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Lauren Marie DePoy Date

Cortical mechanisms and developmental contributions to habit formation

By

Lauren Marie DePoy
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Neuroscience

_____
Shannon Gourley, Ph.D.
Advisor

_____
Leonard Howell, Ph.D.
Committee Member

_____
Stephen Trayenlis, Ph.D.
Committee Member

_____
Mar Sanchez, Ph.D.
Committee Member

_____
David Weinshenker, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Cortical mechanisms and developmental contributions to habit formation

By

Lauren DePoy
B.S., Biology and Psychology, Gettysburg College

Advisor: Shannon Gourley, Ph.D.

An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Neuroscience
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Abstract

Cortical mechanisms and developmental contributions to habit formation

By Lauren Marie DePoy

Adolescence is characterized by high rates of experimental drug use and heightened vulnerability to the development of neuropsychiatric disorders, including substance dependence. Adolescence is also a time of activity-dependent neocortical refinement, consisting of synaptic reorganization and dendritic spine proliferation and then pruning. This reorganization might open a window of vulnerability to insults, such as cocaine exposure. Addiction is characterized by maladaptive decision making, a loss of control over drug consumption, and habit-like drug seeking despite adverse consequences. These cognitive changes likely reflect the effects of repeated drug exposure on prefrontal cortical neurobiology that then further promote drug use. Broadly, this dissertation will focus on the role of the orbitofrontal prefrontal cortex (oPFC) in action-outcome conditioning, which is critical for intact, goal-directed decision making. On the other hand, oPFC damage causes reward-seeking habits, which are increasingly recognized as an etiological factor in the development and maintenance of addiction. I will first show that adolescent cocaine exposure simplifies the dendritic structure of excitatory neurons in the oPFC. Cocaine also eliminates dendritic spines on the same neurons, and I will provide evidence that this structural remodeling is causally associated with adolescent vulnerabilities to developing drug-induced habit-based behavior. I will next shift my focus to receptor subunits that are implicated in addiction-related behavior across multiple species. I will show that silencing β1-integrin, which stimulates downstream signaling partners to coordinate actin dynamics, in the oPFC causes stimulus-response habits and a hyper-sensitivity to conditioned stimuli in a sex- and developmentally-selective fashion. Lastly, I will provide a 'snapshot' of another important prefrontal cortical structure, the medial prefrontal cortex (mPFC), which is known to regulate the acquisition of action-outcome conditioning; however, developmental contributions are unclear. I will show that developmental silencing of $GABA_A\alpha1$ impairs action-outcome conditioning, which is associated with delayed acquisition of a cocaine-reinforced response in cocaine self-administering mice. Together, these results suggest that the normative development of multiple sub-regions of the prefrontal cortex is critical for goal-directed decision making in adulthood. Aberrant structural remodeling or deficiencies in key cytoskeletal regulatory proteins during adolescence, either due to drug exposure or other factors, such as stressor exposure, might contribute to or trigger problematic drug use.

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Lastly, I would like to thank my mentor Dr. Shannon Gourley. When I first met with Shannon to discuss rotating in her lab, we had a fervent conversation about habitual behavior and the prefrontal cortex. She introduced me to the importance of studying adolescent development and her passion was infectious. I knew right away that I wanted to work with her. I joined Shannon's lab as her third graduate student and it has been an amazing ride. Shannon is a dedicated, thoughtful mentor who, above and beyond the skills necessary to be a scientist, has taught me valuable lessons, which I quote to younger students and lab mates frequently: you can't win if you don't play and you are your own best advocate. Shannon is a remarkable scientist and a tremendous role model and I aspire to be like her.

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Chapter 1:

An introduction to the orbitofrontal prefrontal cortex (oPFC)

1.1 Introduction

The oPFC is a subdivision of the prefrontal cortex (PFC) that lies above the orbits of the eyes. The oPFC is considered highly structurally and functionally conserved across species (Preuss, 1995), with the oPFC in rodents and primates not only playing a major role in determining the value of a reinforcer, but also in reward-related behavioral flexibility, inhibiting inappropriate behaviors, as well as in rapidly learning predictive associations between stimuli and associated outcomes when they change (Schoenbaum et al., 2007; Izquierdo and Jentsch, 2012; Izquierdo et al., 2004). The functions of the oPFC have been documented as far back as 1868 in accounts of Phineas Gage by Dr. John Harlow. Gage was a railroad foreman who suffered damage to the oPFC after a demolition accident, wherein a tamping iron was driven through his head. This rod entered his left cheek, behind his left eye and exited the top of his head, extensively damaging the oPFC. After this accident, his behavior became erratic and inappropriate, both sexually and socially (reviewed Damasio et al., 1994). This case study serves as a staggering example of the importance the oPFC in normal everyday decision making.

1.2 Functions of the oPFC

1.2.1 *Regulation of reversal learning*

The oPFC plays a similar role in regulating the behavior of rodents and non-human primates; for example, the classical effect of lesions of the oPFC is impairments in reversal learning (Butter, 1969; Chudasama and Robbins, 2003), an assay of behavioral flexibility and stimulus-outcome associative conditioning that can be tested across primate-rodent species. In this test, animals must discriminate between two stimuli, learning to select one "correct" stimulus, which results in reinforcement, and ignoring another, "incorrect" stimulus. Next, the associations

are reversed; selection of the stimulus that was previously correct is now non-reinforced, and the stimulus that was incorrect is now reinforced.

oPFC lesions disrupt reversal learning in humans, monkeys and rodents. For example, Butter, in 1969, reported that lesions of the orbital surface in rhesus monkeys result in response perseveration, and these results have been repeatedly replicated (*e.g.*, Bachevalier et al., 2011). In rodents, oPFC lesions also impair reversal learning, and impairments can be attributed to increased perseverative responding, as opposed to, for example, response extinction (Ragozzino, 2007; Chudasama and Robbins, 2003; Boulougouris et al., 2007). In humans, bilateral lesions of the oPFC cause a failure to alter response patterns when a stimulus-outcome contingency is reversed; impairments similarly manifest as perseveration of the initial response pattern and a failure to modify responses to stimuli when they no longer result in reinforcement (Hornak et al., 2004).

Behavioral flexibility is necessary for modifying response strategies that fail to result in reinforcement. Reversal learning requires simultaneous inhibition of a previously learned response and deployment of a previously withheld response (Pauli et al., 2012). The oPFC is not critical to the initial stimulus-outcome conditioning itself, since lesions of the oPFC do not impair initial discrimination of reward-associated stimuli and their outcomes. Instead, the oPFC is necessary for altering behavior as contingencies shift (Schoenbaum et al., 2002).

1.2.2 *Regulation of behavioral sensitivity to stimulus-outcome-based reinforcer devaluation*

oPFC lesions not only impair reversal learning, but also impair behavioral sensitivity to stimulus-outcome-based devaluation, another form of stimulus-outcome conditioning. Behavioral sensitivity to outcome devaluation can be examined across species using a variety of techniques. In rodents, outcome devaluation is typically performed using either satiety, wherein a food

outcome is devalued by allowing *ad libitum* access to a food reinforcer prior to test, or conditioned taste aversion, wherein an injection of lithium chloride is paired with the food, causing a sickness sensation. Following satiety or conditioned aversion, subjects will typically reduce responding for a conditioned stimulus associated with the now-devalued food. On the other hand, *insensitivity* to reinforcer devaluation is reflected by persistent responding for that stimulus. oPFC lesions in rodents block the decrement in stimulus-elicited responding typically observed following outcome devaluation (Gallagher et al., 1999; Pickens et al., 2003). Bilateral oPFC lesions in rhesus monkeys also result in aberrant responding for a stimulus associated with a devalued reinforcer (Izquierdo et al., 2004).

1.2.3 *Regulation of actions and habits*

In addition to stimulus-outcome associative conditioning, more recent studies indicate that the oPFC also plays a role in action-outcome associative conditioning. What is action-outcome conditioning? Both humans and rodents can learn to associate specific actions with their outcomes. These actions are considered to be 'goal-directed,' meaning their performance is sensitive to changes in the contingency between the behavior and its outcome. In experimental models, extended training and certain reinforcement schedules can induce a shift away from goal-directed to automated, or 'habitual,' response strategies that are *insensitive* to changes in the action-outcome contingency and instead driven by stimulus-response associations. A classic example for a person who wears glasses is how, when your vision blurs, you push your glasses up on your nose to fix your vision. After repetition, anytime your vision becomes blurred, you might reach to push your glasses up your nose, even if you are not wearing your glasses (for example, if you are instead wearing contact lenses).

The development of habits at the expense of goal-directed response selection is thought to serve an adaptive function by freeing up attentional and higher-order cognitive processes when

one engages in a familiar behavior. On the other hand, behavioral flexibility is necessary for modifying response strategies when familiar behaviors fail to result in reinforcement. Furthermore, the development of intractable reward-seeking habits is widely considered an etiological factor in addiction (Robbins and Everitt, 1999; Jentsch and Taylor, 1999; Everitt and Robbins, 2005).

This dissertation will primarily focus on one assay used to investigate action-outcome decision-making strategies. Action-outcome contingency degradation tests for behavioral sensitivity to changes in the causal *relationship* between an action and its outcome (Hammond, 1980; described Balleine and O'Doherty, 2010). For example, rats can be trained to perform two discrete operant responses for reinforcers, typically food. Then, the likelihood of reinforcement associated with one response is decreased. Preferential engagement of the remaining response, which remains likely to be reinforced, provides evidence of knowledge of the response-reinforcement relationship (Colwill and Rescorla, 1986; Dickinson, 1980; Balleine and O'Doherty, 2010; Yin et al., 2008). By contrast, failures in action-outcome conditioning result in equivalent deployment of both responses, even though one is unlikely to be reinforced.

When subjects cannot alter choice or response patterns based on changes in the likelihood that a given response will be reinforced (for example, in an action-outcome contingency degradation task) or based on outcome value, their behavior is considered habitual. A relatively early study reported that large lesions of the oPFC in female rats do not impair behavioral sensitivity to outcome devaluation in a task devoid of conditioned stimuli (Ostlund and Balleine, 2007). This finding suggested that the oPFC is *not* involved in action-outcome-based decision making, however this perspective contradicts both a classical model of Butter, which argued that failures in extinction conditioning in non-human primates with oPFC lesions reflect habit-based responding (Butter et al., 1963), and two key reports published in 2013, indicating that oPFC damage indeed induces failures in action-outcome conditioning, causing mice to defer to habit-

based behavior. In the first, Gremel and Costa (2013) utilized lesions and chemogenetic approaches to show that inactivation of the ventrolateral oPFC (VLO) caused failures in behavioral sensitivity to outcome value. Similarly, Gourley et al., (2013a) reported that asymmetric lesions disconnecting the VLO and downstream centro-lateral striatum occluded sensitivity to action-outcome contingency degradation. Further, bilateral, oPFC-selective reduction of the pro-plasticity neurotrophin *Brain-derived neurotrophic factor (Bdnf)* impaired behavioral sensitivity to action-outcome contingency degradation and reinforcer devaluation, inducing a bias towards habit-based behavior (Gourley et al., 2013a). This finding has since been replicated (Zimmermann et al., 2015), and site-selective reduction of *Gabra1* and *Fmr1* in the VLO have also been shown to disrupt action-outcome conditioning (Swanson et al., 2015; Gross et al., 2015). These and other findings strongly imply that the rodent oPFC indeed coordinates goal-directed action selection, and that damage to this structure can result in response failures, and consequently, a deferral to familiar, habit-based behavior.

The oPFC is delineated into sub-regions that vary in position along the medial to lateral extent. Isolating the functions of these specific regions of the oPFC may be critical to understanding the various roles of the oPFC in complex behaviors, as oPFC projections are topographically-organized. For example, transitioning from more medial to lateral in their innervation of the striatum. This dissertation will focus largely on the VLO because it projects to the medial striatum, a critical structure for goal-directed action selection. The VLO in rodents is comparable to Broddman's areas 11 and 13 in non-human primates.

1.3 The oPFC and addiction etiology

Addiction is a disorder classically characterized by poor judgment and maladaptive decision making, resulting in compulsive drug use despite adverse consequences (Mendelson et al., 1996; Thomas et al., 2008; Lucantonio et al., 2012). As discussed, the healthy oPFC plays a

critical role in interpreting outcome value (Gremel and Costa, 2013; Stalnaker et al., 2015) and changes in the causal relationship between an action and its outcome (Gourley et al., 2010,2013a). These functions make the oPFC crucial to normative decision-making strategies, which are disrupted by chronic psychostimulant exposure, inducing stimulus-response habits for example (Schoenbaum and Setlow, 2005; Nelson and Killcross, 2006,2013; Nordquist et al., 2007; Corbit et al., 2014; Leong et al., 2016). This is critical because habitual, stimulus-elicited reward seeking is increasingly recognized as an etiological factor in the development and maintenance of cocaine addiction (Robbins and Everitt, 1999; Jentsch and Taylor, 1999; Everitt and Robbins, 2005).

1.4 Dissertation Overview

This dissertation will focus on further examining the role of the oPFC in action-outcome conditioning. I will build on current evidence that damage to the oPFC causes a deferral to stimulus-elicited habits, which are considered a factor in the development and maintenance of addiction (Everitt and Robbins, 2016). In lieu of classically 'damaging' (*i.e.,* lesioning) the oPFC, I will take a more nuanced approach, disturbing actin cytoskeletal structure or using genetic manipulations, with the goal of expanding our understanding of how habit biases form, particularly cocaine-induced habits. Furthermore, since adolescents are particularly vulnerable to drugs of abuse, I will focus on this additional factor, selectively manipulating the oPFC during different stages of development.

I will first show that developmental cocaine exposure simplifies the dendritic structure of excitatory neurons in the oPFC. Cocaine also eliminates dendritic spines on the same neurons, and I will provide evidence that this structural remodeling is causally associated with drug-induced vulnerabilities to developing habit-based behavior. In chapters 4 and 5, I will shift focus to receptor subunits that are implicated in addiction-related behavior across species. I will show

that selective knockdown of *Itgb1*, the gene that encodes β1-integrin, in the oPFC causes stimulus-response habits and a hyper-sensitivity to conditioned stimuli in a developmentally-selective fashion. Lastly, I will provide a glimpse into the importance of another prefrontal cortical region, the medial prefrontal cortex (mPFC), which is known to regulate the *acquisition* of action-outcome conditioning (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Ostlund and Balleine, 2005), but developmental contributions are unclear. I will show that developmental mPFC-selective knockdown of *Gabra1,* encoding the $GABA_A\alpha1$ receptor subunit, impairs action-outcome associative learning, and these failures are associated with the delayed acquisition of a cocaine-reinforced response in cocaine self-administering mice.

Chapter 2:

Adolescent cocaine exposure simplifies orbitofrontal prefrontal cortical dendritic arbors

2.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter describes the effects of adolescent cocaine exposure on the structure of dendritic arbors on excitatory pyramidal neurons in the oPFC. This work was conceptualized by the dissertation author and Dr. Shannon Gourley. Research was conducted by the dissertation author, Dr. Kelsey Zimmermann, and Riley Perszyk, and the document was written by the dissertation author and Dr. Shannon Gourley. Dr. Anthony Koleske provided the mutant mice used herein, guidance, and contributed to editing the manuscript. This chapter is reproduced with minor edits from DePoy LM, Perszyk RE, Zimmermann KS, Koleske AJ, Gourley SL (2014) Adolescent cocaine exposure simplifies orbitofrontal cortical dendritic arbors. *Front Pharmacol* 5:228.

2.2 Abstract

Cocaine and amphetamine remodel dendritic spines within discrete cortico-limbic brain structures including the orbitofrontal cortex (oPFC). Whether dendrite structure is similarly affected, and whether pre-existing cellular characteristics influence behavioral vulnerabilities to drugs of abuse, remain unclear. Animal models provide an ideal venue to address these issues because neurobehavioral phenotypes can be defined both before, and following, drug exposure. We exposed mice to cocaine from postnatal days 31-35, corresponding to early adolescence, using a dosing protocol that causes impairments in an instrumental reversal task in adulthood. We then imaged and reconstructed excitatory neurons in deep-layer oPFC. Prior cocaine exposure shortened and simplified arbors, particularly in the basal region. Next, we imaged and reconstructed orbital neurons in a developmental-genetic model of cocaine vulnerability – the *p190rhogap*+/- mouse. p190RhoGAP is an actin cytoskeleton regulatory protein that stabilizes dendrites and dendritic spines, and *p190rhogap*+/- mice develop rapid and robust locomotor activation in response to cocaine. Despite this, oPFC dendritic arbors were intact in drug-naïve

p190rhogap+/- mice. Together, these findings provide evidence that adolescent cocaine exposure has long-term effects on dendrite structure in the oPFC, and they suggest that cocaine-induced modifications in dendrite structure may contribute to the behavioral effects of cocaine more so than pre-existing structural abnormalities in this cell population.

2.3 Introduction

Cocaine addiction is characterized by maladaptive decision making, a loss of control over drug consumption, and habit-like drug seeking despite adverse consequences. These cognitive changes likely reflect the effects of repeated drug exposure on prefrontal cortical neurobiology that then further promote drug use (Jentsch and Taylor, 1999; Everitt and Robbins, 2005; Torregrossa et al., 2011; Lucantonio et al., 2012). Additionally, *pre-existing* neurobehavioral characteristics in drug-naïve individuals may contribute to drug vulnerabilities (Ersche et al., 2012). Rodents provide an ideal model system to isolate vulnerability factors in drug-naïve organisms and also characterize the *consequences* of cocaine exposure because like humans, rodents will readily self-administer cocaine and engage in complex decision making, as well as relapse-like behavior. Also as in humans, individual differences in behavioral response strategies can serve as phenotypic predictors of addiction-like behaviors such as drug seeking following periods of abstinence (Deroche-Gamonet et al., 2004). Finally, even experimenter-administered, rather than self-administered, cocaine can induce behavioral phenotypes in rodents that are relevant to addiction etiology in humans, *e.g.*, increased propensity to engage in reward-seeking habits (see Chapter 3; Schoenbaum and Setlow, 2005; Gourley et al., 2013b; Hinton et al., 2014).

We recently sought to identify the long-term consequences of cocaine exposure during adolescence, when drug use is often initiated in humans, and when the prefrontal cortex is still developing (Casey et al., 2000; Giedd 2004; Paus et al., 2008). We found that >7 weeks following subchronic exposure during adolescence, cocaine-exposed mice preferentially engaged habit-like

response strategies at the expense of flexible action-outcome-based strategies to acquire food reinforcers (Hinton et al., 2014). Inactivation of the orbitofrontal prefrontal cortex (oPFC) blocks goal-directed action selection in the same task (Gourley et al., 2013a), and adolescent cocaine exposure eliminates dendritic spines in this region (Gourley et al., 2012a). Whether adolescent cocaine exposure regulates oPFC dendrite arbor structure is, to our knowledge, unresolved. Such gross remodeling could contribute to persistent maladaptive decision making following adolescent cocaine exposure and potentially to increased risk of dependence (O'Brien and Anthony, 2005) or decreased likelihood of seeking treatment (Kessler et al., 2001) in individuals who initiate cocaine use in adolescence.

We first quantified the effects of adolescent cocaine exposure on behavioral flexibility in an oPFC-dependent instrumental reversal task and on dendrite arbor structure in adult excitatory deep-layer oPFC neurons. Next, we aimed to evaluate whether *pre-existing* morphological abnormalities in the same neuron population were associated with behavioral vulnerabilities. The model we selected for this experiment was the *p190rhogap*+/- mouse. These mice are deficient in p190RhoGAP, a principal Src substrate in the brain that regulates cell structure through interactions with the RhoA GTPase (Brouns et al., 2000,2001). Drug-naïve *p190rhogap*+/- mice appear at baseline to be behaviorally unremarkable (Gourley et al., 2012a,2013c). Nonetheless, they are highly sensitive to cocaine such that a single injection elicits a sensitization-like response (Gourley et al., 2012a), making them an ideal candidate model by which to isolate pre-existing structural factors associated with subsequent cocaine vulnerability.

Our findings suggest that adolescent cocaine exposure simplifies excitatory oPFC dendritic arbors. By contrast, dendrite arbors appear grossly normal in *p190rhogap*+/- mice and thus, do not obviously account for behavioral vulnerabilities to cocaine in these animals.

2.4 Methods

2.4.1 *Subjects*

All mice were bred on a C57BL/6 background, and those used in anatomical studies expressed *thy1*-derived YFP (Feng et al., 2000) to enable dendrite imaging. YFP-expressing p190RhoGAP-deficient mice (*p190rhogap*+/-) or *p190rhogap*+/+ littermates were also used. *p190rhogap*+/- mice have a ~32-40% reduction in p190RhoGAP protein expression (Brouns et al., 2000). Mice were maintained on a 12-hour light cycle (0700 on), and provided food and water *ad libitum* unless otherwise noted. Procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Emory and Yale University IACUCs, as appropriate.

2.4.2 *Adolescent cocaine exposure*

Cocaine or saline (Sigma) was administered for 5 consecutive days starting at postnatal day (P) 31 (10 mg/kg, *i.p.*, 1 ml/100 g). Then, mice were left undisturbed until P56, at which point they were euthanized for anatomical studies or tested in an instrumental reversal learning task.

2.4.3 *Instrumental reversal learning*

Mice with a history of adolescent cocaine exposure were food-restricted as adults to ~93% of their original body weight and trained to nose poke for food reinforcement (20 mg grain-based pellets; Bioserv) using illuminated Med-Associates conditioning chambers. Training was initiated with a continuous reinforcement schedule; 30 pellets were available for responding on each of 2 distinct nose poke recesses located on opposite sides of a single wall within the chambers, resulting in 60 pellets/session. Sessions ended when all 60 pellets were delivered or at 135 min. Responding on a center aperture was not reinforced. Following 7 days of training, mice

were required to "reverse" their responding to this center aperture to continue to obtain reinforcement. Responding on the previously active apertures was no longer reinforced. These "reversal" sessions were 25 min in duration and used a variable ratio 2 schedule of reinforcement (Gourley et al., 2010). Responses on the active and inactive nose poke apertures were quantified, as were head entries into the magazine where pellets were delivered. One cocaine-exposed mouse consistently generated values two standard deviations above the group mean and was excluded.

2.4.4 *Locomotor monitoring*

In 8-week-old mice, we used a within-subjects design to compare the locomotor response to cocaine between *p190rhogap*+/- and *p190rhogap*+/+ littermates. Mice were administered cocaine (10 mg/kg, *i.p.*, 1 ml/100 g) for 5 sequential days, and then left undisturbed for 7-10 days at which point a "challenge" injection was administered (10 mg/kg, *i.p.*, 1 ml/100 g).

Locomotor activity was monitored using customized Med-Associates chambers equipped with 16 photobeams. Mice were first habituated to the chambers for 1 hour following a saline injection, then cocaine was administered. Total photobeam breaks following the cocaine injection were normalized to those generated in the 30 min following saline injection. During the challenge session, all mice were habituated to the locomotor monitoring chambers for 1 hour without injection, then saline was administered and mice were monitored for 30 min, and finally, cocaine was administered, and mice were monitored for an additional 30 min. Cocaine-elicited photobeam breaks were normalized to those following saline in order to control for conditioned locomotor activation in response to injection. This experiment served to provide evidence that *p190rhogap*+/- mice are hyper-sensitive to cocaine, as we have previously reported (Gourley et al., 2012a), but importantly, mice used for anatomical studies were cocaine-naïve because our goal was to evaluate pre-existing factors that might be associated with cocaine vulnerability.

2.4.5 *Dendritic arbor reconstruction and measurement*

Mice were euthanized, and fresh brains were submerged in 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 50 µm-thick coronal sections on a microtome held at -15°C. These relatively thin sections allow us to image whole deep-layer neurons without background fluorescence that would otherwise obstruct reconstruction. Little is known regarding the typical morphology of these neurons. This may be because traditional Golgi impregnation can spare deep-layer oPFC (Kolb et al., 2008), and while it is conceivable that we under-count dendrites that may have been truncated along the rostro-caudal plane, neurons with clear dendritic arbor truncations were excluded from the analyses.

Neurons were imaged on a spinning disk confocal (VisiTech International, Sunderland, UK) on a Leica microscope. Z-stacks were collected with a 20X 1.4NA objective using a 1 µm step size, sampling above and below the neuron. After imaging, we confirmed at 10X that the image was collected from the oPFC. Most images were collected from the lateral oPFC, however the ventral subregion was also sampled. Neurons contained at least two basal dendritic arbors and a distinct intact apical dendrite, all with at least second-order branching.

Neurons were reconstructed in 3 dimensions by a single experimenter blind to group using Neurolucida (MBF Biosciences). Total dendritic material was measured for apical and basal arbors. To assess dendrite complexity, a 3-D version of a Sholl analysis (Wellman et al., 2007) was performed by measuring the number of dendritic intersections within 10-µm concentric spheres radiating from the soma. 4-11 neurons/mouse from the *p190rhogap*+/- population and 4-8 neurons/mouse from the cocaine-exposed population were imaged, reconstructed, and analyzed. Group sizes were 6-7 mice in the cocaine-exposed population and 4-6 mice in the *p190rhogap*+/- population.

2.4.6 *Statistical analyses*

For instrumental conditioning studies, responding on the active and inactive apertures and magazine head entry rates were compared by ANOVA with repeated measures and group as the independent variable. For locomotor assessments, cocaine-elicited photobeam breaks (calculated as potentiation from baseline) were compared by ANOVA with repeated measures and group as the independent variable. Locomotor counts on the challenge day were compared between groups by unpaired t-test.

For anatomical studies, each mouse contributed a single value – the mean of its multiple neurons – to the analyses. Dendrite lengths were compared between groups by unpaired t-test. Sholl intersections were compared by ANOVA with repeated measures. In the case of interactions, Tukey's post-hoc comparisons were generated; the results of post-hoc comparisons are indicated graphically. $p<0.05$ was considered significant. In one instance, a Kolmogorov-Smirnov comparison was also applied to total dendrite length; in this case, each neuron was considered an independent sample.

2.5 Results

Here we aimed to quantify the effects of adolescent cocaine exposure on oPFC dendrite morphology. We first, however, confirmed that adolescent cocaine had long-term behavioral consequences. Mice were exposed to subchronic cocaine during early adolescence, from P31-35 (Spear 2000), then left undisturbed until adulthood. Cocaine exposure is thought to confer a bias towards inflexible, maladaptive decision-making strategies, so we tested mice in an instrumental "reversal learning" task that is sensitive to chronic cocaine exposure or lesions of the oPFC in adult mice (Krueger et al., 2009; Gourley et al., 2010).

Mice with a history of subchronic saline or cocaine exposure were able to acquire a nose poke response for food reinforcement as adults (interaction $F<1$) (fig.2.1a). Qualitatively, cocaine appeared to modestly decrease overall responding, but this effect did not reach significance [main effect $F_{(1,21)}=3.9$, $p=0.06$], and response rates were equivalent at the end of training. When the response requirement was "reversed" such that mice were required to respond on an aperture at a separate location in the conditioning chamber, mice with a history of cocaine exposure generated fewer responses [main effect of cocaine $F_{(1,20)}=4.1$, $p<0.05$] (fig.2.1b). We identified no effects of cocaine on responding on the previously-reinforced aperture or magazine head entry rate (both $F<1$) (fig.2.1c). This pattern recapitulates the effects of prolonged cocaine exposure in adult mice (Krueger et al., 2009), as well as lesions of the lateral oPFC (Gourley et al., 2010).

We next analyzed the effects of adolescent cocaine exposure on dendrite structure in the adult lateral oPFC. Representative deep-layer neurons from adult mice are shown (fig.2.2a). Note the relatively stellate shape of oPFC pyramidal neurons compared to the more classically pyramidal shape of neurons in other subregions of the prefrontal cortex (further discussed in Kolb et al., 2008; see also Liston et al., 2006; Bortolato et al., 2011).

Total basal arbor length was reduced in cocaine-exposed mice [$t_{(11)}=2.3$, $p<0.05$] (fig.2.2b). Basal arbors were also less complex, as indicated by fewer Sholl intersections 40-100 μm from the soma [interaction $F_{(25,275)}=1.9$, $p<0.01$] (fig.2.2c). As another metric of dendrite length, we measured terminal branches, the segments following the last bifurcations of each dendrite. In this case, terminal branch lengths did not differ [$t_{(11)}=-1.3$, $p>0.2$] (fig.2.2d), consistent with evidence from the Sholl analysis that a history of adolescent cocaine exposure simplifies dendrite arbors in close proximity to the soma (again, fig.2.2c).

Despite differences in basal arbor length and complexity, apical arbors were not significantly affected [for length, $t_{(11)}=1.9$, $p=0.09$; for Sholl intersections, $Fs<1$] (fig.2.2b,e).

We next evaluated dendrite complexity in a developmental-genetic model of cocaine vulnerability – *p190rhogap*+/- mutant mice. We selected these mice because p190RhoGAP is a cytoskeleton regulatory protein implicated in postnatal dendrite stability in the brain (Sfakianos et al., 2007), and we have previously reported that *p190rhogap*+/- mice display augmented sensitivity to cocaine (Gourley et al., 2012a). Specifically, mice develop a sensitization-like response even after exposure to a single relatively low dose, and locomotor activity remains exaggerated over the course of several daily cocaine administrations, an effect that we recapitulate here by administering 10 mg/kg cocaine to mice daily for 5 days [main effect of genotype $F_{(1,20)}$=7.4, p=0.01] (fig.2.3a). Mice were then left undisturbed for one week, after which they were habituated to the locomotor monitoring chambers for one hour, then injected with saline and monitored for 30 min, then finally, injected with cocaine. Wild type mice generated 1.4 times as many photobeam breaks following low-dose cocaine "challenge" relative to saline. By contrast, *p190rhogap*+/- littermates broke >4-fold more photobeams following cocaine exposure [$t_{(19)}$=-2.1, p=0.05] (fig.2.3a).

Adult drug-naive *p190rhogap*+/- mice were crossed with mice expressing YFP, generating YFP-expressing wild type and *p190rhogap*+/- offspring, and allowing us to potentially identify structural predictors of cocaine vulnerable *prior to drug exposure*. When oPFC dendrites from drug-naïve *p190rhogap*+/- mice were imaged and reconstructed, however, we identified no differences in total dendrite length [apical $t_{(8)}$=1.2, p=0.3; basal $t_{(8)}$=0.8, p=0.5] (fig.2.3b). By contrast, basal dendrite lengths differed in *cocaine-exposed* mice above, so as an additional, potentially more nuanced measure, we compared dendrite lengths using a Kolmogorov-Smirnov analysis in which the basal dendrite length from each neuron was considered an independent sample. Even here, we again did not identify differences between wild type and *p190rhogap*+/- mice (D=0.2, p=0.3) (fig.2.3c). Consistent with this outcome, basal arbor complexities did not

differ, as determined by Sholl intersections (interaction F<1) (fig.2.3d), and the length of the terminal branches did not differ [$t_{(8)}$=-1, p=0.3] (fig.2.3e).

When we quantified Sholl intersections for the apical arbor, neither interactions nor main effects were detected [interaction F=1; main effect $F_{(1,22)}$=1.4, p=0.3] (fig.2.3f). Qualitatively, wild type mice appeared to have more complex arbors, but this impression was driven by a single mouse.

2.6 Discussion

The ability of neurons to integrate into networks and regulate behavior is determined in part by the size, shape, and complexity of dendrites. Dendrites can be remarkably plastic – for example, oPFC dendritic arbors remodel following stressor exposure (Liston et al., 2006; Dias-Ferreira et al., 2009) and environmental enrichment (Comeau et al., 2010). Some such modifications may play a role in mood disorders and other psychopathologies involving cortico-striatal circuits (*e.g.*, cocaine addiction), but the characterization of structural modifications that – like drug craving in addiction – persist beyond the period of active drug exposure remains incomplete. We used transgenic mice expressing *thy1*-derived YFP to isolate and reconstruct dendritic arbors of excitatory deep-layer oPFC neurons. We report that arbors remodeled in response to subchronic cocaine exposure in adolescence and were simplified in adulthood. By contrast, dendritic arbors in drug-naïve *p190rhogap*+/- mutant mice – a model of cocaine vulnerability (Gourley et al., 2012a) – were intact, suggesting that the *response* to cocaine, rather than pre-existing structural deficiencies *per se*, is associated with behavioral sensitivity to further drug exposure in these mice.

2.6.1 *Prefrontal cortical dendrites reorganize in response to cocaine*

The effects of amphetamine-like psychostimulants such as cocaine on neural structure have been intensively studied since the seminal reports of Robinson and Kolb describing drug-induced dendrite and dendritic spine elaboration in the nucleus accumbens and medial prefrontal cortex (1997,1999). Within the prefrontal cortex, the vast majority of subsequent research has remained focused on medial wall structures, largely sparing the oPFC; this is despite overwhelming evidence implicating oPFC function in addiction etiology (*e.g.*, see Lucantonio et al., 2012). Currently available data indicate that amphetamine and cocaine *reduce* dendritic spine density in the oPFC (Kolb et al., 2004; Crombag et al., 2005; Muhammad and Kolb, 2011a,b; Gourley et al., 2012a; Radley et al., 2015; but see Ferrario et al., 2005), but effects on dendrite structure remain unclear. We report novel evidence that cocaine exposure simplifies oPFC dendrite arbors, particularly in the basal region. Notably, chronic ethanol exposure does not remodel excitatory oPFC neurons (Holmes et al., 2012; DePoy et al., 2013a), thus the present effects may be selective to stimulants, or potentially cocaine specifically.

In the parietal cortex, amphetamine exposure blocks the dendrite-elaborating effects of environmental enrichment, consistent with our current findings, although amphetamine alone has no consequences (Kolb et al., 2003). Nonetheless, we found evidence of long-term dendrite *simplification* following cocaine. How might we reconcile this apparent contradiction? One difference, in addition to the anatomical, is that cocaine was administered here during the equivalent of adolescence, a period of vulnerability to the development of dependence in humans (Anthony and Petronis, 1995; O'Brien and Anthony, 2005). Recent studies using small-animal magnetic resonance imaging complement ours, revealing that adolescent (though not adult) cocaine exposure results in cortical thinning (Wheeler et al., 2013). Interestingly, overall oPFC volume is *increased* following adolescent cocaine exposure; this increase could conceivably reflect glial responses to cocaine (Bowers and Kalivas, 2003; Haydon et al., 2009), though further investigations are necessary.

Here, oPFC neurons in mice with a history of adolescent cocaine exposure were simplified, particularly in the basal region. This is notable given that adolescent psychostimulant exposure in non-human primates *also* simplifies basal arbors in deep-layer prefrontal cortex (Selemon et al., 2007). A strong trend for a reduction in dendritic spine density was also reported by Selemon et al. (2007); similarly, oPFC dendritic spines are eliminated following adolescent cocaine exposure in the mouse (Gourley et al., 2012a). The amygdala projects to deep-layer prefrontal cortex in both rodents and primates, with neurons terminating on dendritic spine heads of both apical and basal branches (Gabbott et al., 2006; Ghashghaei et al., 2007). Our findings thus indicate that the structural effects of adolescent psychostimulant exposure in critical cortico-amygdala circuits implicated in addiction (see Torregrossa et al., 2011) translate across rodent-primate species.

In tandem with arbor simplification, cocaine exposure impaired performance in an oPFC-dependent instrumental reversal task. In this task, mice are trained to nose poke for food reinforcers in a chamber with multiple response operandi. Once mice have acquired the reinforced response, the location of the reinforced aperture is "reversed," in this case, from the lateral walls of the testing chamber to a center aperture, and mice must redirect responding to this previously non-reinforced aperture. Lesions of the lateral, but not medial, oPFC delay response acquisition, as does chronic cocaine exposure in adult mice (Krueger et al., 2009; Gourley et al., 2010). Conversely, instrumental reversal learning in drug-naïve mice is associated with subsequent cocaine self-administration patterns, with poor reversal performance predictive of higher rates of cocaine-reinforced responding (Cervantes et al., 2013). We report that even *subchronic* cocaine exposure in adolescent mice impaired response acquisition several *weeks* following drug exposure. Similarly, in a water maze reversal, early-adolescent cocaine exposure impairs response acquisition up to 10 days following exposure (Santucci et al., 2004). Together with multiple reports that cocaine exposure also occludes reversal learning based on stimulus-

outcome associative contingencies (see Lucantonio et al., 2012), these findings highlight the long-term negative impact of cocaine on oPFC function, resulting in inflexible habit-like response strategies.

The oPFC also appears to regulate behavioral sensitivity and resilience to contextual stimuli associated with cocaine. For example, prolonged oPFC inactivation enhances context-induced reinstatement of cocaine seeking in rats, sparing drug-seeking behaviors induced by other conditioned stimuli (Fuchs et al., 2004; Lasseter et al., 2009). Thus, the *healthy* oPFC may gate the influence of contextual cues associated with drugs of abuse; repeated cocaine exposure could degrade this function through repeated stimulation of the dopamine D1 receptor for example (Lasseter et al., 2014), simplification of neural structure (fig.2.1), and/or imbalance between D1 and D2, given that D2 is highly expressed on basal arbors that were eliminated here (Brock et al., 1992).

2.6.2 *oPFC dendrite morphology in drug-naïve cocaine-vulnerable mice is intact*

Structural remodeling in the central nervous system is orchestrated by Rho family GTPases including RhoA (Rho), Rac1, and Cdc42, which coordinate the actin cytoskeletal rearrangements required for dendrite elaboration or simplification. Rho activation decreases branch extensions in multiple neural systems (*e.g.*, Li et al., 2000; Wong et al., 2000), and interference with Rho activity promotes arbor growth (*e.g.*, Sin et al., 2002; Couch et al., 2010) or activity-dependent remodeling of dendritic spines (Murakoshi et al., 2011). In the *adolescent* hippocampus, Rho activation causes dendritic arbor retraction, reducing overall length and complexity (Sfakianos et al., 2007).

Rho is inhibited endogenously by p190RhoGAP, which is activated by integrin receptor binding to extracellular matrix proteins (Arthur et al., 2000; Hernandez et al., 2004; Moresco et

al., 2005; Bradley et al., 2006). *p190rhogap*-/- mice are not viable, and while *p190rhogap*+/- mice appear superficially normal, they exhibit significant vulnerabilities to genetic and chemical perturbations. For example, simultaneous heterozygosity for mutations in both p190RhoGAP and the cytoskeletal regulatory protein Abl2/Arg kinase results in increased Rho activity and hippocampal dendritic arbor destabilization, accompanied by novel object recognition deficits (Sfakianos et al., 2007). Further, mice deficient in p190RhoGAP or the upstream effectors β1-integrin or Abl2/Arg kinase are hyper-vulnerable to cocaine, generating a sensitization-like response following a single injection (Gourley et al., 2009a,2012a; Warren et al., 2012).

The *p190rhogap*+/- mouse provides an opportunity to characterize neural morphology in an organism that is behaviorally vulnerable to cocaine *prior to cocaine exposure*. Throughout, we identified no differences in the size or complexity of excitatory deep-layer oPFC neurons between mutants and *p190rhogap*+/+ littermates. These findings suggest that although cocaine exposure remodels the same neuron population, *pre-existing* deficiencies in dendrite arbors do not obviously account for drug vulnerability. Previously, we characterized dendritic spine density on excitatory oPFC neurons in naïve *p190rhogap*+/- mice and *p190rhogap*+/- mice exposed to a subthreshold dose of the stress hormone corticosterone (Gourley et al., 2013c). Corticosterone reduced oPFC spine density in *p190rhogap*+/- mice, and these structural deficiencies emerged in concert with anhedonic-like behavior. Thus, p190RhoGAP may regulate the structural *response* of oPFC neurons to varied pathological insults and thereby impact behavioral outcomes. In line with this perspective, ethanol activates p190RhoGAP and thereby decreases actin stress fiber density in neonate astrocytes exposed to ethanol (Selva and Egea, 2011).

While we did not identify structural *predictors* of cocaine vulnerability in the oPFC, it is important to note that our findings do not preclude the possibility that pre-existing morphological or physiological characteristics in other models contribute to drug vulnerabilities. Additionally, pre-existing characteristics of cell populations in other brain regions – *e.g.*, in the striatum,

amygdala, or other regions of the frontal cortex (Ersche et al., 2012; Winhusen et al., 2013) – may significantly impact drug vulnerability even prior to active drug exposure.

2.7 Summary

The present results contribute to the general perspective that psychostimulant-induced neural remodeling has meaningful behavioral implications. These include potentially adaptive consequences in the case of acute or subchronic drug exposure. For example, cocaine-induced dendritic spine proliferation in the nucleus accumbens has been associated with behavioral resilience (*e.g.*, Smith et al., 2014), and blockade of certain cocaine-induced dendritic spine modifications in the oPFC and nucleus accumbens can *increase – rather than occlude –* sensitivity to subsequent cocaine exposure (Toda et al., 2006; Gourley et al., 2012a; Pulipparacharuvil et al., 2008). Meanwhile, the *correction* of long-term or metaplastic modifications following prolonged cocaine exposure may have behavioral benefits (Giza et al., 2013; Shen et al., 2009). Animal models provide an ideal venue to disentangle these issues, and to determine neurobiological vulnerability and resiliency factors using both prospective and retrospective approaches.

Figure Legends

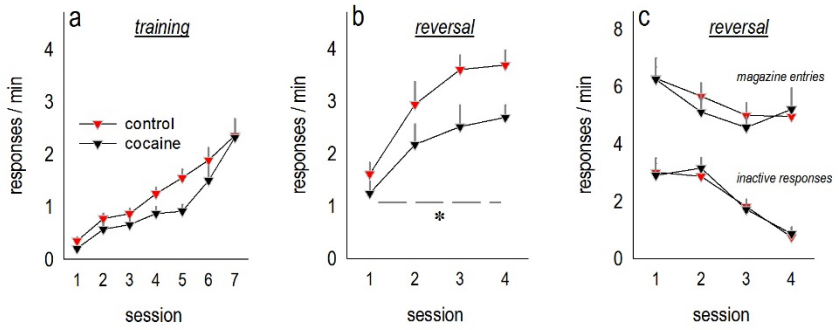

Figure 2.1. Adolescent cocaine exposure impairs instrumental reversal learning in adulthood. (a) Mice were exposed to cocaine or saline from P31-35, then left undisturbed until adulthood, at which point they were trained to nose poke for food reinforcers. (b) When the location of the reinforced aperture within the chamber was then "reversed," cocaine-exposed mice generated fewer responses on the now-active aperture, apparently less able to differentiate between reinforced and non-reinforced responses. (c) Raw response rates on the inactive aperture and magazine are shown. Means+SEMs, *$p<0.05$, main effect of cocaine.

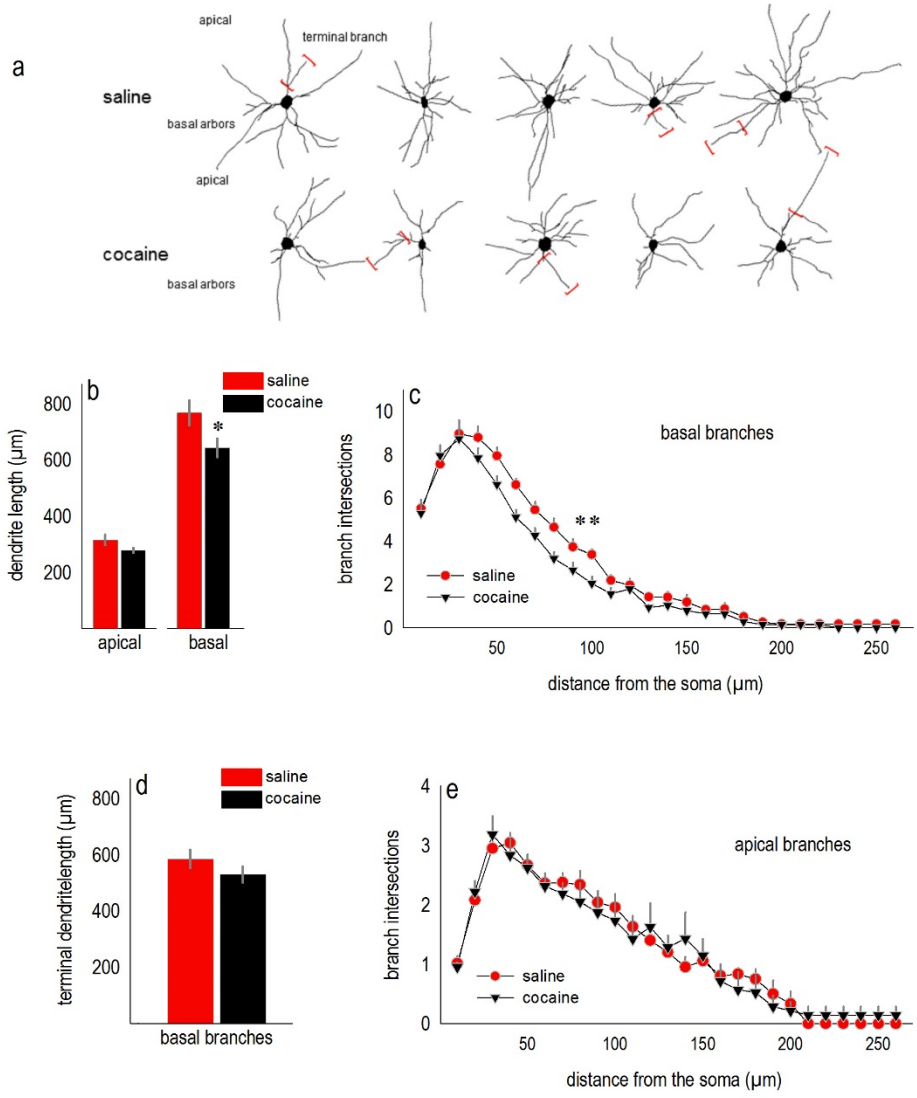

Figure 2.2. Cocaine exposure during adolescence simplifies oPFC dendrite arbors in adulthood. (a) Representative deep-layer oPFC pyramidal neurons are shown. Terminal branches are indicated by red brackets. (b) Total dendrite length in the apical tree was not significantly affected, but basal lengths were reduced. (c) Simultaneously, dendritic arbors on the basal tree were simplified, as indicated by fewer Sholl intersections 40-100 µm from the soma. (d) The total length of basal terminal branches did not significantly differ between groups, consistent with arbor simplification in relatively close proximity to the soma in c. (e) Sholl intersections for

apical arbors did not differ. Means+SEMs. *p<0.05 *vs.* saline, **p<0.05 for 40-100 μm from the soma.

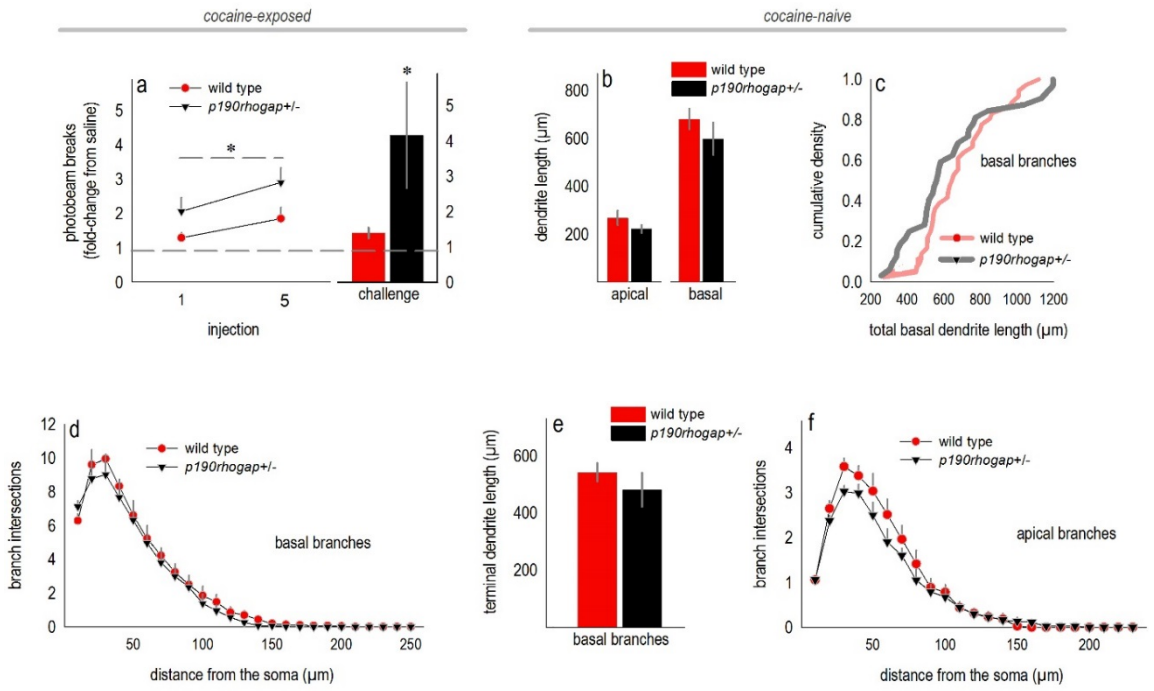

Figure 2.3. oPFC dendrites in drug-naïve *p190rhogap*+/- mice are intact. (a) *p190rhogap*+/- mice are hyper-sensitive to cocaine, as indicated by increased locomotor activity following cocaine injection, relative to saline injection (dashed line at 1), and relative to littermate wild type mice. The bar graph shows that following a washout period, cocaine injection elicited greater photobeam breaks in *p190rhogap*+/- mice than in wild type littermates. (b) We next assessed the morphology of deep-layer oPFC neurons in drug-naïve mice. Length of the apical and basal dendrites did not differ between drug-naïve groups, despite behavioral vulnerabilities to cocaine. (c) Basal dendrites are represented another way – here, total dendrite length for each neuron is represented in a cumulative density function. Again, we identified no significant differences between groups. (d) Sholl analyses of basal arbors also revealed no differences in complexity. (e) Additionally, the length of the terminal arbors did not differ. (f) Sholl analyses indicated that the complexity of apical arbors also did not differ. Means+SEMs. *p<0.05 *vs.* wild type.

CHAPTER 3:

Induction and reversal of adolescent cocaine-induced habits

3.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter describes the effect of adolescent cocaine exposure on dendritic spine density in the oPFC. I will also provide evidence that this structural remodeling is causally associated with drug-induced vulnerabilities to developing habit-based behavior. This work was conceptualized by the dissertation author and Dr. Shannon Gourley. Research was conducted by all of the authors, and the document was written by the dissertation author and Dr. Shannon Gourley. Ms. Courtni Andrews assisted in running some experiments. This chapter is reproduced from DePoy LM, Zimmermann KS, Marvar PJ, Gourley SL (in revision) Induction and reversal of adolescent cocaine-induced habits. *Biol Psych.*

3.2 Abstract

Cocaine use during adolescence increases vulnerability to drug dependence and decreases the likelihood that individuals will seek treatment as adults. Understanding how early-life cocaine exposure influences decision-making processes in adulthood is thus critically important. Adolescent or adult mice were exposed to subchronic cocaine, then behavioral sensitivity to changes in the predictive relationship between actions and their consequences was tested using operant conditioning. Dendritic spines on the principal pyramidal neurons of the orbitofrontal prefrontal cortex (oPFC) were also imaged and enumerated. To determine whether cocaine-induced cellular structural abnormalities in the oPFC were associated with alterations in decision-making strategies, we inhibited the activity of Abl-family and Rho kinases, as well as NR2B-containing NMDA receptors. We also attempted to block the reinstatement of cocaine seeking in cocaine self-administering mice. Adult mice with a history of subchronic cocaine exposure in adolescence develop stimulus-response habits at the expense of goal-directed decision-making strategies and have fewer dendritic spines in deep-layer oPFC. Inhibition of the cytoskeletal regulatory Abl-family kinases in the oPFC recapitulated behavioral deficiencies, while Rho-

kinase inhibition corrected response strategies. Additionally, the NR2B-selective NMDA receptor antagonists ifenprodil and CP-101,606 blocked cocaine-induced habits, and this was dependent on Abl-family signaling in the oPFC. Ifenprodil also mitigated cue-induced reinstatement of cocaine seeking in mice self-administering cocaine. We suggest that adolescent cocaine exposure confers bias towards habit-based decision-making in adulthood via long-term cellular structural modifications in the oPFC. Treatments aimed at mitigating the durable consequences of early-life cocaine may benefit from targeting cytoskeletal regulatory systems.

3.3 Introduction

Adolescent-onset cocaine abuse, relative to adult-onset abuse, increases vulnerability to developing drug dependence and decreases the likelihood that individuals will seek treatment at any point in the lifespan (O'Brien and Anthony, 2005; Kessler et al., 2001). Understanding how early-life cocaine exposure influences decision-making processes in adulthood may inform novel treatment approaches to drug use disorders, yet neurobiological research into the long-term effects of adolescent drug exposure is limited.

"Incubation" typically refers to progressive, time-dependent enhancements in drug craving and sensitivity to drug-related cues and is thought to contribute to the maintenance and persistence of addiction (Grimm et al., 2011; Pickens et al., 2011). Progressive drug-induced modifications in *decision-making* processes may additionally contribute to addiction etiology, however (Everitt and Robbins, 2005; Torregrossa et al., 2011). For example, a bias towards engaging stimulus-response habits, at the expense of goal-oriented behavioral response strategies, is considered an etiological factor in addiction (Everitt and Robbins, 2005; Lucantonio et al., 2012). Accordingly, several independent groups have reported that a history of chronic cocaine or amphetamine exposure induces stimulus-elicited reward-seeking habits in adult rodents (reviewed

DePoy and Gourley, 2015; see Chapter 6). Additionally, drug-induced deficits in reversal tasks appear to incubate in tandem with cocaine-seeking behaviors (Calu et al., 2007).

We aimed to identify whether adolescents are particularly vulnerable to developing cocaine-induced habits, and whether drug-induced habits "incubate." We used action-outcome contingency degradation wherein mice are trained to generate two distinct instrumental responses, then the likelihood that one response will be reinforced is greatly reduced. Rodents that are "goal-directed" will inhibit the response that is no longer likely to be rewarded, evidence of knowledge of the action-outcome relationship (Balleine and O'Doherty, 2010). By contrast, equivalent engagement of both responses reflects a habitual reliance on familiar behavioral patterns. We report that subchronic cocaine exposure in adolescence, but not adulthood, results in a bias towards inflexible habits in adulthood. This phenomenon incubates, in that cocaine-exposed adolescents display intact goal-directed decision-making strategies immediately following cocaine exposure.

The orbitofrontal prefrontal cortex (oPFC) becomes progressively hypoactive during prolonged cocaine withdrawal in humans (Volkow et al., 2011), and lesion studies in rodents indicate that this structure is essential to goal-directed action selection (Gremel and Costa, 2013; Gourley et al., 2013a). We found that adolescent cocaine exposure eliminated dendritic spines in the adult oPFC. Further, inhibition of Abl-family kinases, cytoskeletal regulatory signaling factors highly expressed in the PFC, recapitulated the effects of cocaine. We used these findings as a platform from which to develop intervention strategies to *enhance* new learning regarding the predictive relationships between actions and their outcomes in mice self-administering food or cocaine. Our findings suggest that treatments for individuals first exposed to cocaine as adolescents (by some estimates, 90% of the population (CASA, 2011)) might benefit from targeting learning and memory systems regulated by cytoskeletal signaling factors, *e.g.*, rather than the reinforcing properties of the drug.

3.4 Methods

3.4.1 *Subjects*

Male C57BL/6 mice (Jackson Labs) or transgenic mice expressing *thy1*-derived Yellow Fluorescent Protein (YFP) (Feng et al., 2000) and back-crossed onto a C57BL/6 background were used. Mice were maintained on a 12-hour light cycle (0800 on), experimentally naïve, and provided food and water *ad libitum* unless otherwise indicated. All procedures were approved by the Emory University IACUC.

3.4.2 *Experimenter-administered cocaine, amphetamine*

Cocaine (10 mg/kg), D-amphetamine (3 mg/kg), or saline was administered for 5 consecutive days (Sigma; *i.p.,* 1 ml/100 g). Mice were then left undisturbed until instrumental response training. The timing of injections and training is indicated in table 3.1.

3.4.3 *Instrumental response training*

Adult mice (≥postnatal day (P) 56) were food-restricted to ~90% of their free-feeding body weight. When adolescent (P36) mice were tested, feeding was merely titrated to allow for typical weight gain according to Jackson Laboratory growth trajectories.

Mice were trained to nose poke for food reinforcers (20 mg grain-based Bio-Serv Precision Pellets) in Med-Associates operant conditioning chambers equipped with two nose poke recesses and a separate food magazine on the opposite side of the chambers. Responding was reinforced using a fixed ratio 1 (FR1) schedule of reinforcement in which 30 pellets were available for responding on the 2 distinct nose poke recesses, resulting in 60 pellets/session (5

sessions). The sessions ended at 135 min or when mice acquired all 60 pellets. In our final experiments (those using extended training, CP-101-606 or co-administered ifenprodil and STI-571), these sessions were reduced to 70 min for expediency.

For extended training experiments, mice were reinforced according to a random interval (RI) 30-sec schedule for 2 sessions after FR1 training. Then, an RI-60-sec schedule was used for 3 additional sessions, a schedule commonly used to induce habit behavior in rodents.

Response acquisition curves represent both responses/min, and there were no side preferences throughout.

3.4.4 *Action-outcome contingency degradation*

A modified version of classical action-outcome contingency degradation was used, as in our prior reports (*e.g.,* Gourley et al., 2012b,2013a,b; Swanson et al., 2013), and similar to that of Barker et al. (2013): In a 25-min "non-degraded" session, one nose poke aperture was occluded, and responding on the other aperture was reinforced using an FR1 schedule of reinforcement, as during training. In the 25-min "degraded" session, the opposite aperture was occluded, and reinforcers were delivered into the magazine at a rate matched to each animal's reinforcement rate from the previous session. Under these conditions, only ~7% of pellets are delivered (by chance) within 2 seconds following a response (see Chapter 5). Thus, this response becomes significantly less predictive of reinforcement than the other. These sessions, and which action-outcome contingency was "degraded," were counter-balanced.

The following day, both apertures were available during a 10-min probe test. As is standard practice, the probe test was conducted in extinction. A "goal-directed" response strategy is to preferentially engage the response that is likely to be reinforced, while a habitual response

strategy is to engage both familiar responses equivalently, irrespective of the likelihood of reinforcement (Balleine and O'Doherty, 2010).

In one experiment, mice were then re-trained to nose poke on both apertures using an FR1 schedule of reinforcement for 5 additional sessions, and the action-outcome contingency degradation procedure was repeated.

3.4.5 *Fasudil, ifenprodil, CP-101,606*

Mice were administered fasudil (10 mg/kg, in saline; LC labs); ifenprodil (10 mg/kg, in water; Tocris); CP-101,606 (3 mg/kg, in 20% DMSO+water; Sigma) or the corresponding vehicle (*i.p.*, 1 ml/100g). Doses were determined based on (Swanson et al., 2013; Gourley et al., 2012a; Smith et al., 2011; Higgins et al., 2005). CP-101,606 dosing was also determined based on an attempt to match affinity for the NMDA receptor NR2B subunit to the selected dose of ifenprodil, a less specific antagonist (Rosahl et al., 2006). The timing of injections is described in table 3.1 and throughout the Results section.

3.4.6 *Intracranial infusion, histology*

Treatment groups were assigned by matching mice based on instrumental response rates during training. In initial experiments, mice were anesthetized with ketamine/xylazine immediately following the "degradation" training session. In a subsequent experiment, mice were injected systemically with ifenprodil or vehicle immediately following the "degradation" training session, then anesthetized 30 min later for surgery.

The head was shaved and placed in a stereotaxic frame. The scalp was incised, skin retracted, head leveled, and oPFC coordinates located using Stoelting's digital coordinate system. Two burr holes were drilled, and saline or STI-571 (10 mM in saline; LKT Labs), was infused

into each hemisphere over 2 min in a volume of 0.15 µl at: +2.6 AP/-2.8 DV/±1.2 ML (Gourley et al., 2012a). The wound was then sutured. Mice were allowed to recover for 3 days, then the probe test was conducted.

Mice were then euthanized by rapid decapitation. Brains were submerged in 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 40 µm-thick sections on a microtome held at -15°C. Infusion placements were imaged, and mice with needle tracks that failed to terminate in the oPFC were excluded. A randomly-assigned subset of mice was YFP-expressing, and dendritic spines were imaged in these mice.

3.4.7 *Dendritic spine imaging, quantification*

YFP-expressing mice were euthanized by rapid decapitation. Brains were fixed and sectioned as above. Unobstructed dendritic arbors running parallel to the surface of the section were imaged using a spinning disk confocal (VisiTech International) on a Leica microscope.

Z-stacks were collected with a 100X 1.4NA objective using a 0.1 µm step size, sampling above and below the dendrite. Dendrites were collected from secondary branches within 50-150 µm of the soma in intact mice. All dendrites were 10-40 µm in length. After imaging, we confirmed at 10X that the image was collected from the intended ventrolateral oPFC subregion. Collapsed z-stacks were then analyzed by a single blinded rater using ImageJ. Each protrusion $\leq$4 µm was considered a spine (Peters and Kaiserman-Abramof, 1970), and bifurcated spines were considered singular units.

To generate density values, spine number for each segment was normalized to the length of the segment. Dendritic spine length (the distance from the base of the spine to the tip) and head diameter (the broadest width of the terminal) were also measured.

5-6 dendrites from each mouse were imaged and scored. For density measures, each mouse was considered an independent sample in the case of intact mice, and each dendrite an independent sample in the case of STI-571-infused mice wherein spines were enumerated as a function of their distance from the infusion site. Spine lengths were compared across development in drug-naïve mice (at postnatal day (P) 21, 24, 31, and 60) and between groups of mice exposed to saline or cocaine from P24-28 or P31-35. The average spine length on each dendrite was calculated and compared between time points or groups, as appropriate.

3.4.8 *Cardiovascular function*

Fasudil is used clinically as a vasodilator. Thus, systolic blood pressure and heart rate were measured invasively using radiotelemetry in freely moving mice, as previously described (Marvar et al., 2014). Mice were injected *i.p.* with saline or our behaviorally-active dose of fasudil (10 mg/kg) in a counter-balanced fashion, and heart rate and systolic blood pressure were monitored for 1 hour.

3.4.9 *Intravenous cocaine self-administration*

Naïve mice were trained for 4 sessions to nose poke for food reinforcers using a fixed ratio 1 schedule as in section 3.3.3 (Instrumental Response Training) in Med-Associates conditioning chambers equipped with 2 nose poke recesses. Then, mice were anaesthetized with ketamine/xylazine, and the dorsal and ventral sides were shaved and disinfected. The right jugular vein was exposed by blunt dissection, and a sterile Silastic catheter was placed and then exteriorized posterior to the scapulae. The exit wound was sutured, and mice were housed individually. During the 5-7 day recovery period, catheter patency was ensured by infusing mice daily with 0.05 ml heparinized saline. Subsequently, catheter patency was verified prior to the

first days of cocaine self-administration and extinction training using a 0.03 ml ketamine challenge (15 mg/ml). If mice were insensitive to ketamine at any point, they were excluded.

Cocaine self-administration was tested daily, during which a single nose poke response on the center of 3 nose poke recesses was reinforced with an infusion of cocaine (20 µl; 1.25 mg/ml) delivered through a catheter connected to a swivel holding armored polyethylene tubing. Delivery culminated in extinction of the house light and a 20-sec timeout. Sessions ended when mice self-administered 30 infusions or at 120 min. Responding was considered "stable" when mice self-administered ≥20 mg/kg, with ≥70% responding on the active nose poke, and ≤20% variability in response rate for 2 sequential days, as in our prior investigations (as in Chapter 4 and 5, based on Thomsen and Caine, 2005).

Next, responding was extinguished by placing mice in the conditioning chambers and attaching the catheter tubing, but responding was non-reinforced. Immediately following each session, mice were administered ifenprodil or vehicle. All mice were trained for at least 5 sessions, and mice were considered to have extinguished when response rates on the previously-active aperture were <25% of the last day of training (also as in Chapter 4 and 5, based on Thomsen and Caine, 2005).

Finally, cue-induced reinstatement of cocaine seeking was assessed by presenting mice with a single non-contingent cue (house light off, pump on), followed by contingent cue presentations, but no drug infusion, for 60 min (also as in Chapter 4 and 5, based on Grimm et al., 2002).

3.4.10 *Statistical analyses*

Response rates, dendritic spine densities and lengths, and cardiovascular metrics were compared by *t*-test or ANOVA with repeated measures and Tukey's post-hoc comparisons when

appropriate. Values >2 standard deviations above the mean were considered outliers and excluded. $p<0.05$ was considered significant. Dendritic spine head diameters were compared between groups by Kolmogorov-Smirnov (K-S) tests.

3.5 Results

We aimed to identify whether adolescent mice are vulnerable to developing inflexible reward-seeking habits following cocaine exposure. An overview of experiments is in table 3.1. Mice were first exposed to cocaine from P31-35, early adolescence (Gourley et al., 2012a; Spear 2000; Green and McCormick, 2013). We targeted early adolescence because early-adolescent cocaine use greatly increases risk of later drug dependence (Anthony and Petronis, 1995). Mice were then trained as P56 adults to respond on two distinct nose poke recesses for food reinforcers. All mice acquired the responses, with no differences between groups ($F<1$) (fig.3.1a). A group of mice exposed to cocaine from P24-28 (preadolescence (Spear 2000)) served as a comparison, and throughout, response acquisition curves represent total responses/minute.

An important aspect of goal-directed decision making is selecting between actions that are more, *vs.* less, likely to be reinforced with a desired outcome. Thus, we modified the relationship between one response and the associated outcome by providing the reinforcer associated with that response non-contingently. A goal-oriented response strategy would be to then preferentially generate the remaining response, which remained reinforced (Balleine and O'Doherty, 2010). Saline- and P24-28 cocaine-treated mice indeed preferentially performed the response more likely to be reinforced, a goal-directed response strategy. Mice exposed to cocaine from P31-35, however, failed to differentiate between responses [interaction $F_{(2,26)}=3.8$, $p=0.04$] (fig.3.1b). Thus, cocaine exposure during early adolescence biased response selection towards habit-based strategies in adulthood.

This experiment was next replicated except that mice received cocaine or amphetamine, another psychostimulant, from P31-35. Following instrumental contingency degradation in adulthood, cocaine-exposed mice again responded indiscriminately, habitually, as expected [interaction $F_{(2,24)}$=6.8, p=0.02] (fig.3.1c). Unlike with cocaine, however, amphetamine-exposed mice differentiated between actions that were more, or less, likely to be reinforced.

Some behavioral effects of cocaine become apparent only after a drug washout period. We thus ascertained the effects of cocaine when mice were tested *immediately following* adolescent exposure. There were no effects on response acquisition (F<1) (fig.3.1d), and a main effect of response selection indicated that all mice adopted goal-oriented response strategies following instrumental contingency degradation [$F_{(1,16)}$=8.1, p<0.05] (fig.3.1e).

We next tested the effects of subchronic cocaine exposure in adulthood, exposing mice to cocaine from P56-60. One and 3 weeks later, instrumental response acquisition was unaffected (F<1) (fig.3.1f,h); additionally, all mice adopted goal-oriented response strategies following instrumental contingency degradation [main effects $F_{(1,16)}$=16.4, p<0.001; $F_{(1,18)}$=20.2, p<0.001] (fig.3.1g,i). Thus, subchronic cocaine exposure selectively in adolescence impairs goal-directed action selection, and this effect appears to incubate.

3.5.1 *Dendritic spines remodel following adolescent cocaine exposure*

Dendritic spines on excitatory neurons in deep-layer oPFC were imaged in adulthood (P56) following subchronic cocaine exposure in early adolescence (P31-35) or preadolescence (P24-28). Dendritic spine density was reduced following cocaine exposure during adolescence [$F_{(2,21)}$=4, p<0.05] (fig.3.1j). Further, mice exposed to cocaine during either adolescence or preadolescence had smaller head diameters, suggestive of an immature phenotype (*i.e.*, spines lacking a developed mushroom-like head; both K-S p<0.001) (fig.3.1k).

We have noted over the course of several studies that dendritic spines in deep-layer oPFC lengthen during adolescence, as has also been reported in hippocampal CA1 (Harris et al., 1992). We summarize those findings here [age $F_{(3,77)}=8.1$, $p<0.001$] (fig.3.1l). Dendritic spines collected from adult mice that were exposed to cocaine in adolescence were, however, shorter relative to other groups [$F_{(2,48)}=3.9$, $p<0.03$] (fig.3.1m), an adolescent-like morphology.

3.5.2 *Recapitulation and reversal of cocaine-induced habits*

Do cocaine-induced cellular structural abnormalities contribute to the behavioral effects of cocaine? To address this question, we trained naïve P56 mice to respond for food reinforcers as above, and then "degraded" the relationship between one response and its outcome, also as above. Immediately following the degradation training session, during the presumptive consolidation of new action-outcome learning and memory, we infused into the oPFC saline or STI-571, an Abl-family kinase inhibitor that destabilizes neural structure via disinhibition of the RhoA GTPase (Rho) (fig.3.2a-b) (Koleske 2013). Subsequently, saline-infused mice preferentially generated the response that was most likely to be reinforced. STI-571-infused mice, by contrast, failed to differentiate between the actions that were more, or less, likely to be reinforced and instead relied on familiar habit-based response strategies [interaction $F_{(1,16)}=11.8$, $p<0.01$] (fig.3.2c). Thus, Abl-family kinase signaling appears to be essential to consolidating or retaining new information regarding the predictive relationship between actions and their outcomes.

Dendritic spines were imaged and enumerated following testing. As expected (and like cocaine), STI-571 eliminated dendritic spines on excitatory deep-layer oPFC neurons. Spine elimination was selective to dendrites within 150 μm of the infusion site, within the anatomical boundaries of the ventrolateral oPFC [interaction $F_{(3,94)}=3.2$, $p<0.05$] (fig.3.2d).

We next attempted to *reverse* the effects of early-life cocaine exposure. In this case, we exposed mice to saline or cocaine from P31-35, then trained mice to nose poke as P56 adults as above. We again modified the predictive relationship between a response and its outcome, then administered the Rho-kinase inhibitor fasudil, or the NR2B-selective NMDA receptor (NMDAR) antagonist ifenprodil, immediately following training. Both drugs indirectly *amplify* signaling of Abl-family kinases, in particular Abl2/Arg kinase (fig.3.2e) (Koleske 2013; Lin et al., 2013). Subsequently, control mice preferentially generated the response that was most likely to be reinforced. Mice exposed to cocaine in adolescence did not differentiate between the two responses, responding habitually, also as expected. Both fasudil and ifenprodil *restored goal-directed action selection in cocaine-exposed mice* [interaction $F_{(2,54)}$=4.6, p=0.01] (fig.3.2f). Together, these findings indicate that action-outcome conditioning can be bi-directionally regulated to mimic or block cocaine-induced habits.

Fasudil acts rapidly, with a terminal elimination half-life of <1 hour (Satoh et al., 2001). It is also, at certain doses, a potent vasodilator. To assess cardiovascular function, we administered fasudil to naïve mice bearing indwelling telemeters that measure arterial blood pressure and heart rate. Neither were affected, suggesting that fasudil's actions were not associated with vascular changes (F<1) (fig.3.2g).

3.5.3 *The effects of ifenprodil are Abl-family kinase-dependent*

Ifenprodil has reported protective benefits in animal models of alcohol (Vengeliene et al., 2005), heroin (Shen et al., 2011), and nicotine (Gipson et al., 2013) relapse. Thus, we focused the rest of our studies on this compound. First, we assessed whether the habit-blocking effects of ifenprodil were dependent on activation of Abl-family signaling in the oPFC (fig.3.2h). We exposed mice to cocaine during adolescence, trained them to respond for food reinforcers (all F<1) (fig.3.2i), then decreased the likelihood that one response would be reinforced. As expected,

mice exposed to cocaine in adolescence and then treated with saline in adulthood developed habits, while ifenprodil restored goal-directed response strategies. Simultaneous infusion of the Abl-family kinase inhibitor STI-571 in the oPFC, however, *occluded the effects of ifenprodil*, indicating that the habit-blocking effects of ifenprodil require intact Abl-family signaling in the oPFC [interaction $F_{(1,58)}$=4.3, p=0.04] (fig.3.2j).

Here we utilized a 2x2x2 experimental design, leading to the unexpected discovery that mice exposed to cocaine+STI-571 developed goal-directed response strategies (fig.3.2j). In addition to Abl-family kinases, STI-571 inhibits c-Kit protein kinase and the PDGF receptor (Buchdunger et al., 2000). These off-target actions may contribute to this unexpected blockade of cocaine-induced habits.

In these experiments, ifenprodil was delivered immediately following action-outcome contingency degradation training, during the presumptive consolidation of new learning. Our findings raise the possibility that ifenprodil could also block habits that develop under naturalistic circumstances. To test this possibility, we extensively trained intact mice to induce habits by virtue of prolonged response training ("to be saline" *vs.* "to be ifenprodil" and interaction F<1) (fig.3.2k). Again, ifenprodil was injected immediately following a training session wherein the likelihood of reinforcement was diminished. Subsequently, ifenprodil-treated mice preferentially engaged the response that was most likely to be reinforced, while vehicle-treated mice engaged both responses equally, habitually [interaction $F_{(1,10)}$=10.4, p=0.009] (fig.3.2l). Thus, ifenprodil can block habits induced by either cocaine or extended training.

If ifenprodil is acting on action-outcome memory consolidation/retention, delayed injection should have no effects since consolidation is thought to occur within a narrow time window following a learning event. In a separate group of mice, we delayed ifenprodil treatment for 4 hours following the degradation of the action-outcome relationship ("to be saline" *vs.* "to be ifenprodil" F<1) (fig.3.2m). In this case, all mice displayed habitual response strategies,

suggesting that ifenprodil indeed enhances memory consolidation processes to enable subsequent response flexibility [effect of response $F_{(1,16)}$=1.7, p=0.2; interaction F<1] (fig.3.2n).

3.5.4 *Blockade of cocaine-induced habits with the NMDAR NR2B antagonist CP-101,606*

Ifenprodil is highly active at NMDAR NR2B, but it also has off-target effects. Thus, in a separate experiment, we tested the utility of the more selective NMDAR NR2B antagonist, CP-101,606, in blocking cocaine-induced habits. Again, there were no effects during response training [cocaine $F_{(1,40)}$=2.5, p=0.1; "to be CP-101,606" F<1; interaction F<1] (fig.3.3a). Following action-outcome contingency degradation, control mice preferentially generated the response that was most likely to be reinforced, whereas mice exposed to cocaine in adolescence did not differentiate between the two responses, as expected. Like ifenprodil, CP-101,606 *restored goal-directed action selection in cocaine-exposed mice* [interaction $F_{(1,40)}$=4.8, p<0.05] (fig.3.3b).

Are cocaine-exposed mice incapable of, or delayed in, developing goal-directed response strategies? To address this question, we trained the same mice for 5 additional sessions using an FR1 schedule of reinforcement that would be expected to bias responding towards outcome-sensitive, goal-directed strategies. In the absence of further drug exposure, all groups preferentially generated the response that was most likely to be reinforced following instrumental contingency degradation [response $F_{(1,40)}$=34.1, p<0.001] (fig.3.3c). This finding indicates that adolescent cocaine exposure delays, but does not fully block, the development of goal-directed response strategies.

3.5.5 *Ifenprodil reduces cue-induced reinstatement of cocaine seeking*

Lastly, we assessed whether pairing ifenprodil with action-outcome conditioning could also mitigate relapse-like behavior. Young-adult mice were trained to self-administer cocaine reinforcers via in-dwelling jugular catheters. A main effect of response indicated that mice responded preferentially on the active relative to inactive apertures [$F_{(1,14)}$=9.9, p=0.007], with no differences between mice designated to ultimately receive vehicle *vs.* ifenprodil (F<1) (fig.3.4a). Mice in these groups acquired the same number of cocaine infusions and required the same amount of training to develop stable response patterns [infusions $t_{(14)}$=-0.7, p=0.5; days to acquisition $t_{(13)}$=0.3, p=0.8; to maintenance $t_{(13)}$=-0.7, p=0.5] (fig.3.4b).

We then extinguished responding by withholding the cocaine reinforcer, and immediately following each training session, injected mice with ifenprodil. Ifenprodil had no effects on response extinction (main effect and interaction Fs<1) (fig.3.4c). Following the presentation of cocaine-associated cues, however, ifenprodil-treated mice generated fewer responses [$t_{(13)}$=-2.3, p<0.05] (fig.3.4d). Together, these findings suggest that ifenprodil inhibits both reward-seeking habits and relapse-like behavior by augmenting action-outcome learning and memory.

3.6 Discussion

In cocaine-abusing humans, cocaine craving can progressively increase during periods of drug abstinence, and it can remain high for extended periods (Gawin and Kleber, 1986). Analogous "incubation" phenomena are reported in cocaine-exposed rodents, in which drug-seeking behaviors persist, and are augmented, following cocaine self-administration (Grimm et al., 2011; Pickens et al., 2011). Here we focused on the drug-induced development of so-called "reward-seeking" habits. We found that adolescent mice are more vulnerable to developing cocaine-induced habits than adults. These vulnerabilities appear to incubate, and deep-layer oPFC dendritic spines are eliminated. Habits are *inducible* by blocking Abl-family kinase signaling in the oPFC and *reversible* using strategies that augment Abl2/Arg kinase signaling.

Neuroplasticity in multiple brain regions, including the oPFC, is implicated in the incubation of drug-seeking behaviors (Fanous et al., 2012). Further, a history of psychostimulant exposure eliminates dendrites and dendritic spines on excitatory neurons in this region (see Chapter 2; Crombag et al., 2005; Muhammad and Kolb, 2011a,b; Radley et al., 2015). Here we replicate prior findings that subchronic exposure to cocaine during adolescence reduces oPFC spine counts in adulthood (Gourley et al., 2012a). We further characterize the morphology of remaining spines, revealing that they are shorter, and head diameters are smaller, suggesting an overexpression of stubby-type spines, as opposed to motile thin-type, or mature mushroom-type, spines.

In a previous study using adolescent mice, cocaine was administered during adolescence, then a cocaine "challenge" was given prior to euthanasia in adulthood (Gourley et al., 2012a). This challenge was absent here, and spine densities were nonetheless reduced, indicating that spine elimination can be attributed to early-life drug exposure alone. Remaining spine heads were enlarged in the prior report utilizing a "challenge" injection, but smaller here; thus, previously reported enlargements in head size may be a metaplastic response to cocaine following adolescent exposure. The same population of dendritic spines in mice deficient in the cytoskeletal regulatory protein Abl2/Arg kinase fail to enlarge following cocaine challenge, and these mice rapidly develop cocaine-induced locomotor sensitization and reversal learning deficits (Gourley et al., 2009a,2012a). Together, these findings suggest that cocaine-induced metaplastic head enlargement may be protective, strengthening existing oPFC synapses. This perspective is in line with a general model in which cocaine-induced degeneration in oPFC plasticity contributes to reward-seeking behaviors and failures in impulse control in addiction (see Chapter 6; Torregrossa et al., 2011; Lucantonio et al., 2012; Volkow et al., 2011).

Stimulation of cytoskeletal regulatory factors such as Abl2/Arg kinase can grow or stabilize neural structure by blocking RhoA GTPase signaling, a contractile force on the actin

cytoskeleton (Koleske 2013). Accordingly, STI-571, an anti-cancer drug that inhibits Abl-family kinases (c-Abl and Abl2), increases actomyosin contractility and reduces actin polymerization-based protrusion (Koleske 2013; Buchdunger et al., 2000). oPFC-targeted STI-571 infusion here mimicked the behavioral effects of cocaine, evidence that elimination of oPFC dendritic spines could be a mechanism by which cocaine biases decision-making strategies towards stimulus-elicited habits.

3.6.1 *Blockade of cocaine-induced habits*

Abl2/Arg kinase activity can be amplified by pharmacologically inhibiting the RhoA substrate Rho-kinase, or by silencing NR2B-containing NMDARs, which anchors the Abl2/Arg kinase substrate cortactin in dendritic spines (Lin et al., 2013) (fig.3.2e). We discovered that both Rho-kinase and NMDAR NR2B inhibition, via fasudil and ifenprodil respectively, rescued action selection strategies following cocaine, strengthening new learning regarding the predictive relationship between actions and their outcomes. The more selective NR2B antagonist CP-101,606 recapitulated these effects, suggesting that ifenprodil acts by inhibiting NR2B-containing NMDARs, rather than via off-target influences, *e.g.*, the α1-adrenergic receptor (Chenard et al., 1992). CP-101,606 may have hallucinogenic properties, however (see Nicholson et al., 2007), potentially making it a poor candidate for use as a therapeutic adjunct in treating drug use disorders.

Ifenprodil regulates other cocaine-mediated behaviors. For instance, ifenprodil reduces cocaine-induced psychomotor sensitization and convulsion in adolescent and adult rodents (Witkin and Acri, 1995; Gourley et al., 2012a). It also decreases neural excitability in the ventral tegmental area after cocaine (Yuan et al., 2013). oPFC-targeted infusions of STI-571 blocked the effects of systemic ifenprodil treatment here, indicating that the inhibition of cocaine-induced habits by ifenprodil requires intact Abl-family signaling in the oPFC. Thus, ifenprodil appears to

impact multiple cortico-meso-limbic regions implicated in addiction, protecting against behavioral vulnerabilities to cocaine.

While adolescent cocaine exposure potently regulated outcome-based decision making, subchronic exposure to amphetamine had no effects. In another report, ten days of amphetamine (1 mg/kg) during adolescence (P21-35) increased dendritic spine density in deep-layer medial prefrontal cortex (mPFC) and in the nucleus accumbens (NAc) (Tendilla-Beltrán et al., 2016). However, this effect was observed only in late adolescence not adulthood, unlike with cocaine here. We used a higher dose of amphetamine, 3 mg/kg, which is also within the psychomotor-activating range in mice. Nonetheless, we identified no durable behavioral consequences, reminiscent of the (lack of) spine changes in the report of Tendilla-Beltrán et al., 2016. It is nonetheless possible that other doses of amphetamine could induce habit biases.

Another possible factor is that cocaine inhibits the reuptake of serotonin, while the effects of amphetamine on serotonin reuptake are weak (Ritz and Kuhar, 1989). Further, the suppressive effects of cocaine on adolescent play behavior have been attributed, in part, to actions on serotonin systems (Achterberg et al., 2014). Thus, modifications to serotonin receptor systems may also contribute to behavioral inflexibility following adolescent cocaine exposure. Further experiments are needed to examine a more comprehensive amphetamine dose range and the possible role that serotonergic systems play in the divergent effects of early-life cocaine *vs.* amphetamine on decision-making strategies.

3.6.2 *Ifenprodil reduces cue-induced reinstatement of cocaine seeking*

We extended our investigation to cue-induced reinstatement of cocaine seeking, an animal model of relapse. We used young-adult, rather than adolescent, mice self-administering cocaine. This decision was motivated by evidence that cocaine self-administration in adulthood

decreases dendritic spine density in the mature oPFC (Radley et al., 2015), recapitulating the sustained effects of subchronic cocaine exposure during adolescence (fig.3.1 and Gourley et al., 2012a). Additionally, placing intravenous catheters in adolescent mice poses considerable technical challenges. As in the reinstatement of alcohol-, nicotine-, and heroin-seeking behaviors (Vengeliene et al., 2005; Shen et al., 2011; Gipson et al., 2013), ifenprodil reduced cue-induced cocaine seeking. A key methodological difference between this and prior studies, though, was that we paired ifenprodil with extinction training rather than the reinstatement test in order to model therapeutic intervention strategies in which a pharmacological compound might be paired with behavioral therapy. Mice were tested in the reinstatement test drug-free, and a history of ifenprodil buffered against the reinstatement of cocaine seeking.

Interestingly, ifenprodil did not impact response extinction, even though it reduced reinstatement behavior. This phenomenon is not unprecedented: Extinction training following cocaine self-administration inhibits long-term depression (LTD) in the NAc core (Knackstedt et al., 2010). This plasticity reduces cue-induced reinstatement, but does not impact extinction conditioning itself – as with ifenprodil treatment here. Notably, however, ifenprodil impairs the extinction of conditioned fear (Sotres-Bavon et al., 2007), and infusions into the infralimbic cortex interfere with the extinction of cocaine-conditioned place preference (CPP) (Otis et al., 2014). Ifenprodil and other NR2B-selective antagonists preferentially inactivate NMDARs containing two N2RB subunits, largely sparing heterotrimers containing one NR2A and one NR2B subunit (Paoletti et al., 2013). Accordingly, ifenprodil is primarily active at extrasynaptic sites in the cerebral cortex, a primary source of LTD, such that NR2B-selective NMDAR antagonists can *decrease* LTD (Stocca and Vicini, 1998; Massey et al., 2004; Tovar and Westbrook, 1999). Ifenprodil could therefore have different influences in different brain regions, and also on different forms of conditioning (*e.g.*, stimulus-outcome as in CPP and fear conditioning, *vs.* action-outcome here).

3.6.3 *Regulation of learning and memory systems in combatting cocaine-induced behavioral vulnerabilities*

Throughout the majority of these experiments, infusions and injections were delivered immediately following action-outcome contingency degradation, during a period when mice should be consolidating new information — that a familiar behavior is no longer reinforced with a desired outcome. Delayed ifenprodil injections had no effects, suggesting that ifenprodil enhances this consolidation. In both rats and non-human primates, temporary inactivation of the basolateral amygdala (BLA) during reinforcer devaluation – another commonly-used assay of goal-directed (*vs.* habit-based) decision making – interferes with subsequent goal-directed decision making, while inactivation *during* the probe test has no effects (Wellman et al., 2005; West et al., 2012). Additionally, BLA inactivation attenuates the coding properties of oPFC neurons (Schoenbaum et al., 2003), and BLA projections terminate in deep-layer oPFC (Ghashghei and Barbas, 2002), where spines were eliminated following cocaine (fig.3.1 (Gourley et al., 2012a)). Taken together, these findings suggest that the consolidation of certain forms of outcome-based learning and memory require BLA→oPFC plasticity that then enables the subsequent expression of goal-directed response selection.

We propose a model in which cocaine-induced dendritic spine elimination in the oPFC weakens sensitivity to BLA inputs, weakening goal-directed decision making. Simultaneously, plasticity within a BLA→mPFC circuit may strengthen due to cocaine-induced dendritic spine proliferation in the mPFC (reviewed in Chapter 6, but see Radley et al., 2015). Increased plasticity here would be expected to facilitate the reinstatement of cocaine-reinforced responding following extinction (Stefanik and Kalivas, 2013). These biases would together impair the development of new behavioral patterns in cocaine-abusing individuals seeking to develop and maintain a drug-abstinent lifestyle. Pharmacological inhibition of Rho-kinase (by fasudil) could correct these

deficiencies by structurally stabilizing oPFC synapses and possibly also dendrites (Couch et al., 2010). Ifenprodil may do the same, and it may also interfere with competing inputs from other subcortical structures such as the ventral hippocampus (Flores-Barrera et al., 2014). In addition, ifenprodil could normalize NR2B-mediated signaling in the mPFC, where NR2B is up-regulated by cocaine (Ben-Shahar et al., 2009). A better understanding of the circuit-level consequences of ifenprodil may lead to novel treatment approaches to drug abuse.

Figure and Table Legends

Group	Figure	Age of cocaine exposure	Age of behavioral testing	Post-cocaine injections	Timing of injections	Age of euthanasia & post-mortem procedure
1	1a,b	P24-28	P56			
2	1a,b	P31-35	P56			
3	1c	P31-35, COC or amphetamine	P56			
4	1d,e	P31-35	P36			
5	1f,g	P56-60	P67			
6	1h,i	P56-60	P81			
8	1j,k,m	P24-28	n/a			P56 → image dendritic spines
9	1j,k,m	P31-35	n/a			P56 → image dendritic spines
10	1l	n/a	n/a			P21, 24, 31, and 56 → image dendritic spines
11	2a-d	n/a	P56	intracranial vehicle or STI-571	immediately post-training	~P70 → histology; image dendritic spines
12	2e,f	P31-35	P56	vehicle, ifenprodil or fasudil	immediately post-training	
13	2g	n/a	~P70	vehicle or fasudil	immediately before test	
14	2h-i	P31-35	P56	vehicle or ifenprodil/ intracranial vehicle or STI-571	immediately post-training	~P70 → histology
15	2k,l	n/a	P56	vehicle or ifenprodil	immediately post-training	
16	2m,n	n/a	P56	vehicle or ifenprodil	4 hours post-training; timing adapted from (LaLumiere et al., 2010)	
17	3	P31-35	P56	vehicle or CP-101,606	immediately post-training	
18	4	P63	P56	vehicle or ifenprodil	immediately post-extinction training	

Table 3.1. Summary of experiments. The timing of experimental events is indicated. "P" refers to postnatal day. Injections were systemic unless otherwise noted. "Training" refers to the

conditioning session in which a familiar action-outcome contingency was degraded, except in the final experiment, in which we are referring to extinction training sessions.

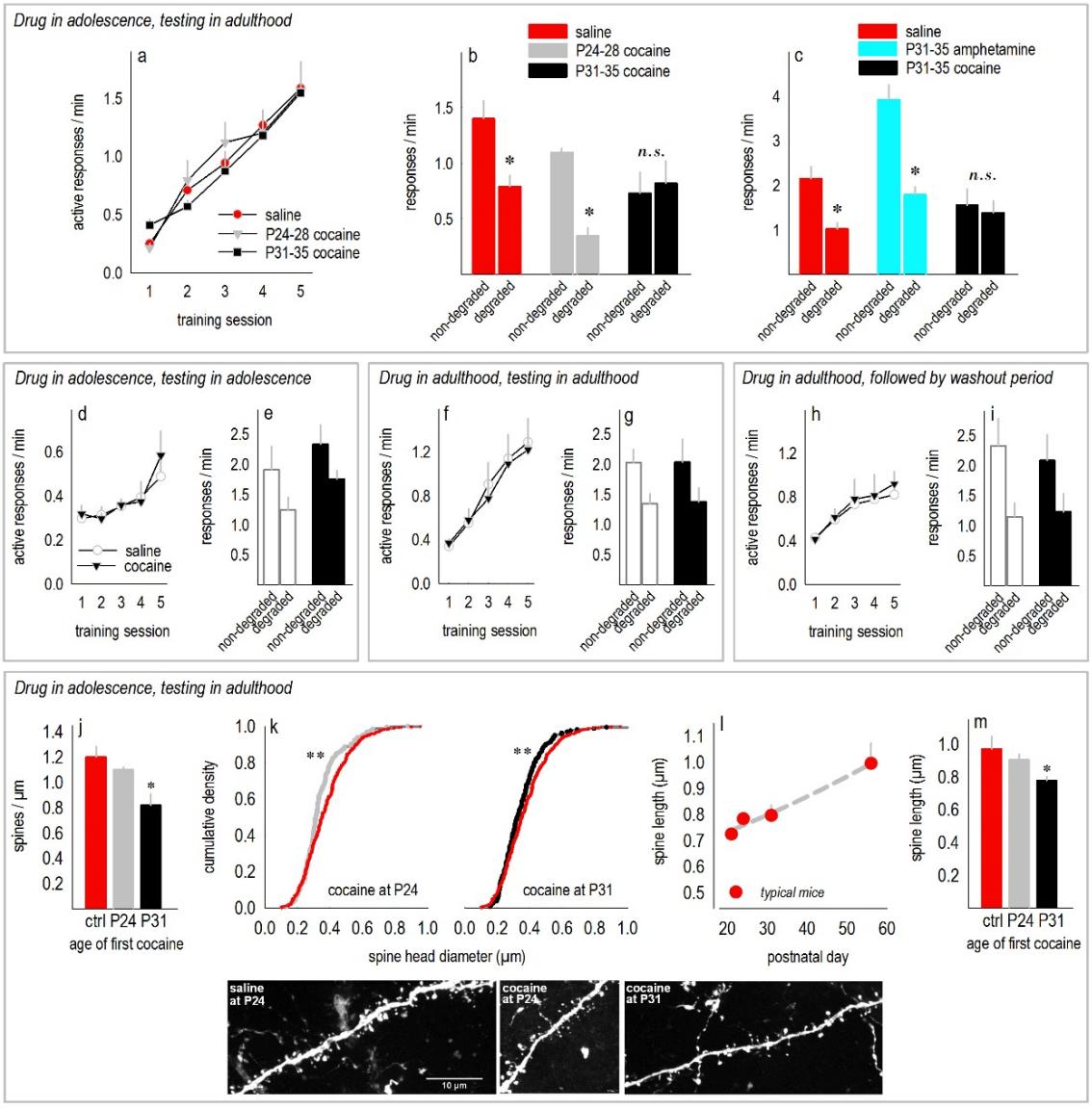

Figure 3.1. Mice exposed to cocaine as adolescents are insensitive to changes in action-outcome contingencies as adults, and oPFC spines are eliminated. (a) We first exposed mice to subchronic cocaine from P24-28 (preadolescence) or P31-35 (early adolescence). We then trained mice to nose poke starting at P56 (young adulthood). Mice acquired food-reinforced nose poke responses. (b) Mice with a history of early-adolescent cocaine exposure were, however, unable to differentiate between responses that were likely, *vs.* unlikely, to be reinforced ("non-degraded" *vs.* "degraded") following instrumental contingency degradation. Instead, these mice

engaged two familiar responses equally, deferring to habit-based strategies. (c) In a separate experiment, we found that unlike cocaine, P31-35 amphetamine did not induce this habit behavior. (d) Separate adolescent mice were exposed to subchronic cocaine, then tested *without* a drug washout period, acquiring the instrumental responses. (e) A main effect of the response choice indicated that these mice could differentiate between the responses that were more, *vs.* less, likely to be reinforced in a goal-directed manner. (f) Similarly, subchronic cocaine exposure in adult mice did not impact instrumental response acquisition, or (g) response selection strategies. (h) A separate group of adult mice was exposed to subchronic cocaine, followed by a prolonged washout period (as in adolescent mice). Instrumental response acquisition was unaffected, and (i) goal-directed action selection was intact (unlike in mice exposed to cocaine in adolescence). (j) Dendritic spines on pyramidal neurons in deep-layer oPFC were imaged in adult mice previously exposed to cocaine from P24-28 or P31-35. Density was reduced following P31-35 exposure. (k) Dendritic spine heads were also smaller in adult mice with a history of cocaine exposure, regardless of age of exposure. (l) Dendritic spines in the oPFC typically lengthen during adolescent development, however (m) mice with a history of adolescent (P31-35) cocaine exposure had shorter spines. Representative dendrites are adjacent. Scale bar=10 μm. Means+SEMs,*$p<0.05$;**$p<0.001$. *n*=8-10/group for behavioral experiments; *n*=5-9/group for morphology experiments.

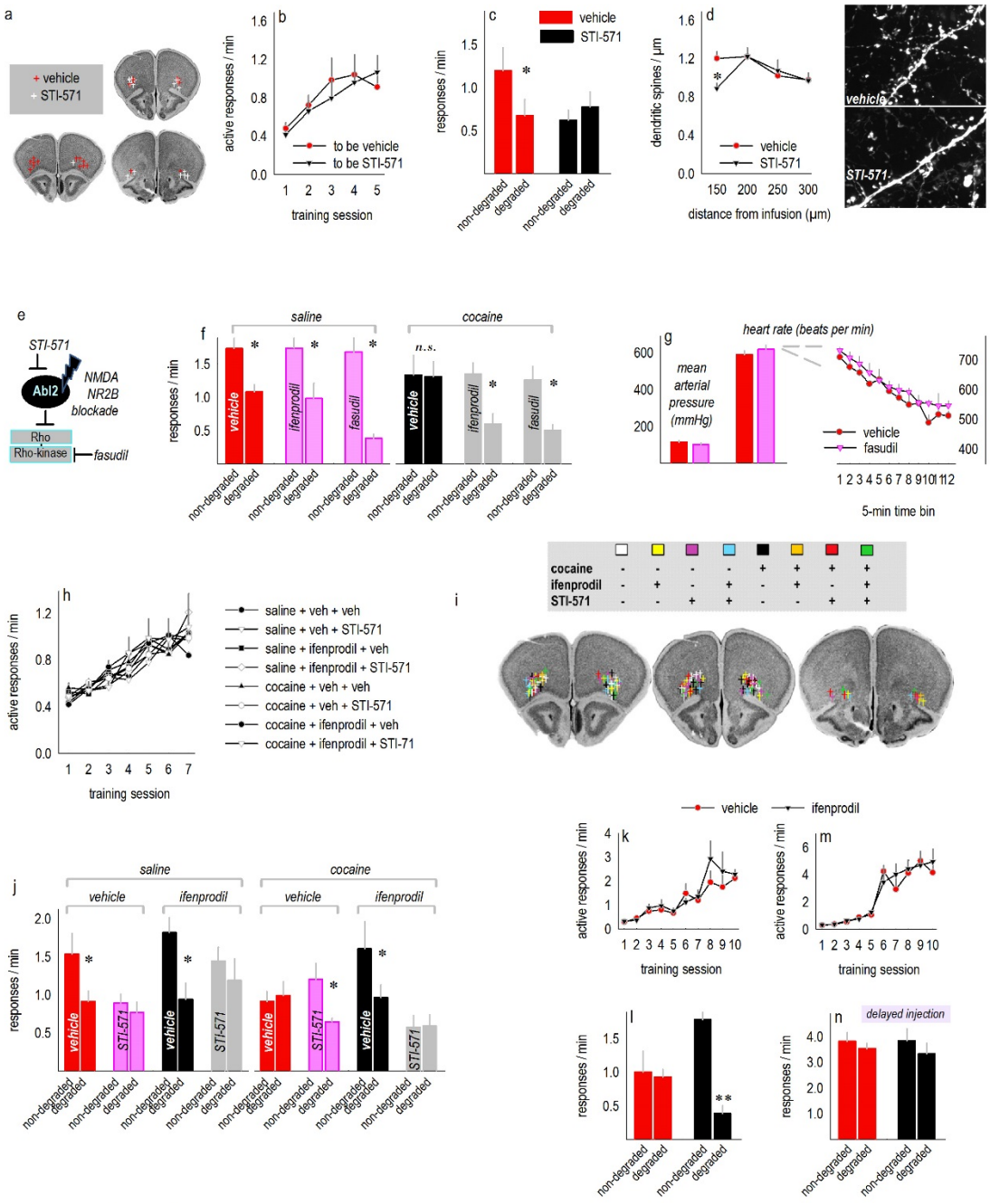

Figure 3.2. Bidirectional regulation of actions and habits. (a) We trained mice to respond for food reinforcers, then infused into the oPFC STI-571, an inhibitor of Abl-family kinases, following instrumental contingency degradation. oPFC infusion sites are represented on images from the Mouse Brain Library (Rosen et al., 2000). (b) Prior to infusion, groups did not differ in response acquisition. (c) STI-571 infusions blocked animals' ability to consolidate a new action-

outcome memory, resulting in an inability to select between responses that are likely, *vs.* unlikely, to be reinforced – a habit-based strategy. (d) Dendritic spines were eliminated within 150 μm of the oPFC infusion site. Representative dendrites are adjacent. (e) Abl2/Arg kinase activity is stimulated by inhibiting NR2B-containing NMDARs, and the inhibition of Rho-kinase also amplifies Abl2/Arg kinase activity. Both events can be induced pharmacologically using ifenprodil or fasudil, respectively. (f) Both ifenprodil and fasudil *rescued* action-outcome memory after adolescent cocaine exposure, as indicated by a goal-directed preference for the response that was more likely, *vs.* less likely, to be reinforced. (g) The effects of fasudil, a potent vasodilator, could not be obviously attributed to actions on cardiovascular systems since this relatively low dose did not impact arterial blood pressure or heart rate. (h) Next, we trained cocaine-exposed mice to respond for food reinforcers, then infused into the oPFC STI-571 in conjunction with systemic ifenprodil treatment. Infusion sites are represented on images from the Mouse Brain Library[65], with (i) no differences between groups in response acquisition. (j) Abl2/Arg kinase blockade occluded the habit-blocking effects of ifenprodil, thus the effects of ifenprodil in blocking cocaine-induced habits require Abl-family signaling in the oPFC. (k) Separate, drug-naïve mice were trained extensively to nose poke for food reinforcers. (l) Control mice developed habit-based response strategies, as expected. Ifenprodil treatment following the degradation of a familiar action-outcome contingency, however, induced goal-directed response selection. (m) We extensively trained another group of mice to respond for food reinforcers. (n) When ifenprodil injection was delayed 4 hours following instrumental contingency degradation, it had no effect, suggesting that NMDAR NR2B blockade enhances the consolidation of action-outcome learning and memory within a 4-hour window. Means+SEMs, *$p<0.05$,**$p<0.001$ following interactions. *n*=5-12/group, except in g (*n*=3/group).

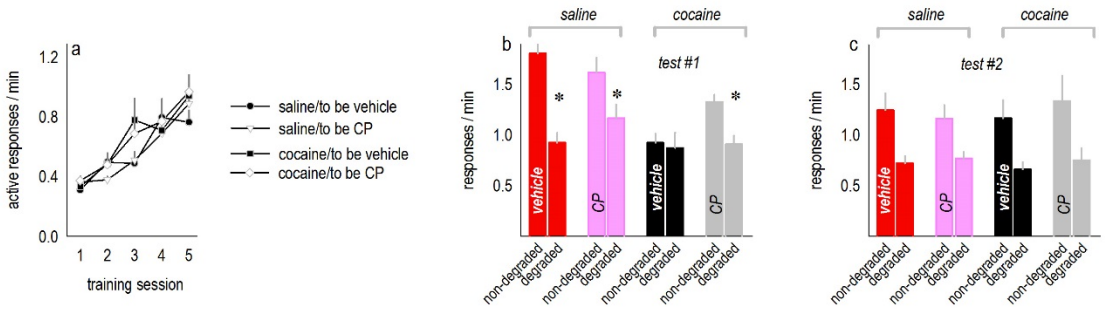

Figure 3.3. The NMDAR NR2B-selective antagonist CP-101,606 occludes cocaine-induced habits. (a) Mice were trained to respond for food reinforcers, with no differences between groups. (b) Following instrumental contingency degradation, mice with a history of cocaine exposure generated habit-based response strategies as expected, not differentiating between responses that were likely, *vs.* unlikely, to be reinforced. CP-101,606 ("CP") blocked these cocaine-induced habits. (c) With additional exposure to instrumental contingency degradation, mice with a history of cocaine exposure were able to express goal-directed response strategies, indicating that cocaine delays, but does not fully block, the development and expression of goal-directed response strategies. Means+SEMs, *p<0.05 following interactions. *n*=8-14/group.

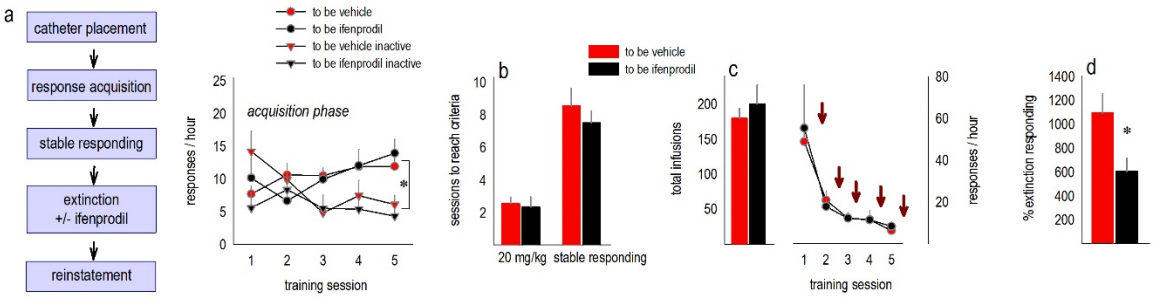

Figure 3.4. Ifenprodil reduces the cue-induced reinstatement of cocaine seeking. (a) Experimental timeline at left. At right, cocaine-reinforced response rates during the response acquisition phase did not differ between mice that would ultimately be treated with vehicle *vs.* ifenprodil. (b) Mice required the same amount of training in order to obtain 20 mg/kg/day (left) and to develop stable response rates (right), regardless of whether they would or would not be ultimately treated with ifenprodil. (c) Further, mice in these two groups acquired the same total number of cocaine infusions (left), and ifenprodil treatment immediately following each extinction training session (arrows) had no effects on response extinction (right). (d) Nonetheless, ifenprodil-treated mice were significantly less likely than vehicle-treated mice to generate cocaine-seeking behaviors when primed with cocaine-associated cues in a reinstatement test. Ifenprodil was not on-board at this time. Means+SEMs, *p<0.05. *n*=7-9/group.

Chapter 4:

Adolescent-onset *Itgb1* knockdown regulates reward-related decision making

4.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter presents evidence that knockdown of *Itgb1*, the gene that encodes β1-integrin, in the oPFC causes stimulus-response habits and increased sensitivity to conditioned stimuli in a developmentally- and sex-selective fashion. The dissertation author contributed to the chapter by designing and conducting the majority of the experiments, analyzing data, and writing the manuscript under the guidance of Dr. Shannon Gourley.

4.2 Abstract

Integrins are heterodimeric cell adhesion receptors activated by extracellular matrix proteins. The β1-integrin is highly expressed at synapses in cerebral cortex and is critical for neuroplasticity. Neuron-selective forebrain-specific knockout of *Itgb1*, encoding β1-integrin, exaggerates cocaine-induced locomotor sensitization and impairs responding in an orbitofrontal prefrontal cortical (oPFC)-dependent reversal task. However, the role of β1-integrin in the oPFC specifically remains unclear. We selectively reduced *Itgb1* in the ventrolateral oPFC and found that *Itgb1* knockdown starting in early adolescence, but not late adolescence, delays the acquisition of food-reinforced instrumental responses and induces biases towards stimulus-response habits at the expense of goal-directed action selection strategies. The introduction of conditioned stimuli "bridging" the action-outcome association eliminated response acquisition differences, suggesting an increased dependence on reward-related stimuli to guide behavioral decision making. Furthermore, *Itgb1* knockdown enhanced Pavlovian fear extinction conditioning and *increased* responding for signaled intravenous cocaine delivery. Together, these findings indicate that β1-integrin tone during an early-adolescent sensitive period is essential for flexible, goal-directed (as opposed to stimulus-elicited) decision making later in life. Interestingly, females were resilient to the behavioral consequences of oPFC-selective *Itgb1* knockdown, potentially due to interactions between β1-integrin and estrogen receptor systems.

4.3 Introduction

Integrins are heterodimeric (α/β) extracellular matrix protein receptors that are widely expressed throughout the mammalian nervous system. β1-integrin is highly expressed in the cortex and hippocampus, and when localized to synapses in the brain, is critical for long-term potentiation (Kramár et al., 2006; Chan et al., 2007). Downstream signaling partners of β1-integrin include cytoskeletal regulatory elements, such as Abl2/Arg kinase, which coordinate actin dynamics (Pinkstaff et al., 1998; Warren et al., 2012; Kerrisk et al., 2013). Actin cycles between monomeric globular (G)-actin and filamentous (F)-actin to regulate the formation, maturation, and morphology of dendrites and dendritic spines. Cytoskeletal regulatory proteins and their actin-binding partners coordinate actin dynamics by regulating the assembly and disassembly of F-actin, processes that can be initiated by β1-integrin signaling.

β1-integrin is critical for dendrite, dendritic spine, and synapse stabilization during postnatal development. For example, β1-integrin inactivation induces dendritic spine loss in the hippocampus (Bourgin et al., 2007). Further, forebrain-selective knockdown of *Itgb1* (encoding β1-integrin) results in dendrite retraction and synapse loss starting in adolescence in the mouse hippocampus (Warren et al., 2012). Both forebrain-specific *Itgb1* knockdown, as well as selective reduction of the β1-integrin substrate Abl2/Arg kinase in the orbitofrontal prefrontal cortex (oPFC) exaggerate cocaine-induced locomotor sensitization and impair responding in an oPFC-dependent reversal task, reminiscent of response patterns in cocaine-exposed humans and non-human primates (Gourley et al., 2009a; Gourley et al., 2012a; Warren et al., 2012; Ersche et al., 2008; Jentsch et al., 2002). Together, these findings suggest that cortical β1-integrin systems might be "protective" in the context of cocaine exposure.

Cocaine also alters integrin receptor systems. For example, hippocampal *LAMB1*, which encodes a subunit of laminin (an integrin ligand), is increased in young men with a history of

binge cocaine use (Mash et al., 2007). In mice, cocaine exposure increases β1-integrin protein levels in the nucleus accumbens (Wiggins et al., 2009). Further, variants in *ITGBL1,* encoding β1 integrin-like protein, are associated with cocaine abuse in humans (Drgon et al., 2010).

Here, we aimed to characterize the behavioral functions of β1-integrin selectively in the oPFC. Using viral-mediated gene silencing, we identify age- and sex-dependent consequences of oPFC *Itgb1* deficiency. In particular, knockdown early, but not late, in adolescence impairs action-outcome conditioning — that is, selecting actions based on their consequences — leading to a deferral to stimulus-elicited habits. These findings reveal an adolescent sensitive period during which β1-integrin tone influences action-outcome decision making later in life. These deficiencies were accompanied by increased responding for signaled cocaine reinforcement (*i.e.,* cocaine reinforcers accompanied by discrete cues), suggesting that cortical *Itgb1* deficiencies could trigger problematic, stimulus-elicited cocaine seeking. Interestingly, females were resilient to *Itgb1* knockdown, which may shed light onto sex differences in β1-integrin function and/or oPFC-dependent decision-making strategies.

4.4 Methods

4.4.1 *Subjects*

Male and female transgenic *Itbg1*^{tm1Efu} mice bred on a mixed strain background (C57BL/6J;129X1/SvJ) were used (Raghavan et al., 2000; Jackson Labs). loxP sites flank exon 3 of *Itgb1*, and Cre-recombinase (Cre) deletes this exon. Mice were maintained on a 12-hour light cycle (0800 on), experimentally naïve, and provided food and water *ad libitum* unless otherwise indicated. All procedures were approved by the Emory University IACUC.

4.4.2 *Intracranial infusion*

Lentiviruses expressed Cre or GFP under the cytomegalovirus (CMV) promoter. Mice were anesthetized with ketamine/dexdomitor at postnatal day (P) 24, 31, or 56, corresponding to pre-adolescence, adolescence, and young adulthood in mice (Spear 2000; Green and McCormick, 2013). Mice were placed in a digitized stereotaxic frame (Stoelting), the head shaved, scalp incised, skin retracted, and head leveled. Two burr holes were drilled, and viral vectors were infused into each hemisphere over 5 min in a volume of 0.5 µl at +2.6 AP/-2.8 DV/±1.2 ML (Gourley et al., 2010). Needles were left in place for ≥5 min prior to withdrawal and suturing. Mice were left undisturbed for 32 days before instrumental response training or intravenous catheter placement.

4.4.3 *Instrumental response training*

Mice were food-restricted to ~90% of their free-feeding body weight. Mice were trained to nose poke for food reinforcers (20 mg, grain-based Bio-Serv Precision Pellets) in Med-Associates operant conditioning chambers equipped with two nose poke recesses and a separate food magazine on the opposite side of the chambers. Responding was reinforced using a fixed ratio 1 (FR1) schedule such that 30 pellets were available for responding on each of 2 distinct nose poke recesses, resulting in ≤ 60 pellets/session. The sessions ended at 70 min or when mice acquired all 60 pellets. Mice were trained for a minimum of 7 sessions or until they acquired all 60 pellets in a 70 min session (maximally 60 sessions).

Response acquisition curves represent both responses/min for the last 7 training sessions, and there were no side preferences throughout.

4.4.4 *Action-outcome contingency degradation*

A modified version of classical action-outcome contingency degradation was used, as in our prior reports (*e.g.*, Gourley et al., 2012b; Gourley et al., 2013a,b; Swanson et al., 2013), and similar to that of Barker et al. (2013): In a 25-min "non-degraded" session, one nose poke aperture was occluded, and responding on the other aperture was reinforced using an FR1 schedule of reinforcement, as during training. In the 25-min "degraded" session, the opposite aperture was occluded, and reinforcers were delivered into the magazine at a rate matched to each animal's reinforcement rate from the previous session. Under these conditions, only ~7% of pellets are delivered (by chance) within 2 seconds following a response (see Chapter 5). Thus, this response becomes significantly less predictive of reinforcement than the other. These sessions, and which action-outcome contingency was "degraded," were counter-balanced.

The following day, both apertures were available during a 10-min probe test. As is standard practice, the probe test was conducted in extinction. Sensitivity to action-outcome relationships is reflected by preferential engagement of the response that is likely to be reinforced. Meanwhile, engaging both familiar responses equivalently, irrespective of the likelihood of reinforcement, reflects a failure in action-outcome conditioning (Balleine and O'Doherty, 2010).

4.4.5 *Stimulus-outcome conditioning*

1) "Bridging cues." Mice were then trained to nose poke for food reinforcers in distinct Med-Associates operant conditioning chambers equipped with three nose poke recesses, and a separate food magazine on the opposite side of the chambers. Responding on the center nose poke recess resulted in the delivery of a 2-sec 2.9 kHz tone, extinction of the house light, presentation of a stimulus light above the magazine, and finally, a pellet. Responding was reinforced according to an FR1 schedule. Sessions ended at 15 min, and mice were trained for 11 sessions. Mice were given *ad libitum* access to food at the end of this test.

2) Auditory fear conditioning and extinction. Mice were tested approximately 1 week following the conclusion of instrumental conditioning. Fear conditioning chambers (Coulbourn) equipped with USB cameras and computer-operated FreezeFrame Software (Actimetrics) were used. Mice were acclimated to the testing room for 1 hour, then habituated to the conditioning chambers for 15 min/day for two consecutive days. Next, mice were placed in the chambers, and after 3 min, they received the first of 5 tone + shock pairings. Each trial consisted of a 30-sec tone (6kHz, 75 dB) co-terminating with a 1 sec, 0.6 mA footshock. The following day, mice were placed in contextually distinct chambers, habituated for 3 min, then presented with 15 30-sec tones over 15 min in the absence of shock. This procedure was repeated over 2 subsequent days. Throughout, the absence of any movement excluding respiration was considered freezing, and was calculated by FreezeFrame software.

4.4.6 *Intravenous cocaine self-administration*

Mice were trained for 4 sessions to nose poke for food reinforcers using an FR1 schedule of reinforcement in Med-Associates conditioning chambers. Thirty pellets were available for responding on 2 distinct nose poke recesses, resulting in a possible 60 pellets per 70 min session. Then, mice were anaesthetized with ketamine/dexdomitor, and the dorsal and ventral sides were shaved and disinfected. The right jugular vein was exposed by blunt dissection, and a sterile Silastic catheter was placed and then exteriorized posterior to the scapulae. The exit wound was sutured, and mice were housed individually. During the 5-7 day recovery period, catheter patency was ensured by infusing mice daily with 0.05 ml heparinized saline. Subsequently, catheter patency was verified prior to the first days of cocaine self-administration and extinction training using a 0.03 ml ketamine challenge (15 mg/ml). If mice were insensitive to ketamine at any point, they were excluded.

Cocaine self-administration was tested daily, during which a single nose poke response on the center of 3 nose poke recesses was reinforced with an infusion of cocaine (20 µl; 1.25 mg/ml) delivered through a catheter connected to a swivel holding armored polyethylene tubing. Delivery culminated in extinction of the house light and a 20-sec timeout. Sessions ended when mice self-administered 30 infusions or at 120 min. Responding was considered "stable" when mice self-administered ≥20 mg/kg, with ≥70% responding on the active nose poke, and ≤20% variability in response rate for 2 sequential days, as in our prior investigations (also as in Chapter 3 and 5, based on Thomsen and Caine, 2005).

Mice were trained for a minimum of 5 sessions, and response acquisition curves represent both active and inactive responses/hour for the first, and last, 5 training sessions.

Next, responding was extinguished by placing mice in the conditioning chambers and attaching the catheter tubing, but responding was non-reinforced. All mice were trained for at least 5 sessions, and mice were considered to have extinguished when response rates on the previously-active aperture were <25% of the last day of training (also as in Chapter 3 and 5, based on Thomsen and Caine, 2005).

Finally, cue-induced reinstatement of cocaine seeking was assessed by presenting mice with a single non-contingent cue (house light off, pump on), followed by contingent cue presentations, but no drug infusion, for 60 min (also as in Chapter 3 and 5, based on Grimm et al., 2002).

4.4.7 *Sucrose consumption*

Passive consumption of a palatable food was assessed approximately 1 week following the test for cue-induced reinstatement of cocaine seeking. Mice were first individually housed with familiar bedding in a quiet room. Then, a 1% (w/v) sucrose solution replaced the drinking

water. Mice were habituated to the sucrose solution for 4 hours, and then the water bottle was removed for 19 hours. Finally, mice were given access to the 1% sucrose solution for 1 hour. Liquid consumption was recorded, and consumption is presented as a percentage of body weight.

4.4.8 *Histology*

After behavioral testing, mice were euthanized by rapid decapitation. Brains were submerged in 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 50 µm-thick sections on a microtome held at -15°C. Infusion sites were verified by immunostaining for Cre as described (DePoy et al., 2013b). Alternatively, GFP was imaged.

4.4.9 *Immunoblotting*

Behaviorally-naïve mice infused with lenti-Cre or lenti-GFP were euthanized approximately 1 month after infusion, matching the start of behavioral testing in our instrumental conditioning experiments. Mice were rapidly decapitated, brains were frozen at -80°C, and then sectioned into 1 mm sections. The oPFC was dissected by a single experimenter using a 1 mm tissue core. Standard electrophoresis techniques were used (as per Swanson et al., 2015): Tissue was homogenized by sonication in lysis buffer (100 µl: 137 mM NaCl, 20 mM tris-Hcl (pH=8), 1% igepal, 10% glycerol, 1:100 Phosphatase Inhibitor Cocktails 2 and 3 (Sigma), 1:1000 Protease Inhibitory Cocktail (Sigma)). Protein concentrations were determined by Bradford colorimetric assay (Pierce), and 50 µg/sample was separated by SDS-PAGE on a 12% gradient tris-glycine gel (Bio-rad). Following transfer to PVDF membrane, membranes were blocked with 5% nonfat milk.

Primary antibodies were anti-CD29 (β1-integrin; BD Transduction Laboratories; Ms; 1:50) and anti-HSP-70 (Santa Cruz; Ms; 1:5000). Immunoreactivity was assessed using a chemiluminescence substrate (Pierce) and measured using a ChemiDoc MP Imaging System (Bio-rad).

4.4.10 *Statistical analyses*

Response rates were compared by ANOVA with repeated measures when appropriate and Tukey's post-hoc comparisons following interactions. Additional metrics (sessions to acquire the food-reinforced responses, sessions to ingest 20 mg/kg cocaine/day, sessions to develop stable response rates, total cocaine infusions, and sucrose consumption) were compared by unpaired t-test. For western blotting experiments, densitometry values were normalized to the corresponding loading control (HSP-70), then normalized to the control sample mean from the same membrane to control for variance between gels, and compared by unpaired *t*-test. Values >2 standard deviations above or below the mean were considered outliers and excluded. $p<0.05$ was considered significant. Trends were considered $0.05<p<.1$.

4.5 Results

To decrease β1-integrin levels, we delivered lentiviruses expressing Cre (or GFP as a control) into the oPFC of 'floxed' *Itgb1* mice. Histological analyses indicated that viral vectors infected primarily the ventrolateral oPFC with some spread into the dorsolateral oPFC and agranular insula (fig.4.1a). Using this protocol, regional β1-integrin levels were reduced to 66% of baseline [$t_{(9)}=1.8$, $p=.05$] (fig.4.1b).

4.5.1 *Itgb1 knockdown impairs action-outcome conditioning and increases the salience of conditioned stimuli*

We aimed to determine whether developmental β1-integrin expression in the oPFC plays a role in reward-related decision-making strategies in adulthood. Lentiviruses can require up to 10 (or more) days to achieve maximal knockdown before behavioral testing can begin (Ahmed et al., 2004; Heldt and Ressler, 2009), and we first aimed to reduce protein levels starting in early adolescence. Therefore, we delivered viral vectors at P24, prior to the onset of adolescence in the rodent, generally considered to be P28 (Spear 2000). Mice were then left undisturbed until P56. Mice were next trained to nose poke on two distinct nose poke apertures for food reinforcers. Throughout, response acquisition curves represent both responses/min. By the final day of training, all mice acquired the responses, however knockdown impaired response acquisition [interaction $F_{(6,108)}$=3.0, p=0.009] (fig.4.2a). Accordingly, knockdown mice required more sessions to reach response criteria (60 pellets within 70 min) [$t_{(18)}$=2.2, p=0.05] (fig.4.2b).

To determine whether this impairment in response acquisition reflects failures in action-outcome conditioning (that is, associating an action with its consequences), we tested mice in an action-outcome contingency degradation task, wherein one response remains reinforced, while the likelihood of reinforcement associated with the other response is greatly reduced. Sensitivity to action-outcome relationships is indicated by preferentially engaging the response that is more likely to be reinforced, while a failure to differentiate between responses indicates a failure in action-outcome conditioning (Balleine and O'Doherty, 2010). Control mice indeed preferentially engaged the response most likely to be reinforced following instrumental contingency degradation, while knockdown mice failed to differentiate between the two responses, evidence of impaired action-outcome conditioning [interaction $F_{(1,18)}$=11.1, p=0.004] (fig.4.2c). An alternative perspective is that *Itgb1* knockdown reduces the hedonic value of the food reinforcer.

However, we identified a trend for *increased,* rather than decreased, passive sucrose intake in an assay of anhedonic-like behavior [$t_{(11)}$=1.8, p=0.09] (fig.4.2d).

If not action-outcome contingencies, what guides responding in *Itgb1*-deficient mice? One possibility is conditioned stimuli associated with the reinforcer (fig.4.2e). To test this hypothesis, we next trained mice in novel chambers to respond on a center, previously unavailable nose poke aperture for a food reinforcer. In this case, auditory and visual stimuli were presented immediately following the response, but one second prior to the reinforcer delivery, "bridging" the action-outcome contingency (fig.4.2f). In this case, mice acquired the response without group differences, suggesting that *Itgb1* knockdown mice were able to utilize discrete cues to optimize responding [interaction F<1; main effect of knockdown $F_{(1,18)}$=1.1, p=0.3] (fig.4.2g).

We next evaluated the effect of *Itgb1* knockdown in Pavlovian fear conditioning and extinction. In this case, a conditioned stimulus is directly linked with an outcome – foot shock during training, and the *absence* of foot shock during extinction testing (see again, fig.4.2e). Thus, Pavlovian fear conditioning and extinction provide another method by which to assess sensitivity to conditioned stimuli. All mice acquired the conditioned freezing response [main effect of day $F_{(5,90)}$=79.2, p<0.001] with no differences between groups during the initial training [interaction $F_{(5,90)}$=1.7, p>0.1; main effect of knockdown $F_{(1,18)}$=2.8, p>0.1] (fig.4.3a). However, mice with *Itgb1* knockdown froze less during extinction training, indicating *enhanced* extinction conditioning [day 1 extinction: interaction $F_{(15,270)}$=2.0, p=0.02; day 2 extinction: interaction $F_{(15,270)}$=2.4, p=0.006; day 3 extinction: main effect of knockdown $F_{(1,18)}$=10.4, p=0.005, interaction F<1] (fig.4.3b-d).

4.5.2 *Implications for illicit drug use*

Together, these patterns suggest that *Itgb1* deficiency increases the salience of conditioned stimuli, while weakening action-outcome conditioning. In the case of extinguishing conditioned fear when a stimulus no longer predicts an aversive outcome, this may be considered advantageous. However, in other circumstances, hyper-sensitivity to conditioned stimuli could be deleterious. One obvious example is drug addiction, which is often conceptualized as drug-seeking behaviors stimulated by conditioned stimuli at the expense of action-outcome associations (Everitt and Robbins, 2016). Thus, we next measured the self-administration of signaled cocaine reinforcers in *Itgb1*-deficient mice. Viral vectors were delivered at P24 as before. To test for potential developmental effects, we infused separate groups of mice at P31, later in adolescence, and at P56, in early adulthood.

One month following viral vector infusion, mice were implanted with indwelling jugular catheters for intravenous cocaine self-administration. Cocaine delivery was accompanied by conditioned stimuli, and during training, *Itgb1*-deficient mice more rapidly differentiated between the active, reinforced response, relative to the inactive response, compared to control mice [interaction $F_{(1,26)}$=8.3, p=0.008] (fig.4.4a), resulting in selective responding for cocaine more rapidly in training than would otherwise be expected. By the end of the cocaine self-administration period, all mice had acquired the cocaine-reinforced response [interaction $F_{(1,26)}$=1.8, p=0.2; main effect of response $F_{(1,26)}$=346.4, p<0.001] (fig.4.4a). We found no differences between groups in the overall number of sessions required to acquire 20 mg/kg/session [$t_{(25)}$=-1.4, p=0.2] (fig.4.4b), nor to reach stable response rates [$t_{(26)}$=-0.64, p=0.5] (fig.4.4b). Furthermore, mice did not differ in the total number of infusions self-administered [$t_{(25)}$=-0.52, p=0.6] (fig.4.4c). We also identified no group differences during extinction conditioning (no conditioned stimuli were present, as is common practice) (main effect of knockdown and interaction Fs<1) (fig.4.4d), nor during cue-induced reinstatement of cocaine

seeking when presented with cocaine-associated stimuli [interaction $F_{(1,26)}$=2.5, p>0.1; main effect of knockdown $F_{(1,26)}$=1.8, p=0.2] (fig.4.4e).

A different pattern was identified in mice with late adolescent-onset knockdown (viral vector infusion at P31). Groups did not differ during the initial response acquisition period [interaction $F_{(1,20)}$=1.2, p=0.3], and a main effect of response indicated that all mice responded preferentially on the active compared to inactive nose poke apertures [$F_{(1,20)}$=122.5, p<0.001] (fig.4.4f). In addition, no differences were identified in during the last 5 days of the response acquisition period [interaction $F_{(1,20)}$=1.6, p=0.2; main effect of response $F_{(1,20)}$=344.1, p<0.001] (fig.4.4f). However, late adolescent-onset *Itgb1* knockdown modestly delayed the stabilization of responding [$t_{(19)}$=-1.9, p=0.07] (fig.4.4g), without impacting the number of sessions to acquire 20 mg/kg [$t_{(19)}$=-1.3, p=0.2] (fig.4.4g) or the total number of infusions taken [$t_{(20)}$=-0.22, p=0.8] (fig.4.4h). No differences were identified during response extinction [main effect of knockdown $F_{(1,20)}$=1.6, p=0.2; interaction F<1] (fig.4.4i), nor during cue-induced reinstatement of cocaine seeking [main effect of knockdown F<1; interaction $F_{(1,20)}$=1.0, p=0.3] (fig.4.4j).

Finally, when the viral vectors were delivered in adulthood, we again identified differential response patterns. First, we found no differences during response acquisition (all F<1) and again, all mice responded preferentially on the active aperture [main effect of response $F_{(1,27)}$=94.5, p<0.001] (fig.4.4k). As expected, no differences were seen during the last 5 days of the response acquisition phase [interaction $F_{(1,27)}$=2.9, p=0.1; main effect of response $F_{(1,27)}$=237.0, p<0.001] (fig.4.4k). We also found no differences in the number of sessions to reach response criteria [sessions to 20 mg/kg $t_{(27)}$=0.38, p=0.7; sessions to stable responding $t_{(27)}$=-0.70, p=0.5] (fig.4.4l) or total number of infusions [$t_{(27)}$=-0.005, p>0.9] (fig.4.4m). Adult-onset knockdown slightly impaired extinction training, increasing responding during the first session [interaction $F_{(4.108)}$=2.8, p=0.03] (fig.4.4n). Knockdown also decreased cue-induced reinstatement of cocaine seeking [interaction $F_{(1.27)}$=6.1, p=0.02] (fig.4.4o).

In sum, *Itgb1* knockdown in the oPFC had developmentally-selective effects, with each age of onset producing different outcomes. Early adolescent-onset knockdown resulted in more rapid selective responding for cocaine, while late adolescent-onset knockdown modestly impaired the stabilization of responding, and adult-onset knockdown decreased the cue-induced reinstatement of cocaine seeking.

4.5.3 *Resiliencies to the effects of oPFC-selective Itgb1 knockdown*

Lastly, we assessed the effect of *Itgb1* deficiency in females, focusing on action-outcome conditioning, as in the beginning of this report. Viral vectors were infused at P24, and despite effects in males, we found no differences in the initial acquisition of the nose poke responses (Fs<1) (fig.4.5a), nor in the number of sessions required to reach response criteria [$t_{(22)}$=0.54, p=0.6] (fig.4.5b). Furthermore, female mice preferentially engaged the response more likely to be reinforced following action-outcome contingency degradation [main effect of response $F_{(1,22)}$=27.2, p<0.001; main effect of knockdown and interaction Fs<1] (fig.4.5c).

Separate groups of female and male mice were also infused with viral vectors later in adolescence, at P31. Again, we identified no differences in response acquisition (Fs<1) (fig.4.5d and g respectively) or the number of sessions required to reach response criteria [females $t_{(25)}$=0.51, p=0.6; males $t_{(11)}$=-0.74, p=0.5] (fig.4.5e and h respectively). All mice preferentially engaged the response that was more, *vs.* less, likely to be reinforced following instrumental contingency degradation [females: main effect of response $F_{(1,25)}$=11.0, p=0.003, main effect of knockdown $F_{(1,25)}$=1.8, p=0.2 , and interaction $F_{(1,25)}$=1.1, p=0.3; males: main effect of response $F_{(1,11)}$=20.3, p<0.001, main effect of knockdown and interaction Fs<1] (Fig.4.5f and i respectively).

Together, our findings suggest that in males, *Itgb1* expression in the ventrolateral oPFC during early adolescence critically organizes reward-related decision making; in its absence, conditioned stimuli take on greater salience and power over response selection strategies.

4.6 Discussion

Integrins are cell adhesion receptors activated by extracellular matrix proteins. In the brain, β1-integrin primarily dimerizes with α3 (Kerrisk et al., 2013), and this dimer is activated by the extracellular protein, laminin. Once activated, β1-integrin stimulates downstream signaling partners, such as Abl2/Arg kinase to facilitate long-term potentiation (Kramár et al., 2006; Chan et al., 2007; Babayan et al., 2012) and coordinate actin dynamics (Warren et al., 2012; Kerrisk et al., 2013). β1-integrin is required for long-term potentiation in the hippocampus, which is considered necessary for the formation of memories, such as object location memory (Kramár et al., 2006; Babayan et al., 2012).

MMP9 is a matrix metalloproteinase that cleaves and degrades the integrin ligand laminin. In the hippocampus, *MMP9* is reduced, and *LAMB1*, encoding a subunit of laminin, is up-regulated in young men with a history of binge cocaine use (Mash et al., 2007). Together, these patterns could result in increased opportunities for the laminin-mediated stabilization of synaptic contacts after cocaine use. In mice, *Itgb1* expression in peripheral blood is stimulated by a combination of social stress and cocaine, which potently induces a preference for a cocaine-associated context (Lo lacono et al., 2016). Through these and other mechanisms, cocaine may strengthen hippocampal synaptic contacts and by extension, drug memory. In a similar vein, repeated cocaine exposure up-regulates β1- and β3-integrin expression in the ventral striatum, and these modifications appear to contribute to the reinstatement of cocaine seeking following drug abstinence (Wiggins et al., 2009,2011).

While hippocampal- and striatal-dependent drug memory and craving are strengthened by repeated cocaine exposure in addiction, inhibitory control over drug seeking is weakened. Thus, one might hypothesize that a *weakening* of integrin systems in the prefrontal cortex could contribute to drug sensitization and the loss of behavioral inhibition in drug use disorders. In humans, variants in *ITGBL1*, encoding β1 integrin-like protein, are associated with cocaine abuse (Drgon et al., 2010). In mice, forebrain-selective knockdown of *Itgb1* exaggerates psychomotor sensitivity to cocaine and impairs responding in reversal conditioning tasks (Warren et al., 2012). Reduction of the β1-integrin substrates Abl2/Arg kinase and p190RhoGAP also exaggerate cocaine-induced locomotor sensitization (Gourley et al., 2009a,2012a), and selective silencing of Abl2/Arg kinase in the oPFC induces reward-seeking habits, a putative factor in the development and maintenance of cocaine abuse (see Chapter 3). Taken together, these findings suggest that β1-integrin systems could regulate vulnerability to cocaine (in the case of naturally-occurring gene variants or fluctuations in gene expression due to stress, for example; Morsink et al., 2006), and additionally, these systems could be *targets* of cocaine — mechanisms by which cocaine strengthens drug memory and craving and weakens inhibitory control systems.

Here we aimed to clarify one piece of this complicated puzzle by determining whether oPFC β1-integrin expression influences an animal's ability to engage in goal-directed response selection strategies (as opposed to stimulus-elicited habits) and propensity to self-administer cocaine. We reveal age- and sex-dependent consequences of *Itgb1* deficiency in the oPFC, demonstrating that developmental β1-integrin silencing could trigger motivated cocaine use in males.

4.6.1 *Impaired action-outcome decision making and increased salience of conditioned stimuli*

Here we first show that β1-integrin is critically important for action-outcome conditioning. Specifically, early adolescent-onset knockdown of *Itgb1* in the oPFC delayed the

acquisition of a food-reinforced instrumental response. This impairment was associated with a failure to later modify response strategies according to action-outcome associative contingencies. Instead, mice deferred to familiar, habit-based behavior. These results cannot simply be explained by *Itgb1* knockdown causing a loss of hedonic value of the food reinforcer because knockdown mice readily consumed a palatable sucrose solution.

Our findings are consistent with prior reports indicating that the oPFC plays a critical role in detecting changes in action-outcome contingencies. For example, lesions (in both mice and non-human primates), temporary inactivation, and elimination of excitatory synapses of the oPFC in mice cause failures in action-outcome conditioning, resulting in a bias towards habit-based response strategies (Gourley et al., 2013a; Jackson et al., 2016; Gremel and Costa, 2013; Swanson et al., 2015). Further, stimulation of inhibitory Gi-coupled Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in the oPFC blocks the retention of action-outcome associations (Zimmermann et al., 2015).

In nonhuman primates, oPFC neurons appear to encode *both* action-outcome and stimulus-outcome contingencies (*e.g.*, Simmons et al., 2008). We thus next sought to determine whether *Itgb1* deficiency affected stimulus-outcome conditioning. To do this, we incorporated conditioned stimuli "bridging" the action-outcome association, and found that the presence of these stimuli eliminated response acquisition deficits in knockdown mice. Next, we used Pavlovian fear conditioning and found that knockdown mice showed enhanced extinction conditioning. This pattern suggests that *Itgb1* knockdown mice were *better* able to use conditioned stimuli to guide responding.

Increased attention to conditioned stimuli could be considered advantageous or detrimental, depending on environmental circumstances. If a stimulus previously associated with threat no longer predicts an aversive outcome (*e.g.,* in fear extinction training), then hyper-sensitivity to conditioned stimuli – and its failure to predict a negative outcome – could be

advantageous, facilitating the extinction of conditioning fear. On the other hand, if a stimulus energizes drug seeking at the expense of other important life activities, hyper-sensitivity to this stimulus could be deleterious. Thus, we next measured the self-administration of signaled cocaine reinforcers and found that early adolescent-onset *Itgb1* deficiency enhanced selectivity in responding for cocaine. Specifically, knockdown mice discriminated between active, reinforced and inactive, non-reinforced responses faster than control mice. This could point to *Itgb1* deficiency as a risk factor for the initiation of problematic cocaine use.

It is important to note that in this report, we used lentiviral vectors, which infect mostly pyramidal neurons, but approximately 1/3 of infected cells would be expected to be glia (Ehrengruber et al., 2001). Since β1-integrin is expressed on glia (Paulus et al., 1993), future studies will need to determine whether glial β1-integrin contributes to these findings.

4.6.2 *Females might be resilient to the loss of β1-integrin*

Here, we report that, unlike males, females with *Itgb1* knockdown in the oPFC starting in early adolescence have no impairments in response acquisition or action-outcome conditioning. Why might females be resilient to the loss of β1-integrin? Women have a larger oPFC than men, lower glucose utilization in this brain region, and a higher density of estrogen and androgen receptors (Goldstein et al., 2001). Estrogen receptor activation stimulates synaptic β1-integrin expression and enhances β1-integrin-dependent synaptic responses and long-term potentiation in the hippocampus (Wang et al., 2016). In our report, we reduce local protein levels by approximately 34%; perhaps the remaining β1-integrin interacts with higher estrogen levels in females to preserve oPFC function.

4.6.3 *Early adolescence is a sensitive period*

β1-integrin silencing in conditional forebrain-selective knockdown mice purportedly has differential effects depending on the time of knockdown (Warren et al., 2012; Chan et al., 2007). Specifically, knockdown starting at approximately embryonic day 11.5 causes deficiencies in hippocampal synapse density that are detectable in adolescence in mice (Warren et al., 2012). Meanwhile, knockdown later in development, starting at approximately P25 has no apparent effects (Chan et al., 2007). Here, we found a similar phenomenon, with only early, but not late, adolescent-onset knockdown in the oPFC impairing action-outcome conditioning. While one cannot equate hippocampal synapse density measures (in Warren et al., 2012 and Chan et al., 2007) with oPFC-dependent behavioral function (here), these findings hint at developmentally- and regionally-selective sensitive periods during which β1-integrin tone has enduring organizational effects on subsequent brain function.

Why might early adolescence be a sensitive period to oPFC *Itgb1* expression? Adolescence is a period of activity-dependent neocortical refinement, consisting of synaptic reorganization and dendritic spine proliferation and then pruning (Bourgeois et al., 1994; Rakic et al., 1994; Anderson et al., 1995). In the mouse oPFC, dendritic spines proliferate until about P31, but pruning does not appear to begin until roughly P39, at least on layer V excitatory pyramidal neurons (Gourley et al., 2012a; Shapiro et al., 2016). Here, our viral vector infusions at P24 would be expected to induce maximal gene knockdown between P34-38, when dendritic spine density is typically very high. In contrast, our infusions at P31 would be expected to induce maximal knockdown between P41-45, during a period of pruning. Silencing β1-integrin during a period when dendritic spine densities should be relatively high (*i.e.,* in early adolescence) could be deleterious, but during a period of pruning (*i.e.,* in late adolescence), less impactful.

This reasoning could also help explain the contrasting, developmental effects of β1-integrin deficiency on intravenous cocaine self-administration. Early, but not late, adolescent-onset *Itgb1* knockdown in the oPFC accelerated discriminative responding for cocaine. In

addition, adult-onset knockdown decreased cue-induced reinstatement of cocaine seeking. Why might adolescent- and adult-onset knockdown have opposing effects? Mice with adolescent-onset knockdown may have experienced some degree of developmental adaptation to the absence of β1-integrin that could have compensated for impaired synaptic plasticity (*e.g.*, long-term potentiation, as discussed above) that would be expected to result from β1-integrin silencing. The mature, adult cortex is much more static by comparison, and exposure to drug-associated cues following cocaine self-administration and extinction can stimulate immediate-early gene expression in the oPFC (Hearing et al., 2008). It may be that rapid integrin-dependent plasticity in the mature oPFC is involved in the reinstatement of cocaine seeking following abstinence. Although this model opposes, or at least complicates, our original hypothesis, it deserves further investigation.

4.7 Conclusions

Prefrontal cortical maturation during adolescent development is thought to open a window of vulnerability to insults. For example, adolescents are particularly vulnerable to drugs of abuse. Adolescence is characterized by both high rates of experimental drug use and heightened vulnerability to the development of substance dependence (Chambers et al., 2003). Adolescent-emergent drug abuse is also associated with increased likelihood of abuse and dependence in adulthood compared to drug abuse that begins later in life (O'Brien and Anthony, 2005). Our findings suggest that loss of β1-integrin during early adolescence, which could occur, for example, as a result of stressor exposure (Morsink et al., 2006), could exaggerate these vulnerabilities by triggering highly motivated drug seeking at the expense of non-drug-related goal-directed activities, specifically in males.

Figure Legends

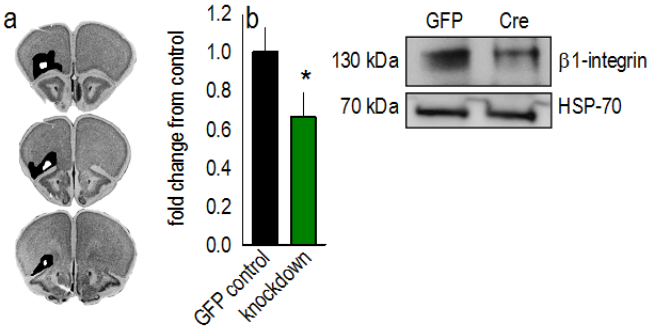

Figure 4.1. Viral-mediated *Itgb1* silencing reduces β1-integrin protein levels. (a) Representations of lentiviral vector infusion sites, with black indicating the largest observed infection, and white the smallest, transposed onto images from the Mouse Brain Library (Rosen et al., 2000). (b) β1-integrin receptor expression was reduced in gross tissue punches collected from the oPFC. Densitometry values were normalized to the corresponding loading control (HSP-70), and representative blots are adjacent. Means+SEMs,*p<0.05,n=5-6.

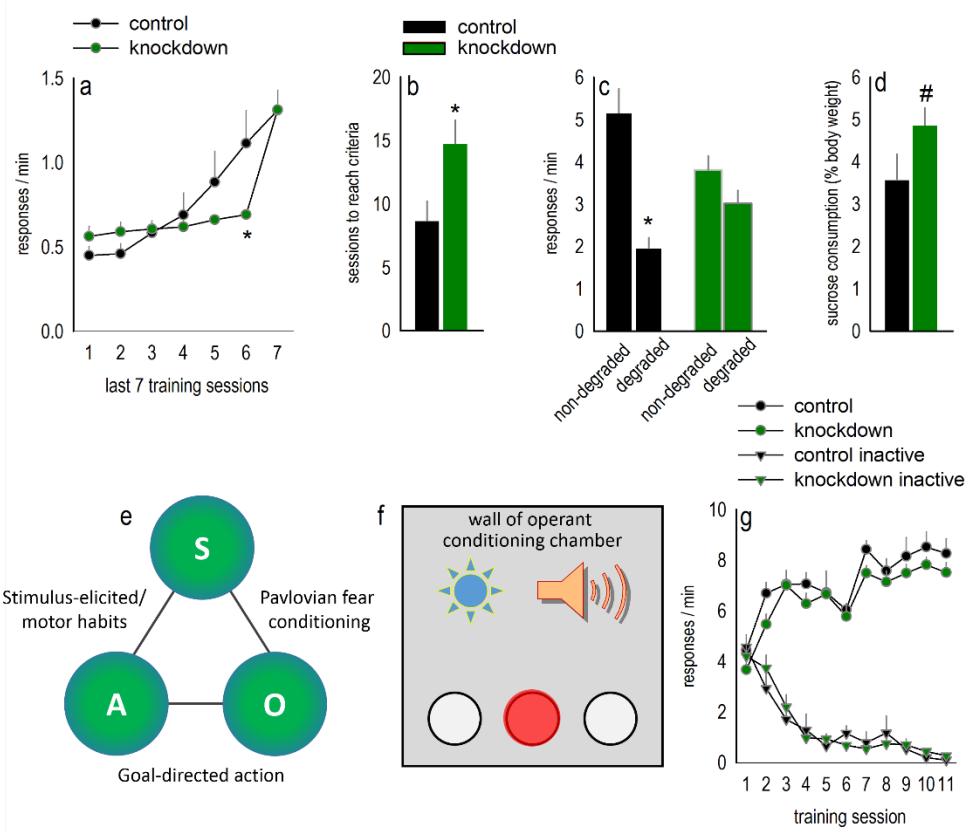

Figure 4.2. Adolescent-onset *Itgb1* knockdown impairs action-outcome conditioning. (a) Mice were trained to respond for food reinforcers on two nose poke apertures. Response rates represent both responses/min. Acquisition of this response was delayed following *Itgb1* knockdown. (b) Accordingly, *Itgb1* knockdown decreased the number of sessions to reach response criteria (60 pellets in 70 min). (c) After training, one nose poke response continued to be reinforced ('non-degraded' contingency), while the likelihood of reinforcement associated with the other nose poke response was greatly reduced ('degraded' contingency). *Itgb1* knockdown impaired action-outcome conditioning, indicated by a failure to differentiate between responses that were likely ('non-degraded'), *vs.* unlikely ('degraded'), to be reinforced. (d) A separate cohort of mice was exposed to a palatable sucrose solution. *Itgb1* knockdown modestly increased sucrose consumption, suggesting that response deficits in a-c cannot be attributed to decreased hedonic valence of a palatable food reinforcer. (e) In addition to action-outcome associations, behavior can be driven by stimulus-outcome and stimulus-action associations. (f) To test whether

Itgb1 deficiency affected stimulus-outcome conditioning, we trained mice in novel chambers to respond on a previously unavailable, center nose poke aperture for a food reinforcer. Here audio and visual conditioned stimuli were added to 'bridge' the action-outcome association. (g) This eliminated group differences in instrumental response acquisition. Means+SEMs,*$p<0.05$, $^{\#}p<0.1$,n=7-13/group.

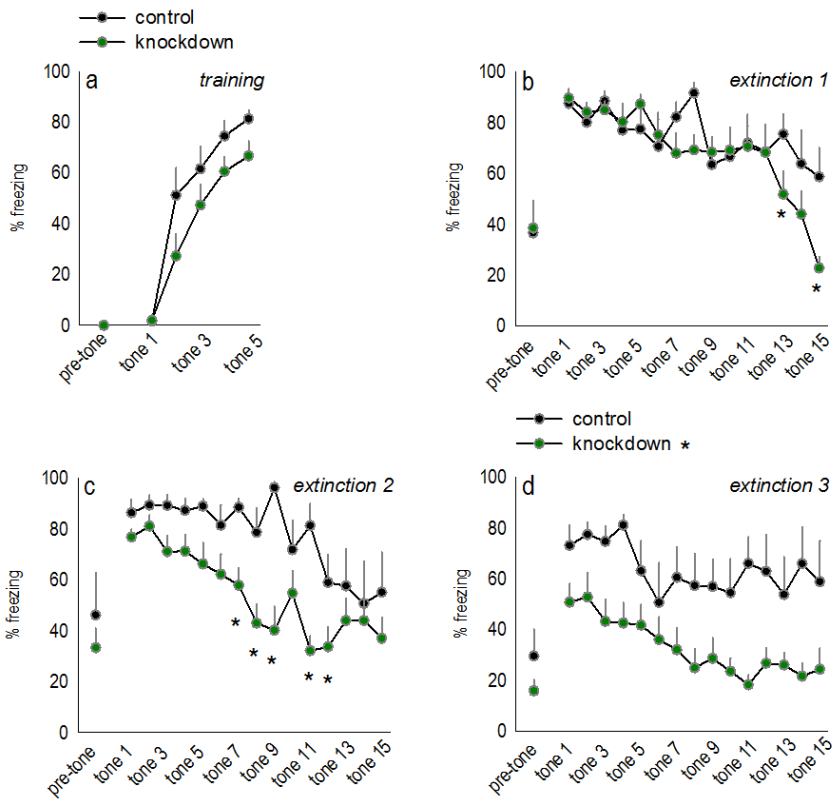

Figure 4.3. Adolescent-onset *Itgb1* knockdown enhances the extinction of conditioned fear. (a) Mice underwent Pavlovian fear conditioning and extinction, a classic example of stimulus-outcome associative conditioning. Conditioned freezing increased during 5 tone-shock pairings. No group differences were observed. (b) During extinction training, *Itgb1* knockdown reduced conditioned freezing, and this effect persisted across 2 subsequent days (c-d). Means+SEMs,*p<0.05,n=7-13/group.

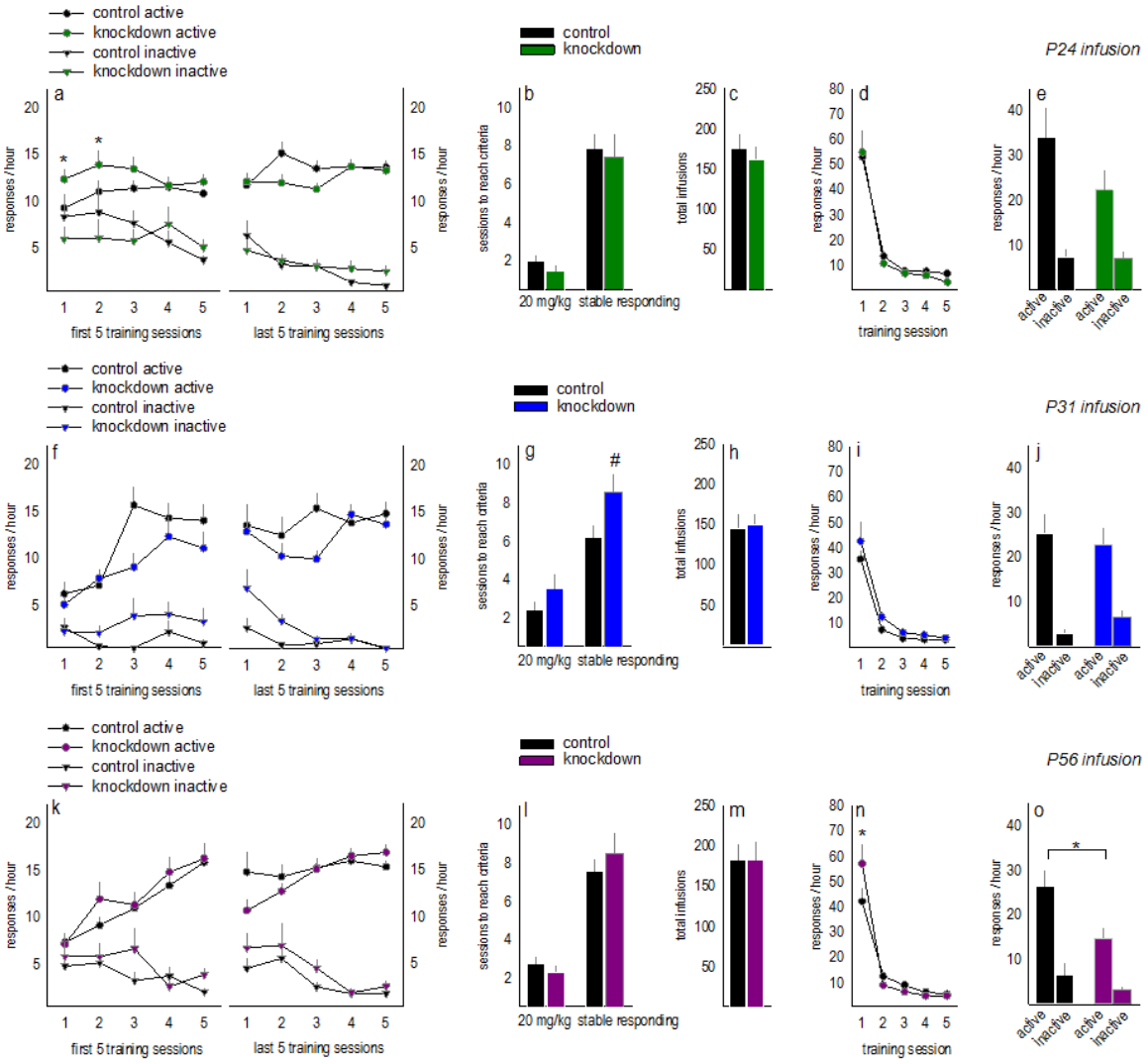

Figure 4.4. Age-dependent effects of *Itgb1* knockdown on intravenous cocaine self-administration. (a) *Itgb1* knockdown starting in early adolescence enhanced responding for a signaled intravenous cocaine delivery. More specifically, knockdown accelerated the selectivity of responding, with knockdown mice discriminating between the active and inactive nose poke apertures on the first 2 days of intravenous cocaine delivery, compared to day 3 for GFP controls. (b) No group differences were identified in the number of sessions required to obtain 20 mg/kg/day (left), nor the number of sessions required to develop stable response rates (right) or (c) total number of cocaine infusions acquired. (d) Responding in extinction was intact, and (e) all mice demonstrated cue-induced reinstatement of cocaine seeking. (f) A separate group of mice

with late adolescent-onset *Itgb1* knockdown was trained to self-administer cocaine, with no group differences. (g) Mice did not significantly differ in the number of sessions required to obtain 20 mg/kg/day (left), however, knockdown modestly increased the number of sessions required to develop stable response rates (right). (h) Nevertheless, knockdown mice did not differ in the total number of cocaine infusions received compared to GFP-expressing controls. (i) Responding in extinction was intact, and (j) all mice demonstrated cue-induced reinstatement of cocaine seeking. (k) Adult-onset *Itgb1* knockdown had no effect on the acquisition of intravenous cocaine self-administration. (l) No group differences were seen in the number of sessions required to obtain 20 mg/kg/day (left), sessions to develop stable response rates (right) or (m) total number of cocaine infusions. (n) Responding in extinction was slightly elevated in adult-onset *Itgb1* knockdown. (o) Adult-onset *Itgb1* knockdown also reduced cue-induced reinstatement of cocaine seeking. Means+SEMs,*$p<0.05$, $^{\#}p<0.1$,$n=10$-15/group.

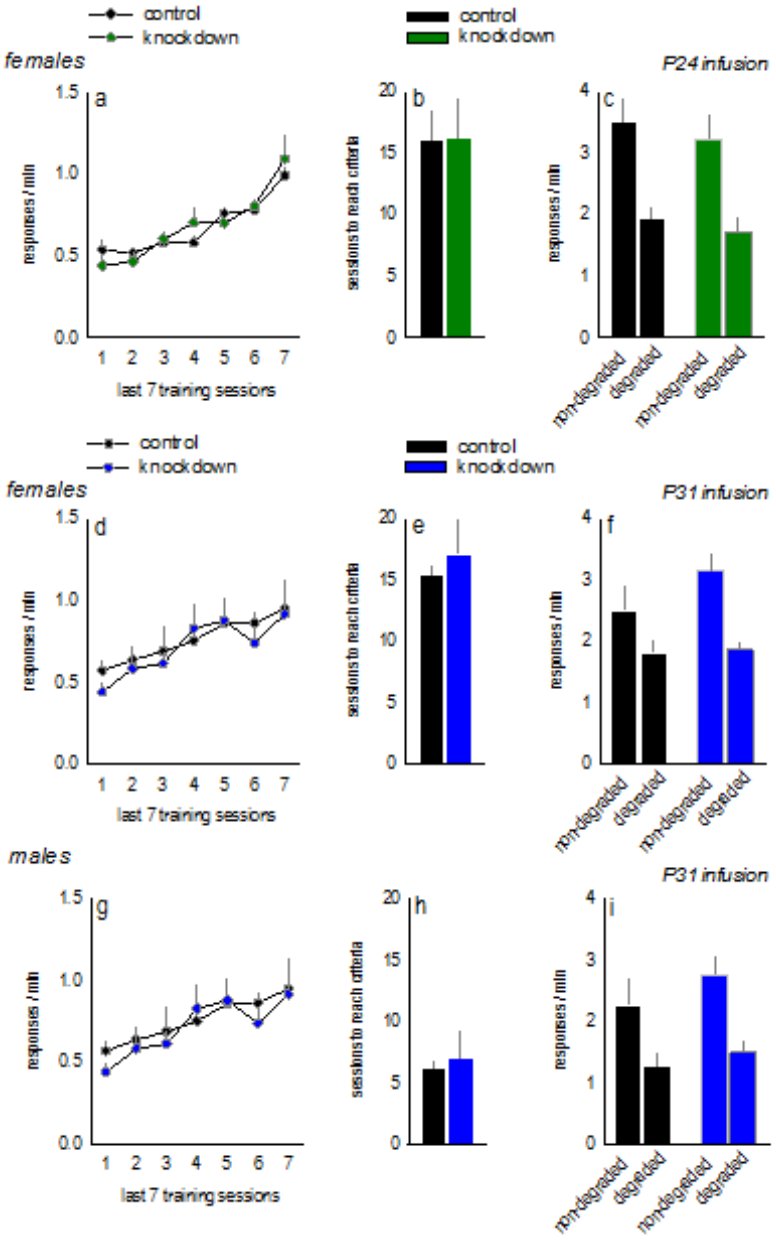

Figure 4.5. Sex- and age-dependent resiliencies to *Itgb1* knockdown. (a) Female mice with early adolescent-onset *Itgb1* knockdown were trained to respond for food reinforcers on two nose poke apertures, with no differences between groups. Response rates represent both responses/min. (b) Early adolescent-onset *Itgb1* knockdown had no effect on the number of sessions needed to reach response criteria in females. (c) After training, one nose poke response continued to be

reinforced (‘non-degraded’ contingency), while the likelihood of reinforcement associated with the other nose poke response was greatly reduced (‘degraded’ contingency). All mice differentiated between responses that were likely (‘non-degraded’), *vs.* unlikely (‘degraded’), to be reinforced, indicating that goal-directed action selection was intact. (d) A separate group of female mice, with late adolescent-onset *Itgb1* knockdown, was trained to respond for food reinforcers. Instrumental response acquisition, (e) the number of sessions to reach response criteria, and (f) response selection strategies were unaffected. (g) Male mice with late adolescent-onset *Itgb1* knockdown were also trained to respond for food reinforcers. Similar to females, response acquisition, (h) sessions to reach response criteria, and (i) goal-directed action selection were intact. Means+SEMs,*$p<0.05$,n=6-7/group for males and 11-16/group for females.

CHAPTER 5:

A snapshot into the medial prefrontal cortex (mPFC): Adolescent-onset $GABA_A\alpha1$ silencing regulates reward-related decision making

5.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter demonstrates that developmental knockdown of *Gabra1*, encoding the $GABA_A\alpha1$ receptor subunit, impairs action-outcome associative learning, and these failures are associated with the delayed acquisition of a cocaine-reinforced response in cocaine self-administering mice. This work was conceptualized Dr. Shannon Gourley, and the research was conducted by all of the authors. Dr. Kerry Ressler provided the transgenic mice used here, and Dr. Ressler and Ms. Elizabeth Hinton provided valuable feedback. The document was written by Ms. Laura Butkovich, the dissertation author, and Dr. Shannon Gourley. This chapter is reproduced with minor edits from Butkovich LM*, DePoy LM*, Allen AG, Shapiro LP, Swanson AM, Gourley SL (2015) Adolescent-onset $GABA_A\alpha1$silencing regulated reward-related decision making. *Eur J Neurosci* 42:2114-2121.

*equal contribution

5.2 Abstract

The $GABA_A$ receptor mediates fast, inhibitory signaling, and cortical expression of the α1 subunit increases during postnatal development. Certain pathological stimuli such as stressors or prenatal cocaine exposure can interfere with this process, but causal relationships between $GABA_A\alpha1$ deficiency and complex behavioral outcomes remain unconfirmed. We chronically reduced $GABA_A\alpha1$ expression selectively in the medial prefrontal cortex (mPFC; prelimbic subregion) of mice using viral-mediated gene silencing of *Gabra1*. Adolescent-onset *Gabra1* knockdown delayed the acquisition of a cocaine-reinforced instrumental response but spared cocaine seeking in extinction and in a cue-induced reinstatement procedure. To determine whether response acquisition deficits could be associated with impairments in action-outcome associative learning and memory, we next assessed behavioral sensitivity to instrumental

contingency degradation. In this case, the predictive relationship between familiar actions and their outcomes is violated. Adolescent-onset knockdown, though not adult-onset knockdown, delayed the expression of goal-directed response strategies in this task, resulting instead in inflexible habit-like modes of response. Thus, the maturation of mPFC $GABA_A\alpha1$ systems during adolescence appears necessary for goal-directed reward-related decision making in adulthood. These findings are discussed in light of evidence that prolonged *Gabra1* deficiency may impair synaptic plasticity.

5.3 Introduction

The gamma-aminobutyric acid A ($GABA_A$) receptor family is made up of ligand-gated chloride ion channels, which mediate fast inhibitory neurotransmission. These pentameric receptors may be composed of any combination of 19 known subunits. The most common, α1, is found in approximately half of $GABA_A$ receptors in the central nervous system, including throughout the cerebral cortex (Olsen and Sieghart, 2009).

During postnatal development, prefrontal cortical $GABA_A\alpha1$ expression levels progressively increase (Fritschy et al., 1994; Hashimoto et al., 2009; Duncan et al., 2010; Datta et al., 2014), and exposure to stressors and drugs of abuse can impinge upon this process. For example, prenatal cocaine exposure decreases postnatal $GABA_A\alpha1$ expression, with impairments first detectable in close proximity to the onset of adolescence (Lu et al., 2009; Huang et al., 2011). As adults, rodents with a history of prenatal cocaine exposure develop anxiety-like behavior and blunted behavioral sensitization to psychostimulants (Crozatier et al., 2003; Lu et al., 2009; Salas-Ramirez et al., 2010; Huang et al., 2011; Wang et al., 2013). A key question, however, is whether adolescent-emergent $GABA_A\alpha1$ deficiency (due to cocaine or other insults) *causally* regulates behavioral outcomes pertinent to human psychopathology. Alternatively, we may ask: What is the role of *typical* $GABA_A\alpha1$ expression during adolescent development?

Here we reduced $GABA_A\alpha1$ expression in the medial prefrontal cortex (mPFC), prelimbic subregion, using viral-mediated gene silencing of *Gabra1*. Viral vectors were initially delivered on postnatal day (P) 31, corresponding to early adolescence in rodents (Spear 2000; Green and McCormick, 2013). Adolescent-onset *Gabra1* knockdown delayed the acquisition of a cocaine-reinforced instrumental response in adulthood, but spared response extinction and cue-induced reinstatement of cocaine seeking, suggesting that healthy mPFC $GABA_A\alpha1$ tone may be essential to developing goal-directed reward-seeking behaviors. To test this perspective, we assessed whether adolescent-onset *Gabra1* knockdown impairs the ability of mice to select responses according to action-outcome contingencies – that is, to select behaviors based on the likelihood that they will be reinforced with a particular outcome. To do so, we used a modified form of classical instrumental contingency degradation in which mice were trained to generate two unique instrumental responses, then the likelihood that one response would be reinforced was greatly reduced. Typical mice preferentially engaged the other response, the one most likely to result in a food reinforcer. Adolescent-onset knockdown, however, delayed action-outcome conditioning, biasing response patterns towards inflexible habit-like strategies that were insensitive to action-outcome contingencies. Interestingly, mice with adult-onset knockdown were largely unaffected in the same task, providing evidence that $GABA_A\alpha1$-dependent maturation of mPFC systems during adolescence optimizes the performance of goal-directed action selection in adulthood.

5.4 Methods

5.4.1 *Subjects*

Transgenic *Gabra1-tm1Geh* mice back-crossed onto a C57BL/6 background were used (see Vicini et al., 2001; Heldt and Ressler, 2010; Jackson Labs). loxP sites flank the α1 exon encoding an essential transmembrane domain, and Cre recombinase (Cre) deletes this domain.

Mice were fed *ad libitum* except during food-reinforced instrumental conditioning when mice were food-restricted and maintained at ~93% of their original body weight to motivate responding. Males were used throughout. All procedures were Emory University IACUC-approved.

5.4.2 *Intracranial surgery*

Cre-expressing lentiviruses were generated as described; intracranial delivery results in reduced $GABA_A\alpha1$ expression within at least 2 weeks (Swanson et al., 2015). Mice were anaesthetized with ketamine/xylazine and placed in a digitized stereotaxic frame (Stoelting Co.,) at P31 or 56, corresponding to early adolescence and early adulthood, respectively (Spear 2000; Green and McCormick, 2013). The scalp was incised, skin retracted, bregma and lambda identified, the head leveled, and coordinates located. Viral vectors expressing Cre or Green Fluorescent Protein (GFP) under the cytomegalovirus (CMV) promoter were infused over 5 min in a volume of 0.5 µl at AP+2.0, DV-2.5, ML+/-0.1 (Gourley et al., 2010). Needles were left in place for 5 min prior to withdrawal and suturing.

5.4.3 *Instrumental response training*

Mice were tested using illuminated Med-Associates conditioning chambers with 2 or 3 nose poke recesses and a separate magazine.

25 days after intracranial surgery, all mice were trained to nose poke for food reinforcers (20 mg Bioserv precision pellets) using a fixed ratio 1 (FR1) schedule of reinforcement; 30 reinforcers were available for responding on each of 2 distinct nose poke recesses, resulting in 60 reinforcers/session. 5-7 daily response acquisition sessions were conducted, as shown, and response rates represent responses on both recesses. We identified no side preferences

throughout. Next, mice were used in either cocaine self-administration or instrumental contingency degradation studies.

5.4.4 *Action-outcome contingency degradation*

Following food-reinforced nose poke training, mice were tested using a modified form of classical instrumental contingency degradation to assess whether they were sensitive to the predictive relationship between a response and the associated outcome. As previously described (Gourley et al., 2012b,2013a,b; Swanson et al., 2013,2015; Hinton et al., 2014), on one day, one of the two nose poke apertures was occluded and pellet delivery was contingent upon responses on the available aperture. Specifically, responding on this aperture was reinforced using a variable ratio 2 (VR2) schedule of reinforcement for 25 min. During a separate session, only the opposite aperture was made available. Here, pellets were delivered independent of animals' interactions with this remaining aperture for 25 min, thus violating, or "degrading," the predictive relationship between nose poking on that particular aperture and pellet delivery. The pellet delivery rate was matched to each animal's mean reinforcement rate from the previous session (also as in the method of Barker et al., 2013). Thus, the action-outcome relationship associated with each response changed relative to the initial nose poke training period, and responding on one aperture became significantly less predictive of pellet delivery than the other. The order of these sessions and location of the "degraded aperture" were counter-balanced.

The following day, both apertures were available for 10 min during a probe test; responding was not reinforced. Preferential engagement of the highly-reinforced response during this probe test is considered "goal-directed," while non-selective responding reflects a failure in action-outcome conditioning (Balleine and O'Doherty, 2010).

Mice that had had surgery during adolescence then received 2 additional nose poke

training sessions, then the contingency degradation procedure was repeated.

5.4.5 *Intravenous cocaine self-administration*

Following food-reinforced nose poke training (described immediately above), mice were implanted with in-dwelling jugular catheters. Mice were anaesthetized with ketamine/xylazine, and the dorsal and ventral sides were shaved and disinfected. The right jugular vein was exposed by blunt dissection, and a sterile Silastic catheter was placed and then exteriorized posterior to the scapulae. The entrance and exit wounds were sutured, and mice were housed individually. During the 5-7 day recovery period, catheter patency was ensured by infusing mice daily with 0.05 ml heparinized saline. Subsequently, catheter patency was tested immediately prior to cocaine self-administration and then again prior to extinction training using a 0.03 ml ketamine challenge (15 mg/ml). If mice were insensitive to ketamine at any point, defined by a failure to lose muscle tone within 10 seconds of infusion, they were excluded.

Then, following the 5-7 day recovery period, cocaine self-administration was tested in contextually distinct conditioning chambers. Mice were tested daily, during which a single nose poke response on the center of 3 nose poke recesses was reinforced with an infusion of cocaine (20 µl; 1.25 mg/ml; Sigma) delivered through a catheter connected to a swivel holding armored polyethylene tubing. Delivery culminated in extinction of the house light and a 20-sec timeout. Sessions ended when mice self-administered 30 infusions or at 120 min. Responding was considered stable when mice self-administered $\geq$20 mg/kg/day, with $\geq$70% responding on the active nose poke, and $\leq$20% variability in response rate for 2 sequential days (also as in Chapter 3 and 4, based on Thomsen and Caine, 2005).

After mice reached these criteria, responding was extinguished by placing mice in the conditioning chambers and attaching the catheter tubing, but responding was non-reinforced.

Mice were considered to have extinguished when response rates on the previously-active aperture were <25% of the last day of training (Kruzich, 2007). All mice were trained for at least 5 days; sessions 1-5 for all mice are shown in the associated figure (also as in Chapter 3 and 4, based on Thomsen and Caine, 2005).

Finally, cue-induced reinstatement of cocaine seeking was assessed by presenting mice with a single non-contingent cue (house light off, pump on), followed by contingent cue presentations, but no drug infusion, for 60 min (also as in Chapter 3 and 4, based on Grimm et al., 2002).

This experiment included 5 intact mice, *i.e.*, mice that did not undergo intracranial surgery. Throughout, GFP-expressing control mice and intact mice did not differ, and their data are combined for statistical and graphical purposes. A total of 18 mice was tested, however 4 control mice died unexpectedly between the initial training and extinction phases.

5.4.6 *Histology*

After behavioral testing, mice were euthanized, and brains were fixed by submersion in chilled 4% paraformaldehyde for 48 hours, then chilled 30% w/v sucrose. Brains were sectioned into 40 μm sections at -15ºC. Infusion sites in knockdown mice were verified by immunostaining for Cre as described (DePoy et al., 2013b). Alternatively, GFP was imaged.

5.4.7 *Immunoblotting*

Separate mice infused with lenti-Cre or lenti-GFP as adults were euthanized approximately 3 months after infusion. Brains were frozen at -80ºC to verify that $GABA_A\alpha1$ protein expression was reduced following lenti-Cre infusion. Standard tissue-punch dissection and electrophoresis techniques were used (as per Swanson et al., 2015). Primary antibodies were

anti-$GABA_A\alpha1$ (Millipore; Rb; 1:500) and anti-HSP-70 (Santa Cruz; Ms; 1:5000), and samples were analyzed in duplicate.

5.4.8 *Statistical Analyses*

Data were analyzed by SPSS. Response rates were compared by ANOVA with repeated measures when appropriate. Following interactions, post-hoc Tukey's comparisons were applied, and results are indicated graphically. For cocaine self-administration experiments, additional metrics (sessions required to ingest 20 mg/kg/day, sessions required to develop stable response rates, and total cocaine infusions) were compared by unpaired *t*-test. For western blotting experiments, densitometry values were normalized to the corresponding loading control (HSP-70) and compared by unpaired *t*-test. Throughout, values lying 2 standard deviations outside of the mean were considered outliers and excluded.

5.5 Results

5.5.1 *Adolescent-onset Gabra1 knockdown delays the acquisition of a cocaine-reinforced response*

To decrease $GABA_A\alpha1$ expression, we delivered Cre-expressing viral vectors to the mPFC of adolescent 'floxed' *Gabra1* mice. Histological analyses indicated that viral vectors infected primarily the prelimbic cortex with some spread to the cingulate cortex (fig.5.1a). Caudally, limited spread into the dorsal infralimbic cortex was detected in a small number of animals, but throughout, the lateral PFC was spared. In 2 mice, Cre was detected in M2, but the majority remained contained within the dorsal mPFC. Two infusions were mis-targeted, and the affected mice were excluded. Using this protocol, $GABA_A\alpha1$ expression was reduced to ~61% of

baseline in homogenized tissue punches [$t_{(23)}$=-2.4, p=0.03] (fig.5.1b), indicating that targeted knockdown reduced regional protein expression as expected.

Viral vectors were infused in early adolescence, at P31 (timing based on Spear 2000). As adults, mice were implanted with indwelling jugular catheters for intravenous cocaine self-administration studies (timeline in fig.5.2a). During the initial training of the cocaine-reinforced response, response rates in knockdown mice lagged, with lower rates on the active, but not inactive, aperture [interaction $F_{(1,48)}$=10.4, p=0.005] (fig.5.2b). (A main effect of session was also detected as expected [$F_{(3,48)}$=2.9, p=0.05], with no overall effect of group [$F_{(1,16)}$=2.4, p>0.1].)

As would be expected by the response acquisition curves, control mice ingested 20 mg/kg more rapidly than knockdown mice [$t_{(15)}$=-2.4, p=0.03] (fig.5.2c, left). Similarly, mice with *Gabra1* knockdown required more training sessions to meet response stability criteria [$t_{(14)}$=-2.2, p<0.05] (fig.5.2c, right).

Mice were trained until they met stability criteria, ensuring that groups ultimately did not differ in the total cocaine infusions self-administered [$t_{(14)}$=-0.26, p>0.8] (fig.5.2d, left). With drug exposure thus matched, we next extinguished the cocaine-reinforced response, and here, response rates did not differ [interaction F<1; effect of group $F_{(1,12)}$=1.5, p>0.2; effect of session $F_{(4,48)}$=16.9, p<0.001] (fig.5.2d, right). Additionally, mice did not differ in the degree of response reinstatement when presented with cocaine-associated stimuli (effect of knockdown F<1) (fig.5.2e). Thus, *Gabra1* knockdown impaired response initiation, but spared cocaine seeking in extinction, as well as relapse-like behavior.

5.5.2 *Adolescent-onset Gabra1 knockdown impairs goal-directed action selection*

Based on the hypothesis that the initial delay in the development of a cocaine-reinforced instrumental response might be associated with impaired action-outcome associative learning and

memory, we generated another group of mice with *Gabra1* knockdown in the prelimbic prefrontal cortex and assessed action-outcome decision-making strategies (fig.5.3a-b). We used a task in which mice were trained to generate 2 operant responses equally, then the likelihood that one response would be reinforced was greatly reduced. Meanwhile, the other response remained reinforced. Preferential engagement of the highly-reinforced response during a subsequent probe test is prelimbic cortical-dependent and considered "goal-directed," while non-selective responding reflects a failure in action-outcome conditioning (Balleine and O'Doherty, 2010).

Response acquisition curves represent both responses/min, and we detected no differences in response rates between groups [interaction $F<1$; effect of group $F_{(1,8)}=2.1$, $p=0.2$; effect of session $F_{(8,64)}=17.6$, $p<0.001$] (fig.5.3c). Next, one nose poke recess was occluded, and responding on the remaining available nose poke aperture was reinforced using a VR2 schedule of reinforcement. In this case, roughly 50% of responses were reinforced, and this percentage did not differ between groups [$t_{(8)}=0.98$, $p=0.4$] (fig.5.3d, left). In another session, the opposite nose poke recess was occluded, and food pellets associated with the other response were delivered non-contingently at a rate yoked to each animal's own reinforcement rate from the previous session. In this case, only ~7% of pellets were delivered (by chance) within 2 seconds of a response. This percentage did not differ between groups [$t_{(8)}=-0.14$, $p=0.9$] (fig.5.3d, right). Response rates generated during these 2 training sessions are shown (fig.5.3e). Control mice appeared to generate the highly reinforced response with greater frequency than the response that was unlikely to be reinforced, while response rates generated by the knockdown mice did not appear to differ between conditions. During this training period, however, neither an interaction effect (which would support this perspective), nor a main effect of response selection (which could refute it), was detected [$F_{(1,8)}=2.5$, $p=0.2$; $F_{(1,8)}=1.8$, $p=0.2$, respectively]. We also detected no effect of group ($F<1$).

During the subsequent probe test, response patterns crystalized, and control mice clearly preferentially generated the response more likely to be reinforced, evidence of goal-directed action selection (fig.5.3f). Meanwhile, mice with adolescent-onset knockdown failed to differentiate between the responses [interaction $F_{(1,8)}$=10.6, p=0.02] (fig.5.3f). Significant main effects were not detected [of response selection $F_{(1,8)}$=3.4, p=0.1; of group $F_{(1,8)}$=3.5, p=0.1].

To determine whether adolescent-onset knockdown delays the acquisition of goal-directed action selection strategies or prevents them entirely, we retrained the nose poke responses (see fig.5.3c, sessions 8,9), and then again "degraded" one action-outcome contingency. In reaction, both groups preferentially engaged the response most likely to be reinforced during training and during the probe test [main effects $F_{(1,8)}$=40.3, p<0.001; $F_{(1,8)}$=11.4, p=0.01 respectively] (fig.5.3g-h). No significant interaction effects were detected during these periods [F<1; $F_{(1,8)}$=1.4, p=0.3, respectively]. Notably, however, as in cases of prelimbic cortical lesions (Corbit and Balleine, 2003), knockdown mice responded less overall during the probe test [main effect $F_{(1,8)}$=17.2, p=0.003] (fig.5.3h), though not during training (F<1) (fig.5.3g).

Thus, adolescent-onset *Gabra1* knockdown in the prelimbic cortex delayed, but did not block, the expression of goal-directed decision-making strategies. As will be addressed in the Discussion, we propose a model in which prolonged *Gabra1* silencing impairs synaptic plasticity in the prelimbic cortex, which then delays the development of goal-directed response selection strategies (fig.5.3i).

5.5.3 *The effects of mPFC Gabra1 knockdown in adolescence and adulthood are dissociable*

To assess whether knockdown had age-specific effects, we next delayed knockdown until adulthood. In this case, histological analyses revealed 2 populations of mice – those with infusions contained within the caudal prelimbic cortex, and mice that were more similar to the

adolescent-onset knockdown group, with broader infection including the rostral prelimbic and a portion of the anterior cingulate cortex (fig.5.4a-b). These groups are referred to as "caudal prelimbic" and "broad prelimbic" and are represented separately. Behaviorally, lenti-GFP control groups did not differ and have been combined.

Mice were food-restricted and trained to generate 2 distinct food-reinforced responses. Again, response acquisition curves represent both responses/minute. Broad knockdown acutely enhanced response rates during training, while caudal prelimbic-selective knockdown had no effects [interaction $F_{(8,108)}$=4.8, p<0.001; main effect of group $F_{(2,27)}$=4.5, p=0.02] (fig.5.4c). A main effect of session was also detected as expected [$F_{(4,108)}$=13.5, p<0.001].

Next, pellets associated with one response were delivered non-contingently. All mice inhibited responding during this period of action-outcome contingency degradation [main effect of response choice $F_{(1,27)}$=23.2, p<0.001; of group $F_{(2,27)}$=2.7, p=0.08; interaction F<1] (fig.5.4d). Subsequently, all mice showed evidence of knowledge of action-outcome associative contingencies, in that they preferentially engaged the response that was most likely to be reinforced; additionally, broad prelimbic infection resulted in higher response rates overall [interaction $F_{(2,27)}$=4.7, p=0.02; main effect of response $F_{(1,27)}$=52.7, p<0.001; main effect of group $F_{(2,27)}$=6.1, p=0.007] (fig.5.4e).

5.6 Discussion

The mPFC develops considerably during adolescence (Spear 2000; Green and McCormick, 2013). Across mammalian species, this process includes the up-regulation of $GABA_A\alpha1$ expression (Fritschy et al., 1994; Hashimoto et al., 2009; Duncan et al., 2010; Datta et al., 2014). Certain pathological stimuli such as prenatal cocaine exposure or early-life stress, by contrast, *decrease* cortical $GABA_A\alpha1$ or *Gabra1* expression when measured immediately prior to

adolescence, in adolescence, or in adulthood (Hsu et al., 2003; Lu et al., 2009; Huang et al., 2011). Conversely, stressor *resilience* is associated with an *enhancement* in mPFC $GABA_A\alpha1$ (Caldji et al., 2000). Nevertheless, the causal effects of adolescent-onset $GABA_A\alpha1$ deficiency on complex decision making pertinent to human psychopathology remain unclear. We report that prelimbic cortical adolescent-onset *Gabra1* knockdown retards the acquisition of a cocaine-reinforced instrumental response. Stimulus-elicited relapse-like behavior is intact in a reinstatement test however, suggesting that healthy mPFC $GABA_A\alpha1$ tone may be essential in particular to developing goal-directed decision-making strategies – that is, selecting actions based on the likelihood of reinforcement, rather than in response to reward-related cues. To test this perspective, we used a modified form of classical instrumental contingency degradation and found that adolescent-onset, but not adult-onset, knockdown indeed delays the development of goal-directed response strategies in adulthood.

5.6.1 *mPFC $GABA_A\alpha1$ regulates cocaine self-administration and goal-directed action selection*

In our first series of experiments, we delivered viral vectors selectively to the prelimbic cortex during early adolescence, causing a site-selective knockdown of *Gabra1*. We then implanted in-dwelling jugular catheters in adulthood for cocaine self-administration studies. Adolescent-onset *Gabra1* knockdown impaired the initiation of cocaine self-administration. Why might this be? First, it is possible that mPFC-targeted *Gabra1* knockdown decreased the initial, though not long-term, reinforcing effectiveness of cocaine, which could account for delayed response acquisition, but intact responding during a reinstatement test. This perspective is based on evidence that both rats and rabbits exposed to prenatal cocaine – which reduces mPFC $GABA_A\alpha1$ expression to roughly 70% of control levels (Hsu et al., 2003; Lu et al., 2009; Huang et al., 2011) – are initially resistant to the locomotor-activating effects of amphetamine, but

locomotor activation after a challenge injection is intact (Lu et al., 2009; Huang et al., 2011; Simansky and Kachelries, 1996; Wang et al., 2013).

A second possibility pertains to evidence that the prelimbic cortex regulates cocaine-seeking behaviors. For example, lesions or inactivation of the prelimbic cortex decrease cocaine-reinforced responding in rodents and interfere with cocaine-conditioned place preference (for excellent review, see Moorman et al., 2014). This is particularly relevant because viral-mediated prefrontal cortical *Gabra1* knockdown, as used here, causes synaptic marker elimination as early as 2 weeks following viral vector infusion (Swanson et al., 2015). Further, *Gabra1*+/- mice have fewer mature, mushroom-shaped dendritic spines – those likely to contain synapses – in the cerebral cortex relative to genetically-intact control mice (Heinen et al., 2003). Lastly, experiments using *in vivo* multiphoton imaging indicate that repeatedly inhibiting $GABA_A$ receptor activity accelerates typical dendritic spine pruning, resulting in a net reduction in dendritic spines in the cerebral cortex (Chen 2014). All together, these findings suggest that *Gabra1* knockdown here may have weakened cocaine-seeking behaviors by destabilizing synaptic plasticity in the prelimbic cortex.

We hypothesized that an additional possibility, which is not mutually exclusive, is that prelimbic cortical *Gabra1* knockdown impairs action-outcome conditioning – that is, associating a specific behavior or behaviors with reinforcement. This could contribute to the delayed development of a cocaine-reinforced response following *Gabra1* knockdown. We tested the possibility that *Gabra1* knockdown weakens action-outcome associative learning and memory using an instrumental contingency degradation task. Mice were first trained to generate two distinct food-reinforced instrumental responses that were equally likely to be reinforced. Then, the likelihood of reinforcement associated with one of the responses was greatly decreased. In reaction, control mice preferentially generated the remaining response, that likely to be reinforced, providing evidence of knowledge of the action-outcome relationship (Balleine and

O'Doherty, 2010; Dickinson, 1980). Mice with adolescent-onset knockdown, however, failed to differentiate between the responses and generated both equally. A failure to modify response strategies following the degradation of an instrumental contingency is thought to reflect a *failure* in action-outcome conditioning or goal-directed decision making; instead, responding is interpreted as stimulus-dependent and reflexive, or "habitual."

As with chronic *Gabra1* knockdown here, lesions of the prelimbic cortex block sensitivity to instrumental contingency degradation, inducing stimulus-elicited habits (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutreau, 2003; Dutech et al., 2011). These deficits are also similar to those resulting from *Gabra1* knockdown in the ventrolateral orbitofrontal prefrontal cortex (VLO; Swanson et al., 2015). The VLO is interconnected with medial wall structures such as the prelimbic cortex in primates, rats, and mice (Sesack et al., 1989; Carmichael and Price, 1996; Ongur and Price, 2000; Gremel and Costa, 2013). Also, both the prelimbic cortex and VLO innervate the dorsomedial striatum, and the VLO additionally has terminals in the central/lateral striatum (Sesack et al., 1989; Schilman et al., 2008; Gremel and Costa, 2013). Instrumental conditioning induces immediate-early gene expression in these dorsomedial and central/lateral striatal compartments (Maroteaux et al., 2014), and studies using lesions or temporary inactivation approaches indicate that, unlike the dorsolateral compartment, the dorsomedial and central/lateral striatum are necessary for selecting actions based on expected outcomes (Yin et al., 2008; Gourley et al., 2013b). We suggest that chronic *Gabra1* knockdown interferes with the integrity of these cortico-striatal circuits and thereby biases response strategies towards reflexive habits at the expense of goal-directed action selection.

Notably, *Gabra1* knockdown mice were ultimately able to develop goal-directed response patterns with additional training, indicating that knockdown delayed, but did not block, action-outcome conditioning. These findings are in agreement with evidence that failures in goal-

directed decision making following permanent or temporary inactivation of the prelimbic cortex are attributable to impairments in the development, as opposed to expression, of outcome-based response strategies (Ostlund and Balleine, 2005; Tran-tu-Yen et al., 2009). Considering our finding that *Gabra1* knockdown also delayed the acquisition of a cocaine-reinforced response, we suggest that intact prelimbic cortical neurocircuits may energize the development of goal-directed behaviors including in the context of drugs of abuse. This would allow for flexible drug seeking as behavioral strategies evolve and ultimately become ingrained.

The *lack* of effect of *Gabra1* knockdown on response extinction may seem to contradict evidence that acute microinfusion of the $GABA_A$ agonist muscimol into the mPFC occludes the extinction of cocaine-reinforced responding (LaLumiere et al., 2010). However, LaLumiere and colleagues (2010) found that infusions targeted to the infralimbic cortex, and not prelimbic cortex, regulate response extinction. Our infusions overwhelmingly targeted the prelimbic cortex and spared response extinction, in agreement with LaLumiere et al. (2010).

Here, we chose to target the prelimbic cortex because it is thought to be critical for response-outcome decision making (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutureau, 2003). The infralimbic and prelimbic cortices are anatomically and functionally distinct, and classically, these two regions oppose each other. In addition to being critical for extinction of cocaine self-administration (see above, LaLumiere et al., 2010), the infralimbic cortex is also required for the acquisition of habitual behavior (discussed in Barker et al., 2014; Killcross and Coutreau, 2003; Coutreau and Killcross, 2003). If we instead targeted the infralimbic cortex, reduced $GABA_A\alpha1$ would be expected to cause goal-directed decision making, as well as impair the extinction of cocaine-reinforced responding.

5.6.2 *The effects of mPFC Gabra1 knockdown in adolescence and adulthood are dissociable*

Remarkably, when we delayed knocking down mPFC *Gabra1* until adulthood, a different behavioral response pattern emerged: First, behavioral sensitivity to instrumental contingency degradation was intact, even in mice with infusions selective to the prelimbic compartment. Given that the mature prelimbic cortex is essential to goal-directed action selection (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutureau, 2003), this outcome was unexpected. Nonetheless, it importantly indicates: 1) that deficiencies following adolescent-onset knockdown are unlikely to reflect the acute effects of knockdown at test in adulthood, but rather the progressive effects of knockdown during a critical period of neocortical development. Additionally: 2) adolescence is a critical period during which even subtle disruptions in mPFC GABA-ergic systems may have long-term maladaptive consequences, and mature cortical systems are by contrast resilient. Finally: 3) given that adult-onset *Gabra1* knockdown in the VLO impairs action-outcome conditioning (Swanson et al., 2015), these findings together raise the possibility that mPFC GABAergic systems are essential to goal-directed action selection during adolescence and early adulthood, but that VLO GABAergic systems "come on-board" or otherwise play a more predominant role later in adulthood.

Response rates during the initial phases of food-reinforced response training were elevated in mice with knockdown broadly distributed throughout the anterior cingulate and rostral and caudal prelimbic cortices. By contrast, mice with knockdown restricted to the caudal prelimbic cortex were unaffected, suggesting that *Gabra1* silencing in the rostral mPFC accounts for increased response rates. Chronic *Gabra1* knockdown decreases synaptic marker expression in the PFC (Swanson et al., 2015), and certain forms of learning and memory are associated with dendritic spine elimination (Lai et al., 2012; Sanders et al., 2012). Thus, *Gabra1* knockdown-mediated plasticity or possibly synapse *clearance* in the rostral prelimbic and/or anterior cingulate cortex may account for augmented response acquisition, but future investigations would be needed to explicitly investigate this possibility.

5.7 Conclusions

The double dissociation between the effects of adolescent- *vs.* adult-onset *Gabra1* knockdown on outcome-based decision making reported here highlights a challenge in basic research – that neurobehavioral studies using genetic or environmental manipulations in adult rodents may be ineffective in informing psychopathologies with neurodevelopmental etiology. As another example, cocaine exposure results in a hypo-metabolic state in the prefrontal cortex of mature rats, while the same cocaine exposure protocol applied in adolescence has the opposite effects (Cass et al., 2013). Despite challenges (*e.g.*, associated with intracranial surgery in adolescent mice and intravenous catheterization of the same subjects later in life, as was performed to complete the studies reported here), experimental approaches applied in developmentally-sensitive ways may more optimally illuminate ontogenic factors in complex behavior.

Figure Legends

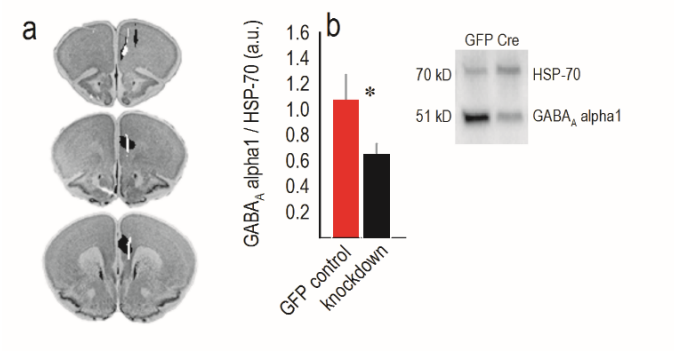

Figure 5.1. GABA$_A$α1 expression is reduced following viral-mediated *Gabra1* knockdown. (a) Histological representations of adolescent infusion sites are transposed onto images from the Mouse Brain Library (Rosen et al., 2000). Black indicates the largest regions affected, and white the smallest. Infusions were bilateral. (b) GABA$_A$α1 receptor expression was reduced in tissue punches collected from the infusion site. Signal was normalized to an HSP-70 loading control, and representative blots are adjacent. n=12-13. Means+SEMs,*p<0.05.

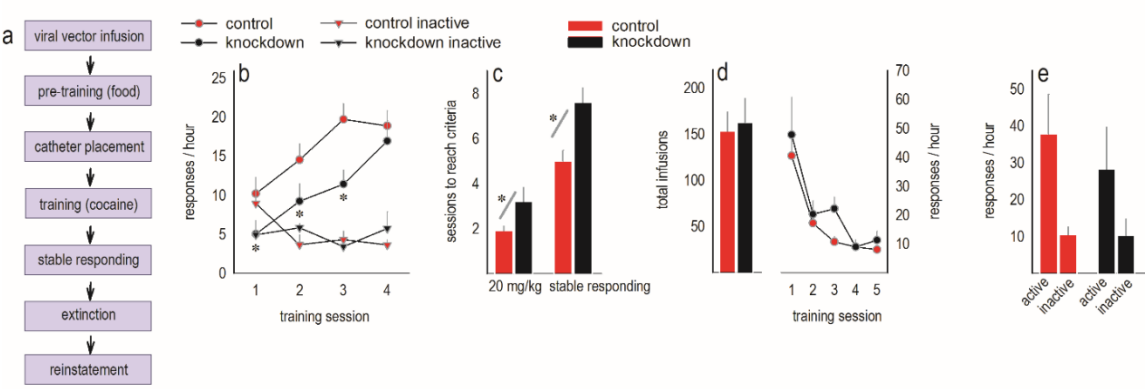

Figure 5.2. Adolescent-onset GABA$_A$α1 silencing retards the acquisition of a cocaine-reinforced response; responding in extinction and cue-induced reinstatement are intact. (a) Experimental timeline. (b) The initiation of cocaine-reinforced responding lagged following *Gabra1* knockdown. Cocaine-reinforced response rates are represented, as are response rates on inactive nose poke recesses. (c) Accordingly, knockdown mice required more sessions to obtain 20 mg/kg/day (left) and to develop stable response rates (right). (d) Nonetheless, knockdown mice ultimately ingested as much cocaine as GFP-expressing counterparts (left), and responding in extinction was intact (right). (e) Additionally, all mice developed cue-induced reinstatement of cocaine seeking. Means+SEMs,*p<0.05. Control n=13, knockdown n=5.

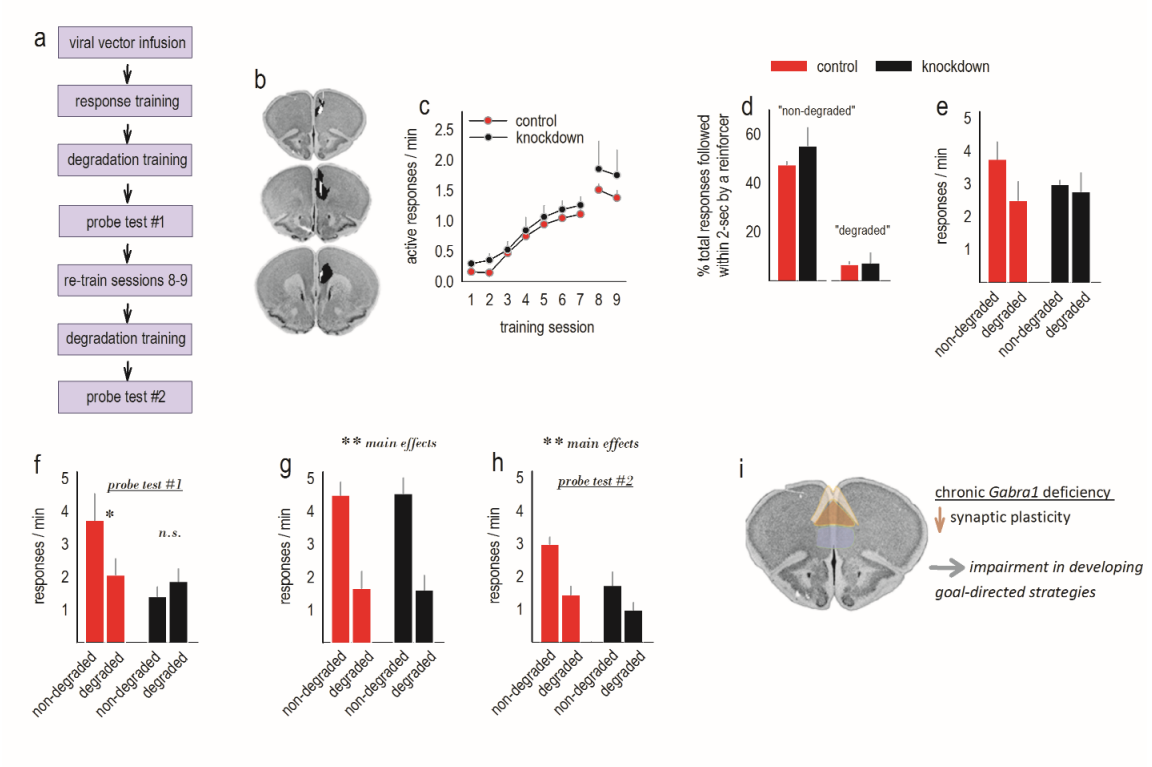

Figure 5.3. Adolescent-onset GABA$_A$α1 silencing impairs goal-directed action selection in adulthood. (a) Experimental timeline. (b) Histological representations of infusion sites are transposed onto images from the Mouse Brain Library (Rosen et al., 2000). Black indicates the largest regions affected, and white the smallest. Infusions were bilateral. (c) Mice were trained to respond for food reinforcers; breaks in the acquisition curves annotate tests for sensitivity to action-outcome contingency degradation. Response rates represent both trained responses/min. (d) Following training, the schedules of reinforcement were modified such that roughly half of responses directed towards one nose poke recess were reinforced, while pellet delivery followed only ~7% of responses directed towards a different nose poke recess. These contingencies are referred to as "non-degraded" and "degraded." (e) Response rates during these training sessions are shown. (f) During a subsequent probe test, control mice preferentially generated the response most likely to be reinforced in a goal-directed manner ("non-degraded"). Mice with *Gabra1* knockdown instead generated both responses at equivalent rates, a failure in action-outcome

conditioning. (g) With additional task experience, both groups generated higher response rates during training sessions when the response was likely to be reinforced. (h) Correspondingly, mice were also ultimately able to select actions that were more, *vs.* less, likely to be reinforced in a probe test. (i) Our model: Chronic *Gabra1* deficiency has been associated with decreased synaptic marker expression and decreased expression of mushroom-shaped spines in the cortex (see Discussion). We suggest that these abnormalities in the prelimbic cortex (highlighted at center) delay the development of goal-directed response strategies. The anterior cingulate (dorsal) and the medial orbitofrontal prefrontal cortex (ventral) are also highlighted on this image from the Mouse Brain Library (Rosen et al., 2000). Means+SEMs,*$p<0.05$,**$p\leq 0.01$. n=5/group.

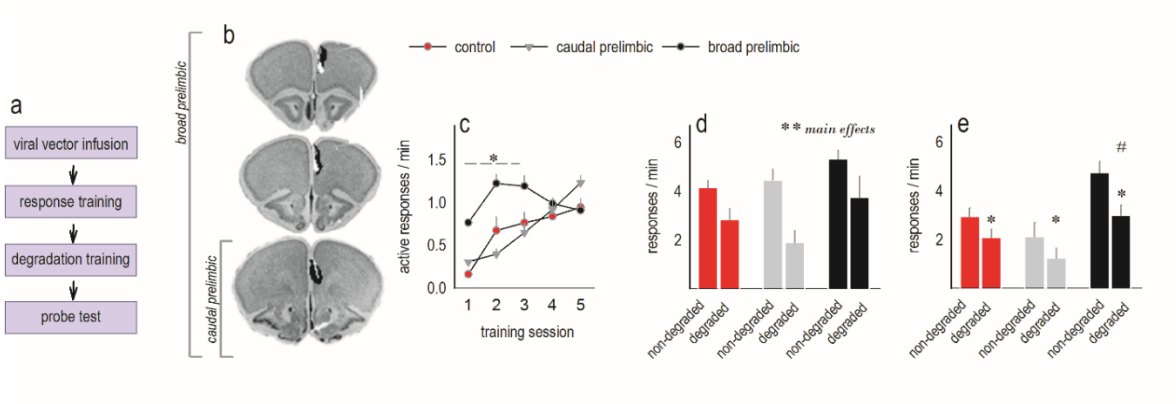

Figure 5.4. Site-selective *Gabra1* knockdown in adulthood does not obviously impair goal-directed action selection. (a) Experimental timeline. (b) Histological representation of viral vector expression imposed onto images from the Mouse Brain Library (Rosen et al., 2000). Infusions were bilateral. Some infections were contained within the caudal prelimbic cortex, and are referred to as "caudal prelimbic." Meanwhile, others were more broadly distributed within the mPFC and are referred to as "broad prelimbic." (c) Acquisition of food-reinforced responses was augmented by broad prelimbic *Gabra1* knockdown, but all mice ultimately responded at equivalent rates. Response rates represent both trained responses/min. (d) Unlike with adolescent-onset knockdown, knockdown mice here inhibited responses that were unlikely to be reinforced during training. (e) Also, knockdown mice could differentiate between responses that were more, or less, likely to be reinforced during a probe test. Mice with broad mPFC knockdown responded more overall. Means+SEMs,*p<0.05,**p<0.001. # signifies higher rates of responding overall in the "broad prelimbic" group, p<0.05. Total control n=14, broad prelimbic knockdown n=8, caudal prelimbic knockdown n=8.

Chapter 6:

Synaptic cytoskeletal plasticity in the prefrontal cortex following psychostimulant exposure

6.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter reviews the effects of psychostimulant exposure on synaptic cytoskeletal plasticity in the prefrontal cortex. The work presented here was conceptualized, organized, researched, and written by the dissertation author with Dr. Shannon Gourley. The chapter is reproduced with minor edits from DePoy LM, Gourley SL (2015) Synaptic cytoskeletal plasticity in the prefrontal cortex following psychostimulant exposure. *Traffic* 16:919-940.

6.2 Abstract

Addiction is characterized by maladaptive decision-making, a loss of control over drug consumption, and habit-like drug seeking despite adverse consequences. These cognitive changes likely reflect the effects of drugs of abuse on prefrontal cortical neurobiology. Here we review evidence that amphetamine and cocaine fundamentally remodel the structure of excitatory neurons in the prefrontal cortex. We summarize evidence in particular that these psychostimulants have opposing effects in the medial and orbital prefrontal cortices ("mPFC" and "oPFC," respectively). For example, amphetamine and cocaine increase dendrite length and spine density in the mPFC, while dendrites are impoverished and dendritic spines are eliminated in the oPFC. We will discuss evidence that certain cytoskeletal regulatory proteins expressed in the oPFC and implicated in postnatal (adolescent) neural development also regulate behavioral sensitivity to cocaine. These findings potentially open a window of opportunity for the identification of novel pharmacotherapeutic targets in the treatment of drug abuse disorders in adults, as well as in drug-vulnerable adolescent populations. Finally, we will discuss the behavioral implications of drug-related dendritic spine elimination in the oPFC, with regards to reversal learning tasks and tasks that assess the development of reward-seeking habits, both used to model aspects of addiction in rodents.

6.3 Introduction

Cocaine addiction is characterized by maladaptive decision making, a loss of control over drug consumption, and habit-like drug seeking despite adverse consequences. These cognitive changes likely reflect the effects of repeated drug exposure on prefrontal cortical neurobiology that then further promote drug use (Jentsch and Taylor, 1999; Robbins and Everitt, 1999; Everitt and Robbins, 2005; Kalivas 2008,2009; Torregrossa et al., 2011,2013; Volkow et al., 2011). Rodents provide an excellent model system by which to characterize the neurobiology of psychostimulant exposure because like humans, rodents will readily self-administer drugs of abuse and engage in complex reward-related decision making, as well as relapse-like behavior. Also as in humans, individual differences in behavioral response strategies can serve as phenotypic predictors of addiction-like behaviors, such as drug seeking following periods of abstinence and despite adverse consequences (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004). *Experimenter*-administered, in addition to *self*-administered, drugs of abuse can also induce behavioral phenotypes in rodents that are relevant to addiction etiology in humans, *e.g.*, increased propensity to engage in reward-seeking habits following cocaine exposure (see Chapter 3; Schoenbaum and Setlow, 2005; Gourley et al., 2013b; LeBlanc et al., 2013; Hinton et al., 2014; Corbit et al., 2014; Schmitzer-Torbert et al., 2015).

At least some of the behavioral effects of drug exposure might be due in part to alterations in the underlying structure of neurons. The estimated 100 billion neurons in the brain are organized by synaptic connections between neurons, the majority of which form on specialized protrusions on dendrites termed “dendritic spines.” Dendrites and dendritic spines can be remarkably plastic – for example, changes in spine structure and synaptic efficacy are thought to provide the cellular substrates of learning, memory, mood, and cognition. Further, research using animal models has repeatedly demonstrated that amphetamine and amphetamine-like

psychostimulants such as cocaine structurally remodel dendrites and dendritic spines within discrete cortico-limbic circuits. For example, landmark studies reported that experimenter-administered amphetamine increases dendritic branch length and spine density in the nucleus accumbens (NAc), as well as on apical dendrites in layer III of the medial prefrontal cortex (mPFC) (Robinson and Kolb, 1997). Cocaine has the same effects in the NAc, and increases dendritic branching and spine density on apical and basal dendrites in layer V of the mPFC (Robinson and Kolb, 1999). Dendritic spines in the mPFC also proliferate after amphetamine or cocaine *self*-administration (Robinson et al., 2001; Crombag et al., 2005; Ferrario et al., 2005). These modifications can persist well beyond the period of active drug exposure, suggesting they may be causally associated with long-term craving, maladaptive decision making, and failures in impulse control associated with addiction.

Here, we will review evidence that drugs of abuse fundamentally remodel prefrontal cortical neurons, and we will highlight relatively recent evidence in particular that cocaine and amphetamine have opposing effects in the orbital, relative to the medial, prefrontal cortex (termed "oPFC" and "mPFC," throughout).

We will then address potential cytoskeletal regulatory mechanisms within the oPFC of cocaine vulnerability and resilience. When relevant, we will use current knowledge regarding the molecular mechanisms of cortical development during adolescence as a framework. Why is this pertinent? Adolescence is characterized by increased risk-taking, and drug use is often initiated in humans during this period (Chambers et al., 2003). In rodents, cocaine self-administration is more likely to escalate in adolescence than in adulthood (Wong et al., 2013). Also during this period, the prefrontal cortex is still developing, which may account for vulnerability to the long-term negative consequences of drugs of abuse and exposure to other pathological stimuli in adolescents (see Chapter 3; Giedd et al., 1999,2004; Casey et al., 2000; Paus et al., 2008; Selemon 2013). Recent studies indicate that certain cytoskeletal regulatory proteins expressed in

the oPFC and implicated in postnatal neural development regulate behavioral sensitivity to cocaine. The isolation of these and other regulatory factors potentially opens a window of opportunity for the identification of novel pharmacotherapeutic strategies and targets in the treatment of drug abuse disorders.

We will conclude by discussing the perspective that drug-induced neural remodeling in the oPFC may be associated with failures in impulse control and maladaptive decision making in addiction. We will focus on so-called "reversal learning" tasks and tasks aimed at characterizing the development and maintenance of reward-seeking habits, behavioral assays commonly used in rodents to model aspects of addiction. The highlights of this review are outlined in Box 1.

Box 1. Review Highlights

- Cocaine and amphetamine cause the structural reorganization of excitatory neurons in the medial and orbital prefrontal cortices, two brain regions implicated in addiction etiology. In particular, repeated psychostimulant exposure causes dendritic spine proliferation in the medial prefrontal cortex (mPFC), while spines are eliminated in the orbitofrontal cortex (oPFC).

- A number of cytoskeletal regulatory proteins expressed in the oPFC impact behavioral sensitivity to cocaine. Several of these targets are additionally involved in postnatal (adolescent) prefrontal cortical development. A better understanding of how drugs of abuse impact structural plasticity during adolescent critical periods may elucidate why adolescents are at heightened risk for the development of drug abuse disorders on the one hand, and help to identify novel targets for intervention on the other.

- Modifications in oPFC neuron structure may be associated with drug-related failures in inhibitory control and the development of inflexible, habitual modes of response, aspects of drug abuse disorders that can be modeled in rodents.

6.4 Psychostimulants remodel dendrites and dendritic spines, with opposite effects in the mPFC and oPFC

This review will focus on the effects of amphetamine and amphetamine-like psychostimulants such as cocaine on neural structure in the frontal cortex, and in this first section, we will highlight the differential effects of cocaine and amphetamine on neurons within the oPFC, relative to the mPFC. We will also briefly discuss the effects of postnatal nicotine exposure in the same regions.

6.4.1 *Drugs of abuse remodel mPFC neurons, causing dendritic spine proliferation*

The mPFC can be separated into the precentral cortex, anterior cingulate cortex, infralimbic cortex, prelimbic cortex, and the medial orbitofrontal prefrontal cortex (fig.6.1a). It can also be divided along the dorsoventral axis into a dorsal portion containing the anterior cingulate and prelimbic cortex and a ventral compartment containing the ventral portion of the prelimbic cortex, the infralimbic cortex, and the medial orbitofrontal cortex (for excellent review, see Heidbreder and Groenewegen, 2003). Although each subregion is anatomically and functionally distinct, cortico-striatal projections arising from the dorsomedial structures, as well as the infralimbic cortex, are implicated in drug-seeking behaviors (Kalivas 2009), hence sustained interest in the field in characterizing drug-related modifications to the structure and function of excitatory neurons in these brain regions.

In an early investigation in gerbils, a single injection of methamphetamine increased dendritic spine density on excitatory neurons in layers III and V of the dorsal mPFC (Dawirs et al., 1991). Using repeated dosing, producing motoric sensitization, Robinson and Kolb (1997) subsequently discovered that amphetamine also increases dendritic spine density in the mPFC in rats. Since then, accumulating evidence indicates that amphetamine (Robinson and Kolb, 1999; Heijtz et al., 2003; Crombag et al., 2005; Muhammad and Kolb, 2011a,b), cocaine (Robinson and Kolb, 1999; Robinson et al., 2001; Ferrario et al., 2005; Frankfurt et al., 2009,2011; Salas-Ramirez et al., 2010; Esparza et al., 2012; Munoz-Cuevas et al., 2013; Rasakham et al., 2014), and (+/-)3, 4-methylenedioxymethamphetamine (Ball et al., 2009) increase dendritic spine density in the mPFC. Deprenyl, which is metabolized into amphetamine, also increases dendrite branching of mPFC pyramidal neurons in Bonnett monkeys (Shankaranarayana Rao et al., 1999), and cocaine exposure increases the thickness and length of prefrontal cortical postsynaptic densities in rats, suggestive of synaptic strengthening (Esparza et al., 2012).

Psychostimulant-elicited dendritic spine proliferation in the mPFC is both rapid and persistent. For example, studies using *in vivo* dendritic spine imaging indicate that cocaine-induced dendritic spine proliferation is detectable as soon as 2 hours after injection and is most robust after the first exposure (Munoz-Cuevas et al., 2013). Meanwhile, *ex vivo* studies have documented elevated dendritic spine counts for up to a month (and longer) following exposure (*e.g.*, Robinson et al., 2001). Interestingly, increased spine counts in drug-exposed animals appear to be attributable to both *de novo* spine proliferation and enhanced survival of spines that were present *prior* to the initial cocaine exposure (Munoz-Cuevas et al., 2013). And importantly, amphetamine and amphetamine-like psychostimulant exposure can result in the formation of new synapses. For example, within the prelimbic cortex, amphetamine increases the number of asymmetric, presumed excitatory, axospinous synapses (Morshedi et al., 2009), and prenatal exposure to cocaine also increases the number of asymmetric spine synapses in the prelimbic cortex (Morrow et al., 2007). Exposure to methylphenidate increases the density of synaptic contacts in the mPFC and also lengthens mPFC dendrites (Zehle et al., 2007; Cavaliere et al., 2012). It is important to note, however, that these synaptic modifications may be part of a temporally-dynamic sequence of events, or possibly dependent on developmental factors, since Rasakham and colleagues (2014) reported evidence of synapse *elimination* in the mPFC following cocaine self-administration in rats.

Repeated psychostimulant exposure causes psychomotor sensitization (reviewed Vanderschuren et al., 2009), and the mPFC regulates this particular behavioral response to drugs of abuse (Pierce and Kalivas, 1997; Steketee 2003; Beyer and Steketee, 2002). Given that many of the studies examining the effects of psychostimulants on dendritic spine density use dosing regimens capable of producing psychomotor sensitization, it has been hypothesized that drug-induced modifications in dendritic spines could be attributed to progressive drug-induced increases in locomotor activity. At least two investigations using a running wheel to dissociate the

effects of locomotor activity from psychostimulant-induced structural modifications, however, indicate that drug-induced dendritic spine proliferation in the mPFC cannot be attributed to locomotor activity (Robinson and Kolb, 1999; Munoz-Cuevas et al., 2013). These and other findings support the perspective that drug-induced remodeling of mPFC neurons contributes to modifications in synaptic plasticity in cortico-cortical and cortico-NAc networks, heightened sensitivity to drug-related cues, and a degradation in the ability of the mPFC to control drug-seeking behaviors in addiction (Kalivas 2009).

Another stimulant, nicotine, also remodels excitatory neurons within the mPFC. As with cocaine and amphetamine, repeated nicotine exposure, followed by a drug washout period, causes dendritic spine proliferation and dendrite lengthening in deep-layer mPFC (Brown and Kolb, 2001). Adolescent nicotine exposure, followed by a subsequent washout period, similarly elongates the basal dendrites of excitatory neurons in the prelimbic cortex, though effects are more modest than following exposure in adulthood; further, nicotine largely spares the infralimbic region (Bergstrom et al., 2008,2010). The structural response of excitatory mPFC neurons to nicotine exposure may be temporally dynamic and/or layer-specific. For example, in another investigation, rats were exposed to nicotine, followed by a washout period. Then, a "challenge" injection was administered, and rats were euthanized the next day (Mychasiuk et al., 2013a). In this case, layer III mPFC dendrites were shorter, rather than elongated. Further, dendritic spines were eliminated. In the same report, nicotine lengthened dendrites in the oPFC and increased spine density (Mychasiuk et al., 2013a). This pattern is opposite that identified following amphetamine or cocaine, as will be discussed in the next section. Clarification of the long-term *vs.* acute responses of both mPFC and oPFC neurons to nicotine exposure will be a key addition to a growing body of literature. For further review regarding current knowledge on this topic, the reader is referred to discussions by Gulley and Juraska (2013) and Kolb and Muhammad (2014).

6.4.2 *Psychostimulant exposure leads to impoverished neurons in the oPFC*

As summarized in the previous section, the rapid and long-lasting effects of amphetamine and amphetamine-like psychostimulants such as cocaine on neural structure have been intensively studied since the seminal reports of Robinson and Kolb describing drug-induced dendrite elaboration and dendritic spine proliferation in the mPFC and downstream NAc (1997,1999). Within the prefrontal cortex, the majority of subsequent research regarding neural morphology has remained focused on medial wall structures, sparing the oPFC. This is despite overwhelming evidence implicating oPFC function in addiction etiology (Volkow and Fowler, 2000; Goldstein and Volkow, 2002; Torregrossa et al., 2008; Lucantonio et al., 2012). For example, the oPFC is hypermetabolic in cocaine-addicted individuals with limited drug abstinence, and plasticity in this region is associated with acute sensitivity to drug-associated cues in rodents (Volkow and Fowler, 2000; Guillem et al., 2010). The oPFC is *hypo*-active following *chronic* cocaine use and withdrawal (Volkow and Fowler, 2000; Goldstein and Volkow, 2002). Further, striatal dopamine D2 receptor binding is reduced in long-term methamphetamine abusers, and receptor occupancy covaries with lower metabolism in the oPFC (Goldstein and Volkow, 2002; Volkow et al., 2011). In rodents, acute and repeated cocaine and amphetamine fundamentally impact the neurobiological make-up of the oPFC, altering for example immediate-early gene and neurotransmitter receptor expression patterns (table 1).

Importantly, the oPFC is thought to be largely structurally and functionally conserved across species (Preuss 1995; Wallis 2012), and the healthy oPFC in both rodents and primates plays a major role in determining reinforcer value and inhibiting inappropriate behaviors, as well as in rapidly learning about predictive associations between stimuli and desired outcomes. These cognitive processes are thought to be impacted in addiction, resulting in increased perceived value of drugs of abuse, in stimulus-elicited drug seeking, and in failures in inhibitory control. In

rodents, oPFC thickness atrophies in response to repeated amphetamine exposure (Muhammad and Kolb, 2011c). With the caveat that this effect is observed in females but not males (Muhammad and Kolb 2011c; see also Wheeler et al., 2013), structural atrophy may be linked to decreased oPFC glucose utilization after even short-term cocaine exposure in monkeys (Lyons et al., 1996), as well as diminished oPFC gray matter and oPFC-dependent cognitive flexibility in long-term cocaine addicts (Franklin et al., 2002; Matochik et al., 2003; Ersche et al., 2008; Moreno-Lopez et al., 2014).

How do psychostimulants impact dendritic spine density and structure in the oPFC? To summarize current evidence, amphetamine and cocaine both *reduce* dendritic spine density on pyramidal neurons within the oPFC (see Chapter 3; Kolb et al., 2004; Crombag et al., 2005; Muhammad and Kolb, 2011a,b; Gourley et al., 2012a; Radley et al., 2015; reviewed by Kolb and Muhammad, 2014) (fig.6.1b-c). In other words, the response of oPFC neurons is opposite that of mPFC neurons. Notably, morphine self-administration and alcohol withdrawal *increase* dendritic spine density and branching in the oPFC (Robinson et al., 2002; McGuier et al., 2015), which has led to the occasional misperception that psychostimulants also induce dendritic spine proliferation in the oPFC, but this generalization appears to be unfounded (for further discussion, see Robinson and Kolb, 2004). Rather, experimenter-administered amphetamine (Muhammed and Kolb, 2011a,b), self-administered amphetamine (Kolb et al., 2004; Crombag et al., 2005), and experimenter-administered cocaine (see Chapter 3; Gourley et al., 2012a) result in dendritic spine elimination in layers III and V of the oPFC. A great strength of reports by Muhammed and Kolb (2011a,b) and Crombag et al. (2005) is that dendritic spines on both mPFC and oPFC principal pyramidal neurons were enumerated *within the same subjects*, providing further compelling evidence that cocaine and amphetamine have opposing structural effects in these brain regions.

Despite multiple studies indicating that dendritic spines on oPFC neurons are eliminated following psychostimulant exposure, it is important to note that Ferrario et al. (2005) found no

evidence of oPFC spine elimination (or proliferation) following short- or extended-access cocaine self-administration. In this case, spine densities in layer III were simply unchanged. Nonetheless, evidence that cocaine eliminates *deep-layer* dendritic spines (see Chapter 3; Gourley et al., 2012a) is provocative, given that: *1)* cocaine causes dendritic spine proliferation in the adjacent mPFC, as already discussed above. *2)* Further, amygdalo-cortical interactions are implicated in addiction-related behaviors (Everitt and Robbins, 2005; Torregrossa and Taylor, 2013), and amygdala projections innervate deep-layer prefrontal cortex (*e.g.*, Gabbot et al., 2006). Thus, cocaine may fundamentally imbalance synaptic plasticity between amygdalo-oPFC and amygdalo-mPFC neurocircuits, contributing to heightened sensitivity to drug-related conditioned stimuli and promoting drug seeking at the expense of engaging inhibitory response strategies. Supporting this perspective, inactivation of amygdalo-mPFC projections in cocaine self-administering rats prevents cue-induced reinstatement of cocaine seeking, an animal model of relapse (Stefanik and Kalivas, 2013).

One report has indicated that early-life (adolescent) cocaine exposure, followed by acute re-exposure in adulthood, eliminates dendritic spines on oPFC neurons and also increases the head diameter of remaining dendritic spines (Gourley et al., 2012a). This is notable because measures such as dendritic spine head diameter directly relate to spine function, as spines with larger heads, mushroom-type spines, are more likely to be synapse-containing (Peters and Kaiserman-Abramof, 1970; Bhatt et al., 2009). And indeed, expression of the post-synaptic marker PSD95 in the oPFC, but not mPFC, is elevated in nonhuman primates euthanized immediately following extended-access cocaine self-administration (McIntosh et al., 2013), suggestive of synaptic strengthening.

To reiterate, early-life cocaine exposure, followed by acute re-exposure in adulthood, *eliminates* oPFC dendritic spines, but increases the head diameter of remaining spines (Gourley et al., 2012a). Cocaine-induced spine head enlargement may reflect a neural response that preserves

or even under some circumstances enhances neuroplasticity in the face of drug-induced spine elimination. Whether this phenomenon is behaviorally 'protective' or deleterious is unclear. For example, blocking F-actin polymerization in the oPFC, needed for dendritic spine enlargement, exaggerates locomotor activity in response to cocaine (Gourley et al., 2012a), suggesting that spine head growth is in some ways protective. On the other hand, acute amphetamine causes cognitive impairments in a food-reinforced instrumental conditioning task, and these deficiencies are associated with acute drug-induced hyper-activity of oPFC neurons (Homayoun and Moghaddam, 2006). In humans, cocaine exposure acutely hyper-activates the oPFC, and this response is associated with drug craving (Volkow and Fowler, 2000).

Future research should clearly dissociate the rapid effects of cocaine on neural structure in the oPFC from the more prolonged, durable consequences since these modifications may have separable influences on behavioral outcomes. For example, acute structural plasticity could contribute to drug craving and disorganized decision making, while the long-term degradation of neural structure following repeated cocaine exposure could contribute to impaired oPFC-dependent impulse control and habit-like drug seeking.

As discussed, repeated cocaine exposure can eliminate dendritic spines on excitatory neurons in deep-layer oPFC. Repeated cocaine exposure also simplifies the dendritic arbors of deep-layer excitatory neurons in the oPFC, reducing overall dendrite length and decreasing branch intersections in Sholl analyses (see Chapter 2; fig.6.1b-c). Chronic ethanol exposure does *not* appear to remodel dendrite arbors of excitatory neurons in the oPFC (Holmes et al., 2012; DePoy et al., 2013a), thus these effects may be selective to amphetamine-like psychostimulants, or potentially cocaine specifically. Interestingly, oPFC dendrite arbors and dendritic spines also remodel following exposure to elevated levels of stress hormones, but in this case, oPFC spines are eliminated while arbors become more complex (Liston et al., 2006; Dias-Ferreira et al., 2009; Gourley et al., 2013c). Thus, even relative to prolonged stressor exposure, cocaine appears to

pose a "double threat" to the dendritic structures of oPFC neurons, simplifying arbors on principal pyramidal neurons *and* eliminating dendritic spines (fig.6.1b-c). In this sense, cocaine is more comparable to prenatal stress, which causes oPFC dendrite regression and spine elimination, detectable multiple weeks following the stressor exposure period (Murmu et al., 2006). Identification of whether common behavioral phenotypes are linked to changes in neural structure may be a fruitful topic of future research.

6.5 Cytoskeletal regulatory factors in the cerebral cortex regulate behavioral sensitivity to cocaine

6.5.1 *Rho regulatory factors*

Dendritic spine morphology is regulated by an underlying actin cytoskeleton, which determines the spine's shape and its signaling properties. Cytoskeletal remodeling of dendrites and dendritic spines is orchestrated in part by Rho family GTPases including RhoA (Rho), Rac1, and Cdc42, which coordinate the actin cytoskeletal rearrangements required for dendrite elaboration or simplification. Rho activation decreases branch extensions in multiple neural systems, while interference with Rho promotes dendrite growth (*e.g.*, Ruchhoeft et al., 1999; Li et al., 2000; Wong et al., 2000; Sin et al., 2002; Sfakianos et al., 2007; Couch et al., 2010). Interference with Rho also promotes activity-dependent remodeling of dendritic spines (Murakoshi et al., 2011). Regulators of Rho activity are thus well-poised to coordinate structural and behavioral responses to cocaine and other drugs of abuse.

One endogenous Rho inhibitor in the brain is p190RhoGAP, which is activated by integrin receptor binding to extracellular matrix proteins and activation of regulatory partners such as Abl2/Arg kinase (Arthur et al., 2000; Hernandez et al., 2004; Moresco et al., 2005; Bradley et al., 2006; Bradley and Koleske, 2009). *p190rhogap*-/- mice are not viable, and while

p190rhogap+/- mice appear superficially normal, they exhibit significant vulnerabilities to genetic and chemical perturbations. For example, simultaneous heterozygosity for mutations in both p190RhoGAP and Abl2/Arg kinase results in increased Rho activity and hippocampal dendritic arbor destabilization, accompanied by deficits in a novel object recognition task (Sfakianos et al., 2007). Further, mice deficient in p190RhoGAP or Alb2/Arg kinase are hyper-vulnerable to cocaine, generating a sensitization-like locomotor response following a single cocaine injection (Gourley et al., 2009a,2012a). Thus, disinhibition of Rho is associated with behavioral vulnerability to cocaine.

Arg-p190RhoGAP interactions are stimulated by integrin receptor binding (Bradley et al., 2006). Integrins are heterodimeric α/β subunit-containing transmembrane receptors that mediate cell adhesion to the extracellular matrix and control signaling pathways that coordinate changes in cytoskeletal rearrangements (Koleske 2013). In neurons, integrins are localized to the synaptic cleft where they initiate biochemical signaling cascades that contribute to synapse maturation, synaptic transmission and plasticity, and dendrite formation and stability. Mice lacking β1-integrin, Abl2/Arg kinase, or both Abl2/Arg kinase and β1-integrin, are deficient in an oPFC-dependent test of behavioral flexibility (reversal learning) (Gourley et al., 2009a,2011,2012a; Warren et al., 2012). This is significant because poor performance in this task in drug-naïve rodents is associated with increased cocaine self-administration (Cervantes et al., 2013). Further, as with Abl2/Arg kinase and p190RhoGAP deficiency, β1-integrin knockdown elicits a sensitization-like locomotor response following a single cocaine injection (Warren et al., 2012).

While the loss of β1-integrin in the forebrain augments cocaine-induced locomotor hyperactivity (Warren et al., 2012), independent investigations have indicated that self-administered cocaine does not impact the expression of β1-integrin in the NAc (Wiggins et al., 2011). Rather, in the NAc, cocaine self-administration regulates β3-integrin expression (Wiggins et al., 2011). Given that drugs of abuse have opposite effects on dendritic spines in the oPFC

relative to NAc, future studies might capitalize on brain region-specific fluctuations in spine regulatory factors, *e.g.*, to develop pharmacological strategies that promote spinogenesis in the oPFC – reversing the effects of repeated psychostimulant exposure – but spare dendritic spine density in the NAc. Such strategies could possibly be combined with those that *reverse* drug-induced spinogenesis in the NAc, but spare oPFC spines. Together, these effects could normalize widespread, divergent structural consequences of psychostimulant exposure, potentially to therapeutic-like ends.

This model presumes, however, that dendritic spine proliferation in the NAc is associated with cocaine seeking behaviors, drug craving, *etc.*, but this may not be the case. For example, knock out of the Rho-GEF Kalirin 7 blocks cocaine-induced dendritic spine proliferation in the NAc and *blunts* cocaine conditioned place preference, a protective-like response. On the other hand, Kalirin 7 knockout mice also develop exaggerated cocaine-induced locomotor sensitization and self-administer more cocaine than wild type mice (Kiraly et al., 2010,2013). For further discourse regarding the complex roles that drug-related dendritic spine proliferation in the NAc may play in cocaine vulnerability *vs.* resilience, see discussions by Chandler and Kalivas (2008), Smith et al. (2014), and others.

Even with these caveats, accumulating evidence indicates that integrin-mediated signaling events are impacted by cocaine exposure in rodents (Wiggins et al., 2009,2011) and possibly involved in cocaine addiction in humans (Mash et al., 2007; Drgon et al., 2010), motivating the authors of the present review to better understand the role of the downstream Abl2/Arg kinase in models of addiction vulnerability. To this end, we have investigated the effects of Abl2/Arg kinase deficiency on the expression of prefrontal cortical dopamine D1- and D2-type receptors. In both cases, Abl2/Arg kinase deficiency results in progressive receptor loss during postnatal development (Gourley et al., 2009a). Additionally, infusion of the Abl-family kinase inhibitor STI-571 selectively in the oPFC mimics behavioral vulnerability to cocaine

observed in *arg*-/- mice, augmenting drug-induced locomotor sensitization and interfering with oPFC-dependent learning and memory (Gourley et al., 2009a,2012a).

At a basic level, dendritic spine morphology is regulated by actin cycling between monomeric globular (G)-actin and filamentous (F)-actin (Cingolani and Goda, 2008; Hotulainen and Hoogenraad, 2010). Chronic cocaine exposure decreases prefrontal cortical levels of F-actin (Toda et al., 2006), consistent with spine elimination in the oPFC following cocaine exposure (Gourley et al., 2012a). Notably, however, modestly *elevated* F-actin and PSD95 levels have also been reported (Esparza et al., 2012). This apparently contradictory finding may simply reflect differences in tissue dissection strategies that resulted in the enrichment of mPFC tissues in the more recent investigation (Esparza et al., 2012) since dendritic spines proliferate in the mPFC following psychostimulant exposure, as discussed in Part 6.4.

Other spine-regulatory factors include transcription factors implicated in cocaine action such as deltaFosB (Nestler 2013). deltaFosB overexpression in the NAc causes dendritic spine proliferation (Robison et al., 2013), and under certain circumstances, deltaFosB induction in the oPFC has protective benefits with regards to the cognitive deficiencies induced by cocaine (Winstanley et al., 2007,2009). These benefits could conceivably be attributable to a proliferative influence on oPFC dendritic spines.

We have noted several instances in which manipulations of neurobiological factors related to dendritic spine stability and density in the oPFC regulate locomotor sensitivity to drugs of abuse. One remaining question thus pertains to *how* the oPFC itself might regulate psychomotor sensitization. The rodent NAc receives projections from the oPFC, but they are quite sparse (Schilman et al., 2008). Further, Winstanley et al. (2009) recently provided indirect but compelling evidence that hyper-sensitization caused by over-expression of deltaFosB in the oPFC was unlikely to be due to effects on the NAc. The rodent oPFC strongly innervates the

dorsal striatum, however (Schilman et al., 2008). This connectivity may be associated with its ability to regulate drug-induced locomotor sensitization.

6.5.2 *Adolescent critical periods and metaplasticity in neural structure*

Adolescence is characterized by increased risk-taking, vulnerability to the development of neuropsychiatric disorders such as drug addiction, and activity-dependent neocortical refinement that culminates in synaptic reorganization and dendritic spine pruning by early adulthood (Bourgeois et al., 1994; Rakic et al., 1994; Anderson et al., 1995; Huttenlocher and Dabholkar, 1997; Spear 2000; Chambers et al., 2003; Bhatt et al., 2009). Synapse and spine stabilization processes may impose a biological "set-point" for prefrontal cortical-dependent neuropsychiatric vulnerabilities and cognitive capacity in adulthood (Crews et al., 2007), and structural stabilization may confer resilience to impulsive decision making, addiction, and addictive-like behaviors in the transition from adolescence to adulthood (Spear 2000; Chambers et al., 2003). Within the prefrontal cortex, certain aspects of oPFC development have a prolonged maturation timecourse (van Eden and Uylings, 1985). This protracted developmental trajectory may result in a substantial window of opportunity for aberrant dendritic spine reorganization and remodeling due to pathological events such as cocaine exposure. On the other hand, 'beneficial' experiences may also impact developmental trajectories. For example, in rodents, early-life housing with same-age conspecifics – increasing the likelihood of social play – appears to facilitate typical dendritic spine pruning in the oPFC relative to housing with adult cagemates (Bell et al., 2010).

The constellation of cytoskeletal regulatory proteins discussed in the prior section – β1-integrin, Arg, and p190RhoGAP – is involved in hippocampal and cortical development during adolescence (Sfakianos et al., 2007; Gourley et al., 2009a,2012a; Warren et al., 2012; Koleske 2013). And as discussed, the absence of Abl2/Arg kinase increases behavioral vulnerability to

cocaine (Gourley et al., 2009a,2011). These patterns suggest that perturbations in normative structural maturation during adolescence increase vulnerability to cocaine. Further support for this perspective comes from additional experiments using Arg-deficient mice, in which oPFC dendritic spines are lost beginning at roughly postnatal day 31 (Gourley et al., 2012a). Psychomotor sensitivity to cocaine is normal at time points preceding dendritic spine loss, and heightened motoric sensitivity emerges only with the onset of dendritic spine elimination associated with Abl2/Arg kinase deficiency. This temporal convergence provides empirical support for the hypothesis that structural instability in adolescence contributes to vulnerability to the drug (Gourley et al., 2012a).

Cocaine-induced locomotor sensitization can be mitigated in adult rodents by treatment with broad-spectrum NMDA receptor antagonists, as well as ifenprodil, an NR2B-selective NMDA receptor antagonist (Kalivas and Alesdatter, 1993; Schumann and Yaka, 2009). Additionally, cocaine exposure can *increase* NR2B expression in the prefrontal cortex (Loftis and Janowsky, 2000). These findings are provocative from a developmental perspective because NR2A signaling stabilizes cortical synapses during early-life critical periods (Quinlan et al., 1999a) and in response to environmental stimuli (Quinlan et al., 1999b; Yashiro and Philpot, 2008), suggesting that *enhancing* NR2A-mediated signaling during adolescent critical periods by *blocking* NR2B may have long-term behavioral benefits. In support of this perspective, ifenprodil treatment in adolescent mice exposed to cocaine blocks the sensitized response to a challenge injection administered in adulthood, 4 weeks later, even in the absence of further ifenprodil treatment (Gourley et al., 2012a).

NR2B blockade can also enhance Abl2/Arg kinase signaling by preventing a substrate, cortactin, from translocating from the dendritic spine (Lin et al., 2013); this mechanism could further contribute to the protective effects of ifenprodil in adolescent mice (see Chapter 3; Gourley et al., 2012a). The authors do not mean to suggest that ifenprodil is *only* effective during

adolescence, however; ifenprodil also has protective benefits in the context of cocaine-, alcohol-, nicotine-, heroin-, and morphine-seeking behaviors in mature rodents, suggestive of potentially widespread applications (see Chapter 3; Vengeliene et al., 2005; Shen et al., 2011; Gipson et al., 2013; Ma et al., 2007,2011).

Of the studies reporting that psychostimulant exposure eliminates dendritic spines on excitatory neurons in the oPFC, the authors of one of these – Gourley et al., 2012a – administered cocaine during the equivalent of early adolescence in rodents, then administered a "challenge" injection in adulthood prior to euthanasia. Dendritic spines were eliminated, and remaining spine heads were enlarged. This experimental design raises the issue of metaplastic sensitivity of oPFC neurons to cocaine exposure. The term "metaplasticity" was originally used to refer to a type of synaptic plasticity in which the history of plasticity at that synapse influenced its response to subsequent stimuli. It has increasingly been applied, however, to biochemical, physiological, and morphological phenomena (Abraham 2008). For example, Shen and colleagues reported that a rat's cocaine exposure history *determines* the structural response of dendritic spines in the NAc to subsequent cocaine injection (Shen et al., 2009). With regards to dendritic spine head enlargement in the oPFC following adolescent cocaine exposure and a subsequent challenge injection in adulthood (Gourley et al., 2012a), our recent experiments indicate that head enlargement *can* be attributed to a *metaplastic* response to cocaine exposure in adulthood following cocaine exposure in adolescence. When cocaine exposure is limited to adolescence, with no challenge injection later in life, head diameters were instead reduced (see Chapter 3). Dissociating these factors is important since the likelihood of occasional cocaine use transitioning to cocaine dependence in humans *peaks* in late adolescence/young adulthood, later than for alcohol and marijuana (Wagner and Anthony, 2002), and developmentally-regulated metaplastic effects of repeated drug exposure on oPFC structure and function may play a role.

6.6 Psychostimulant exposure impacts complex behavior

6.6.1 *Cocaine-related impulsivity and behavioral inflexibility can be modeled in rodents*

In humans, individuals who initiate cocaine use in adolescence are at increased risk of developing dependence (O'Brien and Anthony, 2005) and have decreased likelihood of seeking treatment (Kessler et al., 2001). Interventions aimed at correcting perturbations in the cellular structure of oPFC neurons, particularly during critical adolescent developmental periods, may have beneficial effects, as discussed in Part 6.5. Experiments in this vein may capitalize on tests of impulse control and behavioral flexibility, aspects of oPFC-dependent day-to-day function that are impacted by cocaine exposure in both humans and rodent models. For example, adolescent cocaine exposure impairs response reversal in instrumental and water maze tasks for up to 5 weeks following exposure (see Chapter 2; Santucci et al., 2004,2008), evidence of long-term detriments to oPFC-dependent cognitive and behavioral flexibility. We will conclude our review with a discussion of these and other tasks commonly used to assess the functional consequences of cocaine exposure in rodents.

The term "reversal learning" most commonly refers to tasks in which animals must associate specific stimuli — such as visual cues — with desired outcomes — such as food reinforcers — and then update these stimulus-outcome associations as they are modified by the experimenter. These discrimination-based reversal tasks, like other reversal tasks, require simultaneous inhibition of a previously acquired response and deployment of a previously withheld response. Across multiple species, oPFC lesions impair performance in discrimination-based reversals, and impairments can be attributed to increased perseverative responding, as opposed to, for example, response extinction (Butter 1969; Jones and Mishkin, 1972; Dias et al., 1996; Schoenbaum et al., 2002; Chudasama and Robbins, 2003; Izquierdo et al., 2004; Hornak et al., 2004; Bissonette et al., 2008; further discussed Ragozzino 2007; Izquierdo and Jentsch, 2012). In these tasks, the oPFC is not critical to the initial stimulus-outcome associative

conditioning, since lesions do not impair initial discrimination of reward-associated stimuli and associated outcomes. Instead, the oPFC is necessary for altering behavior as contingencies shift, for using specific information about desired outcomes in the expression of decision-making strategies, for "on the fly" decision making (Stalnaker et al., 2015; McDannald et al., 2014).

oPFC-dependent response reversals can be tested in multiple model systems, and reversal tasks have been used to probe oPFC function following cocaine exposure with a high degree of concordance across species. For example, repeated cocaine exposure in both nonhuman primates and humans impairs reversal performance (Jentsch et al., 2002; Ersche et al., 2008). Furthermore, the severity (amount and duration) of cocaine use correlates with deficits in reversal tasks in humans (Moreno-Lopez et al., 2014). Repeated cocaine exposure also impairs reversal performance in rodents (Schoenbaum et al., 2004; McCracken and Grace, 2013). Of particular note, reversal deficits in cocaine self-administering rats are detectable up to three months following exposure (Calu et al., 2007), further evidence that chronic cocaine exposure persistently degrades oPFC function. Given that cocaine-related dendritic spine elimination in the oPFC also persists multiple weeks beyond the exposure period (see Chapter 3; Gourley et al., 2012a), structural remodeling in this region, coupled with drug-induced irregularities in the electrophysiological and neurochemical properties of oPFC systems (see Izquierdo and Jentsch, 2012; Lucantonio et al., 2012), may be causally associated with cocaine-induced failures in impulse control.

Another reversal task utilized in rodents has been termed "instrumental reversal" conditioning. In this case, mice or rats are trained to nose poke for food reinforcers in a chamber with multiple response operandi. Once animals have acquired the reinforced response, the location of the reinforced aperture is "reversed" to another previously non-reinforced aperture. The initial acquisition of the instrumental response is largely unrelated to reversal performance (Laughlin et al., 2011), and the reversal phase is oPFC-dependent. Specifically, lesions of the

lateral oPFC delay the acquisition of the new response, while lesions of the medial oPFC increase perseverative errors; this can be attributed to energized response rates directed toward the previously-reinforced response because response rates on the newly-reinforced aperture are unaffected (Gourley et al., 2010). Repeated cocaine exposure in mice similarly impairs the acquisition of a new response, characteristic of damage to the lateral oPFC (see Chapter 2; Krueger et al., 2009). DePoy et al. in the same report (see Chapter 2) provided evidence that the same cocaine exposure protocol simplifies dendrite arbors in the lateral and ventrolateral oPFC (recapitulated in fig.6.1b-c here), suggesting that cognitive deficiencies may be associated with these neural rearrangements.

Instrumental reversal conditioning in *drug-naïve* mice is predictive of cocaine sensitivity, in that poor reversal performance is associated with higher rates of cocaine-reinforced responding (Cervantes et al., 2013). This finding suggests that instrumental reversal tasks could be used as a tool to isolate neurobiological factors associated with cocaine vulnerability and resilience in *drug-naïve* organisms.

What factors regulate instrumental reversal learning? oPFC-selective (but not mPFC-selective) knockdown of *Brain-derived neurotrophic factor* (*Bdnf)* impairs response acquisition in an instrumental reversal task in adult male mice (Gourley et al., 2009b,2013a), recapitulating the effects of repeated cocaine exposure. In female mice, oPFC-selective *Bdnf* knockdown results in instrumental responding for food reinforcers that greatly outpaces reinforcer delivery, a possible indicator of impulsive-like behavior (DePoy et al., 2013b). BDNF is essential to dendrite pruning and dendritic spine stability, including in the mature cortex (Woronowicz et al., 2010; Vigers et al., 2012; Reichardt 2006), and accordingly, *Bdnf* knockdown-induced behavioral abnormalities can be corrected by application of a Rho-kinase inhibitor (DePoy et al., 2013b). Rho-kinase is a substrate of the RhoA GTPase, a primary regulator of cell shape throughout the central nervous

system (as discussed in Part 6.5). Thus, oPFC BDNF may facilitate goal-directed decision making via its actions on local structural plasticity.

In a large study, response patterns in an instrumental reversal task were characterized in 51 strains of mice (Laughlin et al., 2011). Among these genetically diverse strains, optimal reversal performance was associated with elevated brain *Synapsin-3 (Syn3)* mRNA levels. *Syn3* codes for synapsin 3, a member of the synapsin family, which anchors synaptic vesicles to the cytoskeletal network of presynaptic terminals. Laughlin and colleagues (Laughlin et al., 2011) propose a model in which *Syn3* regulates dopamine D2 receptor function. This could impact impulse control since reduced D2 activity or expression, due to genetic or pharmacological interventions or individual differences, interferes with reversal performance (Laughlin et al., 2011; Lee et al., 2007; De Steno and Schmauss, 2009; Gourley et al., 2009a). D2 receptors are expressed both pre- and post-synaptically in the prefrontal cortex, and D2 receptor knockout causes dendritic spine elimination in deep-layer prefrontal cortex (Wang et al., 2009). Thus, low D2 levels – *e.g.*, due to genetic perturbations or individual differences in receptor expression levels – may impact addiction-related behaviors by sensitizing oPFC neurons to cocaine-induced dendritic spine elimination.

6.6.2 *Goal-directed decision making and reward-seeking habits*

The oPFC has long been associated with the expression of goal-directed behaviors. For example, oPFC lesions in monkeys and rats resulted in a resistance to the extinction of a food-reinforced response in classical studies (Butter et al., 1963; Kolb et al., 1974). Butter and colleagues (Butter et al., 1963) attributed this finding to the inability of monkeys with lesions to suppress "strong, habitual modes of response."

A bias towards the development of cue-elicited habits at the expense of engaging goal-oriented behavioral response strategies is considered an etiological factor in addiction (Everitt and Robbins, 2005; Pierce and Vanderschuren, 2010; Schwabe et al., 2011). The most common way to test whether reward-related decision making occurs according to outcome-based (goal-directed) *vs.* stimulus-response (habitual) associative contingencies in rodents is by assessing reward-related responding following devaluation of a reinforcer. This can be accomplished by pairing the reinforcer with, for example, temporary malaise induced by post-ingestion injection of lithium chloride. If responding associated with the outcome persists despite devaluation, responding is interpreted as being under the control of a stimulus-response habit, while response inhibition by contrast reflects goal-directed modes of response (Balleine and O'Doherty, 2010). In rodents, responding for cocaine can rapidly assume habitual qualities (Miles et al., 2003; Zapata et al., 2010). Moreover, a *history* of repeated cocaine or amphetamine exposure also results in insensitivity to changes in reinforcer value in food-reinforced tasks, evidence of persistent biases towards stimulus-elicited habits (Schoenbaum and Setlow, 2005; Nelson and Killcross, 2006; Nordquist et al., 2007; Corbit et al., 2014; Leong et al., 2016).

Action-outcome contingency degradation can also be used to classify response strategies. Here, mice or rats are typically trained to generate two distinct reinforced responses, then the likelihood of reinforcement associated with one response is reduced – or "degraded." Rodents and humans that are sensitive to the predictive relationship between actions and their outcomes – that are goal-directed – will inhibit responding associated with non-contingent reinforcer delivery, providing evidence of knowledge of the action-outcome relationship (Dickinson 1980; Balleine and O'Doherty, 2010). By contrast, equivalent engagement of both responses reflects reflexive habits. Using this task, Gourley and colleagues (2013a) found that acute cocaine exposure can interrupt action-outcome learning and memory and thereby bias response strategies towards habit-based, as opposed to goal-directed, responding. Further, this task has been used to provide

evidence that cocaine-induced habit-like response strategies are detectable up to 7 weeks following repeated cocaine exposure in mice (Hinton et al., 2014). The same cocaine dosing protocol simplifies the dendritic arbors of deep-layer excitatory oPFC neurons (see Chapter 2).

These findings indirectly suggested that durable neural remodeling could contribute to a drug-induced bias towards reward-seeking habits. The work conducted for this dissertation project has provided direct evidence for this perspective. Chapter 3 reports that adolescent cocaine exposure reduces dendritic spine density in the oPFC and causes stimulus-response habits. In addition, inhibition of the cytoskeletal regulatory Abl family kinases in the oPFC (with STI-571) impairs action-outcome conditioning, leading to a deferral to stimulus-response habits. Adolescent cocaine-induced habits can be blocked with an NR2B-selective NMDA receptor antagonist, ifenprodil, and simultaneous administration of STI-571 in the oPFC occluded the beneficial effects of ifenprodil. These results indicate that the habit-blocking effects of ifenprodil require intact Abl-family kinase signaling in the oPFC. Lastly, adolescent-onset deficiency in β1-integrin, an upstream effector of Abl2/Arg kinase, in the oPFC also impairs action-outcome conditioning (see Chapter 4). These and other findings described in this dissertation suggest that the long-term degradation of oPFC neuronal structure likely plays a causal role in habit biases following cocaine exposure.

In other investigations, oPFC-selective knockdown of *Bdnf* and surgical disconnection of the oPFC from the downstream dorsal striatum also caused habit behavior, as assessed using action-outcome contingency degradation (Gourley et al., 2013a). This report focused on ventrolateral and dorsolateral oPFC projections targeting the ventrolateral compartment of the dorsal striatum (Schilman et al., 2008; Mailly et al., 2013). Instrumental conditioning — that is, learning that a response produces a reinforcer — increases immediate-early gene expression in this region of the dorsal striatum, supporting the perspective that it is involved in associating a particular behavioral response with a particular reinforcer (Maroteaux et al., 2014).

Abundant evidence indicates that the *medial* compartment of the dorsal striatum — which receives projections from the medial and ventrolateral oPFC (Schilman et al., 2008; Mailly et al., 2013) — is also essential to goal-directed action selection, while the dorsolateral striatum is instead intimately linked with stimulus-response habits (Yin et al., 2008). Consistent with this model, site-selective inhibition of the cytoskeletal regulator Abl2/Arg kinase decreases synaptic marker expression and causes habits when targeted to the dorsomedial striatum, but *breaks* habits when targeted to the dorsolateral striatum (Gourley et al., 2013b). Further, methamphetamine *increases* dendritic spine density in the dorsolateral striatum, associated with stimulus-elicited habits, but *eliminates* dendritic spines (particularly synapse-associated mushroom-shaped spines) in the dorsomedial striatum, tightly coupled with goal-directed response strategies (Jedynak et al., 2007). These structural modifications – in concert with impoverished neural structure within the oPFC – could be causally associated with drug-induced biases towards stimulus-elicited habits.

In non-human primates, oPFC neurons appear to encode both stimulus-outcome and action-outcome associative contingencies (Luk et al., 2013), further implicating the oPFC in goal-directed action selection and suggesting that damage to the oPFC – *e.g.*, through repeated cocaine exposure – could contribute to a reliance instead on stimulus-response habits elicited by reward-associated cues. These cues can include the *context* in which an appetitive reinforcer, such as cocaine, is acquired. Studies using cocaine self-administration approaches indicate that prolonged oPFC inactivation enhances context-elicited reinstatement of drug seeking, sparing drug-seeking behaviors induced by other conditioned stimuli (Fuchs et al., 2004; Lasseter et al., 2009). Independent investigations indicate that oPFC-selective ablation of $GABA_A\alpha1$ receptor subunit expression causes a decrease in synaptic marker expression in the oPFC, and in concert, mice develop reward-seeking habits, but only in the context in which the instrumental response was originally trained (Swanson et al., 2015).

Together, these findings provide evidence that, in the development of adaptive response strategies, the healthy oPFC gates the influence of contextual cues associated with appetitive outcomes. Repeated cocaine exposure could degrade this function to promote the development and maintenance of context-elicited habitual drug seeking through repeated stimulation of the dopamine D1 receptor for example (see Lasseter et al., 2014), dendrite and dendritic spine elimination (as discussed above), and/or imbalance between dopamine D1- and D2-type receptors, given that D2 is highly expressed on basal arbors that are eliminated by cocaine exposure (see Chapter 2; Brock et al., 1992).

6.7 *Conclusions*

Dendrites and dendritic spines can be remarkably plastic, and their structure is sculpted by multiple external stimuli including exposure to drugs of abuse and stressors. Studies aimed at identifying molecular mechanisms of cocaine-induced dendritic spine remodeling – particularly in the NAc – strongly suggest that cellular structural modifications can directly regulate behavioral outcomes. These include potentially adaptive consequences; for example, cocaine-induced dendritic spine proliferation in the NAc has been associated with certain behavioral resiliencies (*e.g.*, Smith et al., 2014), and blockade of cocaine-induced dendritic spine modifications in both the oPFC and NAc can *increase – though in other cases, occlude* – behavioral vulnerability to cocaine (Toda et al., 2006; Gourley et al., 2012a; Pulipparacharuvil et al., 2008; Besnard et al., 2011). Meanwhile, the *correction* of long-term or metaplastic modifications following prolonged cocaine exposure may have behavioral benefits (Giza et al., 2013; Shen et al., 2009).

Animal models, and particularly rodent models, provide a strong venue to disentangle these issues and resolve knowledge gaps: First, rodent models are amenable to determining neurobiological vulnerability and resiliency factors associated with drugs of abuse using both

prospective and retrospective approaches, and using experimental designs and manipulations that can establish *causal relationships* between structural plasticity and behavioral outcomes. Second, we and others argue that the identification of signaling cascades – in addition to specific proteins – that could serve as promising therapeutic targets will be critical (Penzes et al., 2011). Third, developmental critical periods and neurobiological correlates and mechanisms of sex differences in drug vulnerability and resilience may be more rapidly isolated using rodent, rather than primate, systems. A final challenge that could be tackled using rodent models may be the identification of approaches that can normalize drug-induced structural modifications associated with maladaptive behaviors across multiple brain regions. Such studies may benefit from leveraging differential expression of relevant protein targets across structures, and/or attempting to augment new learning regarding the negative consequences of drug self-administration and habitual reward seeking.

Figure and Table Legends

AMPH or COC	Treatment regimen	Target	Effect	Reference
AMPH	Range 1 - 8 mg/kg s.c., 1 injection per day for 4 days, then 4 injections per day for 9 days (40 injections total)	Zif268 (mRNA)	↑	Shilling et al. (2000)
AMPH	0.5 mg/kg/infusion i.v. (1 infusion)	Arc (mRNA)	↑	Klebaur et al. (2002)
AMPH	2.5 mg/kg i.p. (5 daily injections), then 1 mg/kg i.p. challenge	c-fos	↑ with challenge	Nordquist et al. (2008)
AMPH	0.5 mg/kg i.p. (1 injection)	Arc (mRNA)	↑	Banerjee et al. (2009)
AMPH	2.5 mg/kg i.p. (1 injection)	AGS1 AGS3 Rhes (mRNA)	↑ NC NC	Schwendt et al. (2010)
AMPH	Range 1 - 10 mg/kg s.c., 3 injections per day for 4 days (12 injections total)	5-HT2a (mRNA)	↓	Horner et al. (2011)
AMPH	2 mg/kg i.p. (1 injection)	c-fos (mRNA)	↑	Cáceda et al. (2012)
AMPH	1 mg/kg i.p. (14 daily injections)	*Retsat* *Lpar1* *Csrp1* *Bhlhe40* *Dusp6* *Synpo* *Dnajb5* *Ahdc1* (results of gene array)	↑ ↑ ↑ ↓ ↓ ↓ ↓ ↓	Mychasiuk et al. (2013b)
AMPH	Range 1 - 10 mg/kg i.p., 3 injections per day for 4 days (12 injections total)	5-HT2a (mRNA)	NC	Murray et al. (2014)
AMPH; COC	2 mg/kg i.p. (1 injection) 40 mg/kg i.p. (1 injection)	Fos	↑ ↑	Trinh et al. (2003)
COC	0.5 mg/kg/infusion i.v. (multiple infusions self-administered per day for 10 days)	DAT TH	NC NC	Grimm et al. (2002)
COC	0.25 mg/kg/infusion i.v. (multiple infusions self-administered per day for 9-12 days)	Zif268 (mRNA)	↑ in control rats receiving yoked COC-cue pairings	Thomas et al. (2003)
COC	2 mg/kg/infusion i.v. (1 infusion)	c-fos Arc (mRNA)	↑ ↑	Samaha et al. (2004)
COC	0.75 mg/kg/infusion i.v. (2 infusions)	c-fos (mRNA)	↑	Cao et al. (2007)
COC	0.5 mg/kg/infusion i.v. (multiple infusions self-administered per day for 21 days) OR 15 mg/kg i.p. (21 daily injections)	ΔFosB	↑ i.v. and i.p.	Winstanley et al. (2007)
COC	0.75 mg/kg/infusion i.v. (multiple infusions self-administered per day for 28 days) followed by 22 days extinction or abstinence	Fos GluR1 GluR2/3 GluR4	↑ after abstinence NC NC NC	Zavala et al. (2007)
COC	0.4 mg/kg/infusion i.v. (multiple infusions self-administered per day for 22 days; 1 or 6 hour sessions)	DRD2 DRD1 (mRNA)	↓ after long access NC	Briand et al. (2008)

COC	0.6 mg/kg/infusion i.v. (multiple infusions self-administered per day for 10 days) followed by 22 hours abstinence, OR 15 days abstinence, then re-exposure to: 1) a novel chamber; 2) the COC-associated chamber, or 3) the COC-associated chamber and COC-associated cues	Arc BDNF c-fos Zif268 Arc c-fos BDNF Zif268 (mRNA)	↑ after 22 h ↑ with COC, further ↑ with COC-associated environment ↑, but to same degree as drug naïve rats exposed to a familiar context ↑ with COC-associated environment + cues	Hearing et al. (2008)
COC	0.75 mg/kg/infusion i.v. (multiple infusions self-administered per day for 23 days)	Arc (mRNA)	↑	Zavala et al. (2008a)
COC	0.75 mg/kg/infusion i.v. (multiple infusions self-administered per day for 24 days) followed by 24 days extinction or abstinence	Fos	↑ after abstinence	Zavala et al. (2008b)
COC	10 mg/kg i.p. or 40 mg/kg i.p. (1 injection)	c-fos Zif268 (mRNA)	↑ ↑	Caster et al. (2009)
COC	2 mg/kg/infusion i.v. (1 infusion)	Fos	↑	Kufahl et al. (2009)
COC	25 mg/kg i.p. (5 daily injections), then 25 mg/kg i.p. challenge	Zif268 Homer 1a (mRNA)	NC NC	Unal et al. (2009)
COC	15 mg/kg i.p. (4 daily injections)	Zif268	NC	Fritz et al. (2011)
COC	1 mg/kg/infusion, i.v. (multiple infusions self-administered per day for 8 days) followed by cue- or drug-primed reinstatement (10 mg/kg) OR 10 mg/kg i.p. (1 injection)	Arc Zif268 (mRNA)	NC i.p.; ↑ prime NC	Ziólkowska et al. (2011)
COC	15 mg/kg i.p. (4 daily injections)	Zif268	↑	El Rawas et al. (2012)
COC	250 µg/infusion, i.v. (multiple infusions self-administered per day for 11 or 44 days) in high and low impulsivity rats	Zif268 5-HT2c (mRNA)	↓ in high impulsivity rats NC	Besson et al. (2013)
COC	15 mg/kg i.p. (1 injection)	Zif268 (mRNA)	↑	Burton et al. (2013)
COC	0.25 mg/kg/infusion i.v. (multiple infusions self-administered per day for 25 days)	Arc Zif268 (mRNA)	↑ NC	Riedy et al. (2013)
COC	2 mg/kg/infusion i.v. (multiple infusions self-administered per day for 7 days), then 2 mg/kg i.v. challenge	Fos	↑	Kufahl et al. (2015)

Abbreviations: AMPH, *d*-amphetamine; COC, cocaine; i.v., intravenous; i.p., intraperitoneal; s.c., subcutaneous; cue, cue-induced reinstatement; prime, drug-primed reinstatement; NC, no change.

Effects: ↑, increase; ↓, decrease; NC, no change. All results reflect drug-exposed compared to drug-naïve controls.

Table 6.1. Postnatal amphetamine or cocaine exposure alters protein or mRNA expression in the oPFC in rodents.

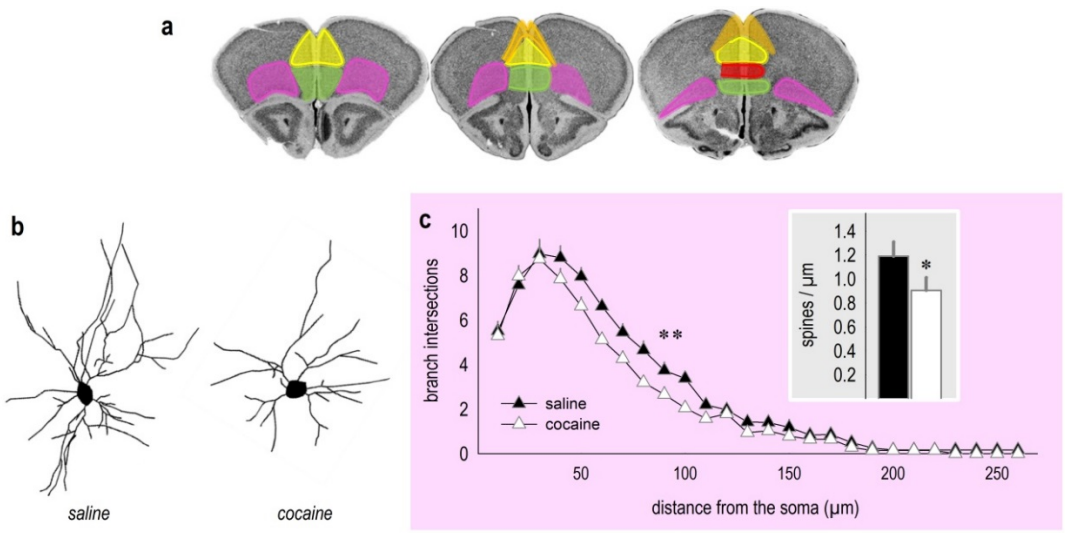

Figure 6.1. Cocaine: A double threat to neurons in the oPFC? (a) Sub-regions of the prefrontal cortex transposed onto coronal images from the Mouse Brain Library (Rosen et al., 2000). Pink represents the oPFC and orange, yellow, red and green represent sub-regions of the mPFC: the anterior cingulate, prelimbic, infralimbic, and medial orbital cortices, respectively. These sections correspond roughly to Bregma +1.98, +2.1, and +2.46. (b) Representative deep-layer oPFC neurons from mice treated with saline or cocaine several weeks prior to euthanasia. (c) Sholl analyses indicate that cocaine exposure decreases branch intersections. **Inset**: Dendritic spines on secondary and tertiary branches are also lost. These findings were originally reported in Chapter 2 (for dendrites) and Gourley et al., 2012a (for dendritic spines), and the reader is referred to these reports for methodological details. Bars and symbols represent means and SEMs, *p<0.05,**p<0.05 40-100 µm from the soma.

Chapter 7:

Summary and Future Directions

7.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter summarizes the results of this dissertation and presents a series of suggested future experiments. The dissertation author wrote this chapter under the guidance of Dr. Shannon Gourley.

7.2 Introduction

The work described in this dissertation was motivated by the perspective that cortical development, particularly in the orbital prefrontal cortex (oPFC), plays a critical role in action-outcome conditioning and subsequent normative goal-directed decision making. Impaired action-outcome associative conditioning, as manifested by stimulus-elicited habits, is considered an etiological factor in the development and maintenance of drug addiction. Here, genetically manipulating or disrupting the structure of the actin cytoskeleton in the oPFC caused a bias towards habitual decision making. This is consistent with previous evidence using oPFC lesions.

7.3 Summary of results and future directions

7.3.1 Chapters 2 and 3

Throughout this dissertation, I selectively manipulated the oPFC, or the medial prefrontal cortex (mPFC), at different stages of development by manipulating addiction-related genes or selectively disturbing the actin cytoskeleton, with cocaine or other pharmacological agents. In Chapters 2 and 3, I showed that adolescent cocaine exposure not only simplified excitatory neuronal arbors in the oPFC, but also eliminated dendritic spines on the same neurons. Moreover, I provided evidence that this structural remodeling is causally associated with a vulnerability to develop drug-induced habits.

Although above I focused on the structural effects of cocaine, how cocaine might be causing these behavioral phenotypes, and underlying structural changes, remains unclear. Given the known pharmacological targets of cocaine, it seems most likely that cocaine is acting through one, or all, of the monoamine transporters. Given the null effects seen with amphetamine in chapter 3, it is possible that cocaine is exerting most of its effects through serotonin, since the effects of amphetamine on serotonin reuptake are weak compared to cocaine (Ritz and Kuhar, 1989). Future experiments could test this hypothesis through a series of parallel experiments using specific monoamine transporter blockers, starting with serotonin, but following up with dopamine and norepinephrine, administered during adolescence in place of cocaine.

Based on these results, we could additionally co-administer cocaine with an agonist for whichever monoamine transporter blocker recapitulated the effects of cocaine most completely. If negating the effects of cocaine at that transporter blocked the effects of cocaine, we would know cocaine is acting through this monoamine system.

If we find that cocaine is acting through the dopaminergic system, we could then test an additional hypothesis. Cocaine increases extracellular dopamine, which causes a complex of protein kinase b (Akt), protein phosphatase2a (PP2A), and β-arrestin2 to form (Beaulieu et al., 2011). This complex inhibits Akt, which then dephosphorylates and activates the downstream glycogen synthase kinase 3 beta (GSK3β) to induce synapse and PSD-95 loss. These effects would cause spine collapse. We could test this hypothesis by co-administering cocaine and lithium, which dissociates complexes that include Akt. Alternatively, we could examine whether cocaine exposure in *β-arrestin2* knockout mice still induces dendritic spine loss in the oPFC.

As discussed, cocaine alters the structure of cortical neurons, and future experiments will examine whether adolescent cocaine exposure is altering synaptic and cytoskeletal proteins in parallel. Importantly, age will be considered as a factor in these experiments, which can be accomplished using immunoblotting. These cytoskeletal regulatory factors vary greatly across

development. Therefore, even if adolescent cocaine does not persistently alter protein expression in the oPFC, an effect that would be detected in adulthood, relevant proteins could still be changing during development, having a long-term impact on neuronal structure.

7.3.2 Chapter 4

Next, I focused on receptor subunits implicated in addiction-related behaviors across species. In chapter 4, I showed that silencing β1-integrin, which coordinates actin dynamics through its signaling partners, in the oPFC causes stimulus-response habits and a hyper-sensitivity to conditioned stimuli in a sex- and developmentally-selective manner.

These experiments utilized lentiviral vectors, which infect pyramidal neurons, as well as glia (Ehrengruber et al., 2001). β1-integrin is also expressed on glia (Paulus et al., 1993), therefore future studies will replicate these experiments using an adeno-associated virus with a calmodulin-dependent protein kinase II (CAMKII) promotor, in order to selectively infect excitatory pyramidal neurons in the oPFC. This will reveal whether glial β1-integrin contributes to the behavioral impairments induced by β1-integrin deficiency in chapter 4.

Why are females resilient to the behavioral effects of *Itgb1* knockdown in the oPFC? As discussed in chapter 4, females might be resilient due to the interaction of β1-integrin and estrogen. We could test this hypothesis by assessing the effects of early adolescent *Itgb1*knockdown on downstream cytoskeletal regulatory factors in females compared to males. If estrogen is overcoming β1-integrin silencing to preserve oPFC function, females might show a lesser effect of knockdown, as demonstrated by increased β1-integrin-mediated activation of downstream signaling partners such as Abl2/Arg. Additionally, we could gonadectomize female mice and/or treat males with estradiol in order to directly examine the effects of sex hormones on the behavioral consequences of *Itgb1* knockdown.

As I alluded to in chapter 4, β1-integrin expression is sensitive to stress, and cocaine is known to cause a stress response, activating the hypothalamic-pituitary-adrenal axis. It is possible that cocaine regulates β1-integrin and its substrates through the glucocorticoid system. As a future direction, we plan to pre-treat mice with metyrapone, a glucocorticoid synthesis blocker, then cocaine. If the effects of cocaine are dependent on the subsequent stress response, then the behavioral and structural effects of cocaine would be absent in adulthood.

7.3.3 Chapter 5

Lastly, I provided a snapshot of another important PFC region, the mPFC, in chapter 5. While the oPFC plays a role in acquisition and retention, the mPFC seems to exclusively regulate the *acquisition* of action-outcome conditioning, but developmental contributions of the mPFC are unclear. I found that developmental mPFC-selective knockdown of *Gabra1,* encoding the $GABA_A\alpha1$ receptor subunit, impaired action-outcome associative learning. Furthermore, these failures are associated with the delayed acquisition of a cocaine-reinforced response in cocaine self-administering mice.

As discussed, particularly in chapters 5 and 6, sub-regions of the PFC can play parallel, synergistic, and complementary roles, or their functions can be diametrically opposed. In future studies, it will be critical to compare and contrast the structure and function of sub-regions in the PFC, particularly during adolescent development, in response to cocaine or cytoskeletal dysregulation. This will shed light onto how the cortex regulates complex decision making, as well as what cortical impairments might be contributing to the heightened vulnerability to developing neuropsychiatric disorders during adolescence.

7.4 Conclusions

Together, these chapters suggest that the normative development of multiple prefrontal cortical sub-regions is critical for goal-directed decision making in adulthood. Aberrant structural remodeling or deficiencies in key cytoskeletal regulatory proteins during adolescence, either due to drug exposure, genetic predispositions, or additional factors, such as stressor exposure, might contribute to or trigger problematic drug use.

Appendix: Complete list of publications to which the author has contributed during her graduate training

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