocentric to allocentric strategy set-shifting

Rats were trained and tested following a procedure modified from Floresco et al. (2006). Briefly, food restriction started 19 ± 1 days after the last injection (P63 ± 1 day for adolescent-exposed, or P122 ± 1 day for adult-exposed). In order to reduce neophobia, each rat was given 30 sucrose pellets (45 mg, unflavored; BioServ, #F06233) in their home cages one day before their initial contact with the maze. For the following behavioral training and testing period, each rat's bodyweight was maintained near 90% of its free-feeding bodyweight. On the first day of habituation, rats were placed in the maze and allowed to freely navigate and consume a total of 20 food pellets (three down the length of each arm and two in each food well). 12 additional pellets were rebaited in the maze (one in the center of each arm and two in each food well) if the initial 20 pellets were consumed before the 15 min session ended. On the second habituation day, arms were baited with 12 pellets. Whenever a rat traveled the entire length of an arm or consumed the two food pellets in the well, the rat was replaced at the entrance of a different arm until all pellets were consumed. For the subsequent habituation, the procedure was similar to habituation day 2 except that only one food pellet was placed in each food well in one session. Each rat received four sessions daily until the rat could finish 4 sessions in under 15 min. This was intended to habituate the animal to repeated handling after consuming food reward. After the rat achieved this criterion on the last day of habituation, the turn bias for the rat was then determined. A rat was placed in a randomly chosen arm as the starting arm and allowed to turn left or right to obtain a pellet. The turn bias of a rat was scored as the first turn it made in that trial, while the direction (right or left) that a rat chose four or more times over seven trials was considered its final turn bias. Egocentric (Experiment 3.1) or allocentric (Experiment 3.2) discrimination training started on the following day.

For the egocentric strategy discrimination, the animal was required to always turn in the opposite direction of its turn bias (left or right), regardless of the spatial cues placed around the maze (Fig. 5.2, top panels). Over the course of training, two arms were randomly used as the start arms to discourage animals from using an allocentric spatial strategy. For example (Fig.5.2 upper panel), an individual rat was started from the arms designated south or west. The choice of starting arms was counter-balanced such that the location of these arms relative to the spatial cues around the maze was varied across animals. On an individual trial, a rat was placed in one of the start arms and required to make the appropriate turn to receive a food pellet. Between trials, the rat was placed back in the holding cage on a cart. The inter-trial interval was kept at 10 s. Rats continued to receive training trials until they reached a criterion of 10 correct consecutive choices, after which they received a probe trial wherein a third arm that had not been used would be the start arm (East as the sample shown in Fig. 5.2). When a rat correctly turned the same direction as required during training then the egocentric strategy phase was completed. If a rat made an incorrect turn, training will be continued until this rat made an additional five correct choices consecutively, followed by another probe trial. This procedure was continued until the rat made a correct choice on the probe trial. The following measures were taken for each rat: (1) number of trials to criterion, which was defined as the total number of test trials completed before a correct choice on the probe trial was made; and (2) number of errors to criterion, which was defined as the total number of incorrect choices made before a correct choice on the probe trial. Rats that did not reach the criterion within 2 hours or 200 trials, whichever occurred first, were removed from the study. 2 male and 4 female rats from adolescent control and 3 female rats from adult control were excluded under this criterion.

For the set-shifting to allocentric strategy, the reward was relocated to one of the arms that had been unbaited in the egocentric phase and the spatial cues around the maze were now useful for predicting the reward location (Fig. 5.2, lower panels). Training was similar to that used in the egocentric strategy phase. Rats were started in two randomly selected arms and the criterion to complete the shifting phase was the same as in the egocentric phase. As suggested by previous studies (Ragozzino et al., 1999; Dias and Aggleton 2000; Ragozzino, 2002; Floresco et al., 2006), the errors made in set-shifting were scored in blocks consisting of 4 consecutive trials. Perseverative errors were scored when a rat made an incorrect choice using the egocentric strategy as required in the previous phase on three or more trials per block. Once a rat made less than three perseverative errors in a block for the first time, all subsequent errors were scored as "regressive" errors if the rat went back to use the egocentric strategy. A never-reinforced error was scored when a rat was not making a perseverative or regressive error but entered an unbaited arm. After reaching the criterion, individual rats were returned to colony room and were given ad libitum access to food.

Experiment 3.2 – Allocentric to egocentric strategy set-shifting

For this experiment, rats were initially required to use allocentric strategy as described above, followed by shifting to egocentric strategy. All other aspects of the testing procedure were identical to those described above.

Electrophysiology

Rats were deeply anesthetized with at least 55 mg/kg of sodium pentobarbital and decapitated between 1 to 9 days after they finished set-shifting (P68-78 for adolescent exposed, P127-137 for adult exposed). Brain slices containing the PL and NAC were prepared as

described in Experiment 2 for fEPSP recording. Slices from each rat were randomly selected for assessing HFS-induced plasticity in the following circuits 1) PL Layer II-Layer V; 2) PL Layer II-NAC; 3) PL layer V-NAC. A single slice was only used for recording in one circuit. For this experiment, the recording electrode was placed in either deep layer of the PL or in the core of NAC approximately 400-600 µm lateral to the anterior commissure, and the stimulating electrode was placed in either superficial or deep layers of the PL. HFS was applied after a stable baseline was obtained for at least 10 min; the post-HFS response was recorded for 30 min.

Data analysis

Because rats in experiment 3.1 and 3.2 went through different behavioral training that may rely on distinct neural substrates (see results and discussion) (Hankosky et al., 2013), we analyzed the data from two experiments separately. Dependent measures used to assess cognitive flexibility included the number of trials to criterion and errors to criterion. Number of errors made in the set-shifting phase were further analyzed based on the error type as described above. Number of trials and errors were analyzed with either three-way (treatment by age by sex) or four-way ANOVA (treatment by age by sex by error type). Significant interactions were followed by Tukey post hoc test. The fEPSP data obtained in slice recordings were analyzed as described in Experiment 2 except that sex was included as a main factor. Significant interaction in the ANOVA of errors was further investigated with tests of linear regression within group. In all of these analyses, we did not find any significant main effect or interaction involving the factor of sex (see Figure 5.2 for an example regarding the potential sex differences typically observed in the current experiment). Therefore we plotted and reported all the analyses collapsed across sex.

Results

Effects of adolescent or adult AMPH exposure on set-shifting

A total of 80 rats (n=17-22/group) were assessed in allocentric to egocentric strategy set-shifting in Experiment 3.1 and a total of 79 rats (n=17-23/group) were assessed in the opposite shifting in Experiment 3.2. A schematic of the experimental design is shown in Fig. 5.1. We first found that control rats took significantly more trials in the egocentric strategy discrimination (Fig. 5.1C; $F_{1, 65}$=34.9, $p < 0.001$). In Experiment 3.1, we did not find any significant group differences in egocentric strategy discrimination or set-shifting to allocentric strategy (Fig. 5.2). In Experiment 3.2, we did not find any significant group differences in the allocentric strategy discrimination (Fig. 5.4). For the set-shifting to egocentric strategy, two-way ANOVA revealed a significant interaction of age by treatment in trials to criterion ($F_{1, 76}$ = 6.575, p = 0.012) and the number of errors to criterion ($F_{1, 76}$ = 4.372, p = 0.040). Post hoc tests suggested adolescent-exposed rats required significantly more trials than the adult-exposed group and made significantly more errors than control and adult-exposed rats. Further analysis of errors based on type using three-way ANOVA revealed a significant main effect of age ($F_{1,210}$=3.98, p = 0.047), error type ($F_{1,210}$=44.8, p <0.001) and a significant treatment by age interaction ($F_{1,210}$=4.96, p = 0.027). Post hoc analysis suggested that the adolescent-exposed group made significantly more regressive errors than the age-matched control and adult-exposed group, and they made significantly more never-reinforced errors than adult-exposed group.

Effect of adolescent or adult AMPH exposure on HFS-induced plasticity in the PL-NAC circuit

We recorded evoked fEPSPs in the PL Layer II-V, PL layer V-NA and PL Layer II-NA in slices from each rat that finished set-shifting in Experiment 3.1 and 3.2, and plotted the data in

Fig. 5.3 (n=17-22 slices/group) and 5.5 (n=17-23 slices/group), respectively. The schematic of electrode placement for these recordings is shown on the left of the two figures. For PL Layer II-Layer V, again we found in the two cohorts that HFS induced LTD in control but not in AMPH-exposed groups. Analysis of the time course data using two-way ANOVA with repeated measures revealed a significant main effect of group (Fig. 5.3: $F_{3, 79} = 47.2$, $p < 0.001$; Fig. 5.5: $F_{3, 75} = 20.4$, $p < 0.001$), time (Fig. 5.3: $F_{38, 2698} = 10.4$, $p < 0.001$; Fig. 5.5: $F_{38, 2850} = 15..3$, $p < 0.001$) and time by group interaction (Fig. 5.3: $F_{114, 2698} = 7.44$, $p < 0.001$; Fig. 5.5: $F_{114, 2850} = 10.2$, $p < 0.001$). Post hoc tests suggest significant group differences between AMPH-exposed groups and their corresponding controls (Fig.5.3 and Fig. 5.5), and between adolescent- and adult-AMPH group (Fig.5.3 and Fig. 5.5). Analysis of the mean slope of the fEPSP from 25-30 min post-HFS revealed a significant main effect of treatment (Fig. 5.3: $F_{1, 79} = 26.2$, $p < 0.001$; Fig. 5.5: $F_{1, 77} = 43.1$, $p < 0.001$) and age (Fig. 5.3: $F_{1, 79} = 4.61$, $p = 0.035$; Fig. 5.5: non-significant) and a significant treatment by age interaction (Fig. 5.3: $F_{1, 79} = 4.23$, $p = 0.043$; Fig. 5.5: $F_{1, 77} = 10.0$, $p = 0.002$). Post-hoc tests suggest a significant difference between the adolescent-exposed group and the age-matched control, and between adolescent- and adult-exposed group. There was also a near-significant difference between adult-exposed group and their age-matched control (Fig. 5.3: $p = 0.057$; Fig. 5.5: $p = 0.082$).

For recordings in the PL Layer V-NAC circuit, we found in the two cohorts that HFS led to LTP-like response in all groups (Fig.5.3 and Fig. 5.5). Two-way ANOVA with repeated measures revealed a significant main effect of time (Fig.5.3: $F_{38, 2698} = 61.3$, $p < 0.001$; Fig. 5.5: $F_{38, 2850} = 39.9$, $p < 0.001$). However, we did not find any group differences.

For recordings in the PL Layer II-NAC circuit, we found in the two cohorts that HFS did not produce any apparent changes in control and adult-exposed groups but led to an increase in

fEPSP in the adolescent-exposed group (Fig.5.3 and Fig. 5.5). Two-way ANOVA of the time course data revealed a significant main effect of time (Fig.5.3: $F_{38, 2546}$= 3.00, p <0.001; Fig. 5.5: $F_{38, 2850}$= 7.18, p <0.001), group (Fig.5.3: $F_{3,79}$= 5.65, p = 0.002; Fig. 5.5: $F_{3, 75}$= 9.68, p <0.001) and time by group interaction (Fig.5.3: $F_{114, 2698}$= 1.92, p <0.001; Fig. 5.5: $F_{114, 2850}$ = 2.27, p <0.001). Post hoc tests suggest significant group differences between adolescent-exposed group and the age-matched control, and a significant or near-significant difference between adolescent- and adult-exposed group (Fig. 5.3: p = 0.083; Fig. 5.3: p = 0.008). Analysis of the mean slope of the fEPSP from 25-30 min post-HFS revealed a significant main effect of treatment (Fig.5.3: $F_{1, 79}$= 14.0, p <0.001; Fig. 5.5: $F_{1, 77}$ = 12.6, p < 0.001), and a significant or near-signifiacnt interaction of treatment by age (Fig.5.3: $F_{1, 79}$= 4.30, p = 0.042; Fig. 5.5: $F_{1, 77}$ = 3.929, p = 0.051). Post hoc tests suggest significant differences between adolescent-exposed group and the age-matched control (Fig.5.3 and Fig. 5.5), and significant or near-significant differences between adolescent- and adult-exposed group (Fig.5.3: p = 0.057; Fig. 5.5: p < 0.05).

To follow up the significant group differences and interactions we found in the behavioral and electrophysiological data, the mean slope of the fEPSP from 25-30 min post-HFS from each individual rats in Experiment 3.2 was correlated with the number of regressive errors they made in the set-shifting. Within-group linear regression analysis suggested that there was no significant correlation between the mean post-HFS response obtained in the PL Layer II-V circuit and regressive errors. However, in both control groups, the post-HFS response recorded in the PL Layer II-NAC circuit was positively correlated with the number of regressive errors (adolescent control: R^2=0.43, p = 0.003; adult control: R^2=0.28, p = 0.023). No significant correlation was found in AMPH-exposed groups (adolescent-exposed: R^2=0.13, p > 0.05; adult-exposed: R^2=0.04, p > 0.05)

Discussion

This study sought to investigate the potential age-of-exposure dependent effect of repeated AMPH on cognitive flexibility and HFS-induced plasticity in the PL-NAC circuit. Our results suggested that repeated AMPH exposure did not affect strategy learning per se in our maze-based paradigm. In the allocentric to egocentric set-shifting, rats that received AMPH during adolescence made significantly more errors and required more trials in comparison to their age-matched control and adult-exposed group. However, this age-of-exposure dependent effect of AMPH was not observed in the egocentric to allocentric strategy set-shifting. Regardless of their behavioral performance, rats that received repeated AMPH exhibited alterations in HFS-induced plasticity in the PL-NAC circuit. Specifically, there was a reduction or lack of HFS-induced LTD in the PL in AMPH-exposed rats. Furthermore, we found that HFS was more likely to produce LTP in either PL or PL layer II-NAC circuit in adolescent-exposed rats compared to other groups. These results are consistent with our previous findings (experiment 2) and demonstrate persistent functional changes in the PL-NAC circuit following repeated AMPH. More importantly, some of these changes were exclusively found in the rats that received AMPH during adolescence and subsequently exhibited significant deficits in behavioral flexibility.

Strategy set-shifting is frequently used in animal studies to investigate cognitive flexibility and it is well established that the mPFC is one of the key brain regions facilitating shifting between different strategies (Floresco et al., 2008; Floresco et al., 2009; Floresco, 2011). Previous studies suggest that disruption of the mPFC does not affect strategy discrimination but severely reduces the ability to suppress using an already learned but no longer appropriate strategy (Floresco et al., 2006; Floresco et al., 2008; Enomoto et al., 2010). Notably, several lines

of evidence suggest that not all strategy set-shifting is mPFC-dependent. The behavioral performance was found to be insensitive to mPFC inactivation when animals were switching from a relatively more difficult strategy to an easier one in multiple versions of set-shifting tasks (Floresco et al., 2008; Ragozzino et al, 2002). This notion that the shifting difficulty (or cost) determines, at least partially, whether mPFC is involved is also supported by findings from non-human primate (Croft et al., 2001) and human studies (Stuss et al., 2000). In the present study, we expected to see age-of-exposure dependent deficits induced by AMPH at least in an mPFC-sensitive version of set-shifting based on the results in Experiment 2. By comparing the performance in the initial discrimination between the control groups in Experiment 3.1 and 3.2, we found that learning the reward location using an egocentric strategy was more challenging. Therefore, the set-shifting to egocentric strategy in Experiment 3.2 was more likely to be mPFC-sensitive while the opposite shifting in Experiment 3.1 was not. Consistent with our hypothesis, a significant behavioral deficit was revealed in the adolescent-exposed group specifically when shifting to a harder strategy in the current study. In contrast, we did not find any significant effect of adult AMPH treatment in neither of these shiftings, which is in line with a previous study that used a very similar allocentric vs egocentric set-shifting design (Featherstone et al., 2008). Taken together, we found that adolescent but not adult AMPH exposure induced a long-lasting deficit in a cognitive flexibility task and the deficit was only observed when the mPFC was likely involved. These results support the notion that mPFC-dependent cognitive functions are more sensitive to drug exposure during adolescence than adulthood. Yet, several studies (Hanskosy et al., 2013; Kantak et al., 2014) found no evident impairment following noncontingent injection or self-administration of AMPHs or cocaine during adolescence in an operant strategy set-shifting. Notably, animals in these studies only shifted from a visual strategy

to a response-based one. Without including the opposite shifting, it is hard to determine the shifting difficulty and whether the shifting was mPFC-sensitive (Westbrook et al., 2018). It is possible that these authors observed a similar phenomenon as we did in experiment 3.1. In addition, differences in the experimental methods between the current study and others may also contribute to the disparity in the results. For example, animals in previous studies either had a more delayed exposure onset (P37; Kantak et al., 2014) or a longer withdrawal (~14 weeks; Hankosky et al., 2013), and had other extensive behavioral testing prior to the set-shifting, all of which may potentially influence or interact with drug-induced plasticity.

Analysis of errors by type in experiment 3.2 revealed that the adolescent-exposed group was more prone to making regressive errors. In addition, the adult-exposed group made slightly less never-reinforced type of errors, at least than their adolescent-exposed counterparts. These behavioral differences again highlight the age-of-exposure dependent effect of AMPH. As suggested in a previous study (Hankosky et al., 2013), strategy discrimination and shifting resemble a probabilistic learning process, in which rats are intermittently reinforced based on their response choice. The adolescent-exposed group made approximately the same amount of never-reinforced errors as control, suggesting that they had intact sensitivity to negative feedback (lose-shift tendency) to parse out irrelevant and incorrect strategies (Floresco et al., 2008). In this regard, the increased tendency to return to a no longer correct strategy (regressive error) in the adolescent-exposed rats could be due to a decreased sensitivity to positive feedback (win-stay tendency; Floresco, 2013) as the rats could not maintain the new strategy (Floresco et al. 2008). This is perhaps consistent with several previous findings that rats decrease their reward sensitivity or expectations following chronic use of psychostimulants (Green et al., 2015; Burton et al., 2018). However, this putative decreased sensitivity to reward in the adolescent-exposed

group appears to only occur during the shifting to, but not the initial learning of, the egocentric strategy. The adolescent-exposed group did not commit more errors than other groups when the egocentric strategy was the first learned (Experiment 3.1). As for the perseverative errors, we did not see any significant group differences, which appears to be inconsistent with our results from Experiment 2 that D_1-mediated inhibition was impaired following AMPH exposure. This is because previous studies have found that that microinfusion of $GABA_A$ or D_1 antagonist or into the mPFC led to an increase specifically in perseverative errors in set-shifting (Floresco et al., 2006, 2008, 2011). In addition, a previous study also suggested that adolescent exposure to AMPH led to increase in perseverative behavior (Hankosky et al., 2013). A caveat though, is that the current study may have a lower chance of detecting perseverative errors in comparison to other strategy shifting studies. In a typical maze-based or operant strategy set-shifting, rats have two turning or lever options whereas in the current study, rats had three arms to choose from (intended to increase the difficulty of the task). So when a rat actually stops persisting on the previous strategy, it still has 50% chance to enter the same arm as if it is making a perseverative error, whereas in the present study, rats had 33.3% chance to do so. Indeed, the average number of perseverative errors in the present study is substantially lower than what has been observed in other maze-based strategy set-shifting (Floresco et al., 2006, 2008, 2011, 2013) such that some perseverative errors might have been counted as regressive. But overall, both perseverative and regressive errors occur from using a previously established strategy or response even when the outcome has become unfavorable. Our results indicate that adolescent but not adult AMPH exposure increases the likelihood of engaging in this type of behavior.

As found in previous studies, inactivation of NAC core or disconnecting the mPFC-NAC circuit specifically increases regressive errors during strategy set-shifting (Floresco et al., 2006,

2008; Floresco, 2011). Thus, the effect of AMPH on the set-shifting could be attributed to specific functional alterations in the mPFC-NAC circuit. Based on this and our results in Experiment 2, we investigated AMPH's effect on the three possible neuronal connections in the PL-NAC circuit and our results revealed unique changes following adolescent exposure. We first replicated the findings in Experiment 2 that HFS induced an LTP but not LTD in the PL Layer II-V in adolescent-exposed rats while adult-exposure produced a dampened LTD. This age-of-exposure dependent effect of AMPH was highly consistent with what we found in rats naïve to behavioral testing in Experiment 2, suggesting that the food restriction and behavioral training in the current experiment likely did not influence AMPH-induced plasticity. In the PL Layer II-NAC circuit, we found that HFS produced LTP only in adolescent-exposed rats. As discussed in the previous chapter, HFS-induced LTD in the PL layer V may likely serve as a gating mechanism that prevents unnecessary output. In this regard, the HFS-induced LTP in the adolescent-exposed group could be interpreted as a loss of such gating so that disinhibited Layer V output cells may be more likely to influence their targets. Consistent with this notion, cells in the NAC appeared to be most responsive to post-HFS stimulation in the PL layer II in the form of an LTP in the adolescent-exposed group. Contrary to this, adult AMPH treatment had either smaller or insignificant impact on HFS-induced plasticity in the PL-NAC circuit. This age-of-exposure dependent difference is in line with a previous study showing that adolescent but not adult cocaine injections abolished the HFS-induced LTD in the HC-mPFC circuit (Cass et al., 2013). Lastly, there was no significant difference in HFS-induced LTP in the Layer V-NAC circuit between groups. Taken together, these results indicate that under normal situations, HFS in the PL Layer II results in an LTD in Layer V and does not further produce significant influences on NAC cells. Following AMPH exposure, though, HFS in the layer II becomes

unable to produce an LTD. Furthermore, the Layer V cells may be even more disinhibited following HFS in rats pre-exposed during adolescence, and perhaps involved in generating an LTP in the PL-NAC circuit.

An advantage of the current experiment is that we were able to analyze the relationship between AMPH-induced changes in synaptic plasticity and set-shifting. Within-group linear regression found that in the control group there was a positive correlation between the post-HFS response (as % of baseline) in the PL layer II-NAC circuit and the number of regressive errors, supporting the notion that this circuit plays a role in strategy selection/execution during strategy shifting (Floresco, 2011). A previous study using single unit recording found that a certain portion of PL cells changed their firing pattern specifically when rats implemented a new behavioral rule in a strategy shifting paradigm. It is likely that the PL is critically involved in coding for the new rule when an individual needs to alter its behavior pattern (Rich and Shapiro, 2011). Our correlation analyses indicate that the behavioral performance during shifting is independent of the plasticity in the Layer V-NAC transmission. However, an attenuated post-HFS response in the NAC to PL layer II input is associated with a tendency of using a new and correct strategy whereas enhanced post-HFS response is associated with a higher chance of using a previously acquired but incorrect strategy. It's worth mentioning that the Layer II pyramidal cells innervate NAC directly and indirectly via Layer V. Therefore, the post-HFS response in the NAC cells elicited by the Layer II input may be a result of plastic changes occurring at multiple loci in the circuit, rather than a direct measure of the level of plasticity in those cells. In this regard, an increase in the post-HFS response in the NAC cells to PL layer II input may indicate a high level of noise coming from the PL and hence a less effective execution of the new rule. This could explain the group differences found in electrophysiology and behavior data in that the

adolescent-expose group exhibited the largest increase in the post-HFS response (LTP) in Layer II-NAC circuit and made more regressive errors than other groups. However, this relationship did not hold well within AMPH-exposed rats as the correlation is near-significant in the adolescent-exposed group and nonsignificant in the adult-exposed group. A possible explanation is that the AMPH-treated animals were guided by alternative strategies and less dependent on utilizing the PL-NAC circuit during the shifting. Lastly but not least, we did not find any significant correlations between HFS-induced plasticity in the PL Layer II-V circuit and regressive error, suggesting that the plasticity in this circuit is dissociable with the behavior that leads to regressive errors. This is consistent with a previous study showing that microinfusion of BMI into the mPFC specifically led to an increase in perseverative errors and had no effect on regressive errors (Floresco et al., 2008). In this regard, the HFS-induced LTP in the adolescent–exposed group would have predicted more perseverative errors, which however, were found not different between groups. As discussed above, this could be due to a relatively low chance of detecting this type of error in the current experimental design.

One of the limitations of the current study is that we were only able to record and analyze populational response. It would be difficult to fully understand the effect of AMPH or fully understand the findings in the current study without knowing the details about what types of cells are affected and how. Although Chapter 4 has addressed some of these issues by investigating AMPH's effect on the physiology of mPFC, it is not clear whether there are significant changes in the NAC. It is known that approximately 95% of the cell population in the NAC consists of medium spiny neurons (MSNs), with the rest being GABAergic and cholinergic interneurons. It would be safe to presume that the fEPSP we recorded in the NAC primarily consisted of the activity in the MSNs. Previous studies have shown that repeated AMPH during adulthood

induces a long-lasting increase in the dendrite length, branching and spine density in MSNs (Robinson and Kolb, 1997). Such finding predicts an enhanced excitability and synaptic efficacy in the MSNs to afferent inputs. However, there has been only a few studies afterwards that investigated the electrophysiological changes in the MSN following repeated AMPH. An early study reported that repeated AMPH increases the electric coupling between MSNs that are innervated by prelimbic input from Layer V, potentially via more gap junctions (Onn and Grace, 2000). In addition, MSNs from AMPH treated animals exhibited increased spontaneous discharge without significant changes in the resting membrane potential (Onn and Grace, 2000). These findings suggest that prelimbic input may elicit more synchronized firing, or, larger responses in the NAC following repeated AMPH exposure (Onn and Grace, 2000). However, a later study found that chronic AMPH treatment during adulthood had no significant effect of HFS-induced LTP in the NAC (Li and Kauer, 2004), suggesting that AMPH-induced physiological changes in the MSNs may not necessarily influence synaptic plasticity. Notably, we also found that adult exposure did not produce any detectable changes in HFS-induced plasticity in the PL-NAC circuit but the adolescent treatment did. Again, the current study focused on the role of exposure age in AMPH-induced plasticity and hopefully, our findings will inspire future studies to continue the investigation on AMPH's effect on NAC physiology and functions in a much more detailed way.

In sum, the present study investigated the age-of-exposure dependent effect of AMPH on cognitive flexibility and HFS-induced plasticity in the PL-NAC circuit. Our results revealed a deficit in strategy set-shifting specifically induced by adolescent drug exposure, which is associated with aberrant PL-NAC circuit function. In contrast, adult AMPH exposure did not produce any significant behavioral changes. These results are in line with several previous

findings in the literature (Featherstone et al., 2008; Hankosky et al., 2013; Kantak et al., 2014) and highlighted the age-of-exposure dependent differences in AMPH-induced plasticity. Converging evidence from human studies suggests that impaired cognitive flexibility is typically associated with chronic psychostimulant use (van der Plas et al., 2009; Fernández-Serrano et al., 2010; van Holst and Schilt, 2011). This particular deficit is suggested to be an important contributor to the pathology of addiction as it could explain why drug users keep going back to their drug-taking behavior when the outcome is no longer in favor of them (Gould, 2010). In addition, a previous study found that rats with relatively low cognitive flexibility were more likely to engage in escalating methamphetamine self-administration (Istin et al., 2017). In the present study, cognitive behavior in the adolescent-exposed group was assessed in their young adulthood, an age range in which humans peak their use of various drugs (Jonathon et al., 2017). In this context, a persistent reduction in behavioral flexibility induced by adolescent drug exposure may predict a heightened vulnerability to continuous and problematic drug use.

Figures

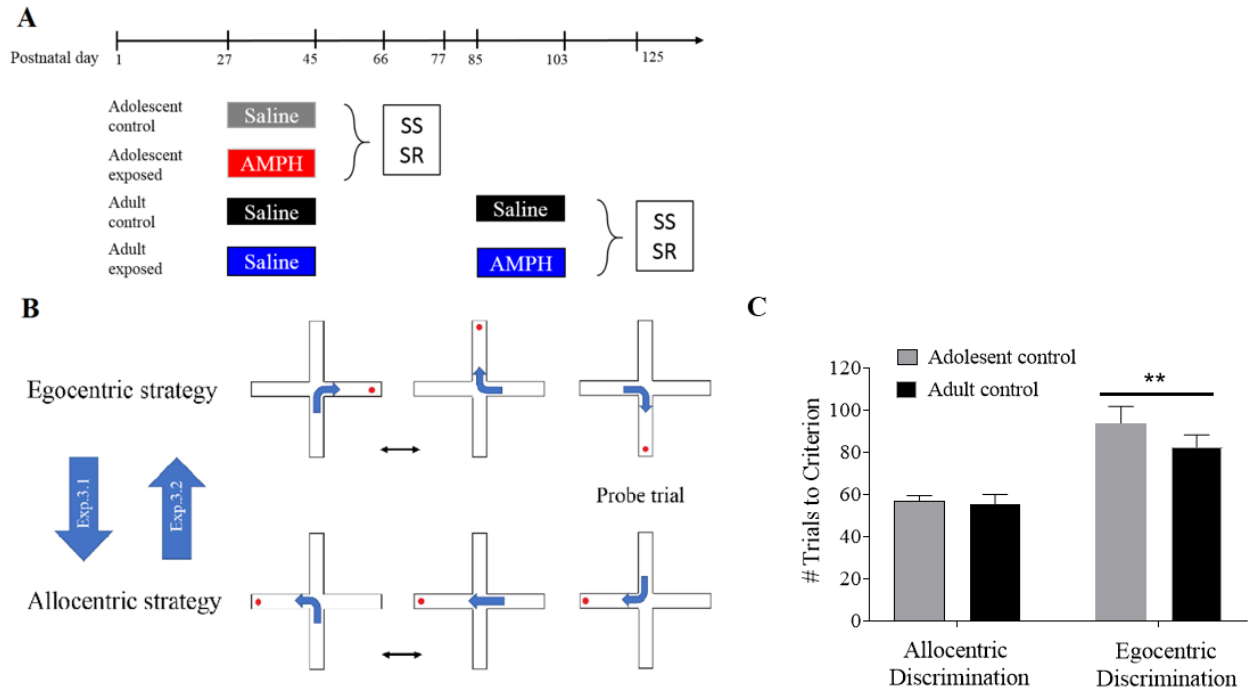

Figure 5.1 **A**, Summary of groups injected (i.p.) with either saline or 3 mg/kg AMPH a total of 10 times (once every other day) during the ages noted for Experiment 3.1 and 3.2. Rats were subjected to set-shifitng (SS) 3 weeks following their last injection and subsequently to slice recordings (SR). **B,** schematic of the behavioral paradigm of set-shifitng between egocentric and allocentric strategy. For egocentric discrimination (upper panel), a rat starts a trial in either south or east arm and always needs to make a right turn to obtain food reward (red dot). After 10 consecutive correct choices, a probe trial starts in the west arm. Training for allocentric discrimination is the same as that of egocentric, exept that the location of food reward is fixed. For experiment 3.1 and 3.2. rats were first required to complete egocentric and allocentric strategy discrimination, respectively, and then to switch to the alternative strategy. **C**, saline injected controls required more trials to criterion in egocentric than allocentric strategy discrimination. $^{**}p < 0.01$ vs. allocentric discrimination.

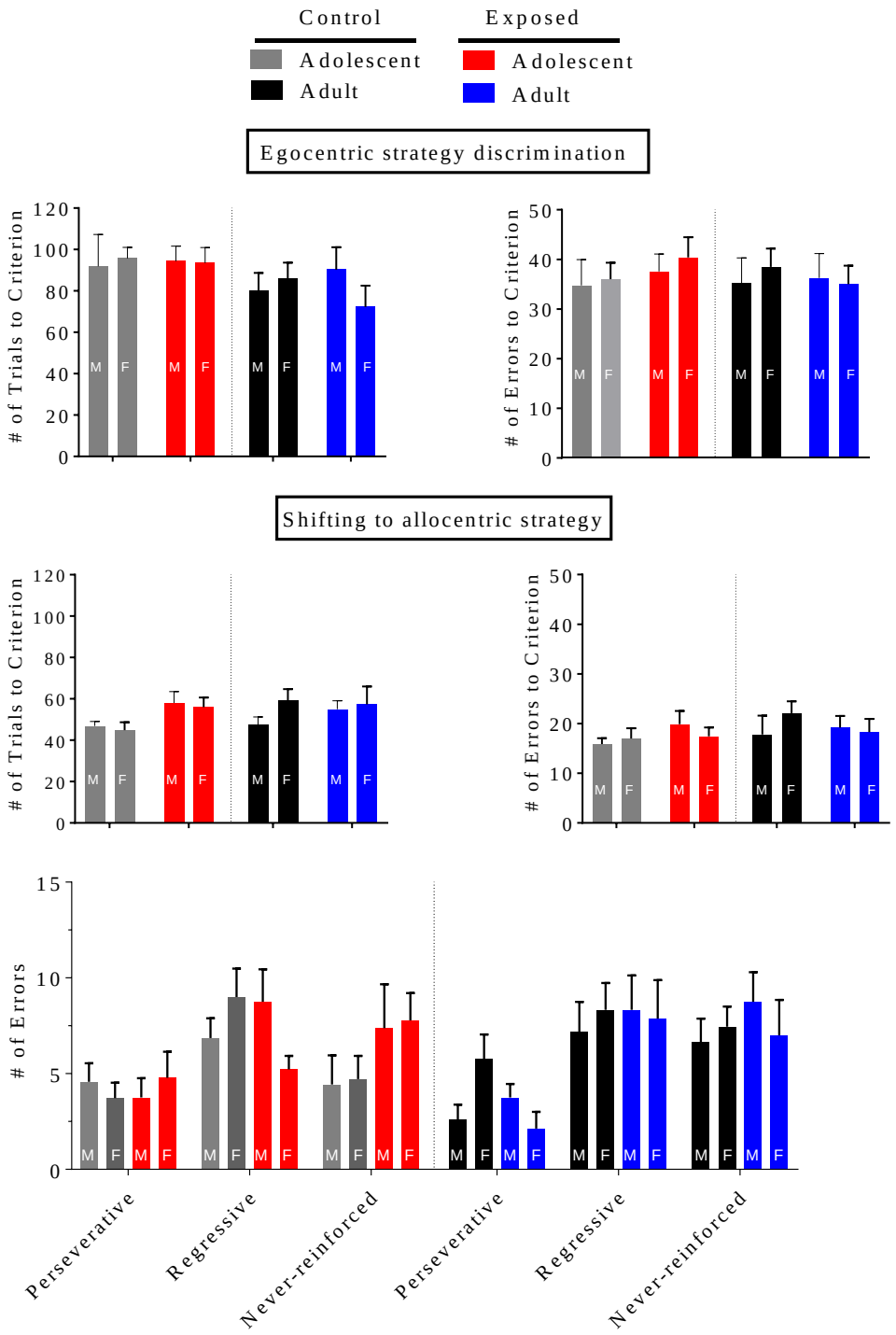

Figure 5.2 Behavioral performance in Experiment 3.1 (n=8-12/group). Number of trials and errors to criterion in egocentric discrimination and set-shifting to allocentric strategy did not differ across groups. M=male; F=Female.

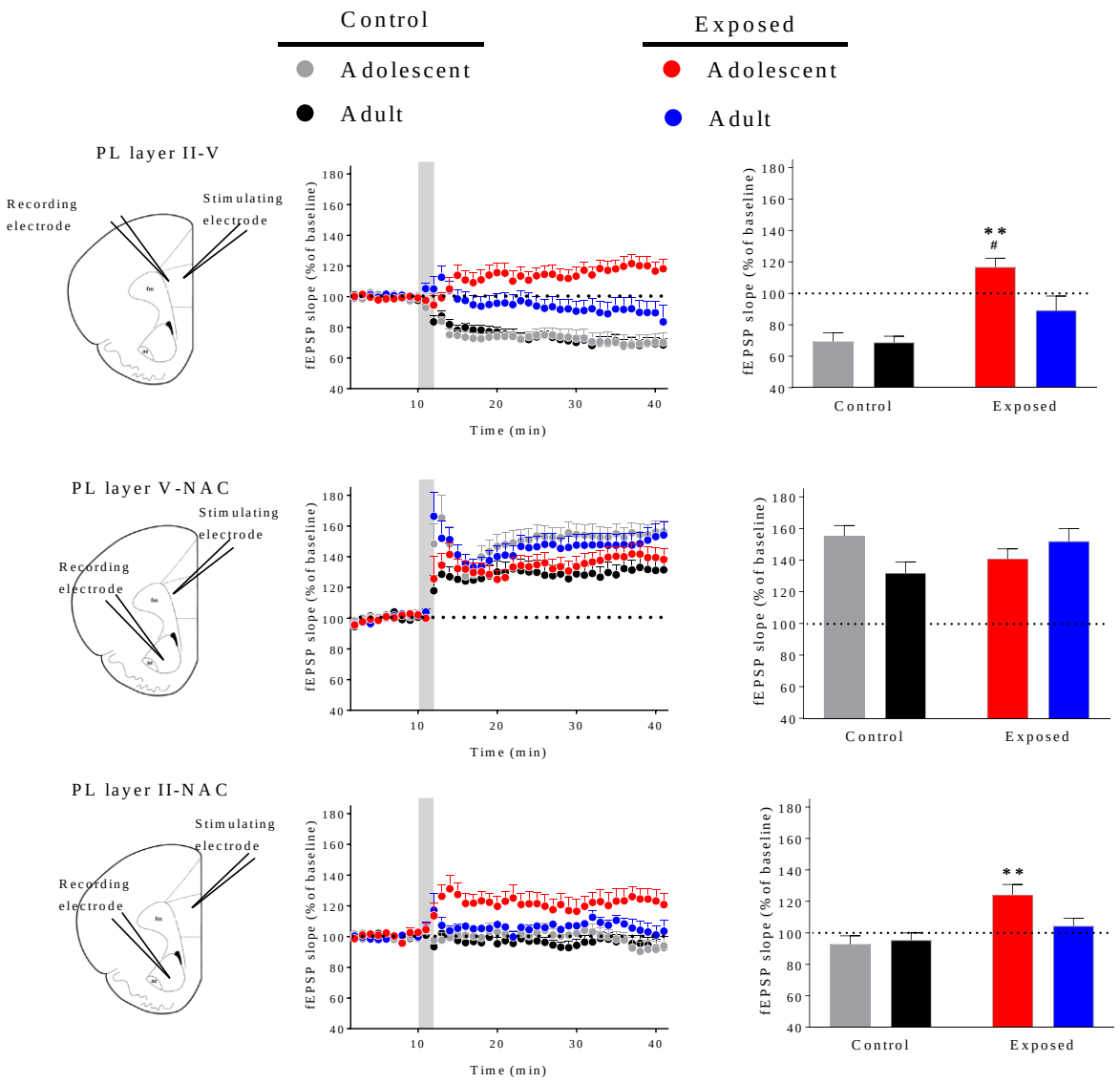

Figure 5.3 HFS-induced plasticity in the PL-NAC circuit in rats in Experiment 3.1. Left: schematic of electrode placement. Middle, time course of the fEPSP slope before and after HFS (shaded vertical bar) in control and AMPH-exposed group (n = 17-22 slices from 17-22 rats per group), $^{**}p < 0.01$, $^{*}p < 0.05$ vs. age-matched control; #$p < 0.05$ vs. adult-exposed group. Right: Mean fEPSP response during the 25-30 min period following HFS for each age group. $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* age-matched control; #$p < 0.05$ vs. adult-exposed group.

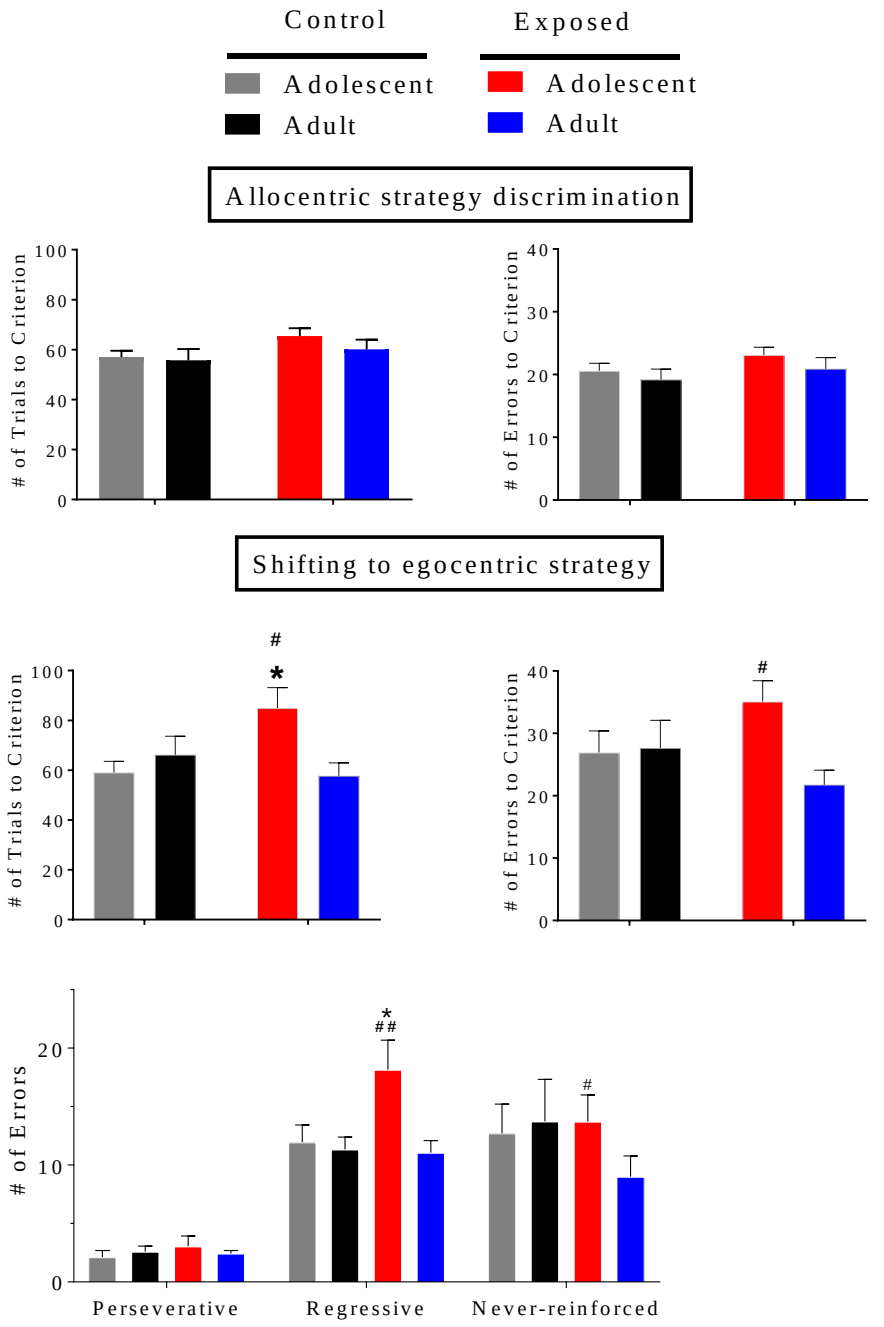

Figure 5.4 Behavioral performance in Experiment 3.2 (n=17-23/group). Number of trials and errors to criterion in allocentric discrimination did not differ across groups. Adolescent-exposed rats required more trials and made more errors in the set-shifting to egocentric strategy. * $p < 0.05$ *vs.* age-matched control; ## $p < 0.01$, #$p < 0.05$ vs. adult-exposed group.

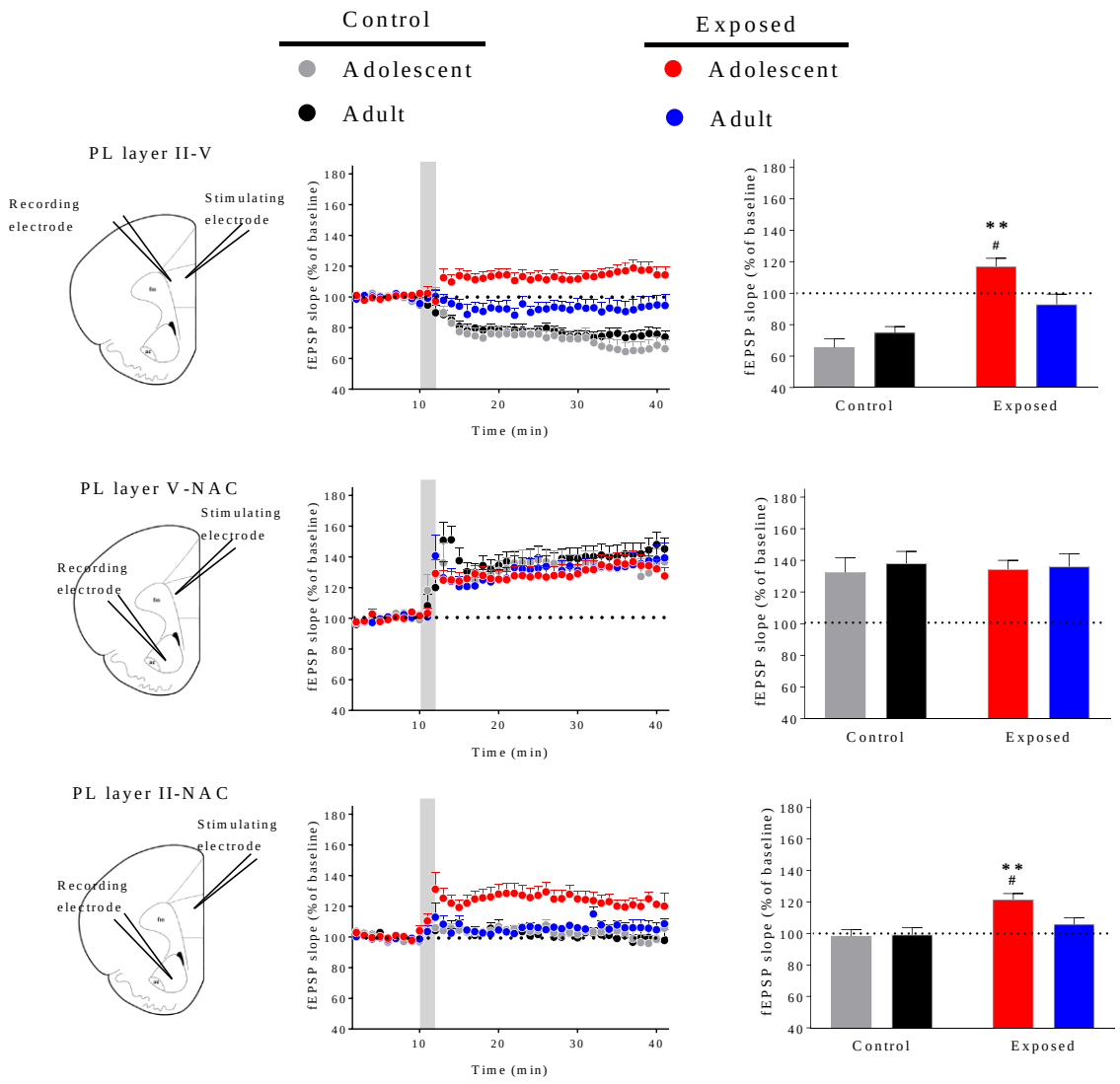

Figure 5.5 HFS-induced plasticity in the PL-NAC circuit in rats in Experiment 3.2. Left: schematic of electrode placement. Middle, time course of the fEPSP slope before and after HFS (shaded vertical bar) in control and AMPH-exposed group (n = 17-23 slices from 17-23 rats per group), $**p < 0.01$, $* p < 0.05$ vs. age-matched control; $\#p < 0.05$ vs. adult-exposed group. Right: Mean fEPSP response during the 25-30 min period following HFS for each age group. $**p < 0.01$, $* p < 0.05$ vs. age-matched control; $\#p < 0.05$ vs. adult-exposed group.

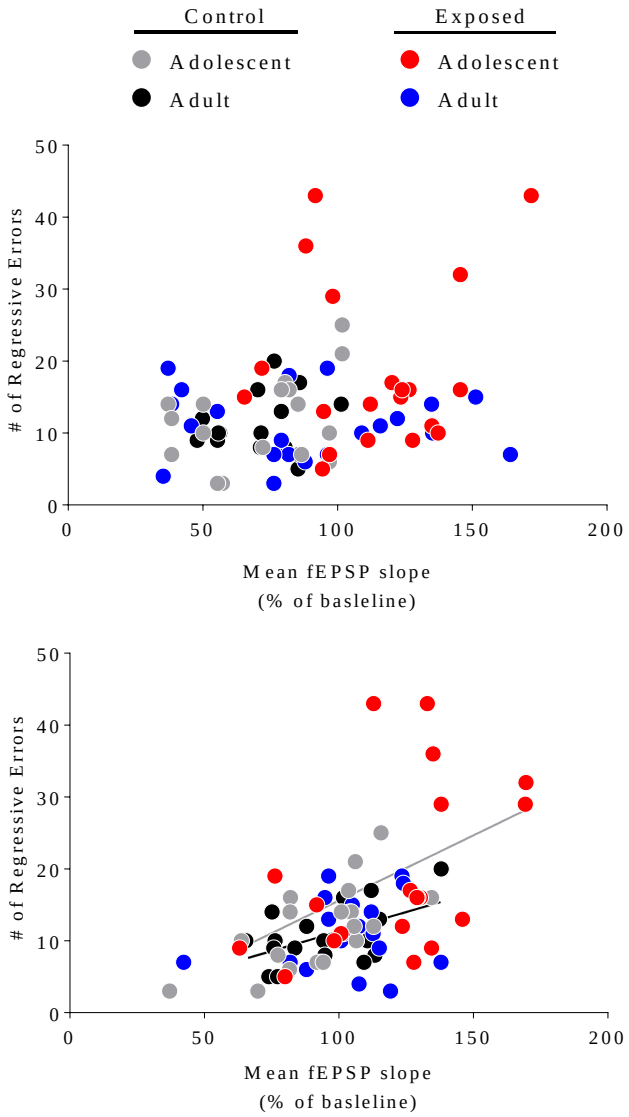

Figure 5.6 Relationship between post-HFS response in the PL Layer II-Layer V (top) and PL layer II-NAC (bottom) and number of regressive errors in Experiment 3.2 (n=17-23/group). Each symbol denotes the value from an individual rat. Lines color-matched for each group indicate the best fit line ($p < 0.05$).

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Chapter 6: General discussion

Empirical evidence suggests that adolescent- compared to adult-onset drug users suffer from more severe deficits in mPFC-dependent cognitive functions and are more likely to be dependent and continue their drug use (Chen et al., 2009; Juraska and Gulley, 2013). To test the hypothesis that the adolescent brain is more sensitive to the detrimental effects of abused drugs, this dissertation focused on the age-of-exposure dependent differences in AMPH-induced neuroadaptations in the mPFC and cognitive behavior. Based on findings from others (Caballero et al., 2014; Cass et al., 2013) and my pilot experiments, we chose to start our investigation by examining HFS-induced LTD in the PL, as the emergence and normal expression of this LTD is suggested to be a critical step in the maturation of the mPFC functioning (Caballero et al., 2016). Our results suggest that HFS-induced LTD has a protracted ontogeny and its nature is likely a dopamine- and GABA-mediated inhibitory mechanism that may critically control the output of the mPFC. Following studies using a non-contingent drug exposure model revealed a significant impairment in the expression of the LTD in the PL following repeated AMPH, with adolescent exposure producing a greater effect. These results indicate that repeated AMPH may lead to reduced inhibitory transmission or decreased dopamine receptor function in the mPFC, which is indeed what we demonstrated in the patch-clamp experiments. Lastly, we determined if the altered LTD following repeated AMPH was associated with abnormalities in the reward circuit (PL-NAC) functioning and goal-directed behavior. Several age-of-exposure dependent difference were found in both electrophysiology and behavior assessment. Taken together, the studies here provided cellular, circuitry and behavioral evidence supporting the hypothesis that adolescent brain is more vulnerable to drug-induced changes.

Not all the results obtained here are in line with this overarching hypothesis that the adolescent brain is more sensitive. For example, we did not see any age-of-exposure dependent differences in AMPH's effect on dopamine-mediated inhibition in the pyramidal cells. It is well known that the dopamine system continues to develop throughout adolescence and young adulthood and is a primary target of psychostimulant drugs like AMPH (Gulley and Juraska, 2013). It was to our surprise that AMPH's effect on dopamine- and D_1-mediated sIPSC change was independent of the timing of exposure. In a separate study though, we examined the effect of bath-application of AMPH as a dopamine releaser, on the sIPSCs activity in the prefrontal pyramidal cells from animals pre-exposed to the same adolescent and adult AMPH treatment as in Experiment 2. Following drug application, sIPSC frequency decreased in the adolescent-exposed group whereas it was unchanged in the adult-exposed group (Paul et al., 2016). This result may explain the age-of-exposure dependent difference in AMPH's effect on HFS-induced LTD. Following the release of endogenous dopamine, inhibitory tone increases in control, stays unchanged in adult-exposed rats, and reduces in the adolescent-exposed group. Notably, the sIPSC activity in the cells from the adolescent-exposed group responded differentially to AMPH-released and bath-application of dopamine, implying that the dopamine release pool may be another target the adolescent drug exposure has impact on. Indeed, besides the drastic changes in the expression (Anderson et al., 2000; Nanxel et al., 2012) and distribution of dopamine receptors (Brenhouse et al., 2008), the amount of dopaminergic fibers (Willing and Juraska, 2016), release potential and varicosity location (Benes et al., 1996) in the mPFC also change during adolescent development. However, the functional significance of all these developmental events in the dopamine system is still largely unknown. A considerable portion of our understanding of how dopamine influences cells in the mPFC comes from studies using young

rodents, typically pre-adolescent (Seamans and Yang, 2004). Very little progress has been made on this topic in the past decade. Our current study revealed important functional changes in the dopamine system and dopamine-dependent cellular function during adolescence. Hopefully, these findings will inspire future work to more carefully characterize these developmental events.

Future directions

The mechanisms underlying the ontogeny and expression of HFS-induced LTD are not entirely clear. For example, it is possible that this LTD is an increased inhibition, decreased excitation or a mix of changes in both excitation and inhibition. Future study should examine HFS-induced changes in specific excitatory and inhibitory synaptic efficacy and how dopamine, and maybe other types of receptors, contributes to the synaptic plasticity. For example, using patch-clamp to measure the evoked inhibitory postsynaptic potential (eIPSP), or the excitatory potential with $GABA_A$ receptor blocked from inside the cell, would delineate the specific role of glutamate and GABA synapse in the forming of HFS. Furthermore, interneurons of different types may contribute differentially to this LTD function. It is suggested that the fast-spiking interneurons provide major inhibitory regulation but it is possible that other types may also play an essential role (Caballero et al., 2016). Future studies are required to determine the more detailed cellular mechanisms. Additional receptors and their signaling pathways should also be examined to determine whether they are involved in the induction of the LTD as these neurotransmitter systems are potential targets of drugs and potentially other environmental adversities to affect the ontogeny of LTD. Last but not least, the current study did not find any sex differences in AMPH's effect on the HFS-induced LTD but there may be potentially sex

differences in the ontogeny that may underlie differential sensitive in this function to drug's disruptive effect.

As mentioned earlier, age-dependent difference in AMPH's effect on inhibition in the mPFC emerged when AMPH was applied to the cells. Future studies should examine D_2-mediated inhibition, for example, sIPSC and eIPSP. Notably, the interneurons have a delayed functional maturation, especially in their responses to dopamine stimulation (Tseng and O'Donnell, 2007). In this regard, their ontogeny and functions may be more sensitive to the impact of drugs. It would be helpful to determine if there are age-of-exposure dependent differences in AMPH's effect on interneuron physiology, for example, cellular excitability and responsivity to specific dopamine agonist following repeated AMPH.

The current work suggested a relationship between synaptic plasticity in the PL-NAC circuit and cognitive flexibility and apparently, the functional significance of HFS-induced plasticity in this circuit requires further investigation. For example, according to several previous studies, the changes we found in adolescent-exposed rats would have been associated with increased perseverative type of behavior (Floresco et al., 2008, 2011), which we failed to observe. Future studies could use a more suitable cognitive task to confirm if there is any relationship between the LTD function and perseverative behavior.

A final consideration

To sum up, the limitations in the current study should be recognized. First, in our rat model exposure to the drug was involuntary (i.e., forced). In this way, the dose and timing of AMPH injection were precisely controlled but the drug intake pattern was quite different from drug use behavior in humans (Spear, 2000). Second, evidence suggests that the onset timing of

drug exposure within the peri-adolescent period plays a critical role in determining drug-induced neuronal and behavioral adaptation (Spear, 2015). The developmental trajectory of many neurotransmitter systems usually proceeds in a non-linear fashion during adolescence (Casey, 2008). In this regard, it is not surprising that the neurobiological or behavioral outcomes resulting from drug exposure depend on when during this development that drugs are introduced to the brain. In the current study, only one treatment onset was included whereas the onset of drug use in human varies. In a separate study though, we found that AMPH treatment starting at P27 and P37 produced differential behavioral and molecular changes in the mPFC (Kang et al., 2016b). Third, we were still able to observe altered LTD and prefrontal D_1 function approximately 4 months after the adolescent AMPH exposure. Four months would be considered a substantially long time in a rodent's life span but without testing, there is no definite answer if these drug-induced adaptations will dissipate eventually. In humans, adolescent development requires years to complete such that it is possible that an individual could have more chances to recover from drug-induced deficits. Taking these limitations into account, it is essential to carefully consider how the AMPH-induced neuroadaptations and behavioral changes we observed in the current study may contribute to the development of drug use problem, which is also influenced by factors such as genetic background, co-morbid mental illnesses, peer influence and other social factors (Chen et al., 2012; Jordan et al., 2014; Lacy et al., 2014; Smith et al., 2014; Strickland and Smith, 2015; Babinska et al., 2016).

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