Prefrontal dopaminergic mechanisms of adolescent cue extinction learning

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Abstract

Addiction and anxiety disorders represent the most prevalent mental illnesses in young people worldwide. Unfortunately, adolescents attain poorer outcomes following extinction-based treatment for these disorders compared to adults. Cue extinction learning involves dopamine signaling via the dopamine 1 receptor (D1R) and dopamine 2 receptor (D2R) in the medial prefrontal cortex. In particular, the infralimbic cortex, a subregion of the medial prefrontal cortex, has been implicated in extinction learning in both adolescent and adult rodents. The prefrontal dopamine system changes dramatically during adolescence. However, the role of prefrontal dopamine in adolescent cue extinction learning is poorly understood. Therefore, this thesis aimed to elucidate the role of prefrontal dopamine in adolescent cue extinction, using cocaine self-administration and fear conditioning in rats.

My first study examined cocaine self-administration and cocaine-associated cue extinction in adolescent versus adult rats. Adolescents displayed a deficit in cocaine-cue extinction learning compared to adults (postnatal day [P]53 and P88 on cue extinction day, respectively). A single infusion of the full D2R agonist quinpirole into the infralimbic cortex prior to extinction enhanced adolescent cue extinction to reduce relapse-like behavior the next day. This effect was recapitulated by a systemic injection of the partial D2R agonist aripiprazole, an FDA-approved drug for the treatment of psychosis with strong translational potential.

My second study examined fear conditioning and extinction in adolescent and adult rats. I first aimed to optimize behavior in late adolescent (P53) and adult (P88) rats during the dark phase of their 12-hour light-dark cycle, to remain consistent with conditions of the previous chapter. However, this produced unreliable behavioral results. In contrast, adolescent rats (P35) consistently display a deficit in long-term fear extinction compared to adults (P88) during the light phase. Infusion of the D1R agonist SKF-81297 into the infralimbic cortex prior to fear extinction had no effect for either age group. However, infusion of quinpirole into the infralimbic cortex significantly enhanced long-term fear extinction in adolescents, whereas it delayed within-session extinction in adults. Interestingly, an acute systemic injection of aripiprazole improved long-term fear extinction in adults.

My final experiments measured prefrontal gene expression for D1R, D2R, and D1R relative to D2R (D1R/D2R ratio) in naïve rats across adolescent development, or following cocaine-cue, or fear extinction. There were no significant differences in prefrontal dopamine receptor gene expression across naïve rats age P35, P53, and P88. Following cocaine-cue extinction, prefrontal D1R gene expression was upregulated in adults but not adolescents. By comparison, following fear conditioning, adolescents showed higher D1R and D1R/D2R ratio gene expression compared to adults. D1R/D2R ratio was modulated in opposite directions following fear extinction learning during adolescence versus adulthood.

These findings show that adolescents are impaired in extinction of emotionally salient cues across both appetitive (drug) and aversive (fear) learning domains. Functional and molecular data provide novel evidence for divergent involvement of prefrontal dopamine in cue extinction learning across adolescent development. Results not only extend understandings of extinction learning in general, but represent an exciting step towards finding new therapeutic targets to facilitate exposure-based therapy in the clinic.

Declaration

This is to certify that:

(i) the thesis comprises only my original work towards the PhD except where indicated in the preface;

(ii) due acknowledgement has been made in the thesis to all other material used; and

(iii) the thesis is less than 100,000 words in length, exclusive of tables, figures, and references.

Signed,

Isabel Catherine Zbukvic

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Preface

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Publications

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Madsen HB, **Zbukvic IC**, Luikinga SJ, Lawrence AJ, Kim JH (2016). Extinction of conditioned cues reduces incubation of craving in adolescent and adult rats. *Neurobiology of Learning and Memory.* E-pub Sep: 1–6. DOI: 10.1016/j.nlm.2016.09.002

Perry CJ, Reed F, **Zbukvic IC**, Kim JH, Lawrence AJ (2016). The metabotropic glutamate 5 receptor is necessary for extinction of cocaine-associated cues. *British Journal of Pharmacology*. Mar 173(6):1085-94. DOI: 10.1111/bph.1343

Kim JH, Perry CJ, Luikinga SJ, **Zbukvic IC**, Brown RM, Lawrence AJ (2015). Extinction of a cocaine-taking context that protects against drug-primed reinstatement is dependent on the metabotropic glutamate 5 receptor. *Addiction Biology*. May 20(3):482-9. DOI: 10.1111/adb.12142.

Perry CJ*, **Zbukvic IC***, Kim JH, Lawrence AJ (2014). Role of cues and contexts on drug-seeking behaviour. *British Journal of Pharmacology*. Oct 171(20):4636-72. DOI: 10.1111/bph.12735 *Co-first author

Presentations

International meetings

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This thesis is dedicated to all those
whose lives have been touched by mental illness

Contents

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Glossary

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
BA	basal amygdala
BLA	basolateral amygdala
BLAST	Basic Local Alignment Search
cDNA	complementary deoxyribonucleic acid
Ce	central amygdala
CET	cue exposure therapy
CPP	conditioned place preference
CR	conditioned response
CS	conditioned stimulus
Ct	threshold cycle
D1R	dopamine 1 receptor
D2R	dopamine 2 receptor
D3R	dopamine 3 receptor
D4R	dopamine 4 receptor
D5R	dopamine 5 receptor
DCS	D-cycloserine
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5
EphB2	ephrin type B receptor 2
EPSC	excitatory postsynaptic current
ERK	extracellular signal regulated kinase
FR	fixed ratio
GABA	gamma-aminobutyric acid
gDNA	genomic deoxyribonucleic acid
GPCR	G-protein coupled receptor
Hip	hippocampus
IL	infralimbic cortex
IPSC	inhibitory postsynaptic current
ITC	intercalated cell
ITI	inter-trial interval
IVC	individually ventilated cage

IVSA	intravenous self-administration
LA	lateral amygdala
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MDF	medium density fiberboard
mGlu5	metabotropic glutamate 5 receptor
MPEP	2-methyl-6-(phenylethynyl)pyridine
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
NAc	nucleus accumbens
NHMRC	National Health and Medical Research Council
NIR	near infra-red
NMDA	N-methyl-D-aspartate
P	postnatal day
PFC	prefrontal cortex
PL	prelimbic cortex
pMAPK	phosphorylated mitogen-activated protein kinase
PPHT	2-(N-Phenethyl-N-propyl) amino-5-hydroxytetralin hydrochloride
PR	progressive ratio
PTSD	post-traumatic stress disorder
RM	repeated-measures
RNA	ribonucleic acid
RT-qPCR	real time-quantitative polymerase chain reaction
US	unconditioned stimulus
VTA	ventral tegmental area

1 Introduction

'*Everybody's youth is a dream, a form of chemical madness*.'

– F. Scott Fitzgerald

Adolescence is a period unlike any other in the human lifespan. In an extraordinarily short number of years, we transition from being almost entirely dependent on our caregivers to having complete independence in the world. Adolescence is often associated with wonderful first experiences, like romantic love and discoveries of self-identity. However, it is also a period of "storm and stress" (Hall 1904). It is perhaps not surprising then that adolescence represents a unique period of risk in terms of mental ill-health (Kessler et al. 2005). More than one in four young Australians experience a mental disorder in a given year, the most common of which are substance use disorders and anxiety disorders (Slade et al. 2009).

Treatment for substance abuse and anxiety disorders for both youth and adult populations often includes behavioral therapy (McNally 2007; Albano and Kendall 2002; Waldron and Kaminer 2004; Carroll and Onken 2005). Compared to medication, behavioral therapy is generally considered safer, and has also been shown to produce better patient outcomes in both adults and youths compared to pharmacological treatment alone (Foa et al. 1999; Pine et al. 1998; van den Brink and van Ree 2003; Resnick et al. 1997). Behavioral therapy for substance abuse and anxiety disorders often involves cue exposure therapy (CET). This treatment is based on the principle of extinction, where the behavioral/emotional response to a cue is reduced by repeated exposure to that cue without any rewarding or aversive consequence. Unfortunately, adolescents typically display poorer outcomes following extinction-based treatments compared to adults (Southam-Gerow et al. 2001; Bodden et al. 2008). To make matters worse, less than one in ten adolescents have received treatment for their substance abuse (Winters et al. 2011), while less than one in five have received therapy for their anxiety (Merikangas et al. 2011). In fact, anxiety disorders and substance abuse ranked first and second for having the biggest gap between prevalence and treatment rates out of all

types of youth mental disorders in the USA (Merikangas et al. 2010). This was identified in part due to financial costs and accessibility of behavioral therapy, which is often more expensive and time consuming compared to medication (Merikangas et al. 2011).

Extinction of emotional reflexes was first described by Pavlov based on his research in dogs (Pavlov 1927). It has since been widely translated in many different species, and extensively in rodents, allowing us to investigate the underlying molecular mechanisms as well as pharmaceutical adjuncts to improve extinction learning. However, such studies of adolescent extinction learning and memory are extremely scarce. As a result, our current understanding of the neural mechanisms of adolescent extinction in relation to addiction and anxiety is woefully incomplete. Therefore, the aim of the present thesis is to elucidate mechanisms underlying adolescent extinction in both appetitive and aversive domains using rodent models. Ultimately, understanding the neuropharmacological mechanisms of adolescent extinction may allow us to develop a way to improve exposure therapy in this vulnerable population.

1.1 Adolescent drug abuse

Adolescence represents a unique period for increased novelty and reward-seeking, as well as a propensity to engage in risk-taking behavior (Spear 2000). It follows that adolescence is often a period of experimentation with illicit drugs (Casey and Jones 2010). In the most recent survey of the health of young Australians aged 16 to 24 years, almost one in five (19%) had used an illicit drug in the last year, equating to an estimated 721,500 young people in Australia (AIHW 2011). In the US, lifetime prevalence of illicit drug use was recently reported at 42.5% of young people aged 17 to 18 years (Swendsen et al. 2012). Of illicit drugs, cocaine use in the US remains particularly high (Degenhardt et al. 2008) and in Australia, cocaine use has risen dramatically over the last two decades. Of a national sample of drug users taken in 2014, 12% reported recent use of cocaine (6 month prevalence) (Stafford and Burns 2015) compared to just 1.3% in 2001 (12 month prevalence) (Loxley et al. 2004). Further, in the most recent survey of Australian drug trends, cocaine was reported as ‘easy’ to obtain nationally (Stafford and Burns 2015). This is of particular relevance given that real or perceived ease of access to illicit drugs is associated with increased risk of use among adolescents (Duncan et al. 2014; Steen 2010; Resnick et al. 1997).

Unfortunately, drug use early in life increases risk for a multitude of problems. This includes increased family and interpersonal problems (Newcomb and Bentler 1988), legal problems associated with selling drugs and drug-related violence (Kaminer and Winters 2010), as well as health risk behaviors not directly to substance use, such as not wearing a seatbelt or carrying a weapon (DuRant et al. 1999). In addition, early drug use is strongly associated with problematic drug use or development of a substance use disorder later in life (Brown et al. 2009; Anthony and Petronis 1995; Warner et al. 2007; Robins and Przybeck 1985; Chen et al. 2009). In fact, age of first drug use inversely predicts the likelihood of subsequent drug dependence, even when duration of total drug use is accounted for (Anthony and Petronis 1995). This means that heightened risk of problems associated with adolescent drug use cannot be explained simply by an increased number of years in which to accumulate them. Rather, adolescence represents a unique period of vulnerability for negative outcomes associated with using and abusing drugs. Together, these findings highlight the importance of research focused on understanding adolescent drug seeking, and improving treatment for addiction specifically in adolescents.

1.2 Adolescent anxiety

Adolescents face many challenges relating to the enormous social, physical, and psychological changes characteristic of this developmental period. With this in mind, it is not surprising that the median age of onset for anxiety disorders most commonly falls during adolescence (McGorry et al. 2011). In fact, anxiety disorders are the most commonly reported (15%) mental disorder in young people aged 16 – 24 years in Australia (AIHW 2011). This is consistent with recent reports that show anxiety is the most frequently experienced mental health problem among youths in the US (Merikangas et al. 2010) and indeed worldwide (Polanczyk et al. 2015). Anxiety disorders encompass a range of conditions, including post-traumatic stress disorder, phobias, separation anxiety disorder, social anxiety disorder, and panic disorder (as well as several others) (American Psychiatric Association 2013). However, all anxiety disorders feature symptoms of excessive and uncontrollable anxiety and fear.

Similar to early substance use, early onset of anxiety disorder is associated with a range of negative outcomes. First and foremost, early development of anxiety disorder is associated with more severe impairment compared to adult onset (Newman

et al. 1996). In addition, youth-onset anxiety disorders constitute a major risk factor for the development of a range of problems later in life (Copeland et al. 2009; Woodward and Fergusson 2001). Specifically, youth-onset anxiety disorders are associated with suicidal ideation and suicide attempts (Sareen et al. 2005), poor education outcomes (Kessler et al. 1995; Van Ameringen et al. 2003), as well as drug dependence and early parenthood (Woodward and Fergusson 2001). Early onset of anxiety disorder has also been consistently shown to correspond with elevated rates of depression and anxiety in adulthood (Last et al. 1987; Pine et al. 1998; Keller et al. 1992), as well as higher comorbidity with other psychiatric conditions (Newman et al. 1996). Similar to the data on substance use, these studies highlight the need for research to understand the mechanisms of adolescent anxiety and its treatment, in order to improve outcomes worldwide.

1.3 Modelling adolescent drug abuse and anxiety

The major behavioral components of substance use disorder and anxiety disorder can be modelled in the laboratory using non-human animals. The use of these models allows us not only to investigate the neural mechanisms of addiction- and anxiety-related behaviors, but also to evaluate the effectiveness of therapeutic interventions such as extinction. Additionally, the use of animal models allows for molecular specificity that is not always possible within the ethical constraints of human research (Ganella and Kim 2014). Importantly, adolescence is a developmental period shared by a number of animal species, and adolescent rodents serve as relevant models of adolescent humans (Spear 2000; Brenhouse and Andersen 2011). Indeed, the adolescent phenotype is observed across a range of mammals, and includes heightened sensitivity to peers, maturing cognitive control, and increased risk taking (Spear 2000). Moreover, there is extensive evidence to suggest that these transitions in behavior relate to specific age-related changes occurring in the brain, such as synaptogenesis and synaptic pruning (Spear 2000; Kolb et al. 2012). Thus, we are able to reasonably employ rats as models of human adolescence to elucidate both the behavioral and neural mechanisms of vulnerability to addiction and anxiety disorders at this age (Brenhouse and Andersen 2011; Kim et al. 2012). In rats, adolescence spans from approximately age postnatal day 28 (P28) to age P55 (Spear 2000; Madsen and Kim 2016).

Intravenous self-administration (IVSA) in rodents is the gold standard paradigm for investigating analogues of the major elements of human drug addiction, including acquisition, maintenance, and relapse-like behavior (**Figure 1.1**). The IVSA paradigm minimizes human intervention, and consequently, has a robust reproducibility index and high face value for the clinical situation. Indeed, the abuse potential of a substance in humans can be predicted from self-administration in rodents (O'Connor et al. 2011). In this model, rats typically learn to perform a specific operant conditioned response (CR, e.g., pressing a lever) to receive a drug infusion via an indwelling intravenous catheter. Rats are placed into an operant conditioning chamber, and presented with two levers: pressing on the 'active' lever results in a drug infusion, whereas the 'inactive' lever has no programmed consequences.

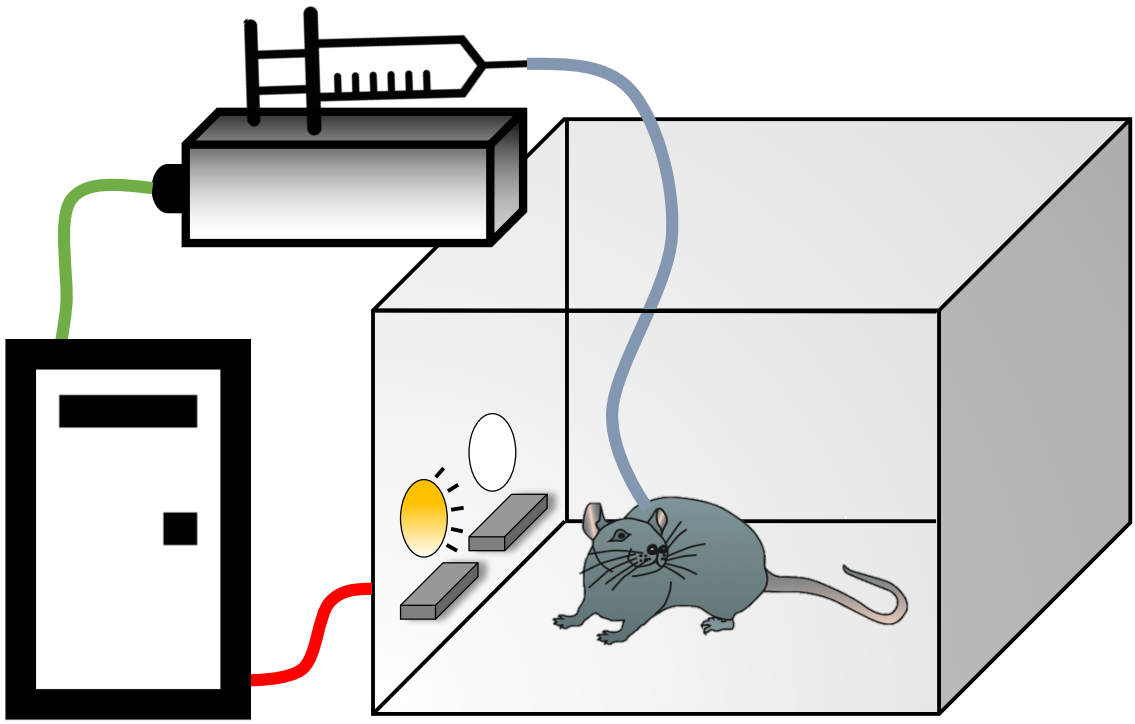

Figure 1.1 *Intravenous self-administration. Pressing on the active lever results in activation of a pump via a computer (left), which delivers an infusion of cocaine via an indwelling jugular catheter. Cocaine infusions are paired with illumination of a cue light, which becomes the drug-associated cue. Pressing on the inactive lever has no programmed consequences, and serves as a control lever.*

The rate at which self-administration behavior is acquired, and discrimination between active and inactive levers are considered measures of the reinforcing effects of the drug. In addition to motivation for the drug itself, IVSA also allows for the study of

drug-associated cues (See 2002). When drug infusions are paired with activation of a discrete cue such as a light, the stimulus becomes a Pavlovian conditioned stimulus (CS) to the drug, which serves as the unconditioned stimulus (US). In the human context, drug-associated cues elicit powerful conditioned responses that are associated with tolerance and withdrawal (Siegel 2005). In addition, cues associated with drugs often trigger cravings that lead to relapse, even after extended periods of abstinence (Fatseas et al. 2015; Gawin and Kleber 1986). Similarly, in the IVSA paradigm, the drug-associated cue will trigger relapse-like behavior in a rodent (See 2002). In fact, there is evidence in rats to suggest that drug-associated cues are more powerful than context or the drug itself in inducing relapse-like behavior following withdrawal (Adhikary et al. 2016).

Pavlovian conditioning also forms the basis of the paradigm most commonly used to model the fear learning typical of many anxiety disorders (Maren 2001). Fear conditioning occurs when the presentation of a discrete cue such as a tone overlaps and/or precedes the presentation of an aversive US such as an electric footshock (**Figure 1.2**). In rodents, the US will initiate a 'freezing' response, a species-specific defense response characterized by the absence of movement except for that required for respiration. This behavior is considered a highly reliable index of fear and, importantly, allows for the quantitative analysis of fear learning and memory (Davis 1990). With repeated pairings, the cue becomes a CS, and the cue alone will come to elicit a freezing response in the absence of any US. Although Pavlovian conditioned fear does not comprehensively model all the elements often involved in anxiety disorders, it is important to understand how learned fear is normally regulated to understand how fear can go astray. Importantly, learned fear is directly involved in the most common forms of anxiety disorders, such as post-traumatic stress disorder and phobias (Rosen and Schulkin 1998), and has been especially implicated in the pathophysiology of youth-onset anxiety disorders (Pine 1999).

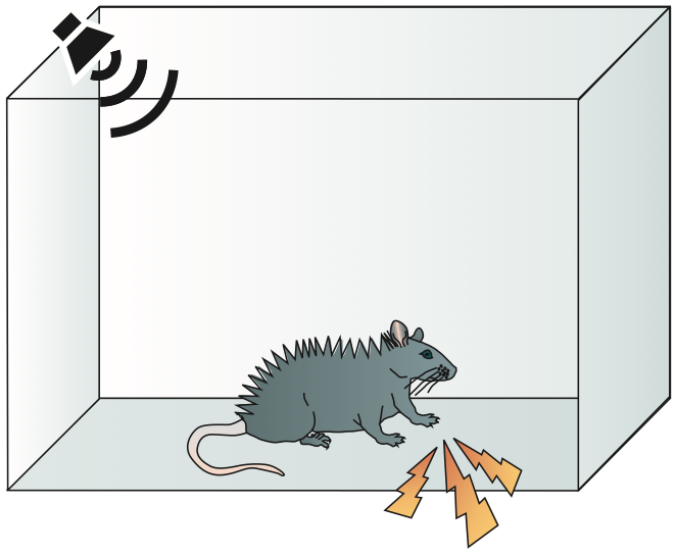

Figure 1.2 *Fear conditioning. Pairing a tone with an electrical footshock will elicit a fear response to the tone by itself.*

1.4 Treatment for drug abuse and anxiety: extinction

In the clinic, cognitive-behavioral treatment for substance use disorder and anxiety disorders in both youth and adult populations often involves CET (McNally 2007; Albano and Kendall 2002; Conklin and Tiffany 2002). CET is based on the principle of extinction, where the frequency and/or magnitude of a response can be reduced by repeated presentations of the cue without any rewarding or aversive outcome (Pavlov 1927). Thus CET for addiction might involve presentation of drug paraphernalia, without the availability of the drug itself (O'Brien et al. 1990). Conversely, an example of exposure therapy to treat post-traumatic stress disorder following military service might include repeatedly listening to helicopter sounds (Rothbaum and Hodges 1999). More broadly, exposure therapy can also involve extinction of an operant response as well as a cue. This is being increasingly seen with the advent of virtual reality, which allows people to complete a behavioral repertoire in a simulated scenario (Krijn et al. 2004; Culbertson et al. 2012; Park et al. 2014). There is also an increasing interest in retrieval-extinction strategies across both drug (Xue et al. 2012) and fear learning (Monfils et al. 2009), including in adolescents (Johnson and Casey 2015), though research is currently ongoing to validate the clinical application of such protocols.

Critically, there is extensive clinical evidence to suggest that adolescents are less responsive to extinction-based therapy for both addiction (Catalano et al. 1990; Perepletchikova et al. 2008; Ramo and Brown 2008; Winters et al. 2011) and anxiety

(Southam-Gerow et al. 2001; Bodden et al. 2008). Moreover, an extinction deficit could help to explain enhanced adolescent vulnerability to these mental disorders. The criteria for both substance abuse and anxiety disorders according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) includes the inability to inhibit or control pathological thought processes (American Psychiatric Association 2013). Thus, one of the hallmarks of addiction is relapse, which is predicted by the uncontrollable urge to obtain the drug. Similarly, one of the characteristic features of any anxiety disorder is uncontrollable worry and fear. It is possible that an inability to control emotional responses in these disorders relates at least in part to impaired extinction. In line with this, post-traumatic stress disorder (PTSD) symptom severity does not correlate with severity of the traumatic event, but is predicted by pre-trauma individual differences in extinction learning (Lommen et al. 2013; Rubin and Feeling 2013). What's more, epidemiological data indicate that persistence of mental health problems among adolescents relates more to recurrence rather than chronicity of youth-onset disorders (Kessler et al. 2012). Therefore, impaired extinction during adolescence may help to explain the enhanced likelihood not only of developing substance abuse or anxiety disorders, but also the elevated chance of having recurring symptoms.

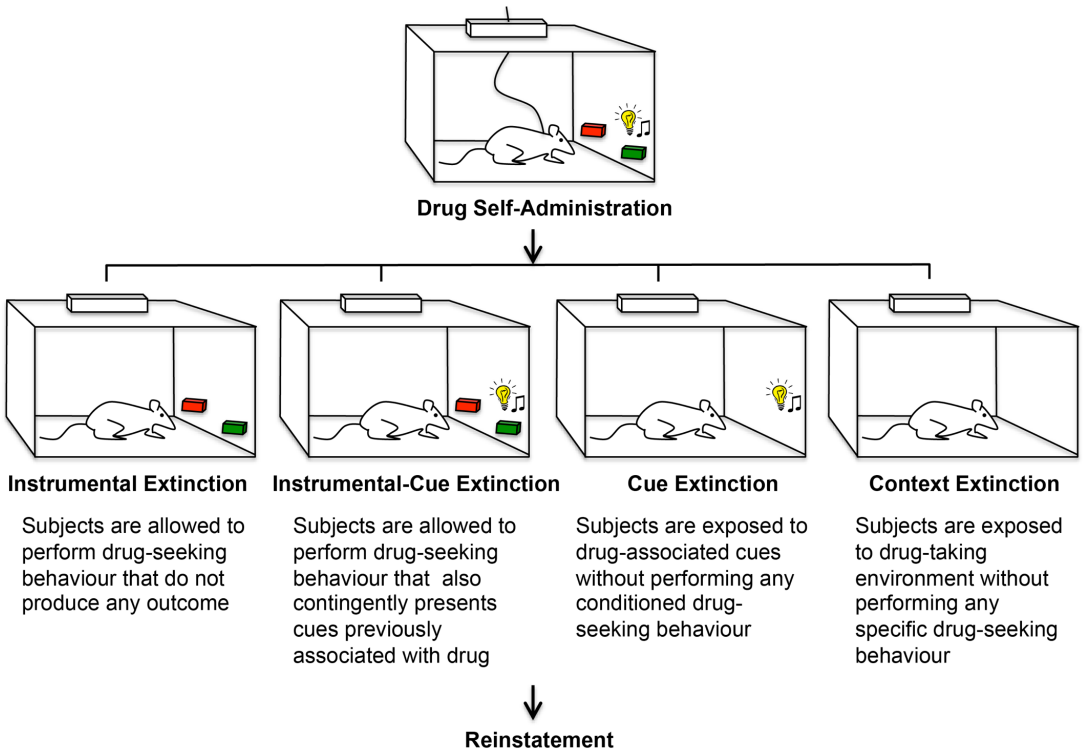

Figure 1.3 *Different forms of extinction using the IVSA paradigm. Figure from Perry et al. (2014).*

Extinction is easily studied in both an IVSA paradigm and a fear conditioning paradigm. In the IVSA paradigm, the CR, CS, and/or the conditioned context can be extinguished separately or in combination (**Figure 1.3**). For instrumental or operant extinction, removing the programmed drug infusion from the active lever will extinguish the operant response (i.e. active lever pressing). For cue extinction, repeated presentations of the cue without the drug reward will extinguish the cue-reward association. Cue extinction can occur alone or, where CS presentations are contingent on operant responding, cue extinction can occur in combination with instrumental extinction. For context extinction, exposure to the environment in which self-administration was acquired will extinguish the context-reward association. In the IVSA paradigm, cue extinction most resembles CET for substance abuse, as it does not involve performing an operant response. Pathological drug-seeking can return when triggered by factors such as re-exposure to the cue or original learning context, stress, drug priming, or simply time elapsed since extinction occurred (Di Ciano and Everitt 2002; Hamlin et al. 2008; Rogers et al. 2008; Venniro et al. 2016). In IVSA, return of drug-seeking behavior following extinction is termed reinstatement, and is characterized by frenetic pressing of the lever previously paired with drug infusions (Bossert et al. 2013).

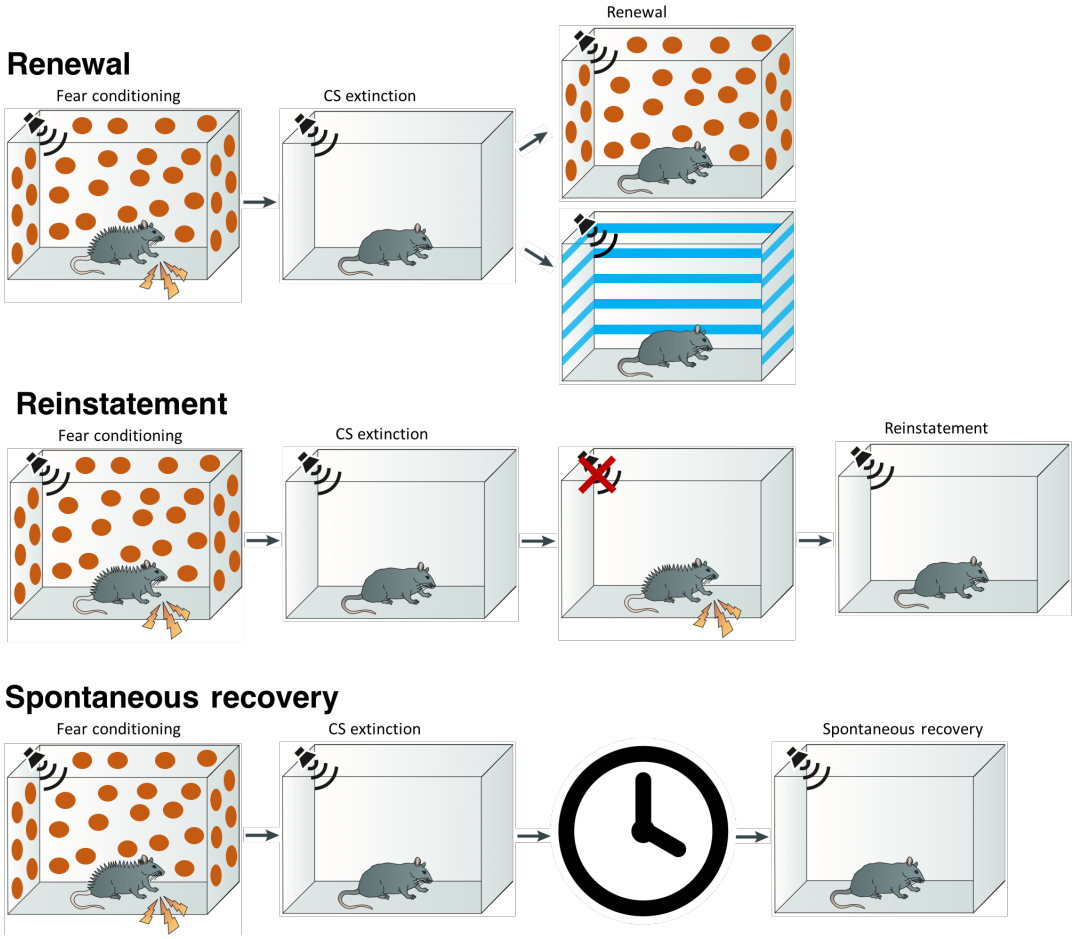

Figure 1.4 *Different relapse-like phenomena in a fear conditioning paradigm.*

In the fear conditioning paradigm, the CR, CS, or context can be extinguished. However, in this model, the CR and CS are extinguished simultaneously, as the CS (tone) is required for the CR (freezing). Thus, repeated presentations of the CS in the absence of the footshock will extinguish the CS-US association. This provides a model of CET used to treat the pathological fear response observed in anxiety disorders. In this paradigm, CS extinction typically occurs in a different context to fear conditioning (**Figure 1.4**), allowing only the CR specific to the CS to be extinguished. This also emulates the common clinical scenario, where treatment is received in a different environment to where trauma was experienced or where triggering events are encountered in everyday life. The footshock-associated context can also be extinguished separately or in combination with the CR and CS, where exposure to the environment in which the conditioned fear response was acquired extinguishes the context-footshock association. Similar to the IVSA paradigm, return to pathological behavior following extinction can also occur in the fear conditioning paradigm (**Figure 1.4**). In this case, relapse-like behavior involves return to high levels of CS-elicited freezing (Goode and

Maren 2014). If the extinguished CS is encountered outside of the extinction context, this can trigger return to fear responding known as renewal (Neumann and Kitlertsirivatana 2010; Bouton 2004). Alternatively, return to CS-elicited freezing can also occur when the US is re-encountered in absence of the CS, defined as reinstatement (Rescorla and Heth 1975). An extinguished fear response can also return merely following the passage of time, a phenomenon defined as spontaneous recovery (Bouton 1993).

The observance of relapse-like behavior in the IVSA and fear conditioning paradigms indicates that extinction memory is generally not as robust as the original memory. Importantly, the same precipitants of relapse-like behavior in rodents are known to trigger return to maladaptive behaviors in humans living with drug addiction or anxiety disorders (Lee et al. 2006; Myers and Davis 2002; Shalev et al. 2002; Maren et al. 2013). On the basis of this, strengthening extinction memory, either through improvements to behavioral treatment or the development of effective pharmacological adjuncts to therapy, may represent a promising strategy to prevent the return of maladaptive behavioral patterns.

1.5 Neurobiology of extinction: a brief overview

Over the past two decades there have been substantial advances in our understanding of the neurobiology underlying extinction. It is now widely accepted that the resulting reduction in CR does not reflect erasure of the original CS-US association (Bouton 2004; Myers and Davis 2002). Rather, extinction involves new learning of a CS-No US association, which exists in parallel with the initial CS-US memory. In line with this, there is strong evidence that extinction produces similar neural changes to conditioning. This similarity is important, as erasure would be expected to produce different neural changes to conditioning, with theories of deconsolidation hypothesizing an important role for long-term depression (LTD) (see Maren 2011 for review). However, pharmacological agents known to enhance long-term potentiation (LTP) have been shown to strengthen extinction memory in both the appetitive and aversive domains (Malenka and Bear 2004; Myers et al. 2011). Furthermore, it has been shown that extinction of conditioned fear can be induced with the direct intra-cranial infusion of a neurotrophic factor involved in conditioning, without the need for any behavioral training (Peters et al. 2010). Evidence that extinction involves strengthening of synapses

suggests that extinction involves new memory, which exists in parallel with the original memory. This explains why return to pathological behavior can occur following extinction training.

Drug- and fear-related extinction learning understandably involves plasticity in different brain regions, as these behaviors fall into distinct learning domains. Evidence shows that drug-related extinction involves the nucleus accumbens (NAc), which forms part of the circuitry involved in reward learning. Following extinction of lever pressing for cocaine, inactivation of the NAc core and shell using a combination of the gamma-aminobutyric acid (GABA) agonists baclofen and muscimol (See et al. 2007) or deep brain stimulation (Vassoler et al. 2013) results in reinstatement of cocaine-seeking behavior in adult rats. Conversely, phosphorylation of a glutamate receptor subunit GluA1 at Ser^{845}, which modulates synaptic plasticity, is increased in the NAc as a whole following contingent cocaine-cue extinction (Nic Dhonnchadha et al. 2013). While the NAc has been implicated in drug-associated operant and cue extinction, fear extinction has been shown to involve the basolateral amygdala (BLA), a region important for processing aversive information. Indeed, in rats intra-BLA infusion of an antagonist at the N-methyl-D-aspartate (NMDA) receptor, purportedly involved in synaptic plasticity, prior to fear extinction impairs expression of extinction learning the next day (Falls et al. 1992). In contrast, intra-BLA infusion of the partial NMDA agonist D-cycloserine (DCS) facilitates otherwise incomplete extinction when tested 24 hours post-extinction training (Kim et al. 2007), while fear extinction increases phosphorylated mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) in the BLA, a marker of neuronal plasticity. Although distinct brain regions are involved in different types of extinction, the general inhibitory nature of extinction learning and memory suggests that at least some of the key structures involved may be shared. In fact, there is a critical region of overlap for appetitive and aversive extinction circuits: the prefrontal cortex (PFC) (for review, see Peters et al., 2009).

1.6 The prefrontal cortex

The PFC is a higher order brain region, responsible for integrating diverse sensory information about both the external state of the world and the internal state of the system (Passingham and Wise 2012; Fuster 2015). This “top-down” processing is

critical for organizing complex behaviors in context and time (Kolb et al. 2012; Miller and Cohen 2001). In line with this, there is strong evidence for the importance of the PFC in acquisition, consolidation and expression of extinction learning. In particular, the infralimbic cortex (IL) has been shown to mediate extinction of both fear- and reward-associated behaviors (see Peters et al. 2009 for review). The IL together with the prelimbic cortex (PL) constitute the medial prefrontal cortex (mPFC), a homologous region in the human and the rodent brain (see Gass and Chandler 2013; Myers and Carlezon 2010; McNally 2014; Millan et al. 2011 for reviews; Gass and Chandler 2013). This is important, as it means the rat is a suitable model to investigate the role of the mPFC in extinction learning and memory.

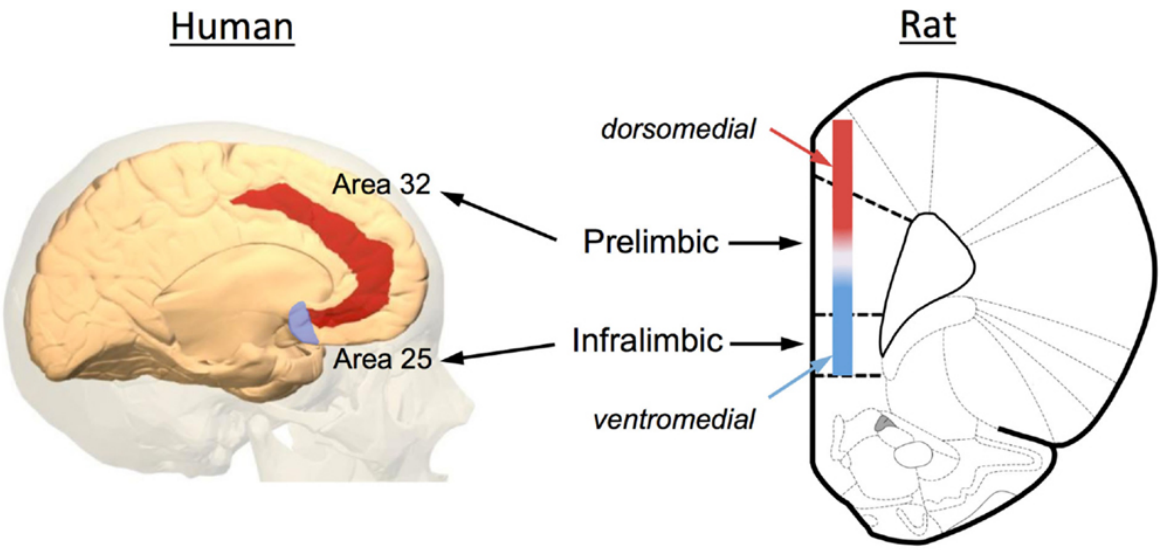

Figure 1.5 *Schematic diagram depicting the PL and IL in the rat and homologous regions in the human brain. Based on inputs from the thalamus, the rat PL region is approximately Brodmann area 32, whereas the IL is analogous to Brodmann area 25. Figure from Gass & Chandler* ***(2013)****.*

Some studies using rodents implicate the IL in the acquisition of drug-associated extinction learning, (**Figure 1.6**). Using the conditioned place preference (CPP) paradigm, in which rodents learn that a distinct context is associated with drug availability, electrolytic lesions of the IL following alcohol (Groblewski et al. 2012) or an amphetamine CPP (Hsu and Packard 2008) blocked subsequent acquisition of extinction. It should be noted that in those studies, lesions also included the PL. By comparison, evidence pointing to a role for the IL in consolidation and expression of drug-extinction memory is much stronger. For instance, intra-IL infusion of $GABA_{A/B}$ receptor agonist cocktail (muscimol/baclofen) immediately following operant extinction

of cocaine-seeking impairs retention of extinction learning when tested following a delay (Peters et al. 2008; Peters 2008). On the other hand, intra-IL infusion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), a specific agonist for the AMPA receptor implicated in neuronal plasticity, immediately following operant extinction of cocaine-seeking enhanced retention of extinction memory (Peters et al. 2008). The IL also appears to be necessary for the expression of drug-associated extinction memory. Acute IL inactivation by GABA agonist cocktail prior to reinstatement enhanced reinstatement of extinguished cocaine-seeking (Ovari and Leri 2008). Similarly, IL inactivation immediately prior to test has also been found to trigger re-emergence of an extinguished heroin CPP (LaLumiere et al. 2010). In contrast, intra-IL infusion of AMPA immediately prior to test inhibited reinstatement of extinguished cocaine-seeking (Peters et al. 2009). Notably, studies involving AMPA/NMDA manipulation are consistent with the idea that extinction involves new learning that requires plasticity in the IL. The IL is thought to regulate extinction of drug seeking via glutamatergic outputs to the NAc shell (Peters et al. 2008). In line with this, disconnection of the IL-NAc pathway following extinction training in rats produces a robust return of cocaine-seeking behavior, resembling that produced by pharmacological IL inactivation (LaLumiere et al. 2010).

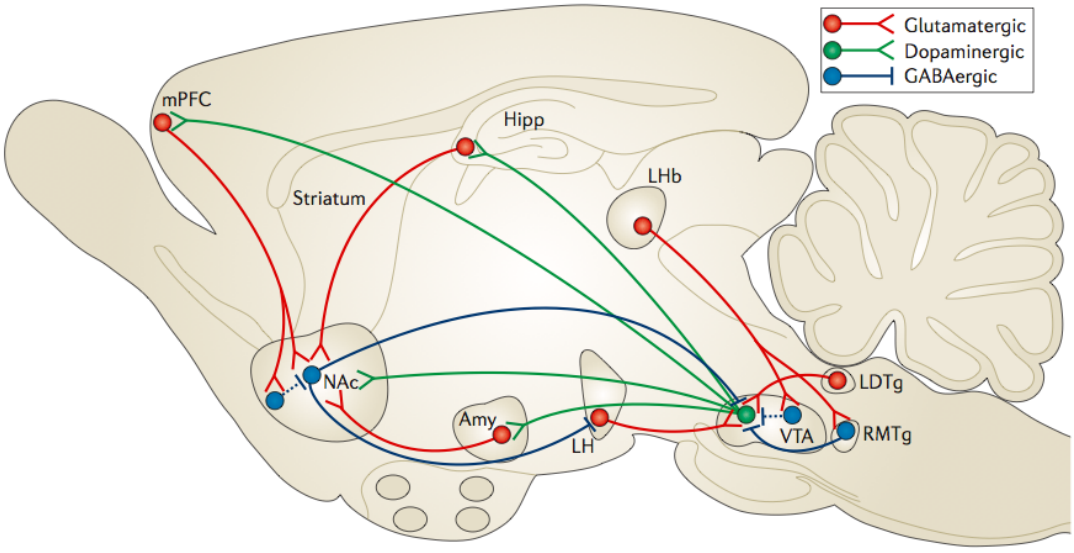

Figure 1.6 *The mesocorticolimbic circuit includes projections from the ventral tegmental area (VTA) to the mPFC, which includes the IL and PL. The mPFC mediates expression of extinction via outputs to the NAc. Schematic adapted from Kauer & Malenka* ***(2007)****.*

Similar to studies of drug-associated extinction, studies of fear extinction using rodents strongly implicate the IL in consolidation and expression of extinction learning, though there is some evidence for a role in extinction acquisition (Morgan and LeDoux 1995; Morgan et al. 1993; Morrow et al. 1999). For instance, lesioning the IL has been shown to increase freezing during fear extinction (Morgan and LeDoux 1995; Morrow et al. 1999), while electrical microstimulation of the IL during fear extinction has been shown to decrease overall freezing during extinction (Milad and Quirk 2002; Vidal-Gonzalez et al. 2006). Optogenetic activation of IL neurons during extinction training has been shown to reduce within-session fear expression and strengthen extinction memory when tested the next day, while silencing had no effect on within-session extinction, but impaired retrieval at test the next day (Do-Monte et al. 2015). These findings suggest a role for the IL in acquisition of fear extinction. However, others have found that rats with lesions of the IL acquire fear extinction within-session, but return to high levels of freezing when tested after a delay compared to sham lesioned rats (Quirk et al. 2000a; Lebron et al. 2004). Similarly, intra-IL infusion of a protein synthesis inhibitor has been found to have no effect on acquisition of fear extinction within-session, but impaired expression of extinction the next day (Santini 2004). Further, post-extinction blockade of IL NMDA receptors (Burgos-Robles et al. 2007) or pharmacological inactivation of the IL (Laurent and Westbrook 2009) reduces expression of extinction the next day. Notably, fear extinction produces NMDA receptor-induced plasticity in the IL (Peters et al. 2009) and increases the intrinsic excitability of IL neurons (Santini et al. 2008), consistent with formation of a new CS-No US memory. Together, these data suggest that the IL is critically involved in consolidation of fear extinction memories.

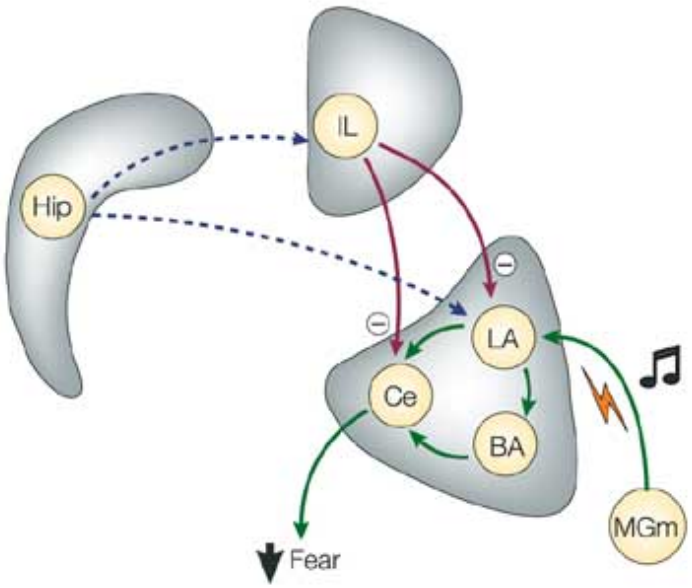

Figure 1.7 *A circuit model of extinction propose that the IL integrates contextual information from the hippocampus (Hip) to mediate expression of extinction learning via excitatory outputs to the LA and BA, which subsequently inhibit the Ce to reduce fear responding. Schematic adapted from Maren & Quirk* ***(2004)****.*

Good expression of extinction memory corresponds to increased neuronal firing in the IL, while poor recovery shows no change in activity in the IL (Milad and Quirk 2002). Inactivation of the IL prior to test has also been found to attenuate an extinguished fear response (Laurent and Westbrook 2009). These findings suggest a role for the IL in expression of extinction. The dominant circuitry model of fear extinction states that the IL mediates fear extinction expression via feedforward inhibition of the amygdala. Ultimately, inhibition of the central amygdala (Ce) suppresses fear responding via outputs to regions such as the thalamus, and thereby promotes expression of extinction (Herry et al. 2010). The IL is thought to inhibit the Ce via excitatory projections to the basal (BA) and lateral amygdala (LA), together the basolateral nucleus (BLA), and/or the intercalated cells (ITCs) of the amygdala (**Figure 1.7**). The BLA and/or the ITCs subsequently inhibit the Ce via local GABAergic connections. Stimulation of the IL has been shown to excite BLA inhibitory interneurons (Rosenkranz and Grace 2002; 2001), while resistance to extinction corresponds to impaired immediate early gene induction in the mPFC and the BLA (Herry and Mons 2004). In addition, extinction learning has been shown to increase early gene expression in the IL and ITCs (Knapska and Maren 2009), though a recent optogenetic study showed that mPFC transmission to ITCs was unchanged following

extinction learning (Cho et al. 2013). The IL may therefore signal the BLA independent of the ITCs. In any case, expression of extinction is widely thought to rely on inhibition of the Ce, as this is the main output nucleus of the amygdala (Veening et al. 1984).

While the IL is a key brain region underlying the consolidation and expression of extinction memory, it is widely accepted that the PL mediates the expression of drug-seeking and conditioned fear (Corcoran and Quirk 2007; Sierra-Mercado et al. 2006; McFarland and Kalivas 2001; McFarland et al. 2004). The PL is thought to control expression of drug-seeking behavior via excitatory projections to the NAc core (Peters et al. 2009). Consistent with this, increased glutamate release in the NAc core is observed during cue- or drug-induced reinstatement of heroin-seeking, and this effect is abolished by inhibiting glutamatergic afferents from the PL (LaLumiere and Kalivas 2008). For conditioned fear, inactivation of the PL has been shown to have no effect on extinction learning or memory (Laurent and Westbrook 2009). In contrast, inactivation of the PL reduces freezing to both a tone and a context previously paired with footshock (conditioned fear), but not to a cat (innate fear) (Corcoran and Quirk 2007). It has therefore been suggested that the PL drives the expression of conditioned fear, via glutamatergic projections to the BA, which in turn excites the CE (Sotres-Bayon et al. 2012).

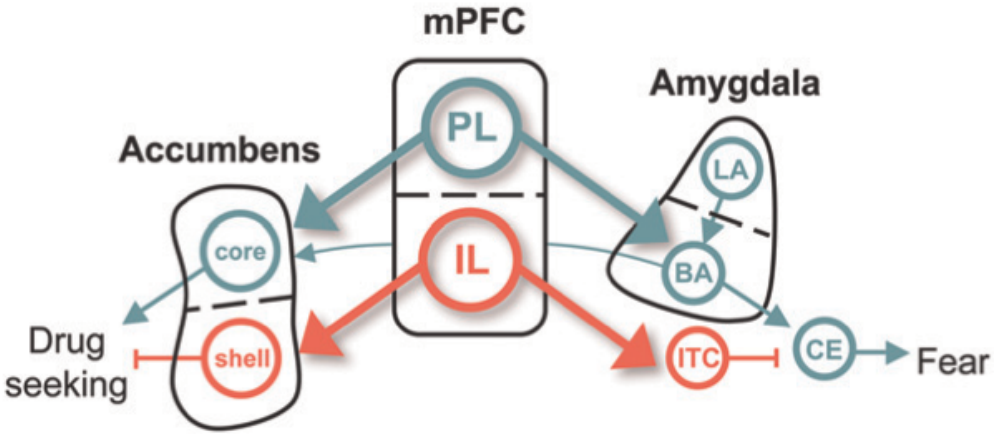

Figure 1.8 *Circuit schematic depicting prefrontal regulation of extinction. Green represents pathways from the PL that activate drug seeking via the NAc core, and promote the expression of fear by the CE via the BA. Red represents pathways from the IL that inhibit drug seeking by the NAc shell, and suppress conditioned fear by the ITCs. Figure from Peters et al.* ***(2009)****.*

Given these divergent roles of the IL and PL in the inhibition and expression of learned behaviors, it has been proposed that these regions supply an "on-off" switch for

the expression of drug-seeking or conditioned fear, especially following extinction (Peters et al. 2009). However, it should be noted that some findings are inconsistent with this theory (Giustino and Maren 2015). For instance, inactivation of the PL has been shown to enhance reacquisition of alcohol seeking (Willcocks and McNally 2013) or reduce conditioned suppression of cocaine seeking in rats (Limpens et al. 2015), while optogenetic activation of PL pyramidal cells was found to decrease cocaine seeking in rats (Chen et al. 2013). By comparison, pharmacological inactivation of the PL has been shown to have no effect on expression of conditioned fear (Bravo-Rivera et al. 2014), while others report that there are no differences between the PL and IL in firing patterns in response to a CS or context associated with a footshock (Baeg et al. 2001). These and other data have led to the idea that at least under some conditions the PL and IL act in concert. Even so, it is predominantly believed that the PL and IL are able to code for opposing behaviors in terms of drug-seeking and conditioned fear.

1.7 Prefrontal dopamine system

The "top down" processing required for successful extinction learning involves precise control of neural networks, in order to integrate and organize dynamic information from multiple inputs, as well as drive output to relevant nodes in the extinction circuitry. This kind of control naturally requires some level of computation involving complex signaling via the major inhibitory and excitatory transmitter systems, including GABA via $GABA_A$ receptors and glutamate via NMDA and AMPA receptors (Miller and Cohen 2001). However, it is now widely recognized that computation in PFC neural networks is actually critically refined by the activity of the dopamine system (see Seamans and Yang 2004 for review).

Dopamine exerts its effects via five G protein-coupled receptors (GPCRs), which are broadly divided into two groups: the include dopamine 1-like receptors (dopamine 1 receptor [D1R] and D5R) and the dopamine 2-like receptors (D2R, D3R, D4R). In rats, D1R and D2R exhibit the highest levels of expression throughout the brain including the cortex (Beaulieu and Gainetdinov 2011), and in humans messenger ribonucleic acid (mRNA) encoding the D1R is the most abundant transcript in the cortex, with D2R the third highest after D4R (Meador-Woodruff 2000). In the prefrontal cortex, dopamine exerts its effects via D1Rs and D2Rs expressed primarily on parvalbumin-positive fast-spiking interneurons (Gorelova et al. 2002; Le Moine and

Gaspar 1998). However, both D1Rs and D2Rs are also expressed on prefrontal pyramidal neurons, allowing dopamine to play a direct as well as an indirect role on prefrontal output (Santana et al. 2009). Being GPCRs, activation of D1R and D2R is able to stimulate a range of second messenger pathways and effector proteins (Seamans and Yang 2004). However, generally, D1R activation tends to enhance the excitability of interneurons, which increases inhibitory postsynaptic currents (IPSCs) in pyramidal neurons. In contrast, D2R stimulation typically reduces IPSCs in pyramidal cells (Seamans et al. 2001b). Thus, the effects of dopamine on PFC networks tend to be bidirectional, depending on whether D1R or D2R signaling is dominant at any given time (Seamans and Yang 2004). Notably, dopamine signaling via D1Rs and D2Rs does not act as an excitatory or inhibitory neurotransmitter, but as a *neuromodulator* (Seamans and Yang 2004). Therefore its precise actions in the PFC depend on a variety of internal and external factors, such as duration of receptor activation, history of activity in the network, and existing membrane potentials (Seamans and Yang 2004; Miller 2000). Extensive evidence now highlights the modulatory importance of dopamine not only for gating incoming sensory information to the PFC, but also for directing PFC output to other brain regions (Del Arco and Mora 2005; 2008). In fact, dopamine signaling in the PFC is crucial for communication with the NAc (Floresco and Tse 2007), as well as with the amygdala (see Abraham et al. 2014 for review). Given the known importance of PFC output to these regions for extinction, it makes sense that studies are beginning to highlight PFC dopamine signaling in both fear- and drug-related extinction learning.

Table 1.1 *Summary of dopamine receptor subtype manipulation effects on extinction learning in adult rodents.*

	Target	Drug	Route/dose	Extinction effects	Test effects	Study
Cocaine	D1 agonist	SKF81297	Systemic (10 mg/kg)	N/A (administered immediately after)	Facilitated	Abraham et al 2016
	D1 antagonist	SCH23390	Systemic (0.4, 1 mg/kg)	N/A (administered immediately after)	Impaired	Fricks-Gleason et al 2012
Fear	D1 agonist	SKF81297	Systemic (1, 3, 10 mg/kg)	Facilitated (1, 3, 10 mg/kg)	Facilitated (10 mg/kg)	Abraham et al 2016
	D1 agonist	SKF38393	Systemic (10 mg/kg)	No effect	No effect	Rey et al 2014
	D1 antagonist	SCH23390	Intra-IL (0.25μg/0.5μL/ hemisphere)	Impaired	Impaired	Hikind & Maroun 2008
	D2 agonist	Quinpirole	Systemic (5 mg/kg)	N/A (administered immediately after)	Impaired	Nader & LeDoux 1999
	D2 agonist	Quinpirole	Systemic (1 mg/kg)	No effect	No effect	Ponnusamy et al 2005
	D2 antagonist	Raclopride	Systemic (0.3 mg/kg)	Impaired (with catalepsy)	Impaired	Mueller et al 2010
	D2 antagonist	Sulpiride	Systemic (20 mg/kg)	Facilitated	Facilitated	Ponnusamy et al 2005
	D2 antagonist	Haloperidol	Systemic (0.05, 0.1, 1 mg/kg)	Impaired	Impaired	Holtzman-Assif et al 2010
	D2 antagonist	Haloperidol	ICV (0.25μg/0.25μL/ hemisphere)	No effect	Impaired	Holtzman-Assif et al 2010
	D2 antagonist	Raclopride	Intra-IL (5μg/0.5μL/ hemisphere)	No effect	Impaired	Mueller et al 2010
	D4 antagonist	L-741,741	Intra-IL (5, 10 ng/μL hemisphere)	No effect	Impaired	Pfeiffer & Fendt 2006
	Dopamine precursor	L-DOPA	Systemic (20 mg/kg)	No effect	Facilitated	Haaker et al 2013

Indeed, presentation of cues previously associated with a natural reward (Feenstra et al. 2001; Wędzony et al. 1996) or a footshock (Milella et al. 2016) have been shown to increase dopamine in the PFC of adult rats. Similarly, in humans with cocaine dependence, presentation of drug-related cues also induces dopamine release in

the PFC (Abraham et al. 2016). This suggests that emotionally salient cues, and by extension cue extinction, involves dopamine efflux in the PFC. It follows that manipulations of dopamine signaling via D1R and D2R have shown effects on extinction learning across both appetitive and aversive domains in adult rodents (**Table 1.1**). One study using a cocaine CPP paradigm showed that pre-extinction systemic treatment with a D1R agonist facilitated extinction in adult rats both within-session and when tested drug-free the next day (Abraham et al. 2016). Conversely, pre-extinction systemic treatment with a D1R antagonist impaired extinction of a cocaine CPP in adult rats (Fricks-Gleason et al. 2012). Similarly, pre-extinction systemic treatment with a D1R agonist enhanced extinction of cued and contextual fear in adult rats (Abraham et al. 2016), though a partial D1R agonist had no effects on extinction acquisition or retrieval (Rey et al. 2014). Intra-IL infusion of a D1R antagonist also impaired long-term fear extinction in adult rats when tested 24 hours later (Hikind and Maroun 2008). Overall, it appears that dopamine signaling via D1R is a key mediator of extinction learning in the adult rat, with a potential mechanism for this effect in the IL.

By comparison, the role of D2R signaling in extinction learning is less clear. For instance, one study showed that pre-extinction systemic treatment with the D2R agonist quinpirole blocked extinction of conditioned fear when tested immediately after extinction (Nader and LeDoux 1999), while another showed largely no effect across a range of doses (0.25, 0.5, 2.0 mg/kg), though one dose (1.0 mg/kg) impaired long-term extinction tested the next day (Ponnusamy et al. 2005). However, pre-extinction systemic or central (intracerebroventricular; i.c.v.) treatment with a D2R antagonist has also been shown to increase freezing across extinction training and when tested the next day (Holtzman-Assif et al. 2010), though others have found pre-extinction D2R antagonism facilitates extinction both within-session and at test the next day (Ponnusamy et al. 2005). One known study that tested an intra-IL D2R antagonist showed impaired fear extinction in adult rats tested the next day (Mueller et al. 2010). To the best of my knowledge, no studies have explicitly examined the role of D2R signaling in drug-related extinction learning.

Overall, there is increasing support from studies using adult rodents for the importance of prefrontal dopamine in mediating extinction learning and memory, with evidence for D1R and D2R as important mediators for this type of learning. It follows

that in cases where extinction fails to reduce either drug seeking or conditioned fear this may be explained by dysfunction of the prefrontal dopamine system, especially in the IL.

1.8 Prefrontal dopamine during adolescence

The PFC undergoes dramatic reorganization during adolescence (Kolb et al. 2012; Spear 2000). Studies in rodents show that adolescent PFC modifications include growth and myelination of both efferent and afferent axons and dendrites, as well as reorganization and maturation of synapses and alterations in neurochemistry including GABAergic activity (Cunningham et al. 2002; 2008; Tseng and O'Donnell 2007a). There is also a strong body of evidence from human studies to suggest the actual volume of PFC decreases during adolescence due to decreases in prefrontal grey matter (Giedd 2004; Paus et al. 2008; Gogtay et al. 2004). It has been proposed that the developmental trajectory of PFC grey matter is in fact "cubic" (Shaw et al. 2008). That is, the PFC gradually develops to peak in volume during pre-adolescent childhood, then shrinks radically during adolescence, and subsequently increases again to stabilize by early adulthood. Importantly, these neurobiological changes in the PFC are thought to contribute to the typical changes in cognition and behavior observed during adolescence (Steinberg 2005). Explicitly, the PFC is proposed to have a similar role in adolescence as in adulthood, but is compromised during the former period in terms of efficiency, speed and capacity (Kim et al. 2011). For these reasons, it has been argued that adolescence represents not a phase of apparent "development" of the PFC, but a period of "maturation" or refinement (O'Donnell 2010; Wahlstrom et al. 2010).

Notably, adolescent changes in the PFC also encompass changes in the dopamine system. Infiltration of dopaminergic fibres from the VTA to the PFC increases constantly through adolescence until early adulthood. This is observed in rodents (Kalsbeek et al. 1988) as well as non-human primates (Rosenberg and Lewis 1995; 1994). In the rat, PFC dopamine synthesis is increased during adolescence (Andersen et al. 1997), while cortical concentrations of dopamine are also reported to peak during adolescence in non-human primates (Brown and Goldman 1977; Goldman-Rakic and Brown 1982). In rats this is accompanied by a peak in D1R gene expression (Garske et al. 2013) and DIR protein expression on glutamatergic projection neurons, at least in the PL of the PFC (Andersen et al. 2000). By comparison, D2R density in rat

PFC has been found to increase constantly until around age P35, when it reaches stable adult levels (Tarazi et al. 1999). Importantly, a D1R-dominant profile has also been reported in human adolescent PFC, where post-mortem *in situ* hybridization revealed significantly greater D1R gene expression in the PFC of adolescents compared to adults, while D2R gene expression showed no differences across these age groups (Weickert et al. 2007; Rothmond et al. 2012). Functional data also point to changes in dopamine activity in the adolescent PFC. For instance, the excitatory effect of D2Rs on PFC interneurons was observed in adult rats but not in adolescents (Tseng and O'Donnell 2007b). Overall, these natural changes in the prefrontal dopaminergic system are thought to play an important role in PFC function during adolescence (Somerville et al. 2010). In fact, these age-related alterations in the dopamine system may help to explain the impaired efficiency of PFC function observed during adolescence (Steinberg 2005). Given the known importance of the PFC and increasing evidence for dopamine signaling in extinction learning, it follows that dopamine dysfunction in the adolescent PFC may help to explain impaired responding to extinction-based treatments for addiction and anxiety disorders at this age.

1.9 Adolescent extinction

Current literature investigating the neurobiology of adolescent extinction learning is extremely limited. One known study showed that extinction of cocaine CPP is impaired in adolescent rats (age P38) compared to adult rats (age P77) (Brenhouse and Andersen 2008). Specifically, adolescents required significantly more days to extinguish and reinstated more robustly to cocaine-associated contextual cues. However, these findings were confounded by age differences in initial conditioning, such that adolescents showed stronger cocaine-associated place preference throughout the experiment compared to adults. In one study using a cocaine IVSA paradigm, adolescent rats (P27-29 on IVSA day 1) displayed increased lever pressing during extinction compared to adult rats (Anker and Carroll 2010). In that study, adolescents also displayed increased drug- and stress-induced reinstatement following extinction, whereas adults responded significantly more following presentation of a drug-associated cue. However, in that study, adolescents acquired stronger self-administration, confounding findings on extinction. Also, a light CS signaled reward delivery in all cocaine self-administration sessions, while during extinction, the CS was absent (i.e., instrumental extinction, **Figure 1.3**). The CS was only turned on again to induce reinstatement, without the CS

ever being extinguished. Therefore, from these studies, specific conclusions cannot be drawn on age differences in drug-related extinction.

Conditioned fear studies during adolescence suggest that aversive extinction learning may be impaired in adolescents due to age-related changes in IL function. In a study that compared cue-elicited fear responding in preadolescent (P24), adolescent (P35) and adult (P70) rats, there was no difference in acquisition or within-session extinction of the conditioned response (Kim et al. 2011). However, when tested next day, adolescent rats showed impairment in extinction memory and returned to high rates of freezing, while preadolescent and adult rats showed low freezing responses. This study also revealed decreased activation of the MAPK/ERK signaling pathway in the IL following extinction in the adolescent group only, suggesting extinction deficits may relate to decreased activation of this region in adolescent animals. The same group showed that systemic administration of a partial NMDA receptor agonist 10 minutes but not 4 hours following extinction training improved extinction retention in adolescents (McCallum et al. 2010). In both studies, doubling the number of extinction sessions improved extinction learning in adolescent rats (Kim et al. 2011; McCallum et al. 2010). In a study that examined fear extinction learning in juvenile (P23), adolescent (P29), and adult (P70) mice, adolescents showed impaired extinction over multiple days compared to both younger and older age groups (Pattwell et al. 2012). The same study showed a similar impairment in fear extinction learning in adolescent humans compared to children and adults. In mice, juvenile and adult extinction learning was accompanied by modifications in glutamatergic synaptic transmission in IL pyramidal neurons. This included increased AMPA/NMDA ratio, and changes in excitatory postsynaptic potentials. Critically, these modifications were not observed in the adolescent IL, further highlighting this region in the potential circuitry underlying adolescent extinction deficits.

Collectively, these results suggest that the observed reduction in expression of fear extinction relates to impairment in the consolidation and retention, rather than the acquisition or expression of extinction learning. In addition, adolescent deficits in extinction consolidation appear to involve age-related differences in IL activity. We know that the PFC undergoes reorganization during adolescence, including changes in dopamine signaling, which contributes to age-related differences in functionality of this

region. Since this system is known to change dramatically during adolescence, it may be these natural maturational changes contribute to extinction impairments at this age. However, this idea has not been extensively explored using animal models. The hypothesis of the present project is that natural developmental differences in the prefrontal dopamine system contribute to deficits in cue extinction learning during adolescence compared to adulthood.

1.10 Project aims

The overall aim of this thesis is to investigate the role of the prefrontal dopamine system in adolescent extinction learning using rat models. Clinically, adolescents display poorer outcomes following extinction-based treatment for substance abuse and anxiety disorders compared to adults. This is consistent with evidence from existing rodent research that adolescents show impairments in extinction learning across appetitive and aversive domains. Adolescence represents a period of significant neurobiological alterations in the PFC, a region known to be critically involved in extinction. This includes changes in the dopamine system, a key modulator of PFC neural networks as well as PFC output. Studies from adult subjects indicate that dopamine in the PFC is involved in extinction learning. However, the role of dopamine in adolescent extinction learning is yet unknown.

Thus, the present thesis examined the role of prefrontal dopamine in adolescent extinction across appetitive and aversive domains. The first study (Chapter 3) investigates adolescent extinction learning and memory in a cocaine self-administration paradigm. The second study (Chapter 4) examined adolescent extinction in a fear conditioning paradigm. The final study (Chapter 5) examined changes in dopamine receptor gene expression in the mPFC of naïve adolescent and adult rats, and in rats that received extinction of cocaine- or shock-associated cue.

2 General Methods

2.1 Subjects

Male Sprague-Dawley rats were bred in-house at the Florey Institute of Neurosciences and Mental Health (Melbourne, VIC, Australia). Stock originated from Animal Resources Centre (ARC; Perth, WA, Australia). The colony was maintained as a standard out-bred line, with breeder stock from ARC brought in every six months. For all experiments, room temperature was maintained at 22 °C with food and water available *ad libitum*. All rats were handled 3 times prior to surgery or commencement of behavioral experiments. Rats were postnatal day (P)34 or P69 ±1 at the commencement of cocaine self-administration (and P53 or P88 on cocaine-cue extinction day). Rats were P35, P53, or P88 ±1 on fear extinction day. Rats were housed 1 – 4 to a cage depending on age and surgery type (refer to 2.2.2 and 2.2.3). For cocaine self-administration experiments, adolescents were group-housed until the day of surgery (P30), and were handled daily from the time of being individually housed, thus minimizing any potential stress of isolation from a young age. Rats in cocaine self-administration experiments were housed in reverse light-dark housing, while rats in conditioned fear experiments were housed in either reverse light-dark or standard housing (details in **Table 2.1**). For all experiments, litter effects were avoided by minimising the number of animals used from single litters, splitting litters across control and treatment groups, and repeating experiments across multiple litters. All experiments were approved by the Florey Animal Ethics Committee, and performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Table 2.1 *Housing conditions for rats across different experiments.*

	Reverse light-dark	**Standard**
Cage type	Open-top cage	Individually-ventilated cage
Light-dark cycle	12:12	12:12
Lights on	7:00 p.m.	7:00 a.m.
Behavioral experimentation	Dark phase	Light phase

2.2 Surgery

2.2.1 Construction of jugular catheters

Jugular catheters were constructed in-house (**Figure 2.1**). The guide cannula consisted of 22-gauge steel tubing extending 5 mm above and 11 mm below a plastic guide pedestal (Plastics One, USA). The 11 mm end was bent using rounded pliers into a right angle arc. Silastic inner tubing (internal diameter 0.51 mm; external diameter 0.94 mm; adult length 14 cm, adolescent length 12 cm; Dow Corning, USA) was soaked briefly in terpene and threaded onto the 11 mm end of the guide cannula up to the level of the pedestal. After drying for 24 hours, Silastic outer tubing (internal diameter 1.47 mm; external diameter 1.96 mm; length 2 cm) was soaked in terpene then sleeved over the inner tubing up to the level of the pedestal and secured using silicone (Dulux Group Pty Ltd, Australia). After drying for 24 – 48 hours, the guide cannula was embedded in dental cement (Vertex, MA, USA) using a custom cast (circle diameter 1.2 cm, depth 3 mm). Squares of polyester mesh (2 cm x 2 cm, Sefar Pty Ltd, Australia) were attached to the back of the dental cement catheter port to provide subcutaneous anchorage. A ball of silicone was applied around the inner tubing 3.0 cm from the vein-entering end for adults and 2.0 cm from the vein-entering end for adolescents. A second, smaller ball of silicone was applied 0.5 cm closer to the vein-entering end of the catheter, and allowed to dry. Catheters were tested for leakage by flushing with saline, and sterilized briefly by washing in 80% v/v ETOH before surgical implantation.

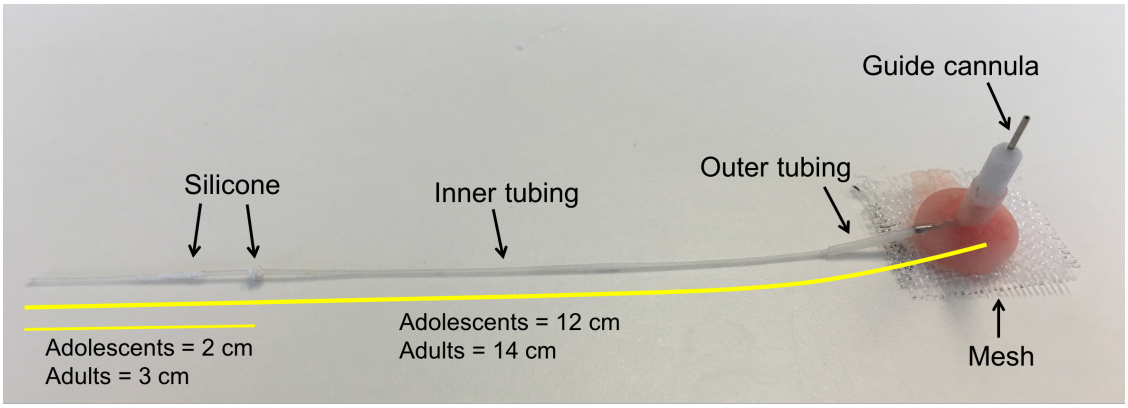

Figure 2.1 *Rat jugular catheter.*

2.2.2 Jugular catheterization

Rats were anaesthetized using isoflurane vaporized in oxygen (5% v/v for induction, 2-3% v/v for maintenance) and injected with meloxicam (3 mg/kg, s.c.) for pain relief. Following the absence of reflex arc activity, incision sites were shaved washed with iodine. A subcutaneous pocket was formed just below the level of the scapulae. The distal end of the catheter was implanted under the skin with the 5 mm section of guide cannula and part of the plastic pedestal protruding through an incision on the dorsal midline. The attached Silastic tubing was guided subcutaneously above the left upper limb and around to the ventral aspect of the animal. The right jugular vein was then isolated and tied off using silk suture (size 4/0; Dysilk, Dynek Pty Ltd, Australia) to prevent haemorrhage. A small incision was made in the vein and the catheter was inserted into the vein until the larger silicone ball was flush with the vein opening. The catheter was anchored in the vein using the same silk suture above and below the silicone ball. All incision sites were closed with surgical suture, cleaned and treated topically with Tricin antibacterial powder (Jurox Pty Ltd, Australia). A single injection of antibiotic (Baytril, Bayer Corporation; 10 mg/kg, i.p.) was administered to prevent infection. For experiments not requiring intracranial cannulation, rats were allowed to recover under observation in a heated (30.2 ˚C) recovery chamber. Following jugular catheterization surgery, all rats were housed individually for the duration of experimentation. To prevent infection and maintain catheter patency, catheters were flushed daily for 2 days following surgery with 0.05 mL of heparinized saline (90 IU/mL; Pfizer, NY, USA) containing 10% Fisamox antibiotic (amoxicillin sodium; Aspen Australia, NSW, Australia). Catheters were then flushed daily with 0.05 mL of 10 and 50 IU/mL antibiotic-heparin solution before and after cocaine self-

administration, respectively. Patency was tested weekly using 0.03 mL of ketamine (100 mg/mL) for adult and 0.02 mL for adolescent rats immediately followed by 0.05 mL of 10 IU/mL antibiotic-heparin solution. Any rat that failed to show loss of muscle tone within 10 seconds was removed from the study.

2.2.3 Intracranial cannulation

If not already anaesthetized from jugular catheterization, rats were anaesthetized using isoflurane vaporized in oxygen (5% v/v for induction, 2-3% v/v for maintenance) and injected with meloxicam (3 mg/kg, s.c.) for pain relief. The scalp was shaved and washed with iodine, then the head secured in a stereotaxic frame (David Kopf Instruments, CA, USA). The scalp was incised at midline and tissue was cleared to reveal the skull. Four screws (PlasticsOne, VA, USA) were implanted in the surface of the skull to provide anchorage for the dental cement cap used to hold the guide cannula in place. Measurements of bregma and lambda were equated to ensure the skull was level, before holes were made in the skull in order to insert a 26-gauge bilateral guide cannula (PlasticsOne, VA, USA). The cannula was secured using dental cement (Vertex, MA, USA), which covered the screws and the bottom half of the cannula pedestal. Once the dental cement had hardened, cannula obturators extending 1 mm below the guide cannulas were inserted and covered with a metal cap. Rats were allowed to recover from the anaesthetic under observation in a heated (30.2 ˚C) recovery chamber. Rats that received intracranial surgery only (i.e., without the jugular catherization) were housed 2 – 4 to a cage. Rats received antibiotic (Baytril, 10 mg/kg, i.p.) daily for 3 days following surgery. Obturators were checked and rats were weighed daily after surgery until behavioral experimentation commenced.

Coordinates used to target the IL were based on a previous study (Orsini et al. 2011) and measurements from the Rat Brain Atlas (Paxinos and Watson 1998). Coordinates were then optimized using an injection of methylene blue (100 nL), where rats received intracranial infusions of dye, then were killed by intraperitoneal injection of sodium pentobarbitone (>100 mg/kg; 1 ml/kg i.p.) and brains processed for visualization of injection sites. Final dorsoventral, mediolateral and anteroposterior coordinates are displayed in **Table 2.2**.

Table 2.2 *Stereotaxic coordinates. Bilateral guide cannula targeted the IL.*

Age	Dorsoventral	Mediolateral	Anteroposterior
Adolescent (P35)	−4.2 mm	±0.6 mm	+3.0 mm
Adult (P88)	−4.6 mm	±0.6 mm	+3.0 mm

2.3 Drugs

2.3.1 Intracranial infusions

The bilateral infusion (0.5 µL/hemisphere) consisted of either vehicle (saline), the selective D1R agonist SKF-81297 (dissolved in saline; 0.1 µg/hemisphere; Sapphire Bioscience, Australia), or the selective D2R agonist quinpirole (dissolved in saline; 1 µg/hemisphere; Tocris, UK) into the IL over 2 minutes. The 33-gauge infusion cannula extended 1 mm below the guide cannula, and remained in place for 2 minutes following the infusion, and then rats immediately underwent cocaine-cue or fear cue extinction training.

At the end of experimentation, cannula placements were validated by an experimenter who was blind to subject treatment. Fresh brains were frozen on dry ice, then 40 µm sections were cut in a cryostat, mounted on slides (Menzel-Glaser, Lomb Scientific, Australia), and stained with cresyl violet (**Figure 2.2**).

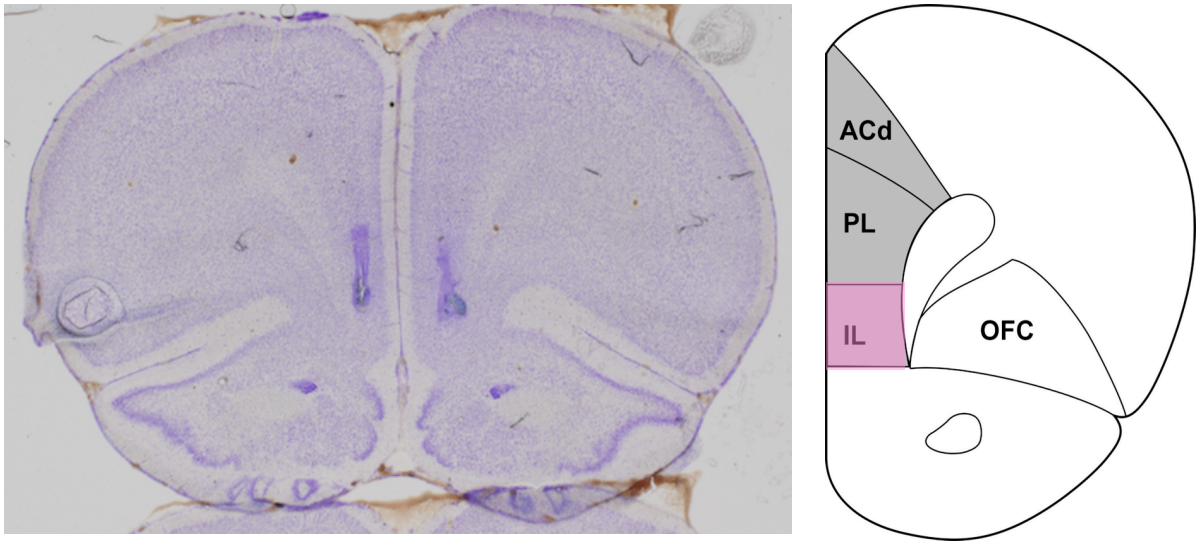

Figure 2.2 *Representative light microscope image showing cannula validation. Coronal brain section stained with cresyl violet shows cannula tracts targeting the IL (pink).*

2.3.2 Systemic injections

For systemic drug injections, rats received a subcutaneous injection 30 minutes before commencement of the cocaine-cue or fear cue extinction session. Injections consisted of either vehicle (5% v/v Tween 80 in saline; Sigma-Aldrich Co., MO, USA) or the partial D2R agonist aripiprazole (Alliance Biotech, India; 5 mg/kg) suspended in vehicle.

2.4 Behavioral procedures

2.4.1 Cocaine self-administration chambers

Cocaine self-administration experiments were conducted in operant conditioning chambers (29.5 x 32.5 x 23.5 cm; Med Associates, VT, USA; **Figure 2.3**). The ceiling, back wall and door of each chamber were made of clear Perspex, and the side walls consisted of aluminium channels to hold metal modular components. The floor was covered with a grid consisting of 4.8 mm diameter stainless steel rods set 16 mm apart, above a catch pan. Two retractable levers 4 cm wide and 6 cm from the grid floor were located 12 cm apart on one side wall. Levers protruded 1.9 cm from their modular panels and were factory set to a tension of 25 g. A white stimulus light (28 V) 2.5 cm in diameter was located directly above each lever. A house light (24 V) was located on the opposite wall, however this was turned off for the duration of all experiments. A vanilla scent in the form of a drop of vanilla essence in a plastic cap was present beneath the grid floor directly below the active lever to serve as a discriminatory cue for the active versus inactive lever. The vanilla scent was present whenever the active lever was presented, even during lever extinction and cue-induced reinstatement when active lever had no programmed consequences. Therefore, it served as discriminative cue for the location of the lever rather than for the presence of cocaine. Pressing on the inactive lever was recorded but had no programmed consequences at any time, and there was no scent beneath the inactive lever. Levers were counterbalanced such that for half of the rats in each condition the active lever was closest to the door and for the other half the active lever was closest to the back wall.

Chambers were enclosed inside medium density fiberboard (MDF) sound attenuation cabinets equipped with ventilation fans, in order to reduce external noise

and visual stimulation. Above the ceiling of each operant chamber, a counterbalanced arm held a flexible swivel attached at one end by polyethylene tubing to a syringe containing cocaine dissolved in saline. Syringes were mounted in a motor-driven pump (MedAssociates, VT, USA) located outside the cabinet. At the other end of the swivel, a length of polyethylene tubing sheathed by a stainless steel tether enabled the syringe to connect each rat's guide cannula. This connection formed a closed system whereby rats would receive drug infusions directly from the syringe that was connected at the other end to the tubing. Chambers had a 5.1 cm diameter hole in the centre of the ceiling with a 0.6 mm access slot from hole to front edge, allowing placement of animal into chamber with tether attached. The operant conditioning chambers and infusion pumps were controlled by MED-PC software custom-written using Trans-IV (MedAssociates Inc, VT, USA). Data were recorded using two Nexlink Intel Core computers (Seneca Data Distributors). Cabinets were housed inside rooms containing red lights, to maintain rats in the dark phase of the light/dark cycle during self-administration sessions.

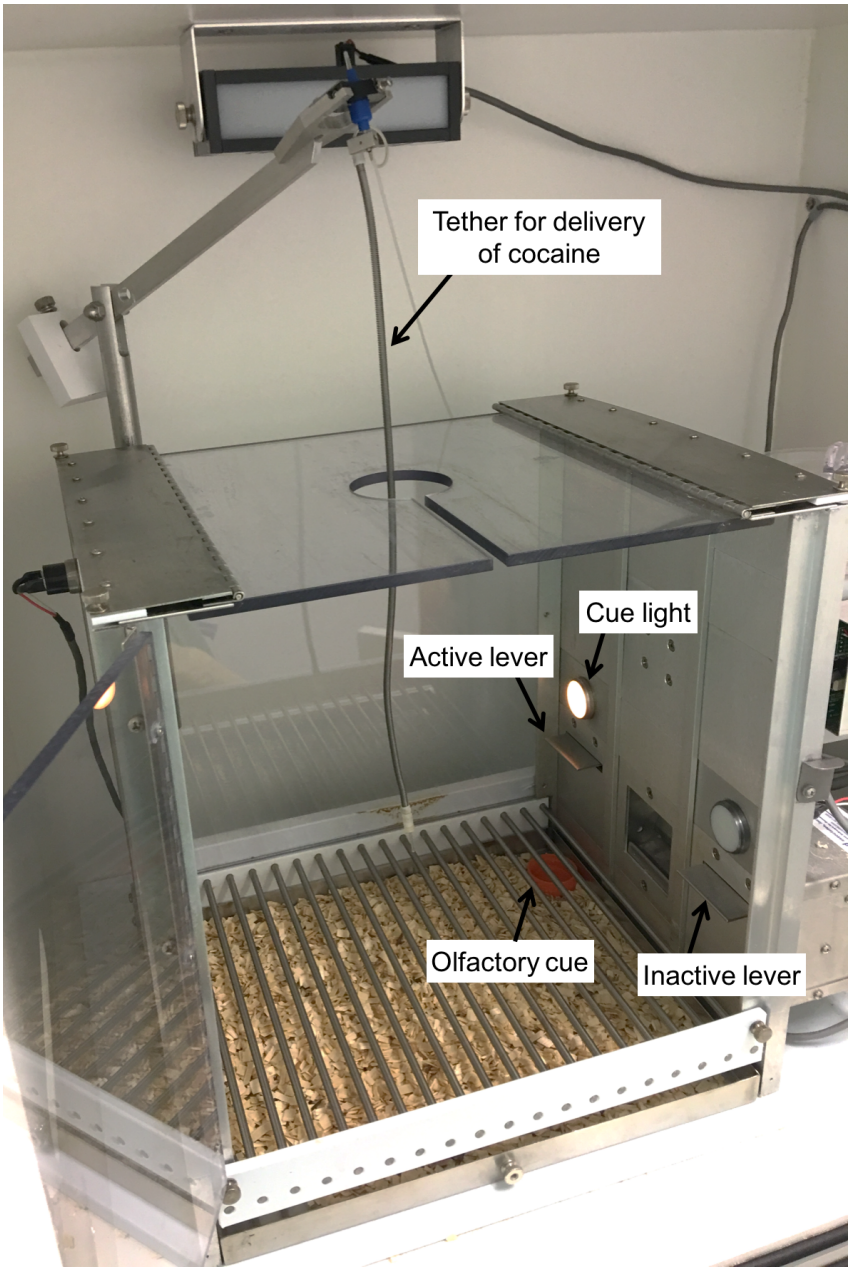

Figure 2.3 *Cocaine self-administration chamber. An olfactory cue is located directly underneath the active lever. Cocaine infusions are paired with illumination of a cue light directly above the active lever.*

2.4.2 Cocaine self-administration behavioral protocol

2.4.2.1 Cocaine self-administration

Rats were trained to self-administer cocaine (cocaine hydrochloride dissolved in saline; Johnson Matthey Macfarlan Smith, Edinburgh, UK) in daily 2-hour sessions. During these sessions, pressing on the active lever resulted in a 50 µL infusion of cocaine (0.3 mg/kg per infusion), which served as the US. Cocaine concentrations were customized for the weight of each rat, updated every 3 days to allow for weight gain over experimentation. Each cocaine infusion occurred over 2.7 seconds, paired with 2.7

second illumination of the light located above the active lever, which served as the CS. Cocaine infusions were followed by a 17.3 s time-out period during which pressing on the active lever was recorded but did not result in cocaine administration. For the first 5 but no more than 7 days, rats self-administered cocaine under a fixed ratio (FR) 1 schedule, where one active lever press led to one cocaine infusion. For the final 5 days of self-administration, responding occurred under FR3, where three active lever presses were required for one cocaine infusion. An increase in lever pressing at FR3 reinforcement schedule would indicate motivated behavior to obtain cocaine, as opposed to random lever pressing. Any rat that failed to self-administer at least 7 infusions of cocaine per session averaged across the last 5 days of self-administration was excluded from subsequent behavioral testing and analyses.

On the penultimate day of self-administration in the first experiment in Chapter 3, approximately half the rats received a single progressive ratio (PR) session. In this session, the number of active lever responses required to receive an infusion increased incrementally. Lever pressing during this session provides a measure of motivation to self-administer a drug, by measuring how many lever presses an animal is willing to make for one infusion (Farid et al. 2012). The PR session ran for a maximum of 4 hours, but terminated automatically if no lever pressing occurred for more than 1 hour. For the experiment where rats received the PR session, rats went back onto FR3 for one 2-hour self-administration session the next day.

2.4.2.2 Lever extinction

The day after the final cocaine self-administration session, rats commenced daily 1-hour lever extinction sessions. In these sessions, pressing on either the active or inactive lever had no programmed consequences. Thus pressing on the previously active lever did not result in a cocaine infusion or illumination of the CS light. Lever extinction occurred daily for seven days.

2.4.2.3 Cocaine cue extinction

The day after the final lever extinction session, animals received a single cocaine-cue extinction session without levers present. This session was designed to model CET for substance use disorder, which typically involves presentations of drug-associated cues in the absence of drug-taking behavior (Conklin and Tiffany 2002). Following a 2-

minute baseline period, the 2.7 second CS light above the previously active lever was presented every 30 seconds 120 times. For some experiments, a control group did not receive cue extinction but were handled for 2 minutes by the experimenter (Pre-extinction) while an experimental group did receive cocaine-cue extinction (Post-extinction). Since there was no lever present for cue extinction, there was no vanilla scent in chambers for this session. For experiments where rats were assigned to different groups for cocaine cue extinction (e.g. No Cue Extinction vs Cue Extinction; Vehicle versus Drug), groups were balanced for operant responding during self-administration and lever extinction.

2.4.2.4 Cue-induced reinstatement

For Chapter 3, the day after cue extinction or no cue extinction rats underwent a single 1-hour cue-induced reinstatement session, where pressing the previously drug-paired (active) lever resulted in illumination of the CS light above that lever under an FR3 schedule. If a rat failed to press the previously drug-paired lever within the first 2 minutes of the reinstatement session, the CS illuminated automatically once (2.7 seconds). There was no cocaine present for the cue-induced reinstatement session.

2.4.3 Fear conditioning chambers

All fear conditioning experiments used fear conditioning chambers (31.8 x 25.4 x 26.7 cm) equipped with the near infra-red (NIR) VideoFreeze system (Med Associates, VT, USA; **Figure 2.4**). A grid floor consisting of 4.8 mm stainless steel rods set 16 mm apart allowed delivery of electric footshocks. A speaker positioned in one wall of each chamber was used to produce a tone (5000Hz, 80dB). Chambers were housed in MDF cabinets insulated with acoustical soundproof foam to minimize external noise. A ventilation fan in each cabinet produced low-level constant background noise. Chambers contained an NIR fear conditioning system and a monochrome video camera equipped with 8.0 mm lens and NIR pass filter was attached to the inside of each cubicle to record behavior. All tone and footshock presentations were controlled and recorded by VideoFreeze software (Med Associates, VT, USA). Fear memory was quantified by levels of freezing behavior, defined as a motion threshold of less than 50 pixel changes for a minimum of 1 second duration using the VideoFreeze software (Med Associates, VT, USA). These criteria show high concordance with manual

scoring as previously described (Ganella et al. 2016). Time spent freezing is expressed as a percentage of the total duration of CS presentation.

Two separate rooms representing two different experimental contexts housed 4 conditioning chambers each. In one context, the back wall of the chambers was covered with a plastic spot-patterned cover and a tray containing woodchip bedding was located underneath the grid floor (**Figure 2.4A**). In this context, chambers were cleaned with eucalyptus-scented disinfectant before each session and a white houselight remained on in each chamber for the duration of all sessions. In the other context, chambers were fitted with a curved white wall that covered the sides and back wall of the chamber, trays beneath the grid floor contained paper towel, houselights were off for the duration of all sessions and a red light was on in the room (**Figure 2.4B**). Chambers in this context were cleaned with ethanol (80% v/v in water) before each session. The two contexts served as conditioning or extinction/test contexts in a counterbalanced manner.

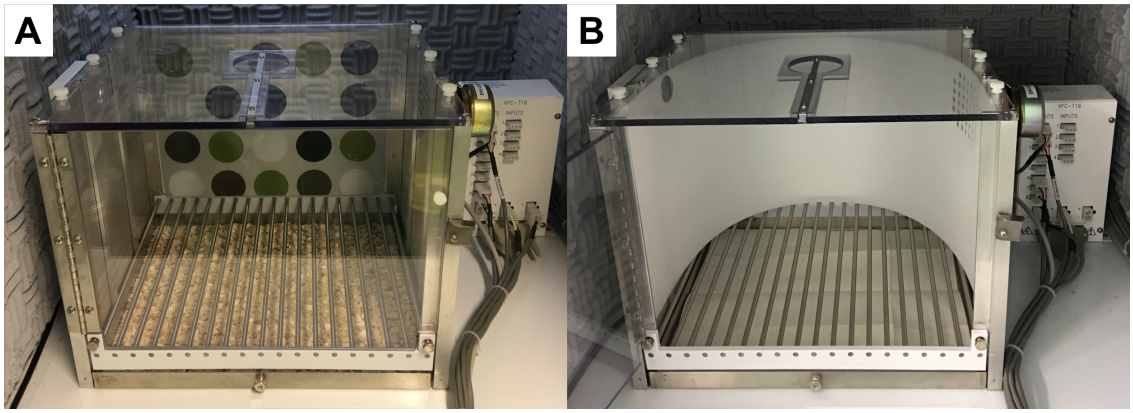

Figure 2.4 *Fear conditioning chambers. Contexts differed in terms of lighting, odor, wall shape/pattern, and bedding.*

2.4.4 Fear conditioning behavioral protocol

2.4.4.1 Fear conditioning

Rats were placed in the chambers and their baseline level of freezing was recorded for 2 minutes. The CS tone (80 dB) was then presented for 10 seconds and co-terminated with a 1 second footshock (0.6 mA). There were three CS-US pairings and the inter-trial interval (ITI) between each pairing was 85 then 135 sec. Following the last CS-US pairing, rats remained in the chambers for 2 minutes before returning to their home cages.

2.4.4.2 Fear extinction

The day after fear conditioning, rats received extinction in the context different to conditioning. Baseline freezing was measured for the first 2 minutes, followed by 30 CS alone trials with a 10-second ITI. For the gene expression experiment in Chapter 4, control groups did not receive fear extinction but were handled for 2 minutes by the experimenter.

2.4.4.3 Test

For Chapter 4, rats were tested 24 hours following extinction in the same context as extinction training. Baseline freezing was measured for the first minute, followed by a 2-minute presentation of the CS alone. Rats remained in the chambers for 1 minute before returning to their home cages.

3 Role of dopamine 2 receptors in impaired drug-cue extinction in adolescent rats

3.1 Introduction

Adolescence represents a unique period of vulnerability for the development of substance use disorders (Spear 2000). Adolescent drug users are less responsive to behavioral treatment interventions such as CET, based on the principle of cue extinction (Conklin and Tiffany 2002). Adolescents are also more likely to relapse following re-exposure to cues associated with the drug-taking experience compared to adults (Catalano et al. 1990; Perepletchikova et al. 2008; Winters et al. 2011; Ramo and Brown 2008). Poorer outcomes following extinction-based treatment, in addition to increased likelihood of relapse on re-exposure to drug-related cues, may be explained by differences in drug-cue learning during this unique developmental period. As discussed in Chapter 1, studies using adult and adolescent rats suggest that the salience of drug-associated cues is mediated by D1R activity on glutamatergic projections from the PL of the mPFC to the NAc core (Kalivas and Duffy 1997; Brenhouse et al. 2008). By comparison, evidence from adult rodents suggest that extinction learning is largely controlled by projections from the IL of the mPFC to the NAc shell, and may involve D1R and/or D2R signaling (Peters et al. 2008; Haaker et al. 2013; Mueller et al. 2013).

The mPFC undergoes dramatic alteration during adolescence (Kolb et al. 2012), including changes in the dopamine system (Somerville et al. 2010). Critically, developmental differences in prefrontal dopamine have been implicated in adolescent drug-context extinction learning (Brenhouse et al. 2010). However, to the best of my knowledge, the role of prefrontal dopamine in extinction of a discrete drug-associated cue has never been previously investigated. Extinction of a discrete cue associated with drug self-administration, as well as cue-induced relapse-like behavior, can be examined using the IVSA paradigm in rats. This model represents an effective way to not only examine the neurobiology of drug-cue extinction, but to test pharmacological adjuncts

that might improve cue extinction learning to reduce relapse-like behavior. The following paper, published in *Cerebral Cortex* in June 2016 (Volume 26, Issue 6), examines cocaine self-administration, operant extinction, and cocaine-associated cue extinction using an IVSA paradigm in adolescent and adult rats (P35 and P70 on commencement of self-administration, respectively). This paper also investigates the efficacy of intra-IL infusions of the full D2R agonist quinpirole or systemic injections of the partial D2R agonist aripiprazole on cocaine-cue extinction learning in adolescent rats.

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Original Article

ORIGINAL ARTICLE

Role of Dopamine 2 Receptor in Impaired Drug-Cue Extinction in Adolescent Rats

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Abstract

Adolescent drug users display resistance to treatment such as cue exposure therapy (CET), as well as increased liability to relapse. The basis of CET is extinction learning, which involves dopamine signaling in the medial prefrontal cortex (mPFC). This system undergoes dramatic alterations during adolescence. Therefore, we investigated extinction of a cocaine-associated cue in adolescent and adult rats. While cocaine self-administration and lever-alone extinction were not different between the two ages, we observed that cue extinction reduced cue-induced reinstatement in adult but not adolescent rats. Infusion of the selective dopamine 2 receptor (D2R)-like agonist quinpirole into the infralimbic cortex (IL) of the mPFC prior to cue extinction significantly reduced cue-induced reinstatement in adolescents. This effect was replicated by acute systemic treatment with the atypical antipsychotic aripiprazole (Abilify), a partial D2R-like agonist. These data suggest that adolescents may be more susceptible to relapse due to a deficit in cue extinction learning, and highlight the significance of D2R signaling in the IL for cue extinction during adolescence. These findings inspire new tactics for improving adolescent CET, with aripiprazole representing an exciting potential pharmacological adjunct for behavioral therapy.

Key words: adolescence, aripiprazole, dopamine, extinction, infralimbic cortex

Introduction

Drug addiction is a chronic, relapsing mental disorder characterized by loss of control over drug use, compulsive drug-seeking, and continued use despite serious adverse consequences (Camí and Farré 2003). It has been argued that mental disorders such as addiction should be defined as developmental disorders, due to the unique likelihood of onset during teenage and young adult years (Insel 2009). Indeed, adolescent drug users show higher resistance to therapeutic interventions and increased probability to relapse compared with adults, especially when faced with cues associated with the drug taking experience (Catalano et al. 1990; Perepletchikova et al. 2008; Ramo and Brown 2008; Winters et al. 2011).

Common behavioral treatments for addiction such as cue exposure therapy (CET) aim to reduce the craving elicited by drug-associated cues, based on the principle of extinction (Conklin and Tiffany 2002). Preclinical research using adult and adolescent rats suggests that the salience of drug-associated cues is strongly mediated across development by dopamine 1 receptor (D1R) activity on glutamatergic projections from the prelimbic cortex (PrL) of the medial prefrontal cortex (mPFC) to the core of the nucleus accumbens (NAc) (Kalivas and Duffy 1997; Brenhouse et al.

2008). By comparison, studies using adult animals suggest that extinction learning is largely controlled by projections from the infralimbic cortex (IL) of the mPFC to the NAc shell, and may involve D1R and/or dopamine 2 receptor (D2R) signaling (Peters et al. 2008; Haaker et al. 2013; Mueller et al. 2013). Adolescence constitutes a period of dramatic maturation of the mPFC, including alterations in dopamine receptor density (Andersen et al. 2000), fiber infiltration (Kalsbeek et al. 1988), and dopamine availability (Wahlstrom et al. 2010). However, current understandings of adolescent extinction learning, particularly the significance of the mPFC, are relatively incomplete.

Based on clinical findings that adolescent drug users are more resistant to extinction-based therapies and more liable to cue-induced relapse, we hypothesized that adolescent vulnerability to addiction relates to a deficit in cue extinction. When re-exposed to environmental stimuli associated with the drug experience, this deficit would increase the likelihood of compulsive return to drug-seeking and drug taking for this population. To investigate this theory, we developed a preclinical paradigm that separates the critical components of adolescent drug abuse liability, namely: motivation to self-administer, amenability to therapeutic intervention (cue extinction), and propensity to relapse. Using this model, we also aimed to reduce relapse-like behavior by pharmacologically manipulating dopamine signaling in the IL at the time of cue extinction. Importantly, targeting cue extinction has stronger translational potential compared with targeting reinstatement, since relapse is difficult to pre-empt due to its unpredictability in the human scenario. We found that cue extinction was able to significantly reduce cue-induced reinstatement in adult rats but not in adolescents. We also observed that acutely enhancing D2R signaling in the adolescent IL by microinfusion of the D2R agonist quinpirole enhanced cue extinction learning to reduce subsequent cue-induced reinstatement the next day. A similar effect was observed following acute systemic treatment with the partial D2R-like agonist aripiprazole (Abilify). These results present aripiprazole as a promising adjunct to improve exposure-based therapy for adolescent drug users.

Materials and Methods

Animals and Surgery

Male Sprague-Dawley rats ($N = 72$; bred in-house) were individually housed under a 12:12 light/dark cycle (lights off 7 a.m.) with food and water available ad libitum. All testing was conducted during the dark phase. Rats were group-housed and handled 3 times prior to surgery. Rats were individually housed immediately following surgery for the duration of experimentation. Rats were aged postnatal day (P)34(±1) (adolescent) or P69(±1) (adult) at the commencement of self-administration. All procedures were performed in accordance with the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

For all experiments a catheter was implanted into the right jugular vein. Rats were anesthetized with isoflurane vaporized with oxygen and injected with meloxicam (3 mg/kg, ip). Catheters were constructed in-house as described previously (Kim et al. 2014) and consisted of guide cannulas (22 gauge, PlasticsOne, VA, USA) and three layers of Silastic tubing (adult length 14 cm; adolescent length 12 cm; Dow Corning, USA). Catheters were flushed daily for 2 days following surgery with 0.05 mL of heparinized saline (90 IU/mL; Pfizer, NY, USA) containing 10% Fisamox antibiotic (amoxicillin sodium; Aspen Australia, NSW, Australia). For the duration of experiments catheters were flushed daily with 0.05 mL of 10 and 50 IU/mL antibiotic-heparin solution before and after cocaine self-administration, respectively.

For the quinpirole experiment a double guide cannula (26 gauge, PlasticsOne) bilaterally targeting the IL (AP, +3.0 mm; ML ± 0.6 mm; DV −4.2 mm) was implanted stereotaxically (David Kopf Instruments, CA, USA) following jugular catheterization. The cannula was secured to the skull using dental cement (Vertex, MA, USA) combined with 4 anchoring screws (PlasticsOne). Obturators extending 1 mm below the guide cannula were checked and rats were weighed daily.

Adult Versus Adolescent Self-Administration, Extinction, and Reinstatement

Experimental design is depicted in Figure 1.

Cocaine Self-Administration

Rats were trained to self-administer cocaine (cocaine hydrochloride dissolved in saline; Johnson Matthey Macfarlan Smith, Edinburgh, UK) in standard operant conditioning chambers (29.5 × 32.5 × 23.5 cm; Med Associates, VT, USA) equipped with two retractable levers and a cue light above each lever. House lights remained off. Pressing on the active lever resulted in a 50 μL infusion of cocaine (0.3 mg/kg per infusion—concentration of cocaine dissolved in saline was customized for the weight of each rat updated every 3 days) over 2.7 s by activation of a pump (Med Associates). Infusions were paired with 2.7 s of illumination of the light located above the active lever, followed by a 17.3 s time-out period. A vanilla scent was present beneath the grid floor below the active lever to serve as a discriminatory cue for the active versus inactive lever. The vanilla scent was present whenever the active lever was presented, even during lever extinction and cue-induced reinstatement when active lever had no consequences, therefore, it was a mere discriminative cue for the location of the lever. Pressing on the inactive lever had no programmed consequences at any phase of experiment.

For all experiments, daily 2-h self-administration sessions were conducted. For the first 5 but no more than 7 days, rats received cocaine under a fixed ratio (FR) 1 requirement. For the final 5 days of self-administration, responding occurred under FR3. This was to ensure that lever pressing by rats was for cocaine, which would be indicated by an increase in lever pressing at FR3 reinforcement schedule. Patency was tested weekly using 0.03 mL of ketamine (100 mg/mL) for adult and 0.02 mL for adolescent rats immediately followed by 0.05 mL of 10 IU/mL antibiotic-heparin solution. Any rat that failed to show loss of muscle tone within 10 s was removed from the study. Any rat that failed to self-administer at least 7 infusions of cocaine/session averaged across the last 5 days of self-administration was removed from the study.

On the penultimate day of self-administration in the first experiment, approximately half the rats received a single progressive ratio (PR) session in which the number of active lever responses required to receive an infusion increased incrementally. Lever pressing during PR session indicates the animal's motivation to self-administer a drug by measuring how many lever presses an animal is willing to make for an infusion (Farid et al. 2012). The session ran for a maximum of 4 h, but terminated automatically if no response was made for 1 h. On the final day of self-administration rats went back onto FR3 for one 2-h session.

Lever Extinction

The day after the final self-administration session, rats received daily 1-h lever extinction session for 7 days, where pressing

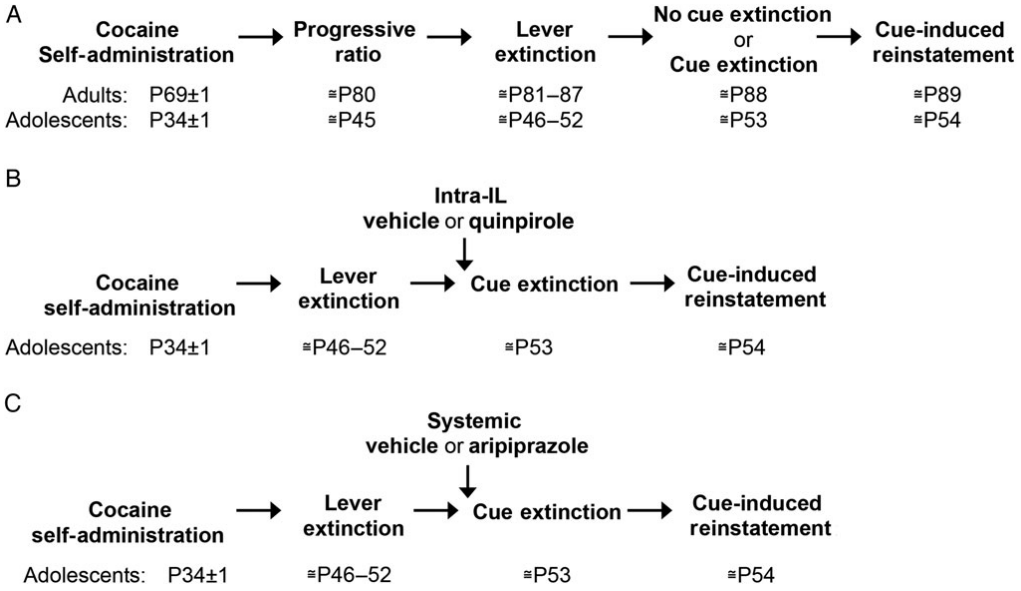

Figure 1. Experimental design. (A) Adult and adolescent rats underwent cue-paired cocaine self-administration. In the first experiment only, rats underwent a single PR session prior to the final day of self-administration. Rats then received lever extinction in absence of the cue. Rats were divided into groups for handling (No Cue Ext) or cue extinction (Cue Ext). Rats were tested the next day for cue-induced reinstatement. (B) Adolescent rats underwent cocaine self-administration and lever extinction as per the first experiment. Prior to cue extinction, rats received an infusion of vehicle or quinpirole (5 μg per hemisphere) into the infralimbic cortex (IL). Rats were tested the next day for cue-induced reinstatement. (C) Adolescent rats underwent cocaine self-administration and lever extinction as per the first two experiments. Prior to cue extinction, rats received a systemic injection of either vehicle or aripiprazole (5 mg/kg). Rats were tested the next day for cue-induced reinstatement.

either lever had no consequences. In other words, pressing on the previous active lever did not result in a cocaine infusion or a cue light illumination.

Cue Extinction

The day after the final lever extinction session, animals received a single cue extinction session without any levers present. This was to model CET in the clinic that typically does not involve re-enactment of drug taking actions but presentations of drug-associated cues. Following a 2-min baseline period, the 2.7 s cue light above the previously active lever was presented every 30 s 120 times. In the first experiment, rats were randomly assigned to one of two groups: a cue extinction group (Cue Ext) and a group that did not receive cue extinction but were handled for 2 min (No Cue Ext). In subsequent experiments all rats received cue extinction. Since there was no lever present for cue extinction, there was no vanilla scent present for this session.

Cue-induced Reinstatement

The day after cue extinction, rats were tested for cue-induced reinstatement. For 1 h, pressing the previously active lever resulted in illumination of the light above the lever under an FR3 schedule. If no lever response was made within the first 2 min, the cue light above the active lever illuminated automatically once. Reinstatement data greater than 5 standard deviations (SD) from the group mean were considered statistical outliers and were excluded from all analyses as previously reported (Kim et al. 2014). Overall, 2 rats from the quinpirole group in experiment 2, 1 rat from the vehicle group and 2 rats from the aripiprazole group in experiment 3 fell into this criterion and were excluded from the entire analyses. There were no rats that displayed reinstatement data >5 SD below their group mean.

Adolescent Intra-IL Quinpirole

A separate group of adolescent rats underwent cocaine self-administration followed by lever extinction as described for the first experiment. All rats then underwent cue extinction the following day. Prior to cue extinction, rats were treated using a bilateral intra-IL infusion. The infusion (0.5 μL per hemisphere) consisted of either vehicle (saline) or quinpirole (dissolved in saline; 2 μg/μL; Tocris, UK), and took place over 2 min. The infusion cannula extended 1 mm below the guide cannula, and remained in place for 2 min following the infusion. All rats then received cue extinction. All rats were tested for cue-induced reinstatement the next day. At the end of experimentation fresh frozen brains were sectioned and processed with cresyl violet to visualize cannula placement which are depicted in Fig. 4A.

Adolescent Systemic Aripiprazole

In the same design as the previous experiments, a separate group of adolescent rats underwent cue-paired cocaine self-administration, followed by lever extinction. All rats then underwent cue extinction the following day. Rats were treated with a systemic injection 30 min before the cue extinction session. The subcutaneous injection consisted of either vehicle (5% v/v Tween 80 in saline; Sigma-Aldrich Co., MO, USA) or aripiprazole (Alliance Biotech, India; 5 mg/kg; dose based on Feltenstein et al. 2007) suspended in vehicle. All rats were tested for cue-induced reinstatement the next day.

Data Analysis

Active lever presses with cocaine infusions and during the time-out period were summed into “active lever responses”. Self-administration, lever extinction, and reinstatement data

were analyzed using mixed-design repeated-measures analysis of variance (ANOVA). Significant interactions were followed up with further ANOVAs or *t*-tests as appropriate. Lever discrimination and PR data were analyzed using independent *t*-tests. Statistical tests were conducted using SPSS (IBM Corp., New York, USA), with acceptance for significance at $P \leq 0.05$.

Results

No Age Differences in Cocaine Consumption, Motivation to Self-Administer, or Lever Extinction

There was no difference between adult and adolescent rats in cocaine self-administration (Fig. 2). Analyses of active lever response data revealed a significant main effect of self-administration Day [$F_{9,\ 360} = 23.3$, $P < 0.05$], but no effect of Age, and no interaction between Day and Age (*F*s < 1). Consistent with this, analyses of reward data revealed a significant main effect of Day [$F_{9,\ 360} = 8.8$, $P < 0.05$], with no effect of Age and no interaction (*P*s > 0.05). Inactive lever response data showed no effect of Day or Age, and no interaction (*P*s > 0.05). There also was no effect of Age on total active responses made over the PR session [$t_{(38)} = 1.1$, $P = 0.3$], or on PR breakpoint ($t < 1$).

Lever extinction was also similar across age groups (Fig. 2*D*). Analyses of active lever responses revealed a significant main effect of lever extinction Day [$F_{6,\ 240} = 7.0$, $P < 0.05$] but no effect of Age and no interaction (*F*s < 1). This suggests that both adults and adolescent animals learned to inhibit drug-seeking over days, that is, lever extinction occurred. The same analyses of inactive lever response data revealed no effect of Day, Age, and no interaction (*P*s > 0.05).

Age Differences in Cue-Induced Reinstatement

To analyze cue reinstatement we performed a 4-way ANOVA comparing active versus inactive lever pressing (Lever Type) on the last day of extinction versus cue reinstatement (Day), in different ages (Age) and cue extinction conditions (Cue Extinction) (Fig. 3*A*). This revealed significant main effects of Lever Type, Day, and Age (*P*s < 0.05). There were also significant interactions between those factors and Cue Extinction (*P*s < 0.05) hence analyses were split for each lever type for different age groups.

For adults, analyses of active lever revealed a main effect of Day [$F_{1,\ 22} = 12.8$, $P < 0.05$], and an interaction between Day and Cue Extinction [$F_{1,\ 22} = 4.8$, $P < 0.05$], but no main effect of Cue Extinction [$F_{1,\ 22} = 3.6$, $P = 0.07$]. Post hoc paired-sample *t*-tests of active lever responses comparing final lever extinction day versus cue-induced reinstatement revealed a significant difference between days for No Cue Ext adults [$t_{(12)} = 3.8$, $P < 0.05$], however, no difference for Cue Ext adults [$t_{(10)} = 1.1$, $P = 0.3$] (Fig. 3*B*). By comparison, analyses of adolescent active lever data revealed a

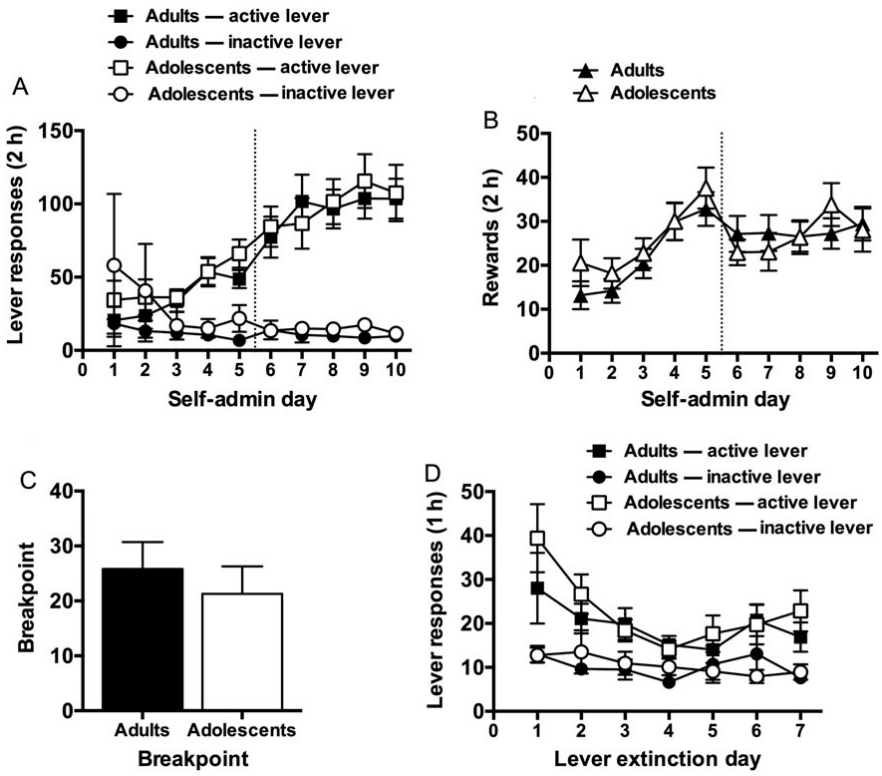

Figure 2. Cocaine self-administration was similar for adult and adolescent rats. Responding occurred on a FR 1 for the first 5 days, and increased to FR3 for the final 5 days of self-administration (broken line). (*A*) Mean (±SEM) daily lever responses. Responding on the active lever increased for both age groups over self-administration days ($P < 0.05$), while responding on the inactive lever remained low. (*B*) Mean (±SEM) daily rewards, that is, cocaine infusions (0.3 mg/kg per infusion) increased for both age groups over self-administration days ($P < 0.05$). (*C*) Once stable cocaine self-administration was established responding on a PR of reinforcement was similar across age groups, with adult and adolescent rats showing a similar number of maximum consecutive active lever presses to obtain a cocaine infusion (breakpoint). (*D*) Mean (±SEM) active lever responses decreased over lever extinction days ($P < 0.05$), with no difference between age groups by final lever extinction day. Inactive lever responding remained low relative to active lever responding across days. Adult $n = 24$; adolescent $n = 18$.

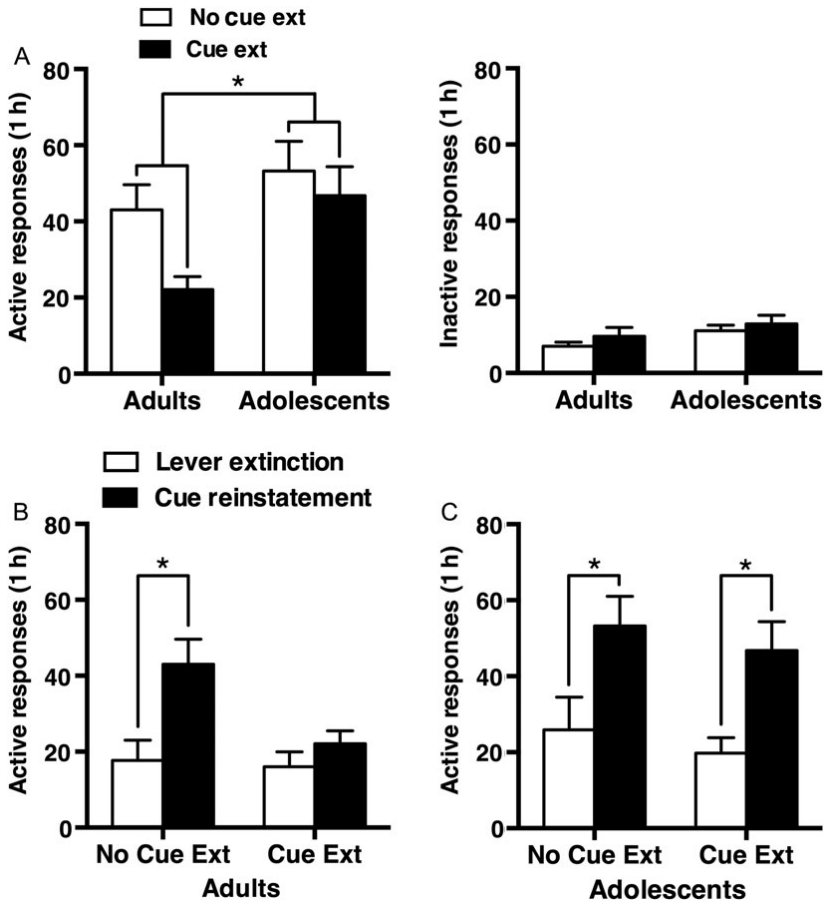

Figure 3. Age differences in cue-induced reinstatement following cue extinction. (*A*) Mean (+SEM) active lever responses made over one hour cue-induced reinstatement differed depending on age (*$P < 0.05$, main effect of Age, significant interaction of Age and Cue Extinction). There was no effect of day or cue extinction on inactive lever responding for both adults and adolescents. (*B*) Compared with the final day of lever extinction, adults that did not receive cue extinction (No Cue Ext) significantly reinstated cocaine seeking behavior following re-exposure to a drug-associated cue the next day (*$P < 0.05$, effect of Day in the No Cue Ext group) while adults that received cue extinction training did not. Adult No Cue Ext $n = 13$; adult Cue Ext $n = 11$. (*C*) Adolescents reinstated to the cue regardless of whether cue extinction training was received or not (*$P < 0.05$, main effect of Day). Adolescent No Cue Ext $n = 9$; adolescent Cue Ext $n = 9$.

significant main effect of Day [$F_{1,16} = 16.0$, $P < 0.05$], but no effect of Cue Extinction or an interaction ($Fs < 1$) (Fig. 3*C*). Analyses of inactive lever data found no effect of Day, Cue Extinction, and no interaction for either adults or adolescents ($Ps > 0.05$). These results indicate that cue extinction effectively reduced cue-induced reinstatement in adults but not in adolescents, and that this effect was not due to a generalized decrease in lever pressing activity.

Intra-IL Quinpirole or Systemic Aripiprazole at Cue Extinction Reduces Cue-Induced Reinstatement in Adolescent Rats

A separate group of adolescent rats underwent cocaine self-administration and lever extinction, and received an intra-IL infusion of the D2R-like agonist quinpirole (1 μg per hemisphere) or vehicle immediately prior to cue extinction. Rats were tested for cue-induced reinstatement the next day. Brains were processed for cannula placement verification following reinstatement and data from any rat with a cannula outside the IL were excluded from all analyses (Fig. 4*B*).

Treatment groups were comparable prior to intracranial infusion as indicated by analyses that showed no effect of Treatment (vehicle versus quinpirole) on any measure of cocaine self-administration or lever extinction ($Ps > 0.05$). Analyses of active lever on final lever extinction versus cue-induced reinstatement showed significant main effects of Day [$F_{1,\ 12} = 12.0$, $P < 0.05$], Treatment [$F_{1,\ 12} = 9.6$, $P < 0.05$], and a significant interaction [$F_{1,\ 12} = 6.1$, $P < 0.05$]. Post hoc *t*-tests revealed a significant increase in active lever responding at cue reinstatement compared with lever extinction for vehicle-treated rats [$t_{(7)} = 3.5$, $P < 0.05$], indicating that cue extinction was ineffective in this group. By comparison, there was no such difference across days for quinpirole-treated rats [$t_{(5)} = 2.4$, $P = 0.06$] (Fig. 4*B*). Analyses of inactive lever responses showed no effects ($Ps > 0.05$) (Figure). Together these results show that enhancing D2R activity in the IL improved cue extinction learning and thereby significantly reduced cue-induced reinstatement the next day.

We then aimed to replicate our quinpirole results using a pharmacological adjunct to cue extinction with strong translational potential. We chose aripiprazole, a widely used atypical antipsychotic with D2R partial agonist activity (Hirose and

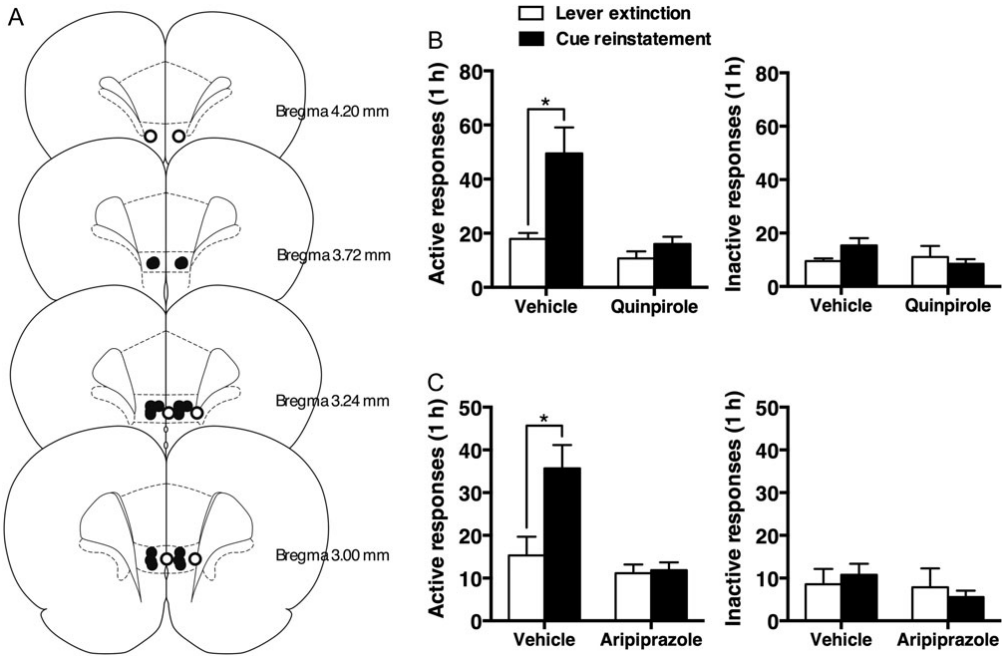

Figure 4. Enhancing D2R signaling at the time of cue extinction reduces cue-induced reinstatement in adolescent rats the next day. (*A*) Coronal sections illustrating intracranial cannula placements show that 14 rats had successful cannula tips within the infralimbic cortex (hits; filled circles) (misses; empty circles) (Paxinos and Watson 2013). (*B*) Analyses of mean active lever responses (+SEM, left panel) indicate that adolescent rats that received vehicle at the time of cue extinction showed reinstatement the next day (*$P < 0.05$), while rats that received quinpirole (5 μg per hemisphere) did not. Mean responses on the inactive lever (+SEM, right panel) remained low for both vehicle- and quinpirole-treated rats across final lever extinction and reinstatement test. Vehicle $n = 8$; quinpirole $n = 6$. (*C*) Adolescent rats that received vehicle at the time of cue extinction displayed cue reinstatement the next day (*$P < 0.05$), whereas rats that received aripiprazole (5 mg/kg) did not. Responding on the inactive lever remained low for both vehicle- and aripiprazole-treated rats over final lever extinction and reinstatement days. Vehicle $n = 9$; aripiprazole $n = 7$.

Kikuchi 2005). A separate group of adolescent rats underwent cocaine self-administration and lever extinction, and then received a systemic injection of aripiprazole (5 mg/kg) or vehicle 30 min prior to cue extinction. Cue-induced reinstatement was tested the next day.

Groups were comparable prior to treatment as indicated by analyses that showed no effect of Treatment (vehicle versus aripiprazole) on any measure of cocaine self-administration or lever extinction (*P*s > 0.05). Analyses of active lever responses made on the final lever extinction day versus cue reinstatement showed a significant main effect of Day [$F_{1, 14} = 10.5$, $P < 0.05$] a significant main effect of Treatment [$F_{1, 14} = 8.1$, $P < 0.05$], and a significant interaction [$F_{1, 14} = 9.1$, $P < 0.05$]. Post hoc paired-samples *t*-tests revealed a significant difference in active lever responses made on the final lever extinction day compared with cue reinstatement in vehicle-treated adolescents [$t_{(8)} = 3.7$, $P < 0.05$]. By comparison, there was no such difference across days in aripiprazole-treated rats ($t < 1$). Analyses of inactive lever responses made on final operant extinction day versus cue reinstatement revealed no effects (*P*s > 0.05) (Fig. 4C). These data indicate that acute aripiprazole at the time of cue extinction significantly reduced cue-induced reinstatement the next day, without affecting general lever responding.

Discussion

Understanding adolescent drug-cue extinction is critical to developing more effective treatment strategies for this vulnerable population. Our results show that adolescents are impaired in reducing cue-induced reinstatement following the extinction of cocaine-associated cue compared with adults. That is, we observed that adolescent rats that received cue extinction returned to drug-seeking when challenged with the cue the next day. By comparison, adult rats that received the same cue extinction session showed a significant decrease in cue-induced reinstatement. We found that the observed adolescent deficit in cocaine-associated cue extinction was ameliorated by acutely enhancing D2R signaling at the time of cue extinction training, with a potential mechanism for this effect identified in the IL of the mPFC. These results not only add to our understanding of the significance of the IL in adolescent drug-cue extinction learning, but also inspire novel approaches to improving adolescent exposure-based therapy in the clinical setting.

Adolescent Sensitivity to Drug-Associated Cues

In the present study, adolescent rats displayed impaired reduction of cue-induced reinstatement following cue extinction compared with adult rats. That is, while adult rats that received cue extinction training showed significantly reduced relapse-like behavior the next day, adolescent rats reinstated drug-seeking regardless of cue extinction training. Importantly, there were no observed age differences in extinction of lever pressing that were conducted in the absence of the cue. While one previous study shows that adolescent rats display increased responding during lever extinction following cocaine self-administration compared with adults, those results are confounded by age differences in overall cocaine consumption prior to lever extinction

(Anker and Carroll 2010). Adolescent rats have also been reported to show lower (Li and Frantz 2009) and equal (Schramm-Sapyta et al. 2011) responding during lever extinction. Those studies differed from our study in terms of methodology such as housing. Notably, there is evidence to suggest that individual housing from P21 effects anxiety and drug-seeking behavior during adulthood (Hall et al. 1998), although there were no adult isolated control groups. In the present study, all animals were bred and born in our facility, and rats assigned to adolescent groups were group housed until day of surgery (~P30), and were handled daily from the time of being individually housed, thus minimizing the potential stress of isolation from a young age. Therefore, our study suggests that acquisition, consolidation, and retrieval of operant extinction learning in the absence of the cocaine-associated cue occurs similarly in both adult and adolescent rats, as consecutive extinction sessions produced decreases in lever pressing for both ages. Thus the age difference in drug-associated extinction learning appears not to relate to operant responding, but to the extinction of drug-associated cues that is inferred from cue-induced reinstatement data. This specific age difference on drug-associated cue may be due to the dissociation in brain regions important for operant versus cue learning (Millan et al. 2011; Perry et al. 2014; Torregrossa et al. 2010).

In the present study, both adult and adolescent rats that did not receive cue extinction training displayed robust cue-induced reinstatement the next day. This is consistent with preclinical research in adult animals that shows re-exposure to the drug-associated cue triggers relapse-like behavior (Shaham et al. 2003). In previous preclinical investigations of adolescent drug self-administration, evidence for adolescent sensitivity to cue-induced reinstatement is mixed (Li and Frantz 2009; Anker and Carroll 2010). In those studies the drug-associated cue was never separately extinguished from lever responding, making interpretation of drug-cue sensitivity difficult. In one known study investigating adolescent extinction of drug-associated environmental cues, adolescent rats took longer to extinguish cocaine-associated contextual cues and displayed stronger reinstatement to those cues in a conditioned place preference paradigm (Brenhouse and Andersen 2008). Those findings are consistent with present results, which show for the first time in a self-administration paradigm that adolescents also display a deficit in extinction of a discrete cue associated with a self-administered drug. By comparison, our findings in adult rats are consistent with previous studies that show cue-alone extinction following lever-alone extinction reduces cue-induced reinstatement in adult rats (Torregrossa et al. 2010, 2013). It would be of great interest clinically for future studies to examine whether cue extinction without any lever extinction sessions reduces reinstatement of drug-seeking behaviors in adult rats, since it has already been shown that exposure to the cocaine-associated context can reduce drug-induced reinstatement in the absence of lever extinction (Kim et al. 2014).

Importantly, the present findings in adolescent rats directly model clinical evidence that CET is less effective in adolescent drug dependents (Catalano et al. 1990; Perepletchikova et al. 2008; Ramo and Brown 2008; Winters et al. 2011), which logically corresponds to higher relapse rates following therapy in this population (Ramo and Brown 2008). It should be noted that we did not observe enhanced cue-induced reinstatement per se in adolescents compared with adults, which may appear inconsistent with some human data that report adolescent humans show increased sensitivity to reward-associated cues in general (May et al. 2004; Ernst et al. 2005; Somerville et al. 2010). Critically, one study has specifically observed that adolescent drug users are more likely to relapse following craving induced by drug-associated cues (Ramo and Brown 2008), whereas, adult drug users are more likely to relapse when experiencing a negative physiological state such as withdrawal (Ramo and Brown 2008). In those studies, drug users did not necessarily undergo CET, whereas in our preclinical study, rats received lever and cue extinction. Therefore, we propose that adolescent vulnerability to addiction is at least partially due to a deficit in cue extinction that leads to increased likelihood of relapse, a hallmark of addiction. Combined with human research showing adolescent sensitivity to drug-associated cues, the present findings strongly suggest that drug use during adolescence leads to the formation of robust drug-cue associations that are difficult to extinguish.

It is important to note that cue extinction training was not given until late adolescence in the present study (Fig. 1). In the rat, P28–P56 is widely accepted as adolescence, with P70 as the onset of young adulthood (Spear 2000; Amorós-Aguilar et al. 2015; Saul et al. 2015). This relatively small developmental window is one of the reasons that preclinical adolescent addiction research is difficult to carry out. In the present study, self-administration occurred during early to mid-adolescence, and cue extinction occurred during late adolescence (~P53). We propose that self-administration during adolescence is most clinically relevant in terms of our model, and that cue extinction treatment during late adolescence still provides valuable insight into the effects of substance use during that vulnerable period.

The IL and Adolescent Cue Extinction

We observed that acutely enhancing D2R signaling in the IL of the mPFC during cue extinction reduces cue-induced reinstatement in adolescent rats. The IL was selected as a putative brain region important for adolescent cue extinction as a number of studies highlight a role for the IL in extinction of both aversive and reward-associated cues in adults (Peters et al. 2009). Indeed, in preclinical addiction studies, the IL has been strongly implicated in the extinction of operant responding (e.g., lever responding) and drug-associated contextual cues (Millan et al. 2011). However, the circuitry underlying extinction of discrete drug-associated cues is less clear. One study found that adult cue extinction was enhanced by systemic injection of the NMDA partial agonist D-cycloserine (DCS) (Torregrossa et al. 2010). Interestingly, this effect was observed via microinfusion into the NAc but not the mPFC (Torregrossa et al. 2010), though it may be that a lack of effect in the mPFC was due to targeting the whole region rather than the IL. In contrast, results of two studies examining contingent cue extinction in adult rats have suggested a role for the mPFC as a whole (Nic Dhonnchadha et al. 2013) and the IL specifically (Nic Dhonnchadha et al. 2012). However, those findings are confounded by lever pressing during cue extinction. Overall current understandings of the neural mechanisms underlying drug-cue extinction learning are relatively poor, as preclinical addiction research has largely ignored this aspect of addiction-related behaviors.

In fact, the neural basis of drug-associated cue extinction may be better understood from studies of fear extinction. While the vast majority of preclinical addiction literature focuses on extinction of operant and not cue memory, studies of conditioned fear focus largely on cue extinction in the absence of operant responding (Peters et al. 2009). Importantly, the IL has been implicated in fear extinction in both adults (Quirk and Mueller 2007) and adolescents (Kim et al. 2011). Furthermore, adolescents show a deficit in the consolidation of fear extinction learning comparable to the deficit in cocaine-cue extinction learning

observed in the present study (Kim et al. 2011; Pattwell et al. 2012; Ganella and Kim 2014). Our findings demonstrate for the first time that dopaminergic signaling via D2R in the IL is important for drug-cue extinction learning during adolescence. This is consistent with findings from fear conditioning in adult rats that show infusion of the D2R antagonist raclopride into the IL impaired retrieval of extinction the next day (Mueller et al. 2010). While further investigation is required to fully elucidate the neural basis of adolescent versus adult cue extinction learning in light of PFC maturation into late adolescence through adulthood, the present findings add invaluable novel data to this growing area of research, highlighting a role for dopaminergic signaling in the IL.

Translation to the Clinic: Aripiprazole

We sought to replicate the effect of intra-IL quinpirole using a pharmaceutical adjunct to cue extinction with strong translational potential. Therefore, we tested the effectiveness of aripiprazole, which is presently FDA-approved for the treatment of psychosis. We found that systemic administration of aripiprazole prior to cue extinction reduced relapse-like behavior in adolescents the next day.

Importantly, aripiprazole is already widely used in the treatment of psychosis not only for its efficacy but also because of its favorable safety profile and good tolerability (DeLeon et al. 2004). These factors make aripiprazole a compelling candidate for use in addiction treatment and in fact, aripiprazole is already in clinical trials for the treatment of cocaine dependence (Kim and Lawrence 2014). However, long-term use of aripiprazole in conjunction with abstinence has not generally shown beneficial results in non-psychotic patients (Brunetti et al. 2012). Evidence from preclinical relapse studies points to the benefits of short-term targeted use, with acute administration of aripiprazole reducing cocaine self-administration (Sørensen et al. 2008; Thomsen et al. 2008), as well as cue-induced and drug-primed reinstatement of cocaine-seeking following lever extinction (Feltenstein et al. 2007) or abstinence (Feltenstein et al. 2009). However, it should be noted that in those studies, treatment with aripiprazole occurred prior to reinstatement testing and drug-associated cues were never extinguished. Our results represent novel evidence for the efficacy of aripiprazole to block relapse specifically by improving cue extinction learning. This has important potential clinical implications, as therapy offers a controlled target for pharmacological intervention compared with relapse, which is often highly unpredictable.

Mechanism of Treatment Effects

We propose that the effects of quinpirole and aripiprazole in the present study are likely modulated through the D2R postsynaptically in the IL (Santana et al. 2009). Previous studies indicate that quinpirole decreases excitatory postsynaptic potentials (EPSPs) in PFC pyramidal cells both directly and by recruitment of local interneurons, consistent with post-synaptic D2R activation (Tseng and O'Donnell 2007). Similarly, the neuropsychological effects of aripiprazole involve D2R in the mesocorticolimbic dopamine pathway, which includes the IL (Stahl 2001; Burris 2002). However, aripiprazole differs from typical D2R agonists, as it does not produce motor behaviors associated with postsynaptic D2R activation. For instance, acute treatment does not induce contralateral rotation in striatal-lesioned rats or hyperlocomotion in reserpinized striatum-lesioned mice (Kikuchi et al. 1995). However, these experiments used adult rodents, and maturational differences in adolescent PFC may be associated with different drug effects. Importantly, aripiprazole is not a full D2R agonist but a partial D2R agonist. This means that when extracellular dopamine levels are low, it can act as a postsynaptic D2R agonist (Stahl 2001). Since the adolescent PFC is characterized by decreased dopamine availability (Wahlstrom et al. 2010), aripiprazole is likely acting as an agonist at postsynaptic D2Rs at this age. Computational models of PFC networks suggest that when D2R signaling is dominant, the PFC is in an "open gate" state where multiple inputs can have simultaneous representations in working memory (Seamans and Yang 2004). We suggest that activation of postsynaptic D2Rs shifts adolescent IL networks toward this more flexible state. In this way, acutely enhancing D2R signaling during cue extinction improves learning of the new inhibitory cue-no reward association.

It should be noted that while aripiprazole displays robust preferential binding to the D2R in both rats (Natesan et al. 2006) and humans (Mamo et al. 2007), it also exhibits partial agonist activity at the serotonin receptors 5HT1A and 5HT7 (DeLeon et al. 2004). Importantly, mPFC dopamine signaling is strongly mediated by the serotonin system (Benes et al. 2000). Indeed, serotonin fibers have been shown to interact with both dopamine afferents and gamma-aminobutyric acidergic interneurons in the mPFC (Taylor and Benes 1996), and to modulate the infiltration of fibers to this region (Taylor et al. 1998). This is of particular relevance given the infiltration of dopaminergic fibers occurring in the PFC during adolescence (Kalsbeek et al. 1988). However, "acute" treatment with aripiprazole during adolescence is unlikely to profoundly alter the course of dopamine afferent connectivity in the mPFC either directly or via serotonin modulation.

Importantly, the effects of treatment in the present study are not likely due to nonspecific effects during cue extinction such as sedation or stress. In fact, a single infusion of quinpirole into the mPFC has been shown to produce an anxiolytic response in mice tested drug-free the next day, with no effect on any anxiety measure at the time of treatment (Wall et al. 2003). In addition, a single intra-mPFC infusion of quinpirole has been found to produce no effect on locomotion compared with saline (Beyer and Steketee 2000). Studies using acute systemic aripiprazole in mice have similarly found no effect on locomotor activity (Viana et al. 2013). In fact acute aripiprazole has been shown to improve cognition in rats in terms of attentional functioning and response control (Carli et al. 2010), both of which are important in cue extinction learning. Acutely enhancing D2R signaling in the IL at the time of cue extinction therefore represents a promising tactic to enhance learning effectiveness per se and thereby reduce subsequent relapse to drug-associated cues.

Conclusion

Adolescence represents a unique period of risk for developing mental disorders, including drug addiction (Spear 2000). Our findings strongly suggest that adolescent vulnerability to addiction is explained at least in part by deficits in cue extinction that may lead to enhanced liability to relapse to drug-associated cues. Importantly, the present findings directly model clinical evidence that adolescent drug users are more resistant to exposure-based therapies and liable to relapse, especially to cues associated with the drug-taking experience, compared with their adult counterparts (Ramo and Brown 2008). The present study highlights a role for the D2R in the IL of the mPFC in mediating effective cue extinction learning in the adolescent rat. Since the neural correlates of adolescent behaviors are often conserved across species (Spear 2000), these findings inspire novel tactics

for pharmacologically enhancing extinction-based therapies for drug users who started during their adolescent years. Tailoring treatments to adolescent users will hopefully break the cycle of addiction for many living with substance abuse disorders.

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Notes

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4 Role of dopamine 1 and 2 receptors in adolescent versus adult fear extinction

4.1 Introduction

In the previous chapter, I showed that compared to adults, adolescent rats display a deficit in extinction of a discrete cue associated with cocaine. Enhancing dopamine signaling via D2R at the time of cocaine-cue extinction improved extinction learning in adolescent rats to reduce relapse-like behavior the next day, with a potential locus of action for this effect identified in the IL of the mPFC. These results show for the first time that prefrontal dopamine is a key mediator of adolescent cue extinction learning in an appetitive learning domain. However, it is not clear from those results whether dopamine signaling is involved in extinction of other emotionally salient cues during adolescence and/or adulthood. The present chapter therefore aimed to further investigate the role of prefrontal dopamine in cue extinction learning by examining extinction of conditioned fear in adolescent versus adult rats.

Fear conditioning is frequently employed in a laboratory setting to model the fear learning typical of many anxiety disorders (Maren 2001). Importantly, learned fear is directly involved in the most common forms of anxiety disorders (Rosen and Schulkin 1998), and has been especially implicated in the pathophysiology of youth-onset anxiety disorders (Pine 1999). The fear conditioning paradigm allows us to model extinction learning. In this paradigm, fear to a stimulus can be reduced by repeated presentations of that stimulus without any aversive outcome. This is relevant since treatment for anxiety disorders in both youth and adult populations often involves extinction-based treatments (McNally 2007; Albano and Kendall 2002). Unfortunately, clinical data show that adolescents attain poorer outcomes following CBT compared with children, even when family participation is accounted for (Southam-Gerow et al. 2001; Bodden et al. 2008). It has therefore been suggested that adolescent resistance to exposure-based therapies relates to poor responses to extinction at this age (Hartley and Casey 2013; Kim and Ganella 2015). This notion is based on several studies that have

shown impaired extinction of conditioned fear in both adolescent rodents (Kim et al. 2011; McCallum et al. 2010; Pattwell et al. 2012) and adolescent humans (Pattwell et al. 2012). In order to improve adolescent responses to extinction-based therapy for anxiety, it is necessary to clarify the neural basis of extinction during this unique maturational period.

Extinction learning and memory involves plasticity in the mPFC (Peters et al. 2009; Kim et al. 2011). The mPFC changes dramatically during adolescence (Casey et al. 2008), and there is evidence to suggest that age-related differences in mPFC activity contribute to adolescent deficits in fear extinction across both rodents and humans (Pattwell et al. 2012; Kim et al. 2011). In particular, dopamine signaling in the mPFC displays a unique maturation profile during adolescence (O'Donnell 2010; Wahlstrom et al. 2010). For instance, the density of dopaminergic fiber infiltration of the PFC increases throughout adolescence until early adulthood in rodents (Kalsbeek et al. 1988) and non-human primates (Rosenberg and Lewis 1995). Dopamine synthesis also peaks in the PFC during adolescence (Andersen et al. 1997), along with dopamine receptor density in the PFC (Tarazi and Baldessarini 2000). It is possible that these and other age-related changes in prefrontal dopamine signaling contribute to adolescent impairments in fear extinction learning. However, this theory has never been directly tested.

Studies using adult rodents are beginning to highlight a role for prefrontal dopamine in the extinction of conditioned fear (see Abraham et al. 2014 for review). Presentation of cues previously associated with a footshock has been shown to increase dopamine in the PFC of adult rats (Feenstra et al. 2001), suggesting extinction may involve dopamine efflux in this region. Pre-extinction systemic treatment with SKF-81297, which is an agonist at D1R, the most highly expressed dopamine receptor in the PFC, enhanced extinction of cued and contextual fear (Abraham et al. 2016), while intra-IL infusion of the D1R antagonist SCH-23390 impairs long-term fear extinction (Hikind and Maroun 2008). Consistent with this, transgenic mice lacking D1R show normal fear conditioning but delayed extinction up to 90 days post-conditioning (El-Ghundi et al. 2001). In comparison, one study has shown systemic treatment with the D2R agonist quinpirole also impairs extinction learning (Nader and LeDoux 1999), whereas another study found no effect (Ponnusamy et al. 2005). However, pre-

extinction systemic or central (intracerebroventricular; i.c.v.) injection of the D2R antagonist haloperidol has also been found to increase CS-elicited freezing during extinction and at test the next day (Holtzman-Assif et al. 2010). Still others report that pre-extinction D2R antagonism with sulpiride facilitates extinction both within-session and at test the next day (Ponnusamy et al. 2005), while one known study investigating intra-IL D2R antagonism using raclopride found impaired long-term fear extinction in adult rats the next day (Mueller et al. 2010). Overall, the precise role of prefrontal dopamine receptors, especially D2R, in adult fear extinction remains equivocal. Furthermore, the role of prefrontal dopamine in adolescent fear extinction is unknown.

In the rat, adolescence is widely accepted as spanning from P28 to P55 (Spear 2000; Kerstetter and Kantak 2007; O'Neill et al. 2015). In the previous chapter, cocaine-cue extinction occurred at P53, which falls into late adolescence, while adult rats received cocaine-cue extinction at P88. Rats were housed in reverse light-dark conditions (lights off at 7 a.m.), with all behavioral experimentation conducted during the dark phase of the cycle. In an effort to remain consistent with the design of the previous chapter, the first experiment in the current chapter investigated extinction of conditioned fear in late adolescent rats (P53) and adult rats (P88) housed in reverse light-dark conditions, with fear conditioning and extinction occurring during the dark cycle. Across the rodent fear conditioning literature, behavioral experiments are typically carried out during the light phase of a 12-hour light-dark cycle (McGuire et al. 2012). It is not surprising then that all known studies of fear extinction in adolescent rodents have been conducted during the light phase (Baker and Richardson 2015; Kim et al. 2011; Pattwell et al. 2012; McCallum et al. 2010; Pattwell et al. 2016). These studies have found that adolescents display a deficit in fear extinction learning compared to adults, consistent with findings from humans (Pattwell et al. 2012). Therefore, I first aimed to recapitulate the previously observed adolescent versus adult phenotype of fear extinction, with an effort to match the age and housing conditions of adolescent and adult rats with the previous chapter. I then examined the effect of acutely enhancing IL D1R or D2R signaling at the time of fear extinction in both adolescent and adult rats. Finally, I investigated the effect of acute systemic treatment with aripiprazole, an antipsychotic with partial agonist activity at D2Rs (Burris 2002). Dopamine receptor agonists were chosen due to the clinical significance of existing FDA-approved dopamine receptor agonists, including aripiprazole, which are more

readily administered compared to dopamine receptor antagonists in adolescent humans (Kirino 2012). Thus, results from the present chapter have strong translational potential for improving extinction-based treatments for anxiety, as well as adding to literature on the mechanisms of extinction learning across development.

4.2 Methods

4.2.1 Animals

Refer to Chapter 2.1 for details. Briefly, male Sprague Dawley rats (n=271) were bred in-house. Rats in the first experiment were P53 (late adolescent) or P88 (adult) on fear extinction day, and were housed under reverse light-dark conditions (lights on 7 p.m.). Rats in subsequent experiments were P35 (adolescent), P53 (late adolescent), or P88 (adult) on fear extinction day, and were housed under standard conditions (lights on: 7 a.m.).

4.2.2 Surgery

Refer to Chapter 2.2.3 for details.

4.2.3 Drugs

Refer to Chapter 2.3.1 and Chapter 2.3.2 for details.

4.2.4 Fear conditioning

Refer to Chapter 2.4.3 and 2.4.4 for details. For all experiments rats were fear conditioned as per Chapter 2.4.4.1. In this chapter, different experiments involved varying amounts of extinction training. For each of these protocols, baseline freezing was measured for the first 2 minutes, followed by a number of 10 second CS presentations (ITI = 10 seconds). Different extinction protocols were as follows:

30 CS Extinction – 30 CS presentations

60 CS Extinction – 60 CS presentations

90 CS Extinction – 90 CS presentations

60 × 2 CS Extinction – two sessions consisting of 60 CS presentations conducted on the same day, with approximately 60 minutes between sessions during which rats remained in their home cage.

Two-day 60 CS Extinction – two sessions of 60 CS presentations conducted separately on consecutive days.

For subsequent experiments, rats received 30 CS presentations. All rats were tested 24 hours after extinction training as per Chapter 2.4.4.3.

4.2.5 Data analysis

Behavioral data were analyzed using one-way or repeated-measures (RM) analysis of variance (ANOVA). Significant interactions were followed by ANOVA or t-tests as appropriate. Statistical tests were conducted using SPSS, with acceptance for significance at $p \leq 0.05$. All extinction data were analyzed in blocks of 5 CS presentations.

4.3 Results

4.3.1 Behavioral experimentation in the dark phase produces unreliable results

The first part of this chapter aimed to optimize the behavioral protocol for investigating fear extinction during the dark phase of the light-dark cycle. Adolescent (P53) and adult (P88) rats housed under reverse light-dark conditions (lights off 7 a.m.) were fear conditioned with 3 trials of tone (CS) paired with an electric footshock (US) as described previously (Kim et al. 2011; McCallum et al. 2010; Pattwell et al. 2012). In the first experiment, I employed a 30 CS Extinction protocol (**Figure 4.1**) because extinction consisting of 30 CS presentations has been shown to be sufficient to reduce within-session freezing in both age groups but expose a long-term extinction deficit in adolescents (Kim et al. 2011; McCallum et al. 2010).

Table.4.1 *Mean ± SEM baseline freezing for 30 CS Extinction during the dark phase. There was no effect of Age for any session. P53 n = 9, P88 n = 8. Freezing is expressed as a percentage of the total duration of CS presentation.*

Session	P53	P88
Conditioning	0.1 ± 0.1%	0.4 ± 0.4%
Extinction	27 ± 8%	20 ± 9%
Test	22 ± 10%	42 ± 14%

Baseline freezing during conditioning, extinction, and test for this experiment are summarized in **Table 4.1**. ANOVA showed no difference between age groups at conditioning, extinction (Fs<1), or test [$F_{(1, 16)}=1.5$, $p=0.2$]. Analyses of CS-elicited freezing across 3 CS-US pairings showed an effect of Conditioning trial [$F_{(2, 30)}=44.4$,

p<0.05], with no overall effect of Age [$F_{(1, 15)}$=3.6, p=0.07], and no interaction [$F_{(2, 30)}$=1.6, p=0.2]. Thus, both age groups increased CS-elicited freezing over conditioning (**Figure 4.1A**). For 30 CS Extinction the next day, analyses revealed an effect of Extinction block [$F_{(5, 75)}$=5.0, p<0.05], with no effect of Age and no interaction (Fs<1). Although there was an overall effect of Extinction block, freezing at the final block was very high (~76% for both ages; **Figure 4.1B**). Lastly, RM ANOVA of final extinction block versus test showed no effect of Day ($F_{(1, 15)}$=1.5, p=0.2] with no effect of Age and no interaction (Fs<1) (**Figure 4.1C**). Taken together, fear conditioning, 30 CS extinction, and test during the dark cycle appears to produce similar levels of extinction in adolescent and adult rats, under these conditions.

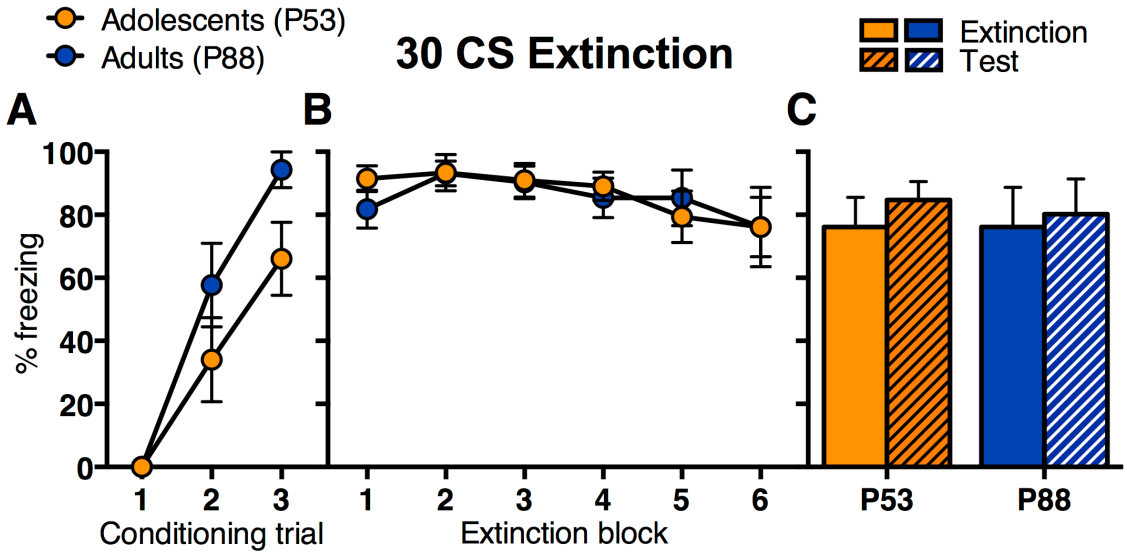

Figure 4.1 *Fear conditioning and 30 CS extinction during the dark phase. (A) Late adolescent (P53) and adult (P88) rats increased CS-elicited freezing during conditioning. (B) Freezing remained high across extinction and (C) at test the next day for age groups. P53 n = 9, P88 n = 8. Data represent mean ±SEM.*

Because CS-elicited freezing remained high across 30 CS presentations and at test the next day in the preceding experiment, I tried doubling extinction to 60 CS presentations in new groups of P53 and P88 rats (**Figure 4.2**). Baseline freezing levels for this experiment are summarized in **Table 4.2**. ANOVA showed no difference between age groups at conditioning or test (Fs<1). Inconsistent with the previous experiment, however, P53 rats showed higher freezing at extinction baseline compared to P88 rats [$F_{(1, 39)}$=5.0, p<0.05].

Table 4.2 *Mean ± SEM baseline freezing expressed as a % of CS duration for 60 CS Extinction during the dark phase. *p<0.05, significant effect of Age. P53 n = 20, P88 n = 20.*

Session	P53	P88
Conditioning	0.2 ± 0.1%	0.1 ± 0.1%
Extinction*	33 ± 7%	13 ± 6%
Test	16 ± 7%	17 ± 7%

For conditioning, RM ANOVA showed an overall effect of Conditioning trial [$F_{(2, 76)}$=124.6, p<0.05], with no effect of Age (F<1) and no interaction [$F_{(2, 76)}$=1.5, p=0.2] (**Figure 4.2A**). For extinction the next day, RM ANOVA showed an effect of Extinction block [$F_{(11, 418)}$=93.9, p<0.05], with an effect of Age [$F_{(1, 38)}$=9.1, p<0.05] and an interaction [$F_{(11, 418)}$=4.6, p<0.05]. Due to the significant interaction, post-hoc t-test for each block of extinction was employed, which showed significant age differences at blocks 3 to 8 (ps<0.05). Thus P53 displayed higher levels of CS-elicited freezing in the middle of extinction training, but similar freezing to P88 rats at the start and again by the end of extinction training (i.e., transiently delayed extinction; **Figure 4.2B**). Analyses of final extinction block versus test revealed an effect of Day ($F_{(1, 38)}$=81.1, p<0.05] with an effect of Age [$F_{(1, 38)}$=5.1, p<0.05]. The interaction approached significance [$F_{(1, 38)}$=3.3, p=0.08] (**Figure 4.2C**). These results suggest that both age groups showed spontaneous recovery of freezing 24 hours after extinction training and that adolescent rats froze more than adult rats in extinction and test.

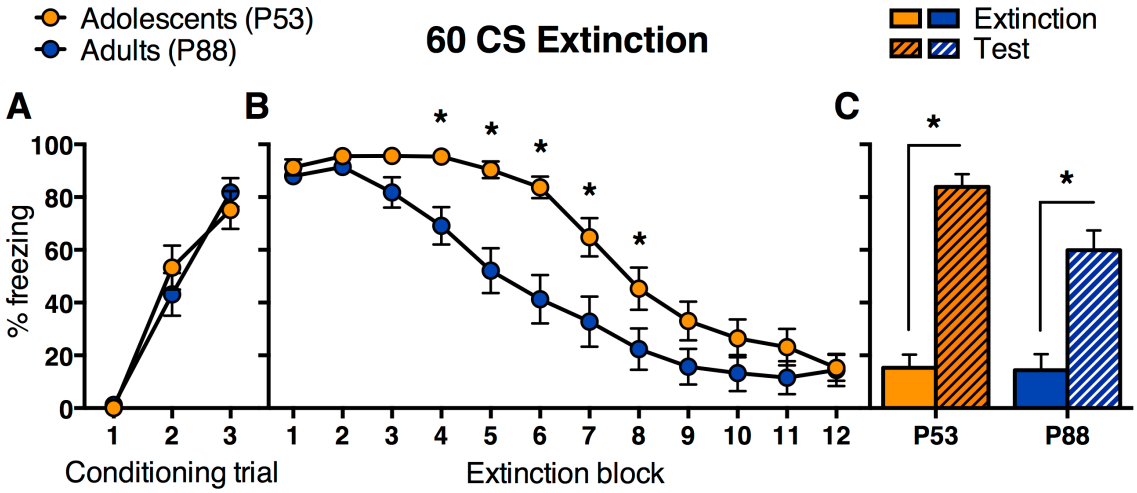

Figure 4.2 *Fear conditioning and 60 CS extinction during the dark phase. (A) Both late adolescent (P53) and adult (P88) rats increased freezing over conditioning. (B) Both age groups reduced CS-elicited freezing over extinction training, and (C) both age groups showed spontaneous recovery of freezing the next day. P53 n = 20, P88 n = 20. Data represent mean ±SEM. *p<0.05.*

Because 60 CS presentations was still insufficient to reduce spontaneous recovery at test the next day, especially in adult rats where spontaneous recovery is rarely observed, I then investigated increasing the amount of extinction training to 90 CS-alone presentations (**Figure 4.3**). Baseline freezing data at each session for this protocol are summarized in **Table 4.3**. ANOVA showed no difference between age groups for conditioning, extinction (Fs<1) or test [$F_{(1, 19)}$=2.4, p=0.1].

Table 4.3 *Mean ± SEM baseline freezing expressed as % of CS duration for 90 CS Extinction during the dark phase. There was no effect of Age for any session. P53 n = 9, P88 n = 11.*

Session	P53	P88
Conditioning	0.0 ± 0.0%	0.2 ± 0.2%
Extinction	9 ± 5%	19 ± 9%
Test	6 ± 5%	27 ± 12%

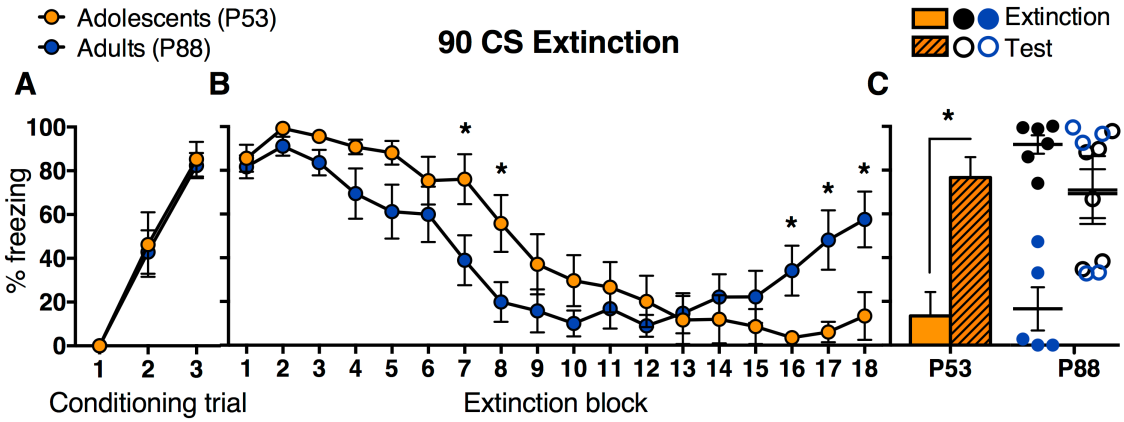

Figure 4.3 *Fear conditioning and 90 CS Extinction during the dark phase. (A) Both late adolescent (P53) and adult (P88) rats increased freezing over conditioning. (B) Both age groups reduced CS-elicited freezing over extinction training. (C) Some adult rats fell asleep by the end of extinction (black circles). All rats showed high levels of freezing at test the day after extinction training. P53 n = 9, P88 n = 11. Data represent mean ±SEM. *p<0.05.*

For conditioning there was an overall effect of Conditioning trial [$F_{(2, 36)}$=67.2, p<0.05], with no effect of Age and no interaction (Fs<1) (**Figure 4.3A**). The next day, there was an overall effect of Extinction block [$F_{(17, 306)}$=21.9, p<0.05], and an interaction between Extinction block and Age [$F_{(17, 306)}$=3.6, p<0.05], but no effect of Age (F<1). Post-hoc t-tests per block showed age effects at blocks 7 to 8, and 16 to 18 (ps<0.05). Specifically, P53 rats displayed increased CS-elicited freezing compared to P88 rats near the middle of extinction, while the opposite occurred over the final few blocks of extinction (**Figure 4.3B**). RM ANOVA of final extinction block versus test revealed an effect of Day ($F_{(1, 18)}$=12.4, p<0.05], with a significant interaction [$F_{(1, 18)}$=5.6, p<0.05] but no effect of Age [$F_{(1, 18)}$=3.0, p=0.10] (**Figure 4.3C**). Post-hoc paired t-tests revealed a difference between Final extinction block and Test for adolescents [$t_{(8)}$=-4.7, p<0.05] but not adults (p>0.05). Because the lack of difference between extinction and test for adults appeared to be due to an increase in freezing at the end of extinction, I examined video recordings of extinction sessions. This revealed that what was reported as freezing was actually rats falling asleep. When adult rats that fell asleep were excluded from analyses, RM ANOVA of final extinction versus test showed an effect of Day [$F_{(1, 12)}$=27.6, p<0.05], but no effect of Age and no interaction (Fs<1). Notably, there was no difference in CS-elicited freezing at test between adult rats that fell asleep and those that did not (t<1). Thus it is likely that both P53 and P88 rats returned to higher levels of freezing at test compared to the end of 90 CS Extinction.

Due to rats falling asleep by the end of 90 CS Extinction, I then tried two sessions of 60 CS presentations run consecutively on the same day (**Figure 4.4**). Baseline freezing data per session for this protocol are summarized in **Table 4.4**. ANOVA showed no difference between age groups for conditioning [$F_{(1, 19)}$=2.2, p=0.2], extinction session, or test (Fs<1).

Table 4.4 *Mean ± SEM baseline freezing as a % of CS duration for 60 × 2 CS Extinction. There was no effect of Age for any session. P53 n = 10, P88 n = 10.*

Session	P53	P88
Conditioning	0.0 ± 0.0%	0.2 ± 0.1%
Extinction A	22 ± 9%	12 ± 9%
Extinction B	22 ± 8%	16 ± 9%
Test	3 ± 3%	10 ± 10%

For conditioning, there was an overall effect of Conditioning trial [$F_{(2, 36)}$=86.5, p<0.05], with an overall effect of Age [$F_{(1, 18)}$=4.5, p<0.05], but no interaction [$F_{(2, 36)}$=2.0, p=0.1]. Thus, inconsistent with the previous two experiments, here P53 rats displayed higher levels of CS-elicited freezing compared P88 rats during conditioning overall (**Figure 4.4A**). In the first extinction session there was an overall effect of Extinction block [$F_{(11, 231)}$=48.6, p<0.05], with no effect of Age [$F_{(1, 21)}$=1.6, p=0.2] and no interaction [$F_{(11, 231)}$=1.5, p=0.2) (**Figure 4.4B**). Again, this is inconsistent with findings from the previous two experiments, where extinction involved interactions between Age and Block. The second session similarly showed an overall effect of Extinction block [$F_{(11, 198)}$=42.2, p<0.05], with no effect of Age and no interaction (Fs<1) (**Figure 4.4C**). RM ANOVA of final extinction block versus test showed was an effect of Day (F(1, 18)=8.4, p<0.05], with no effect of Age [$F_{(1, 18)}$=2.4, p=0.1] no interaction [$F_{(1, 18)}$=1.3, p=0.3]. This suggests higher freezing on test day compared to final block of extinction for both adolescent and adult rats (**Figure 4.4D**).

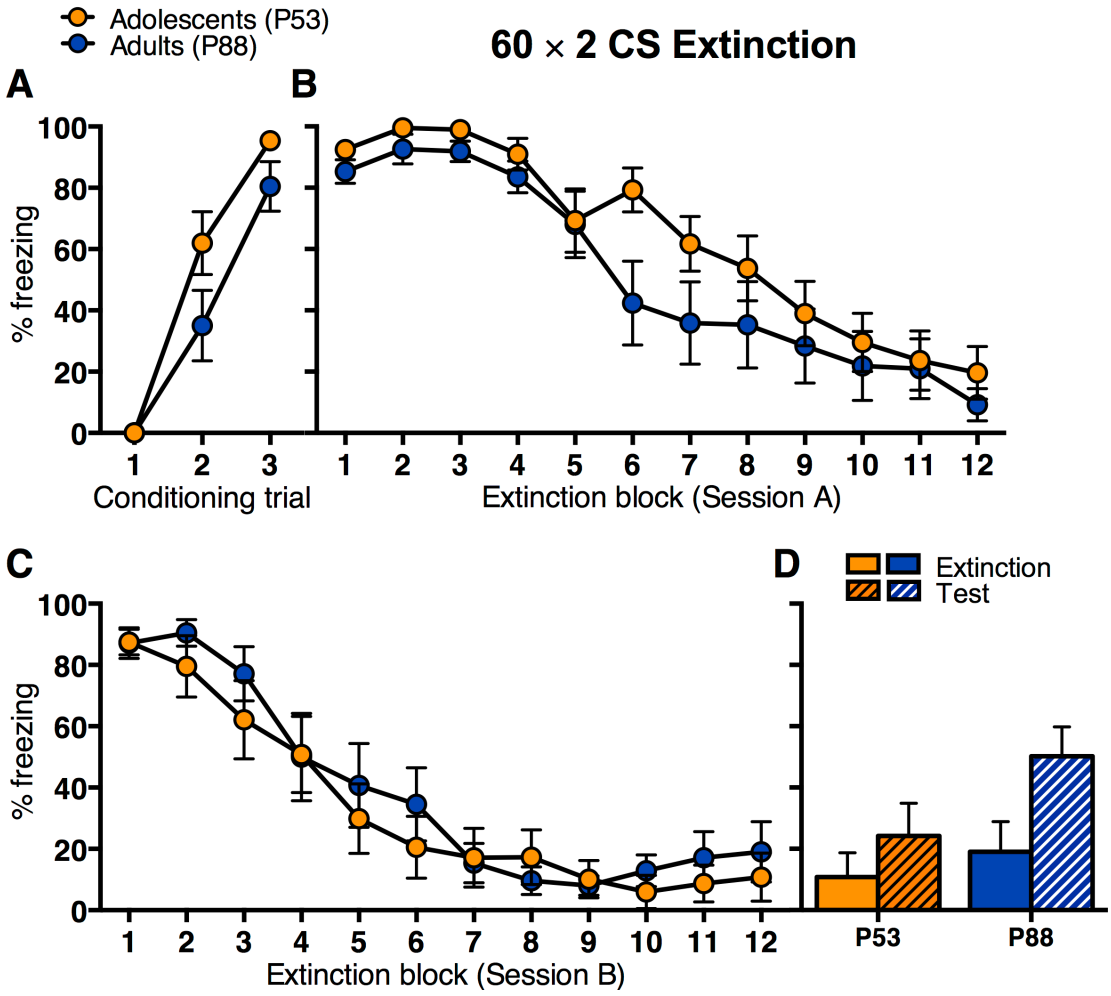

Figure 4.4 *Fear conditioning and 60 × 2 CS Extinction during the dark phase. (A) Both late adolescent (P53) and adult (P88) rats increased freezing over conditioning. Both age groups reduced CS-elicited freezing over 60 CS presentations in (B) the first session and (C) the second session. (C) Rats showed higher levels of freezing at test compared to final block of extinction training. P53 n = 10, P88 n = 10. Data represent mean ±SEM.*

Finally, I investigated the effect of one 60 CS Extinction per day over two consecutive days. Baseline freezing data for this experiment are summarized in **Table 4.5**. ANOVA showed no difference between age groups for conditioning and extinction (Fs<1), or test [$F_{(1, 18)}$=1.2, p=0.3].

Table 4.5 *Mean ± SEM baseline freezing as a % of CS duration for Two-day 60 CS Extinction during the dark phase. There was no effect of Age for any session. P53 n = 9, P88 n = 10.*

Session	P53	P88
Conditioning	0.0 ± 0.0%	0.1 ± 0.1%
Extinction Day 1	36 ± 10%	29 ± 11%
Extinction Day 2	26 ± 10%	38 ± 13%
Test	3 ± 2%	13 ± 8%

Analyses of conditioning revealed an overall effect of Conditioning trial [$F_{(2, 36)}$=69.6, p<0.05], with no effect of age (F<1) and no interaction [$F_{(2, 36)}$=2.6, p=0.1] (**Figure 4.5A**). This is similar to findings from previous protocols but inconsistent with findings from the last experiment (60 × 2 CS Extinction). Analyses of within-session extinction on the first day showed an effect of Extinction block [$F_{(11, 198)}$=41.2, p<0.05], with no effect of Age and no interaction (F<1) (**Figure 4.5B**). This is inconsistent again with previous findings, most notably with data from the 60 CS Extinction protocol, which one would expect to be replicated here. The second day similarly showed an overall effect of Extinction block [$F_{(11, 198)}$=14.0, p<0.5], with no effect of Age and no interaction (Fs≤1) (**Figure 4.5C**). For final extinction block compared to test, there was no effect of Day [$F_{(1, 17)}$=2.2, p=0.2] and no effect of Age (F<1), but an interaction between Day and Age [$F_{(1, 17)}$=5.3, p<0.05]. Post-hoc paired t-tests per age showed that adults displayed spontaneous recovery of CS-elicited freezing the next day [$t_{(9)}$=-2.8, p<0.05], while adolescent freezing remained low (t<1).

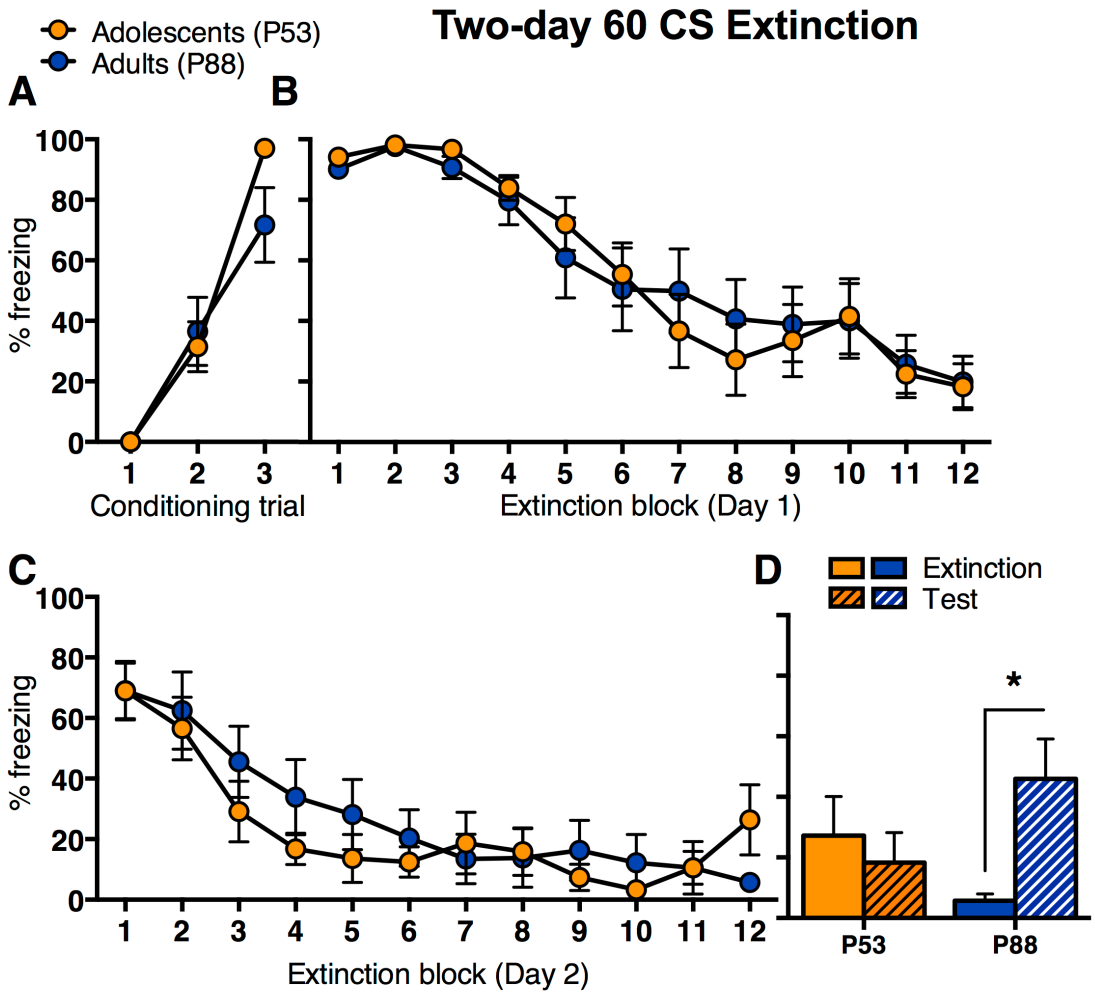

Figure 4.5 *Fear conditioning and 60 CS extinction per day for two days during the dark phase. (A) Both late adolescent (P53) and adult (P88) rats increased freezing over conditioning. (B) and (C) Both age groups reduced CS-elicited freezing over 60 CS presentations over two separate days. (D)Adult rats showed higher levels of freezing at test compared to final block of extinction training. P53 n = 9, P88 n = 10. Data represent mean ±SEM. *p<0.05.*

Across all the different extinction protocols tested in the dark phase, none reliably showed consistent findings during conditioning, extinction, and test. It should be noted that across the five extinction protocols tested, multiple (eight) cohorts of animals were used. These cohorts underwent behavioral experimentation on different dates, according to availability of rats from our breeding colony. To help elucidate whether these results related to individual variability across subjects, I also conducted analyses of CS-elicited freezing with Cohort as a factor across all rats trained and tested in the dark phase

Analyses of conditioning data showed an overall effect of Conditioning trial [$F_{(2, 210)}$=347.8, p<0.05] and a significant interaction between Conditioning trial and Cohort [$F_{(14, 210)}$=1.8, p<0.05], between Cohort and Age [$F_{(2, 105)}$=8.1, p<0.05], and between Conditioning trial, Cohort, and Age [$F_{(4, 210)}$=3.5, p<0.05]. Conditioning data were then analyzed per age group to determine whether one or both age groups were contributing cohort effects (**Table 4.6**). For adolescents, RM ANOVA showed an overall effect of Conditioning trial [$F_{(2, 104)}$=159.0, p<0.05], with a significant effect of Cohort [$F_{(4, 52)}$=5.6, p<0.05] and a significant interaction [$F_{(8, 104)}$=3.9, p<0.05]. For adults, RM ANOVA of CS-elicited freezing showed an overall effect of Conditioning trial [$F_{(2, 106)}$=193.6, p<0.05], but no effect of Cohort and no interaction (Fs<1). Thus although both age groups showed an increase freezing response across conditioning, the level of CS-elicited freezing over conditioning trials was inconsistent across cohorts for late adolescent rats (P53).

Table 4.6 *Results of RM ANOVA for conditioning across multiple cohorts of rats during the dark phase. *p<0.05.*

	P53	P88
Conditioning trial	*	*
Conditioning trial*Cohort	*	
Cohort	*	

Because extinction in each experiment involved at least 30 CS presentations (i.e., 6 blocks), I was also able to investigate the robustness of CS-elicited freezing across the first 30 CS presentations during extinction by analyzing with a factor of Cohort, to determine whether this behavior was consistent across multiple groups of subjects. RM ANOVA across all extinction protocols showed an effect of Extinction block [$F_{(5, 525)}$=63.8, p<0.05], an interaction between Extinction block and Cohort [$F_{(35, 525)}$=1.6, p<0.05], Extinction block and Age [$F_{(5, 525)}$=5.1, p<0.05], and between Extinction block, Cohort, and Age [$F_{(10, 525)}$=4.2, p<0.05]. There was also an overall effect of Age [$F_{(1, 105)}$=8.9, p<0.05] and an interaction between Cohort and Age [$F_{(2, 105)}$=3.5, p<0.05]. To further investigate which age group was contributing to cohort effects, extinction was also analyzed separately for each age group (**Table 4.7**). For adolescents, RM ANOVA showed a main effect of Extinction block [$F_{(5, 260)}$=20.7, p<0.05] and an interaction between Extinction block and Cohort [F(20, 260)=1.9, p<0.05] but no overall effect of Cohort (F<1). Analyses of adult data similarly showed a main effect of Extinction block [$F_{(5, 265)}$=45.1, p<0.05] and an interaction between

Extinction block and Cohort [$F_{(25, 265)}$=2.4, p<0.05] but no overall effect of Cohort [$F_{(5, 53)}$=1.6, p=0.2]. This suggests that across all experiments, adolescents and adults displayed cohort variability in within-session extinction behavior at least for the first 30 CS presentations.

Table 4.7 *Results of RM ANOVA for initial 30 CS presentations during extinction across multiple cohorts of rats during the dark phase. *p<0.05.*

	P53	P88
Extinction block	*	*
Extinction block*Cohort	*	*
Cohort		

Because not all protocols used multiple cohorts for each age group, and different extinction parameters theoretically should produce different results at test, I was not able to analyze each extinction protocol, or final extinction block versus test, with a factor of Cohort. However, these results clearly suggest cohort effects. I concluded that conducting fear conditioning, extinction, and test during the dark phase can produce inconsistent data, especially in adolescent rats.

4.3.2 Behavior in the light phase: adolescents consistently display extinction deficits

Previous studies that report a fear extinction deficit in adolescent compared to adult rodents have all been conducted during the light phase of the light-dark cycle. I hypothesized that behavioral experimentation during the dark phase may be producing the observed variability in data, as well as the spontaneous recovery observed in adult rats. Therefore, the next study aimed to optimize robust fear conditioning and extinction during the light phase using rats age P35, P53, and P88 on extinction day. The P35 age group was added because I hypothesized that P53 rats may be showing variability due to some individuals being closer to adult maturity than others, as P53 falls close to the end of the adolescent developmental period. The age P35 was chosen based on previous studies of adolescent fear extinction in rats (Kim et al. 2011; McCallum et al. 2010), and a consensus in rat literature that P35 falls within adolescence (Spear 2000; Madsen and Kim 2016).

Table 4.8 *Mean ± SEM baseline freezing a % of CS duration for 30 CS Extinction during the light phase. There was no effect of Age across any session. P35 = 11, P53 n = 23, P88 n = 13.*

Session	P35	P53	P88
Conditioning	0.4 ± 0.2%	0.2 ± 0.2%	0.0 ± 0.0%
Extinction	25 ± 9%	14 ± 5%	5 ± 2%
Test	3 ± 2%	6 ± 2%	4 ± 4%

Baseline freezing data for each session are summarized in **Table 4.8**. There was no difference between age groups for baseline at conditioning [$F_{(2, 44)}$=1.1, p=0.3], extinction [$F_{(2, 44)}$=2.3, p=0.1], or test (F<1). Rats were conditioned with 3 CS-US pairings (**Figure 4.6A**). RM ANOVA of CS-elicited freezing during conditioning showed an effect of Conditioning trial [$F_{(2, 88)}$=62.4, p<0.05], with no effect of Age [$F_{(2, 44)}$=1.5, p=0.2] and no interaction [$F_{(2, 88)}$=2.1, p=0.09]. Thus, age groups similarly increased CS-elicited freezing across CS-US pairings.

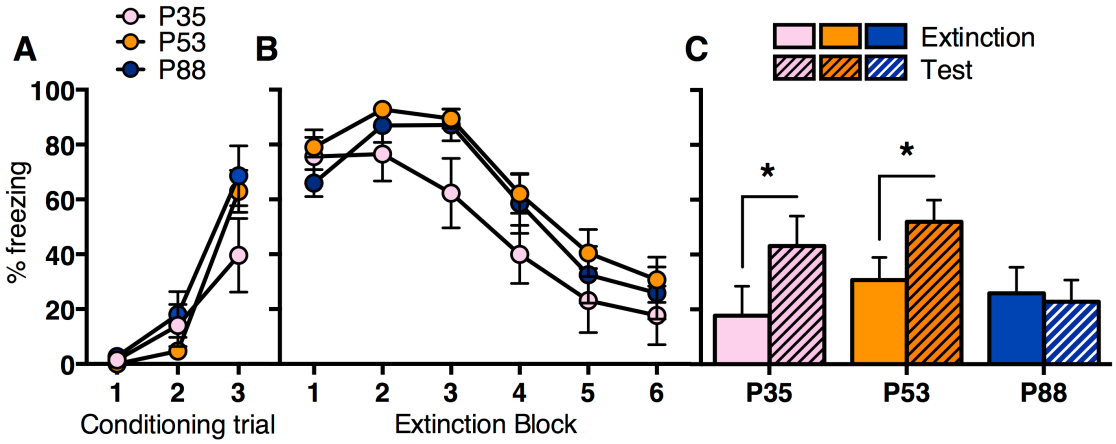

Figure 4.6 *Fear conditioning and 30 CS extinction during the light phase. (A) Adolescent (P35), late adolescent (P53) and adult (P88) rats increased freezing over conditioning. (B) All age groups reduced CS-elicited freezing over extinction training. (C) CS-elicited freezing was higher at test compared to final block of extinction for adolescent but not adult rats. P35 = 11, P53 n = 23, P88 n = 13. Data represent mean ±SEM. *p<0.05.*

The next day, rats underwent fear extinction consisting of 30 CS-alone presentations (**Figure 4.6B**). RM ANOVA showed an effect of Extinction block [$F_{(5, 220)}$=56.8, p<0.05], with no effect of Age [$F_{(2, 44)}$=1.8, p=0.2] and no interaction (F<1). Thus, all age groups similarly decreased freezing over 30 CS presentations. Twenty-four hours after extinction training, rats were tested for long-term CS-elicited freezing. RM ANOVA of final extinction block versus test showed an effect of Day [$F_{(1, 44)}$=12.5, p<0.05] and an interaction between Day and Age [$F_{(2, 44)}$=4.4, p<0.05], but no overall

effect of Age [$F_{(2, 44)}$=1.1, p=0.3]. Post-hoc paired t-tests found a significant difference in freezing at extinction versus test for P35 [$t_{(10)}$=3.0, p<0.05] and P53 [$t_{(22)}$=3.3, p<0.05] rats but not P88 rats (t<1).

In this experiment conducted during the light phase, multiple cohorts of rats were also used according to availability from the breeding colony. Thus, I also analyzed CS-elicited freezing across conditioning, extinction, and test with a factor of Cohort. RM ANOVA of CS-elicited freezing during conditioning showed an overall effect of Conditioning trial [$F_{(2, 72)}$=58.5, p<0.05], with no effect of Cohort [$F_{(6, 36)}$=2.1, p=0.08) or Age (F<1) and no interactions between Cohort x Conditioning trial [$F_{(12, 72)}$=1.2, p=0.3], Age x Cohort, Age x Conditioning trial and Age x Cohort x Conditioning trial (Fs<1). Thus all age groups consistently showed an increase in CS-elicited freezing during conditioning regardless of cohort.

Analyses of extinction showed an overall effect of Extinction block [$F_{(5, 180)}$=63.8, p<0.05], with no overall effect of Age [$F_{(1, 36)}$=2.6, p=0.1] and no interactions between Age x Cohort, Age x Extinction block or Age x Cohort x Extinction block (Fs<1). However, there was an overall effect of Cohort [$F_{(6, 36)}$=3.5, p<0.05], with an interaction between Cohort and Extinction [F(30, 180)=2.3, p<0.05]. To further investigate which age group may be contributing to cohort variability, I also analyzed extinction separately for P35, P53, and P88 **Table 4.9**). For P35 rats, there was an overall effect of Extinction block [$F_{(5, 40)}$=12.1, p<0.05], with no effect of Cohort [$F_{(2, 8)}$=2.9, p=0.1], and no interaction (F<1). For P53, there were significant effects of Extinction block [$F_{(5, 90)}$=37.5, p<0.05], Cohort [F(4, 18)=3.8, p<0.05], and an interaction [$F_{(20, 90)}$=3.2, p<0.05]. For P88 rats, there was an effect of extinction [$F_{(5, 50)}$=18.3, p<0.05], but no effect of Cohort (F<1) and no interaction [$F_{(10, 50)}$=2.0, p=0.053]. These results strongly suggest that the late adolescent (P53) group is the most unreliable age group during extinction compared to P35 and P88 rats.

Table 4.9 *Results of RM ANOVA for extinction across multiple cohorts of rats during the light phase. P35 = 11, P53 n = 23, P88 n = 13. *p<0.05.*

	P35	P53	P88
Extinction block	*	*	*
Extinction block*Cohort		*	
Cohort		*	

Cohort analyses of freezing during final block of extinction and test showed an overall effect of Day [$F_{(1, 36)}$=26.9, p<0.05], an overall effect of Cohort [$F_{(6, 36)}$=2.8, p<0.05, and an overall effect of Age [$F_{(1, 36)}$=4.6, p<0.05], with an interaction between Day and Cohort [$F_{(6, 36)}$=5.1, p<0.05]. There was no interaction between Age and Cohort [$F_{(2, 36)}$=2.0, p=0.2], Age x Day [$F_{(1, 36)}$=1.7, p=0.2], or Day x Age x Cohort (F<1). To examine the possibility that a particular age group was contributing to this variability, I also analyzed extinction versus test data per age (**Table 4.10**). P35 rats showed an effect of Day [$F_{(1, 8)}$=8.2, p<0.05] but no Cohort effect [$F_{(2, 8)}$=1.5, p=0.3], and no interaction (F<1). P53 rats showed an effect of Day [$F_{(1, 18)}$=25.7, p<0.05], with an interaction between Day and Cohort [$F_{(4, 18)}$=9.2, p<0.05] as well as an overall effect of Cohort [$F_{(4, 18)}$=4.1, p<0.05]. Rats age P88 showed no effects (Fs<1). Thus extinction and test results from P35 and P88 rats were robust across different cohorts of rats, however, P53 rats varied across cohorts.

Table 4.10 *Results of RM ANOVA of freezing during final block of extinction versus test (Day) across multiple cohorts of rats during the light phase. * indicates significant effect (p<0.05). P35 = 11, P53 n = 23, P88 n = 13.*

	P35	P53	P88
Day	*	*	
Day*Cohort		*	
Cohort		*	

4.3.3 Difference in baseline freezing in the dark versus the light phase

To help elucidate whether increased adult freezing at test in the dark phase was due to increased basal anxiety during this phase, I compared freezing levels at baseline for conditioning, extinction for rats that received 30 CS Extinction during the dark phase or the light phase (**Table 4.11**). Baseline freezing was minimal at conditioning for all age groups and conditions (refer to **Tables 4.1 – 4.10**). ANOVA of baseline freezing at Extinction for P53 and P88 rats showed no effect of Age or Phase (Fs<1), and no interaction [$F_{(1, 49)}$=1.2, p=0.3]. ANOVA of baseline freezing at Test showed no effect of Age [$F_{(1, 49)}$=2.2, p=0.1], with no interaction between Age and Phase [$F_{(1, 49)}$=3.1, p=0.09], but an overall effect of Phase [$F_{(1, 49)}$=19.0, p<0.05]. This suggests that level of freezing was higher during baseline at test for both P53 and P88 rats. It is worth noting that baseline freezing at test for P35 rats during the light phase was low (3%, **Table 4.8**).

Table 4.11 *Mean ± SEM baseline levels of freezing for Conditioning, Extinction (30 CS presentations) and Test conducted during the dark or light phase. * indicates significant main effect of Phase ($p<0.05$). P53 dark n = 9, light n = 23; P88 dark n = 8, light n = 13.*

	P53		P88	
	Dark	Light	Dark	Light
Conditioning	0.1 ± 0.1%	0.2 ± 0.2%	0.4 ± 0.4%	0.0 ± 0.0%
Extinction	27 ± 8%	14 ± 5%	20 ± 9%	5 ± 2%
Test*	22 ± 10%	6 ± 2%	42 ± 14%	4 ± 4%

4.3.4 Intra-IL quinpirole enhances adolescent fear extinction

My first series of experiments aimed to optimize protocol to capture the previously observed adolescent deficit in long-term extinction, in order to investigate the molecular mechanisms involved in this phenotype. Behavioral experimentation during the light phase revealed a reliable deficit in long term cue extinction in P35 compared to P88 rats. Therefore, all subsequent fear experiments were conducted in the light phase of the light-dark cycle using rats age P35 and P88 on extinction day. To understand a potential role for prefrontal dopamine in adolescent versus adult fear extinction, my next experiments targeted IL dopamine signaling using agonists of D1R or D2R. Rats were fear conditioned with three CS-US pairings. The next day, rats received an infusion of vehicle, the D1R agonist SKF-81297 (0.1 ug/side), or the D2R agonist quinpirole (1.0 ug/side) directly into the IL immediately prior to extinction training consisting of 30 CS presentations. Rats were tested for CS-elicited freezing the next day. Cannula placements were verified after behavioral experimentation (**Figure 4.7**). No drug effects were observed in data from rats with cannula outside the target region (*p*s > 0.05).

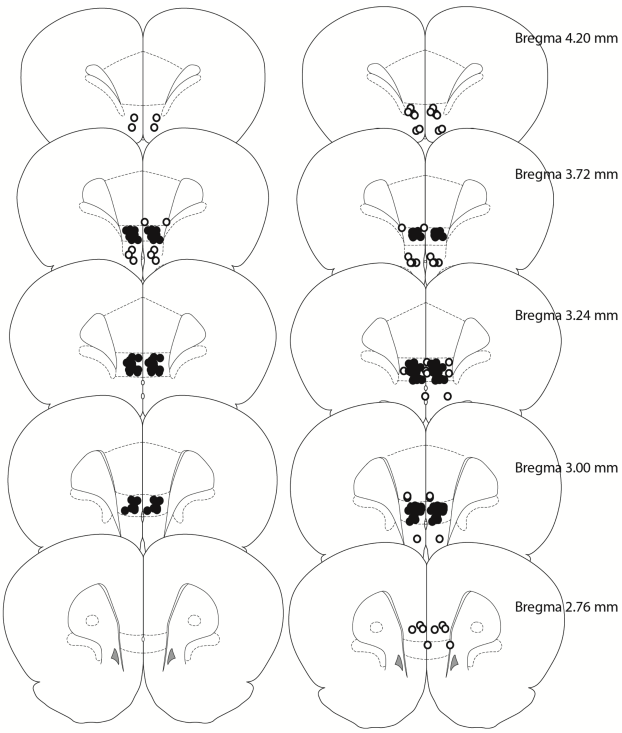

Figure 4.7 *Coronal sections illustrating intracranial cannula placements in rats age P35 (adolescents, left) and P88 (adults, right). Bilateral cannula targeted the infralimbic cortex (IL). Hits (filled circles) P35 n = 36, P88 n = 41, and misses (empty circles) P35 n = 6, P88 n = 15.*

Baseline freezing data for all sessions are summarized in **Table 4.12**. Note that infusions were given before extinction training only. ANOVA showed no effect of drug on baseline freezing for adolescent rats at extinction or test (Fs<1), or for adult rats at extinction (F<1) or test [$F_{(2, 38)}$=1.3, p=0.3].

Table 4.12 *Mean ± SEM baseline freezing for Conditioning, Extinction, and Test sessions for adolescent and adult rats that received intra-IL vehicle, SKF-81297, or quinpirole prior to extinction. There was no effect of Drug at any session. P35 vehicle n = 19, SKF-81297 n = 8, quinpirole n = 9; P88 vehicle n = 18, SKF-81297 n = 10, quinpirole n = 13.*

	Adolescent (P35)			Adult (P88)		
	Vehicle	SKF-81297	Quinpirole	Vehicle	SKF-81297	Quinpirole
Conditioning	0.3 ± 0.2%	0.1 ± 0.1%	0.5 ± 0.5%	0.8 ± 0.6%	0.6 ± 0.5%	0.5 ± 0.4%
Extinction	3 ± 1%	6 ± 5%	6 ± 2%	4 ± 2%	10 ± 8%	11 ± 5%
Test	13 ± 6%	8 ± 8%	5 ± 4%	6 ± 3%	16 ± 10%	4 ± 2%

Adolescents displayed a significant increase in CS-elicited freezing across conditioning (**Figure 4.8A**). RM ANOVA of CS-elicited freezing, [$F_{(2, 70)}$=13.5, p<0.05]. The next day, all adolescents showed comparable extinction with no differences between drug groups (**Figure 4.8B**). RM ANOVA of CS-elicited freezing

showed an effect of extinction block [$F_{(5, 165)}$=16.9, p<0.05], with no effect of drug and no interaction (Fs<1). Interestingly, adolescent rats that received in intra-IL vehicle or SKF-81297 at the time of extinction froze significantly more compared to the end of extinction when tested the next day, while adolescents that received intra-IL quinpirole did not (**Figure 4.8C**). RM ANOVA of freezing during the final block of Extinction compared to test revealed an effect of day [$F_{(1, 33)}$=6.3, p<0.05] and an interaction between day and drug [$F_{(2, 33)}$=3.5, p<0.05], with no overall effect of drug (F<1). Post-hoc paired t-tests found a significant difference in freezing levels at extinction versus test for vehicle [$t_{(18)}$=3.6, p<0.05] and SKF-81297 [$t_{(7)}$=2.6, p<0.05], but not for quinpirole (t<1). Thus, acutely enhancing IL D2R signaling at the time of extinction improved long-term extinction in adolescents.

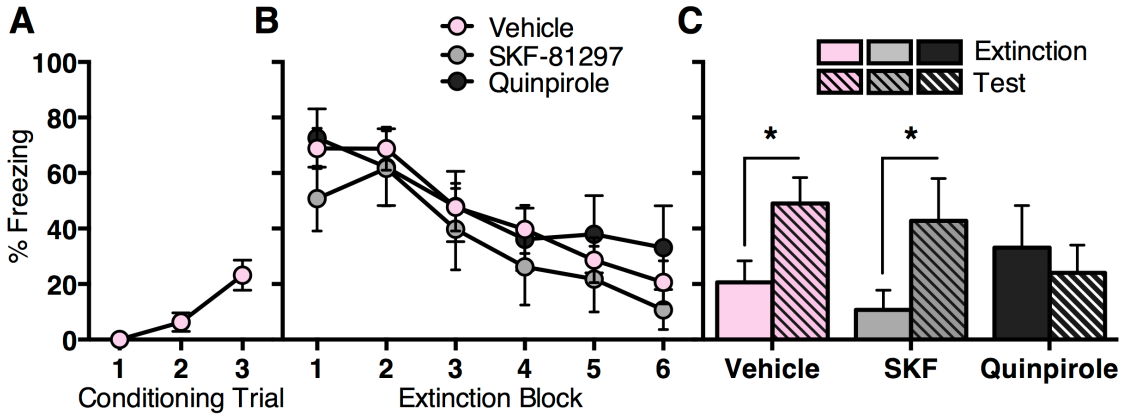

Figure 4.8 *Intra-IL infusions of a D1R agonist (SKF-81297 [SKF]) or a D2R agonist (quinpirole) had different effects on long-term extinction for adolescent rats. (A) Adolescents displayed an increase in CS-elicited freezing across fear conditioning. (C) Acutely manipulating IL D1R or D2R signaling had no effect on extinction within-session. (D) Adolescents that received in intra-IL vehicle or SKF-81297 at the time of extinction returned to high levels of CS-elicited freezing when tested the next day, while adolescents that received intra-IL quinpirole did not. Vehicle n = 19, SKF-81297 n = 8, quinpirole n = 9. Data represent mean ±SEM. *p<0.05.*

Adults also displayed a significant increase in CS-elicited freezing during conditioning [$F_{(2, 80)}$=75.1, p<0.05] (**Figure 4.9A**). The next day, acutely manipulating IL D2R signaling in adults had a transient effect only during extinction, however all adults inhibited freezing to a comparable level by the end of extinction training (**Figure 4.9B**). Acutely manipulating IL D1R signaling in adults had no effect on within-session or long-term extinction compared to vehicle. RM ANOVA showed an effect of extinction block [$F_{(5, 190)}$=24.9, p<0.05] and a block x drug interaction [$F_{(10, 190)}$=2.0, p<0.05], but no overall effect of drug [$F_{(2, 38)}$=1e.6, p=0.2]. When the interaction was

examined with post-hoc one-way ANOVA of individual extinction blocks, an effect of drug at extinction block 5 only was revealed [$F_{(2, 38)}$=3.3, p<0.05], suggesting that intra-IL quinpirole transiently delayed within-session extinction for adults. Comparison of extinction versus test 24 hour later showed no effect of drug treatment (**Figure 4.9C**). RM ANOVA showed an effect of day [$F_{(1, 38)}$=13.0, p<0.05], with no effect of drug [$F_{(2, 38)}$=1.0, p=0.4], and no interaction [$F_{(2, 38)}$=1.4, p=0.2]. Thus, increasing IL D1R or D2R signaling at the time of extinction had no effect on long-term extinction in adults.

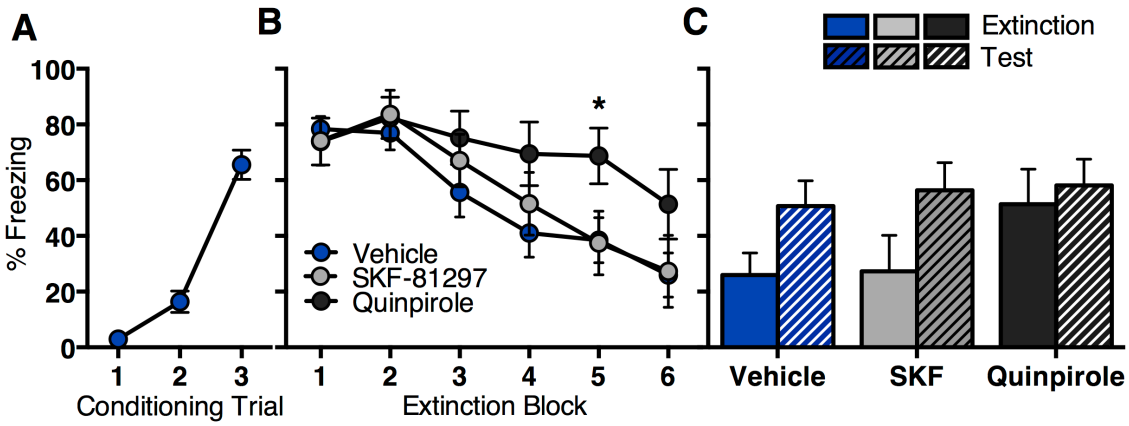

Figure 4.9 *Intra-IL infusions of a D1R agonist (SKF-81297 [SKF]) or a D2R agonist (quinpirole) had different effects on within-session extinction for adult rats. (A) Adults displayed an increase in CS-elicited freezing across fear conditioning. (B) Acutely manipulating adult IL D2R signaling transiently impaired within-session extinction, however all adult rats inhibited CS-elicited freezing to a comparable level by the end of extinction training, irrespective of intracranial drug treatment. (C) Enhancing IL D1R or D2R signaling at the time of extinction training had no effect on long-term extinction in adults. Vehicle n = 18, SKF-81297 n = 10, quinpirole n = 13. Data represent mean ±SEM. *p<0.05.*

4.3.5 Systemic aripiprazole enhances adult fear extinction

In the previous experiment I showed that acutely activating prefrontal D2Rs at the time of extinction can enhance extinction learning in adolescent rats. Our lab has recently found that this effect is replicated in adolescent rats by treatment with a systemic injection of the D2R partial agonist aripiprazole (Ganella et al., under review). This finding has strong translational potential, especially since aripiprazole is already FDA approved for clinical use in psychosis. However, here I showed that intra-IL quinpirole impaired within-session extinction in adult rats, therefore, I determined if aripiprazole had similar effects in adult rats. Adult (P88) rats first underwent fear conditioning, then the next day received a systemic injection of aripiprazole (5 mg/kg) or vehicle 30 minutes prior to extinction (**Figure 4.10**). CS-elicited freezing was tested the day after

extinction training. Baseline freezing data are summarized in **Table 4.13**. Note that drug was administered before extinction training only. ANOVA showed no effect of drug on baseline at extinction or test (Fs<1).

Table 4.13 *Mean ± SEM baseline levels of freezing for Conditioning, Extinction (30 CS presentations) and Test for adult rats that received vehicle or aripiprazole prior to extinction. There was no effect of drug at any session. Vehicle n = 15, aripiprazole n = 16.*

	Vehicle	Aripiprazole
Conditioning	0.1 ± 0.1%	0.1 ± 0.1%
Extinction	18 ± 6%	16 ± 5%
Test	12 ± 6%	15 ± 8%

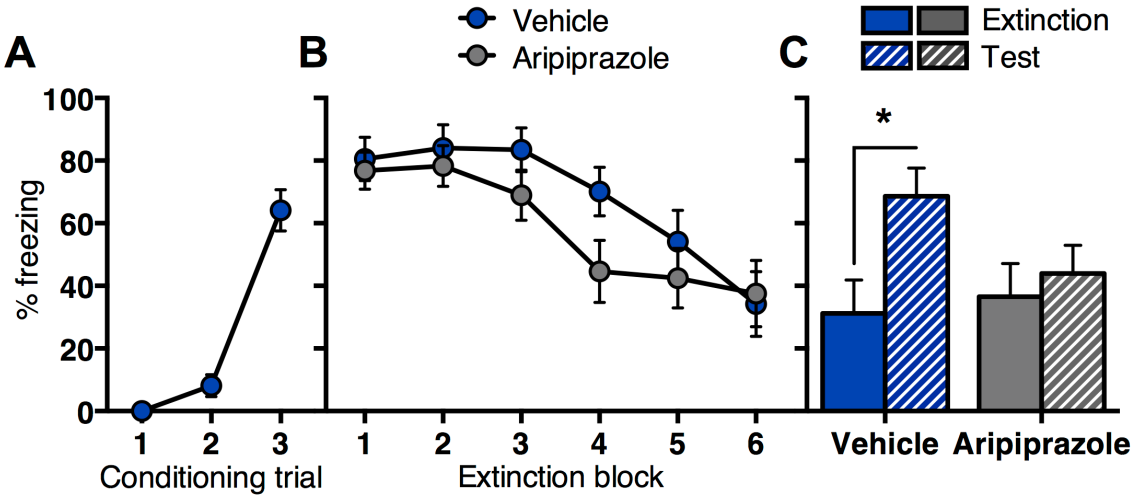

Figure 4.10 *Systemic treatment with aripiprazole improved long-term extinction for adult (P88) rats. (A) Adults showed increased CS-elicited freezing during fear conditioning. (B) Rats treated with vehicle or aripiprazole showed a similar decrease in CS-elicited freezing over extinction. (C) Rats treated with vehicle at the time of extinction showed high levels of freezing the next day. Spontaneous recovery was prevented in rats treated with aripiprazole at extinction. Vehicle n = 15, aripiprazole n = 16. Data represent mean ±SEM. *p<0.05.*

RM ANOVA showed that adult rats increased CS-elicited freezing during conditioning, with an overall effect of Conditioning trial [$F_{(2, 60)}$=65.7, p<0.05] (**Figure 4.10A**). Analyses of CS-elicited freezing during extinction showed an effect of Extinction block [$F_{(5, 145)}$=22.15, p<0.05], with no significant effect of Drug (F=1) and no interaction [$F_{(5, 145)}$=1.6, p=0.2]. Thus, acute treatment with a systemic injection of aripiprazole had no effect on within-session extinction behavior (**Figure 4.10B**). RM ANOVA of freezing at the final Extinction block compared to test revealed an effect of Day [$F_{(1, 29)}$=12.1, p<0.05], with no effect of Drug [$F_{(1, 29)}$=0.6, p=0.4], but an interaction between Day and Drug [$F_{(1, 29)}$=5.4, p<0.05]. Post-hoc paired t-tests found a

significant difference in freezing levels at extinction versus test for vehicle [$t_{(14)}$=4.1, p<0.05], but not aripiprazole (t<1) (**Figure 4.10C**). Thus, systemic treatment with aripiprazole at the time of fear extinction improved long-term extinction tested the next day in adult rats.

4.4 Discussion

In the present chapter I investigated extinction of conditioned fear in adolescent and adult rats. Adolescent rodents have previously been reported to display a deficit in long-term extinction compared to adult rodents (Kim et al. 2011; Pattwell et al. 2012; McCallum et al. 2010), consistent with data from adolescent humans (Pattwell et al. 2012). However, when I tested late adolescent (P53) and adult (P88) rats during the dark phase of the light-dark cycle in the present chapter, adolescent extinction deficit over and above adults was not observed. In addition, conditioning and extinction varied over different cohorts of rats when measured during the dark phase. Notably, fear extinction in late adolescent rats (P53) also showed variability when measured during the light phase. By comparison, I found that during the light phase younger adolescent rats (P35) displayed a robust deficit in long-term fear extinction compared to adults (P88), consistent with many previous findings examining extinction across adolescence.

Following optimization of protocol to investigate adolescent fear extinction deficits, I then aimed to examine a role for dopamine signaling in adolescent and adult fear extinction learning. I found that D1R agonist infusion in the IL at the time of extinction had no effects on within-session or long-term extinction for either adolescents (P35) or adults (P88). In contrast, intra-IL D2R agonist infusion at the time of extinction improved long-term extinction in adolescent rats, whereas it delayed extinction acquisition in adult rats. These data show for the first time that prefrontal dopamine signaling mediates adolescent fear extinction, and that its role in extinction is dissociated during adolescence versus adulthood. Interestingly, acute pre-extinction systemic treatment with the partial D2R agonist aripiprazole, an FDA-approved anti-psychotic, improved long-term extinction in adults. These findings highlight dopamine signalling as a potential pharmacological target for improving extinction learning during exposure-based therapy for anxiety disorders, and suggest the partial D2R agonist aripiprazole may provide benefits for adults as well as adolescents.

4.4.1 Behavioral experimentation during different phases of the light-dark cycle produces different results across adolescent development

The first experiment in this series aimed to optimize a protocol for investigating extinction of conditioned fear in late adolescent (P53) and adult (P88) rats during the dark phase of a 12:12 light-dark cycle. These housing conditions and age groups were chosen in order to remain consistent with conditions of Chapter 3, where I observed a deficit in extinction of a cocaine-associated cue in late adolescent compared to adult rats. In the present chapter, adolescent rats showed high levels of freezing at test 24 hours after 30, 60, or 90 CS presentations during fear extinction, but low levels of freezing following two sessions of 60 CS presentations either on the same day or two separate days. By comparison, adult rats (P88) exhibited spontaneous recovery of freezing during the dark phase irrespective of how many CS presentations were administered during extinction training.

Previous literature where behavioral experimentation was conducted during the light phase shows that 30 CS presentations is sufficient to reduce freezing to the CS at test the next day in adult (P70) rats compared to adolescent (P35) rats (Kim et al. 2011; McCallum et al. 2010). It has also previously been shown that extinction consisting of 60 CS presentations reduces spontaneous recovery in adolescents (P35) (Kim et al. 2011; McCallum et al. 2010). This was not observed here during the dark phase with late adolescent rats. The present findings are the first to my knowledge to examine late adolescent fear conditioning and extinction during the dark phase of the light-dark cycle. Across the relatively limited literature on adult fear conditioning during the dark phase, some have found no effect of time-of-day on acquisition of cued fear (Valentinuzzi et al. 2001). However, others have found decreased recall of cued and conditioned fear when trained and tested during dark phase compared to light phase (Chaudhury and Colwell 2002; Valentinuzzi et al. 2001; Eckel-Mahan et al. 2008). In the present study, adult rats exhibited spontaneous recovery of freezing during the dark phase regardless of how much extinction training was received. However, both adult and late adolescent rats showed variability in within-session extinction, making test results difficult to interpret. The following sections discuss possible explanations for increased behavioral variability during the dark phase.

4.4.1.1 Age

Analyses reveal that late adolescent rats showed particularly inconsistent data compared to the other ages. It is possible at P53, some individual rats may have been closer to adult maturity than others. Indeed, it is generally accepted that rats are considered adult at P60 (Spear 2000). Approaching this age means there may have been larger individual differences in behavior and learning and/or memory, especially for extinction, within and across cohorts. By comparison, the widely-accepted conservative age range for adolescence in rodents is P28 – 42 (Spear 2000; McCormick and Mathews 2010). Thus, most would agree that P35 falls within the range for adolescent development in terms of physiology and cognition. While it should be noted that a number of studies suggest puberty in male rats does not occur until P39 – 47 as measured by physiological markers (see Sengupta 2011 for review), measures of hormonal, physical, and behavioral markers suggest that adolescent-typical behavior is not necessarily dependent on physiological markers of puberty (Vetter-O'Hagen and Spear 2011). However, given the scarcity of information on conditioned fear and extinction across development, it would still be interesting for future studies to investigate behavior of rats across more adolescent age groups.

4.4.1.2 Locomotion and eating

Variability in extinction learning during the dark phase may relate to variability in patterns of locomotion and eating. Rats are nocturnal animals, and both adolescent and adults display similarly increased locomotion during the dark phase versus light phase (Kayyal et al. 2015). However, rats display relatively high variability in their motor rhythms (Refinetti 2006; Tang et al. 2007), and a number of studies confirm that rats show asymmetrical distributions of locomotion during the dark phase (Refinetti 2006; Borbély and Neuhaus 1978; Johnson and Johnson 1991). Rats also show peaks in eating at the beginning and end of the dark phase (Strubbe et al. 1986). I did take care to ensure conditioning, extinction, and test occurred at the same time each day within cohorts. However, between cohorts, the time of behavioral experimentation could differ up to several hours due to availability of equipment. Large fluctuations in locomotor activity and eating could mean marked differences in motivation and attention across this period, which may have contributed to the inconsistencies in conditioning freezing observed.

4.4.1.3 Circadian rhythm and sleep

Increased variability during the dark phase may also relate to disrupted circadian rhythms. Circadian rhythms have been identified as a key mediator of learning and memory processes (Walker and Stickgold 2006; Mahan and Storm 2009; Smarr et al. 2014; Krishnan and Lyons 2015). Though rats were only removed from the reverse light-dark room for the duration of behavioral testing, light pollution from the experimenter entering the room on repeated occasions could vary considerably between cohorts. Notably, light during the usual dark phase can disrupt circadian rhythms in rats for a number of physiological parameters (Dauchy et al. 2015), as well as behavioral measures (Bedrosian et al. 2013). Long-term memory processes are especially vulnerable to the effects of altered circadian rhythms, as shown by disruption of cognitive performance by phase shifting (Winocur and Hasher 1999; Devan et al. 2001; Chaudhury and Colwell 2002).

In addition, rats tested during the dark phase would be less likely to benefit from sleeping after behavioral testing compared to rats tested during the light phase. There is now extensive evidence that sleep plays an important role in learning and memory processes (Walker and Stickgold 2006; Smarr et al. 2014; Maquet 2001; Diekelmann and Born 2010). Moreover, sleep deprivation in rodents immediately following training has been shown to impair consolidation of contextual fear memory (Graves et al. 2003; Hagewoud et al. 2010; 2011), as well as Morris water maze (Smith and Rose 1996) and object recognition tasks (Palchykova et al. 2006).

4.4.1.4 Innate anxiety

Baseline analyses suggest both late adolescent and adult rats may exhibit increased anxiety during the dark phase compared to the light phase, as baseline freezing was high at test for both age groups of rats during the dark phase. Inconsistent with our data, one previous study found that light vs dark phase had no effect on innate anxiety as measured by an array of tests (standard and unstable elevated plus maze, holeboard, open field) in adult rats (Jones and King 2001). It has also been reported that adult rats in fact show decreased anxiety during the dark phase when tested for innate anxiety levels on the elevated plus maze (Verma et al. 2010). Therefore, I believe that increased baseline freezing in the present chapter was due to learned fear rather than innate

anxiety. This is especially plausible since baseline freezing was <1% at conditioning across light and dark phases in both adolescents and adults.

Notably, sensitivity to pain in rats is highest during the dark phase (Christina et al. 2004). Thus, fear conditioning during the dark phase in the present study may have caused increased sensitivity to shock, in turn increasing basal anxiety/generalized freezing across contexts for both adolescent and adult rats. Additionally, a previous study showed that an electric footshock administered during the dark phase did not change plasma levels of the stress hormone corticosterone in adult rats until 6 hours later, whereas it caused an immediate dramatic increase that plateaued to control levels after just 1 hour during the light phase (Retana-Márquez et al. 2003). Since corticosterone levels are considered an index of physical and/or psychological stimuli intensity (Pitman et al. 1988), rats might more easily discriminate the context in which electric footshock was administered during the light phase despite showing a decreased pain threshold. Therefore, behavioral experimentation during the light phase may improve context specificity and/or discrete cue-footshock associative learning.

4.4.1.5 Housing

Differences in housing conditions may also have contributed to increased variability in rats tested during the dark phase. Rats under a reverse light-dark cycle were housed in open-top cages, while rats under standard light-dark conditions were housed in individually ventilated cages (IVCs). Pilot experiments from our laboratory showed that open top housing leads to less consistent data compared to IVC housing, putatively due to the easy transfer of smells and sounds through the open top cages. This is consistent with findings that show altered behavioral phenotypes in transgenic mice housed in IVCs compared to open-top cages (Logge et al. 2013).

4.4.2 Adolescent deficits in fear extinction

Behavioral experimentation during the light phase of the 12:12 light-dark cycle revealed that adolescent rats (P35) display a reliable deficit in long-term extinction compared to adult rats (P88). This finding that retention of extinction is related to the age at extinction is consistent with previous studies that report impaired fear extinction in adolescents compared to adults in both rodents (Pattwell et al. 2012; Kim et al. 2011; McCallum et al. 2010) and humans (Pattwell et al. 2012). These findings of extinction

deficits during adolescence recapitulate clinical evidence that extinction-based therapy for anxiety is less effective in adolescents compared to other ages (Southam-Gerow et al. 2001; Bodden et al. 2008), providing a laboratory platform to investigate mechanisms and potential therapeutic avenues.

It should be noted that in the experiments involving drug manipulations, there was a spontaneous recovery of extinguished freezing 24 hours following extinction in adult rats. This behavior was not observed in our or other groups' previous studies (Quirk 2002; McCallum et al. 2010; Kim et al. 2011; Orsini et al. 2013), and was not observed in the first light cycle experiment across multiple cohorts of adult rats in the present chapter. A careful examination of the literature found that infusion of saline or vehicle into the IL or PL, but not other brain regions, before extinction may cause this effect in adult rats (Sierra-Mercado et al. 2011). This small spontaneous recovery due to vehicle infusion appears to also be present even when the freezing was well extinguished to baseline (i.e., ~0%), and when vehicle has also been infused during test to provide identical physiological contexts for extinction and test (Laurent and Westbrook 2008). A degree of spontaneous recovery has also been previously observed in adult rats that received a systemic injection of Tween-80 prior to extinction but not test (Harris and Westbrook 1998), though a similar effect has also been observed for systemic injections of saline or water prior to extinction and not test (Sotres-Bayon et al. 2007). It is possible that in these and the present study, return of freezing in adults may be a display of renewal. Indeed, renewal is known to occur not only on re-exposure to conditioning context, but also on exposure to novel context (Neumann and Kitlertsirivatana 2010). However, the majority of adult extinction studies without drug manipulation report low levels of freezing following extinction. Therefore, I believe that the results of the final experiments are idiosyncratic, and do not affect the overall interpretation of data.

4.4.3 Dopamine signaling and fear extinction

In the present study, I observed that the selective D1R agonist SKF-81297 (0.1 ug/side) had no effects on within-session or long-term extinction in either adolescents or adults. This is consistent with previous findings in adult male rats showing that pre-extinction systemic injection of the partial D1R agonist SKF38393 had no effect on fear extinction or retrieval in (Rey et al. 2014). By comparison, findings from studies using D1R

antagonists show that intra-IL infusion of SCH23390 impairs fear extinction (Hikind and Maroun 2008), and that systemic injection impairs extinction of cocaine-associated contextual cues (Fricks-Gleason et al. 2012). Present findings are the first to my knowledge to investigate the effect of an intra-IL infusion of a full D1R agonist on extinction in either adult or adolescent rodents. It may be that IL D1R signaling is necessary but not sufficient for extinction during adulthood and adolescence. Although a systemic injection of a D1R agonist has been shown to enhance extinction of cued and contextual fear in adult rats, the anatomical targets of that effect were not clear (Abraham et al. 2016).

By comparison, the role of IL D2R activity appears to be dissociated across age. I showed for the first time that in adolescent rats, pre-extinction quinpirole (1.0 ug/side), blocked relapse of extinguished freezing 24 hours later. These data are consistent with findings from Chapter 3 that show intra-IL quinpirole improves extinction of a discrete cocaine-associated cue in adolescent rats. Our lab has also recently shown that a systemic injection of the D2R partial agonist aripiprazole enhances adolescent fear extinction in a similar manner to intra-IL quinpirole (Ganella et al., under review). Those findings are also consistent with Chapter 3, where aripiprazole enhanced extinction of a cocaine-associated cue to reduce relapse-like behavior the next day in adolescent rats. Overall, it appears that acutely enhancing IL D2R signaling can improve extinction learning in adolescent rats across both appetitive (drug) and aversive (fear) domains.

In contrast, intra-IL quinpirole produced a delay in the acquisition of extinction in adult rats, with no effect on long-term extinction. Consistent with this, a previous study showed that adult rats systemically pre-treated with quinpirole still showed high levels of CS-elicited freezing after 10 non-reinforced CS presentations compared to adult rats treated with water (Nader and LeDoux 1999). Similarly, pre-extinction systemic quinpirole dose-dependently impaired long-term fear extinction (30 CS presentations) in adult rats compared to vehicle (Ponnusamy et al. 2005). On the other hand, pre-extinction systemic treatment with the D2R antagonist sulpiride has been shown to facilitate extinction in adult rats (Ponnusamy et al. 2005). Interestingly, intra-IL infusions of the selective D2R agonist 2-(N-Phenethyl-N-propyl) amino-5-hydroxytetralin hydrochloride (PPHT) has been found to increase error in a working

memory task by enhancing perseverative tendencies (Druzin et al. 2000), suggesting that enhancing D2R signaling in the adult brain may disrupt the function of prefrontal neural networks known to be involved in working memory.

However, others have shown that decreasing D2R signaling is *detrimental* for extinction in adult rats. Specifically, intra-IL or systemic treatment with the D2R antagonist raclopride (Mueller et al. 2010) or systemic or ICV treatment with the D2R antagonist haloperidol (Holtzman-Assif et al. 2010) at the time of extinction impaired retrieval of fear extinction the next day in adult rats. Disparities in the findings may be due to the functional specificity, pharmacological selectivity of agonists and antagonists used, dose and/or route of administration. While quinpirole is also capable of binding D3R (Gehlert et al. 1992), its primary mechanism of action is via the postsynaptic D2R (Bowery et al. 1994; Tseng and O'Donnell 2007a). One study of binding using human kidney cells actually found that quinpirole exhibited higher affinity for the D3R compared to the D2R (Robinson et al., 1994), however in the mammalian (canine) brain, quinpirole has been found to display greater selectivity for the D2R compared to D3R (Seeman and Schaus 1991). It is also worth noting that D2Rs also show higher expression in the PFC compared to D3Rs (Larson and Ariano 1995). By comparison, both haloperidol and sulpiride show high affinity for D3Rs and D4Rs, as well as D2Rs (Bowery et al. 1994; Martelle and Nader 2008). Notably, haloperidol has also been reported to bind the D1R (Köhler et al. 1985; Seeman and Ulpian 1988). Raclopride shows higher affinity for the D2R than sulpiride but also binds D3R with relatively high affinity (Martelle and Nader 2008). Further research is still required to delineate the precise functional role of prefrontal D2R signaling in fear extinction across development. Importantly, I show for the first time that acutely stimulating IL D2R signaling with a full D2R agonist can have different effects on learning and memory across maturation.

Importantly, I found that systemic treatment with the partial D2R agonist aripiprazole had no effect on within-session extinction, but actually reduced spontaneous recovery of CS-elicited freezing the next day in adult rats. To my knowledge, this is the first evidence that aripiprazole can improve consolidation of fear extinction learning in adult rats. Indeed, oral administration of aripiprazole has previously been found to abolish cognitive deficits in measures of attention and

response control in adult rats (Carli et al. 2010). Thus, aripiprazole may be improving extinction by improving attention and/or memory consolidation more broadly. Notably, aripiprazole has also been found to improve long-term fear extinction in adolescent rats (Ganella et al., under review). These and the present findings suggest that partial agonist activity at the D2R receptor, which can include increasing or decreasing activation depending on intrinsic dopamine signaling, may be beneficial to extinction learning during both adolescence and adulthood. Importantly, as discussed in the previous chapter, aripiprazole is favored clinically not only for its efficacy in treating psychiatric symptoms, but also because it is well-tolerated by patients (DeLeon et al. 2004). This makes aripiprazole an exciting candidate for use as an adjunct to extinction-based treatment for anxiety disorders, with results from the present study suggesting potential beneficial effects for adults as well as adolescents (Ganella et al., under review).

4.4.4 Conclusion

Together with findings of the previous chapter, these data suggest that adolescents are generally impaired in extinction of emotionally salient cues, with evidence for long-term deficits across an aversive (fear) as well as appetitive (drug) domain. Here I also provide novel functional data suggesting potentially different involvement of prefrontal D2R signalling in fear extinction across development. While more work is required to elucidate the precise involvement of prefrontal dopamine receptor signalling in extinction learning, present findings provide important steps for understanding extinction learning to improve exposure-based therapy for both adolescents and adults.

5 Dopamine receptor gene expression in the prefrontal cortex across adolescent development, and following cocaine-cue or fear extinction

5.1 Introduction

The previous chapters demonstrate that adolescent rats are impaired in extinction of a discrete cue associated with cocaine, or with an electric footshock. I showed that cocaine-cue extinction prevents cue-induced reinstatement of drug-seeking the next day in adult (P88) but not adolescent (P53) rats, while fear extinction attenuates the return of freezing behavior the next day in adult (P88) but not adolescent (P35) rats. In addition, I showed that enhancing IL dopamine signaling with the full D2R agonist quinpirole improved cue extinction learning in adolescents in both a cocaine-cue and fear paradigm, but delayed adult fear extinction learning. In contrast, systemic treatment with the partial D2R agonist aripiprazole enhanced cocaine-cue extinction in adolescents and fear extinction in adults. Interestingly, enhancing D1R signaling at the time of extinction training had no effect on fear extinction for adolescents or adults, under the present conditions. These findings show for the first time that modulating prefrontal dopamine signaling can have different effects on extinction learning across adolescent development, and highlight the dopamine system as a potential pharmacological target to improve extinction learning in exposure-based therapy for either drug addiction or anxiety disorders. Divergent effects of intra-IL infusions of the full agonist quinpirole suggest that adult and adolescent prefrontal networks are distinct in terms of dopamine signaling.

In order to elucidate how age differences in the prefrontal dopamine system may contribute to age differences in extinction learning, the present chapter investigates innate dopamine receptor gene expression in the mPFC. Using real-time quantitative polymerase chain reaction (RT-qPCR) analysis, I measured mPFC D1R and D2R gene expression in naïve rats across adolescent development, as well as in adolescent and adult rats following cocaine- or fear-cue extinction.

Dopamine exerts its effects via five distinct receptors, which are subdivided into two families: D1-like and D2-like receptors (Andersen et al. 1990). The D1-like subfamily comprises D1R and D5R, and the D2-like includes D2R**,** D3R and D4R. All belong to the superfamily of GPCRs which feature seven highly-conserved transmembrane domains (Missale et al. 1998). The most abundant dopamine receptor subtypes in the central nervous system are D1R and D2R (Jaber et al. 1996), with both showing expression in the mPFC (Vincent et al. 1993). As members of different subfamilies, D1R and D2R show distinct profiles in terms of downstream signal transduction and physiological effects (Beaulieu and Gainetdinov 2011; Jackson and Westlind-Danielsson 1994) as reviewed in the Introduction of this thesis. However, D1R and D2R also show important similarities, which have implications for visualization and quantification in brain tissue. For instance, sequence similarity searching using the Basic Local Alignment Search Tool (BLAST) database reveals that D1R and D2R share 77% of their amino acid sequence (Agostino 2012). It follows that D1R and D2R display somewhat similar ligand binding profiles (Levey et al. 1993). This means that commercially available antibodies for D1R and D2R are liable to display cross-reactivity. In fact, antibodies against GPCRs are notoriously unreliable, owing at least in part to high levels of homology even across broad GPCR groups (Michel et al. 2009; Hutchings et al. 2010). Immunostaining of D2R in particular has historically shown conflicting results across previous literature, with some studies reporting extensive labelling throughout all layers of cortex (Ariano et al. 1993), while others have shown little to no staining (Levey et al. 1993; Sesack et al. 1994).

Therefore, I determined that a more reliable proxy measure of D1R and D2R involvement in mPFC function across adolescence and during extinction would be by quantification of gene expression using real-time quantitative polymerase chain reaction (RT-qPCR). In this method, total ribonucleic acid (RNA) is extracted from the target brain region, and reverse transcribed into complementary deoxyribonucleic acid (cDNA). This serves as a template for PCR quantification, which is achieved using primers specifically designed to amplify the cDNA of the target gene of interest. In this case, genes of interest were *Drd1*, which codes for D1R, and *Drd2*, which codes for D2R. During each PCR cycle, the amount of target DNA doubles, which increases the fluorescence of a DNA-binding dye. In RT-qPCR, the intensity of this fluorescence is measured after every cycle (Pfaffl 2001). The cycle number at which the fluorescence

exceeds the threshold for detection is called the threshold cycle (Ct) (Schmittgen and Livak 2008). The expression of the gene of interest is then calculated by comparison with an internal control gene or housekeeping gene (Pfaffl et al. 2004). This gene is chosen based on its stable expression across experimental conditions. By normalizing to a housekeeping gene, any difference in gene expression between experimental groups can be attributed to treatment, rather than differences in absolute initial levels of cDNA.

In order to quantify gene expression using RT-qPCR, a number of factors must be considered. Isolation of high quality RNA, primer specificity, selection of a stable housekeeping gene, and appropriate data analysis are crucial elements for accurate results. Thus, given that no one in our laboratory yet had previously used this technique in rats, my first aim was to optimize several of these experimental steps to ensure my RT-qPCR experiments would generate reliable results. I then implemented this optimized protocol to examine prefrontal dopamine receptor gene expression in naïve rats age P35, P53, and P88. In a second experiment, I measured mPFC D1R and D2R gene expression in adolescent (P53) and adult (P88) rats before or after cocaine-cue extinction. Finally, I measured mPFC D1R and D2R gene expression before or after fear extinction in adolescent (P35) and adult (P88) rats.

5.2 Materials and methods

5.2.1 Subjects

Refer to Chapter 2.1 for details. Briefly, male Sprague Dawley rats (N = 100) were bred in-house. For the naïve RT-qPCR experiment, rats were housed in standard housing as described in **Table 2.1** and did not undergo any behavioral testing but were handled 3 times prior to tissue collection. Rats were P35±1 P53±1, or P88±1 at tissue collection day. For the cocaine-cue extinction experiment, rats were individually housed under reverse light-dark conditions as described in **Table 2.1**. Rats were aged P34±1 or P69±1 at the commencement of cocaine self-administration (and P53±1 or P88±1 on cue extinction day). For the fear extinction experiment, rats were housed 3 - 4 per cage in standard housing as per **Table 2.1**. Rats were aged P35±1 or P88±1 on extinction day.

5.2.2 Behavioral protocol

5.2.2.1 Surgery

Refer to Chapter 2.2.2. for details.

5.2.2.2 Cocaine Self-Administration, Lever Extinction, and Cue Extinction

Refer to Chapter 2.4.1 and Chapter 2.4.2 for details.

5.2.2.3 Fear Conditioning and Extinction

Refer to Chapter 2.4.3 and 2.4.4 for details.

5.2.3 RT-qPCR protocol

5.2.3.1 Tissue collection

Rats in the cocaine-cue extinction experiment were handled (Pre-extinction) or received cue extinction (Post-extinction) and killed either immediately after (0h) or the next day (24h). This way, cocaine-cue brains were microdissected at ~1.5 hrs from the onset of extinction or at a time when cue-induced reinstatement test would occur. Rats in the fear extinction experiment were handled (Pre-extinction) or received extinction (Post-extinction) and killed two hours later.

Tissue collection included both the IL and the PL of the mPFC (**Figure 5.1**). The IL and PL are adjacent structures with no observable anatomical landmark separating them to the naked eye (Paxinos and Watson 1998), therefore I determined that combining these regions would be the best approach to minimize the chance of including some of one region in samples of the other, and vice versa. Thus we were able to confidently quantify dopamine receptor gene expression in the mPFC as a whole, rather than make tentative conclusions from samples of IL and PL individually. When collecting mPFC sections, I was mindful of the distribution of receptors across the layers of cortex. While D1Rs are expressed diffusely across layers II, V and VI of the cortex, D2Rs are reportedly localized almost exclusively to Layer V (Santana et al. 2009). This gave a further rationale for not 'punching out' the IL separately from PL, which can only include layers I, II, and III (Cruz et al. 2015). Thus I ensured that the sections taken included the region as close as possible to the visible white matter of the corpus callosum, in order to be confident that the deeper D2R-containing cortical layers were included in all samples, in addition to more superficial cortical layers I-IV.

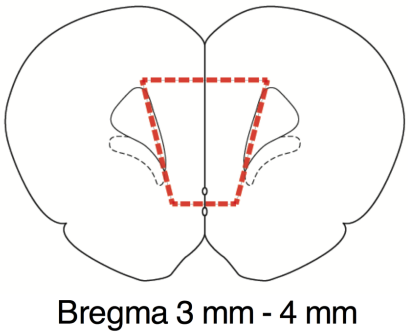

Figure 5.1 *Coronal section illustrating mPFC collected for RT-qPCR analyses. Section within broken line indicates microdissected tissue.*

The mPFC of each hemisphere was micro-dissected using equipment cleaned with 100% ethanol, RNAse zap (Qiagen) and diethylpyrocarbonate (DEPC)-treated water prior to use and between each rat. Equipment was kept on a metal plate on wet ice to stay cool during tissue collection procedures. Each rat was terminally anaesthetized by intraperitoneal sodium pentobarbitone injection (100 mg/kg) and the brain was rapidly removed and washed in cold sterile saline. The brain was then positioned ventral side up in a metal rat brain matrix (World Precision Instruments, FL, USA), which had been pre-cooled on wet ice. The brain was positioned such that the rostral surface of the brain was flush with the rostral end of the brain matrix. Razor blades that had been pre-cooled were lowered simultaneously at intervals 3 and 4 mm from the rostral end of the matrix, to make a coronal slice spanning approximately 3 – 4 mm from bregma (Paxinos and Watson 1998). This section was removed and positioned rostral side up on the cooled metal plate, and the mPFC of each hemisphere was micro-dissected using metal razor blades. Collected mPFC sections from left and right hemispheres were combined and placed into a 1.7mL sterile Eppendorf tube. Samples were frozen over liquid nitrogen, then stored at -80°C until required for RNA extraction and isolation.

5.2.3.2 RNA isolation

Total RNA was extracted from each sample using the RNeasy Mini kit (Qiagen, NL). QIAzol lysis reagent (900 µl, Qiagen, NL) was added to micro-dissected tissue and homogenized using a fixed blade variable speed Tissue-Tearor (Biospec Products Inc.) for approximately 30 seconds or until tissue was emulsified. gDNA eliminator (100 µl) was then added to the homogenate, followed by 180 µl of chloroform. The sample was then centrifuged at 12,000 *g* for 15 minutes (4 °C) to separate RNA, DNA and protein into fractions. All subsequent steps were carried out at room temperature, with

centrifugation at 8,000 *g* for 15 seconds unless otherwise stated. The upper RNA-containing aqueous phase was collected and combined with an equal volume of 70% ethanol (v/v in DEPC-treated water). The solution was mixed thoroughly, then passed through an RNeasy spin column. Buffer RWT (700 µl) was passed through the spin column once, then Buffer RPE (500 µl) was passed through twice (the second time for 2 minutes) to wash the column membrane prior to elution of the RNA with RNase-free water (35 µl). Aliquots (10 µl) of each sample were taken for assessment of concentration and purity. One ul per sample was analysed using a Nanodrop ND-2000c Spectrophotometer (Thermo Scientific, USA), to obtain 260/280 and 260/230 values. To confirm integrity of eluted RNA and identify gDNA contamination, a sample (1 µg of RNA) from each treatment group was made up to 20 µl in RNAse-free water and denatured at 70°C for 5 min, then run on a 0.8% agarose gel at +100V for ~40 minutes. RNA bands were detected by UV transillumination and imaged with the BioDoc-ITTM Imaging System (Ultra-Violet Products Limited). Intact 28S and 18S ribosomal RNA bands were visualized and an approximate mass ratio of 2:1 indicated that RNA was intact for each sample. RNA samples were stored at -80°C until required for reverse transcription.

5.2.3.3 Reverse transcription PCR

For each sample, 1 µg of total RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems) with random hexamers. Reverse transcription (RT) reactions were performed using the PCR Thermal Cycler Dice (Takara Bio Inc., JP). Reactions (20 µl) containing RT master mix and template RNA were conducted under the following conditions: 25°C for 10 minutes, 42°C for 30 minutes, 95°C for 5 minutes and held at 4°C (**Table 5.1**). Following RT, neat cDNA was diluted 1:6 in nuclease-free water for use in qPCR reactions. Samples containing cDNA products were stored at -20°C until required.

Table 5.1 *Conditions for reverse transcription PCR reactions.*

Stage	Temperature	Duration
Primer annealing	25 °C	10 min
Extension	42 °C	60 min
Reaction termination	85 °C	5 min
Hold	4 °C	∞

5.2.3.4 Real-time quantitative PCR

Gene expression was measured by RT-qPCR using the ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA; **Table 5.2**). Reactions were performed in triplicate in 96- or 384-well plates and run using standard conditions: 2 minutes at 50 °C, 10 minutes at 95 °C, and 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. A product dissociation melt curve was then produced to ensure there was only one PCR product per reaction. Each well contained cDNA plus master mix consisting of SYBR Green Mastermix (Applied Biosystems, USA), forward and reverse primers, and DNase- and RNase-free water. Amount of PCR product for every amplification cycle was quantified by measuring the fluorescence of DNA-intercalating fluorophore SYBR Green. Data were analyzed using ViiATM 7 software (Applied Biosystems, USA).

Table 5.2 *Conditions for RT-qPCR reactions.*

Stage	Cycles	Temperature	Duration
DNA polymerase activation	1	50 °C 95 °C	2 min 10 min
PCR	40	95 °C 60 °C	15 sec 1 min
Melt (dissociation) curve	1	95 °C 60 °C 95 °C	15 sec 1 min 15 sec

5.2.3.5 PCR primer sequence design

Primers were designed using Primer3 software (Rozen and Skaletsky 1999). The primer sequences for endogenous control genes and target genes are provided in **Table 5.3**. It should be noted that the D2R exists in two alternatively spliced isoforms: the D2R short form and the D2R long form (Usiello et al. 2000). These isoforms have distinct functions in vivo, with the short form showing pre-synaptic autoreceptor activity and the long form showing mainly post-synaptic activity (Usiello et al. 2000). In the current study, primers were designed to amplify mRNA for both short and long isoforms of D2R from total prefrontal RNA, therefore results for D2R refer to total amount of both forms.

Table 5.3 *Primer sequences for RT-qPCR relative gene expression analyses*

Gene	Accession no.	Forward primer (5'→3')	Reverse primer (3'→5')
Hprt1	NM_012583.2	CTGGTGAAAAGGACCTCTCG	TCCACTTTCGCTGATGACAC
Actb	NM_031144.3	CTAAGGCCAACCGTGAAAAGAT	AGAGGCATACAGGGACAACACA
Gapdh	NM_017008.4	CTACCCCCAATGTATCCGTTG	AGCCCAGGATGCCCTTTAGT
Drd1	NM_012546.3	CCTTCGATGTGTTTGTGTGG	GGGCAGAGTCTGTAGCATCC
Drd2	NM_012547.1	TCCTGTCCTTCACCATCTCC	GACCAGCAGAGTGACGATGA

5.2.3.6 Internal control gene validation

In RT-qPCR experiments, several parameters can affect quantification of gene expression, including integrity of the RNA, loading error, primer performance, or inhibitory factors of the tissue. This means that a 'full procedural control' (Pfaffl et al. 2004) or housekeeping gene is required for all reactions. In order for this gene to act as an effective internal control, its expression must remain constant under different experimental conditions. However, the expression of several endogenous control genes have been shown to vary depending on treatment, stress, and brain region (Vandesompele et al. 2002). Therefore, three widely-used candidate control genes were assessed for use across behavioral conditions and age groups: Actin β *(Actb)*, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*). To assess candidate housekeeping genes, I used cDNA from cocaine-cue extinction rats, as these subjects had experienced the most extensive behavioural and neurochemical manipulations and had tissue collected at two different experimental time points. This group was therefore deemed most appropriate to determine whether housekeeping gene expression was indeed stable across treatment and age.

Neat cDNA was taken from 13-14 rats in each age group (adolescent and adult) comprising 3-4 rats per treatment group (No Ext 0h, Ext 0h, No Ext 24h, and Ext 24h) and pooled within age and treatment groups. Any differences between the treatment groups or age groups were then assessed using RT-qPCR. For this experiment, reactions were performed in triplicate in 96-well plates under standard conditions as described above. Thus neat cDNA was diluted 1:6, then 4 µl of dilute cDNA was added to wells followed by mastermix containing 10 µl SYBR Green Mastermix, 0.8 µl each of the forward and reverse primers (10 µM) made up to 20 µl with DNAse and RNAse free water. RT-qPCR was conducted as described above. Unpaired t-tests were used to assess the difference in fold change between age groups and treatment groups, for each

candidate internal control gene. Genes were also assessed using algorithms designed specifically to validate the stability of potential endogenous control genes. The web-based tool RefFinder (Kim et al. 2010; Xie et al. 2012) integrates several computational programs, BestKeeper (Pfaffl et al. 2004), NormFinder (Andersen et al. 2004) and GeNorm (Vandesompele et al. 2002), along with the comparative $\Delta\Delta C_T$ method (Silver et al. 2006) to compare and rank candidate housekeeping genes based on C_T values from qPCR reactions.

5.2.3.7 Primer efficiency assessment

To ensure that relative gene expression analyses were reliable, I first determined that primers for the endogenous control and target genes amplified cDNA with similar efficiencies across a range of cDNA concentrations. If primer efficiency varies according to total starting amount of cDNA in a reaction, quantification of relative expression will be inaccurate. An optimal primer will have an efficiency of 100%, which would double the amount of amplicon with each PCR cycle. However, primer efficiency between 75 – 120% is generally considered adequate for qPCR analyses (Buh Gašparič et al. 2008). Here, I used a serial dilution of cDNA to calculate primer efficiency and therefore determine optimal primer concentration for subsequent experimental qPCR analyses.

Briefly, RT-qPCR was performed on serial dilutions of equally pooled cDNA from rats across experimental groups. Pooled cDNA was first diluted to 1:6, from which subsequent serial dilutions of 1:2, 1:5, 1:10, 1:20, 1:50 and 1:100 were made. Reactions were performed in triplicate in 96-well plates under standard conditions. Each well contained 4 µL of cDNA and master mix containing 10 µL SYBR Green Mastermix plus 0.8 µl of forward and reverse primers (10 µM), made up to 20 µL total volume with DNase- and RNase-free water. The average threshold cycle (Ct) value of each triplicate (Y-axis) was plotted against the log of the cDNA dilutions (X-axis) for both the endogenous control gene and the target gene primers. Linear regression analysis was performed using GraphPad Prism 6.0 Software to determine the line of best fit, from which the gradient (m) could be calculated. This value was used to calculate amplification efficiency (E) for each gene, using the equation:

$$E = 10^{(1/m)} \times 100$$

5.2.4 Statistical analyses

Experimental gene expression data were analyzed using two-way ANOVA for each target gene, with Treatment (Pre-extinction vs Post-extinction) and Age (Adolescent vs Adult) as between subjects factors. Significant interactions were followed by t-tests as appropriate. Analyses of behavioral data were conducted using RM ANOVA, ANCOVA, ANOVA, and/or t-tests as appropriate. Statistical tests were conducted using SPSS. Any F or t value less than 1 was summarized as F or t <1. Acceptance for significance was determined at $p \leq 0.05$.

5.3 Results

5.3.1 Internal control gene validation

A housekeeping gene (HKG) was chosen by measuring mPFC expression of *Actb*, *Gapdh*, and *Hprt1* in adolescent (P53) and adult (P88) rats from pre- and post-cocaine-cue extinction groups, with tissue collected at 0h and 24h time points. Analysis of relative gene expression was performed using the $2^{-\Delta\Delta C}{}_T$ method (Schmittgen and Livak 2008; Livak and Schmittgen 2001). Following reverse transcription, neat cDNA was diluted 1:6 for use in RT-qPCR reactions. Triplicate C_T values were determined for each rat, which were averaged to a mean C_T value. The difference between the mean C_T value of the gene of interest (GOI) and the mean C_T value of the housekeeping gene was defined as ΔC_T, such that:

$$\Delta C_T = C_{T\,GOI} - C_{T\,HKG}$$

To compare the effect of Treatment i.e. Pre-extinction versus Post-extinction, the mean ΔC_T for the GOI and the HKG for Pre-extinction rats was calculated and subtracted from the ΔC_T for each rat to generate individual $\Delta\Delta C_T$ values:

$$\Delta\Delta C_T = (C_{T\,GOI} - C_{T\,HKG}) - (C_{T\,GOI\ average\ Pre\text{-}extinction} - C_{T\,HKG\ average\ Pre\text{-}extinction})$$

This formula was also used to investigate the effect of Age on potential internal control gene expression, where the mean ΔC_T for adult rats was calculated and subtracted from the ΔC_T for each rat to generate individual $\Delta\Delta C_T$ values:

$$\Delta\Delta C_T = (C_{T\,GOI} - C_{T\,HKG}) - (C_{T\,GOI\ average\ Adult} - C_{T\,HKG\ average\ Adult})$$

The formula $2^{-\Delta\Delta Ct}$ was then used to determine the relative fold change in housekeeping gene expression for treatment groups relative to Pre-extinction, or for age groups relative to adult rats.

Analyses of C_T values across treatment groups at the 0h time point showed no significant difference in expression of any of the internal control genes tested (*Actb* p=0.94, *Gapdh* p=0.97, *Hprt1* p=0.67). Comparison of C_T values across treatment groups at 24h likewise showed no significant difference in expression of any of the internal control genes tested (*Actb* p=0.76, *Gapdh* p=0.66, *Hprt1* p=0.10). Comparison of C_T values across age groups also showed no differences (*Actb* p=0.32, *Gapdh* p=0.06, *Hprt1* p=0.18). Based on these analyses, it appears that *Gapdh* has the greatest change in expression between age groups compared to *Actb* and *Hprt1*, as the age effect for *Gapdh* approached statistical significance. However, C_T values alone do not control for total RNA in the reaction.

Therefore, I tested each candidate as an internal control gene and measured the relative fold change in gene expression of the remaining two genes using the $2^{-\Delta\Delta Ct}$ method (**Figure 5.2**). Analyses of fold change differences between treatment groups using unpaired t-tests showed no significant differences between Pre-extinction and Post-extinction at either the 0h or 24h time point (ps>0.05).

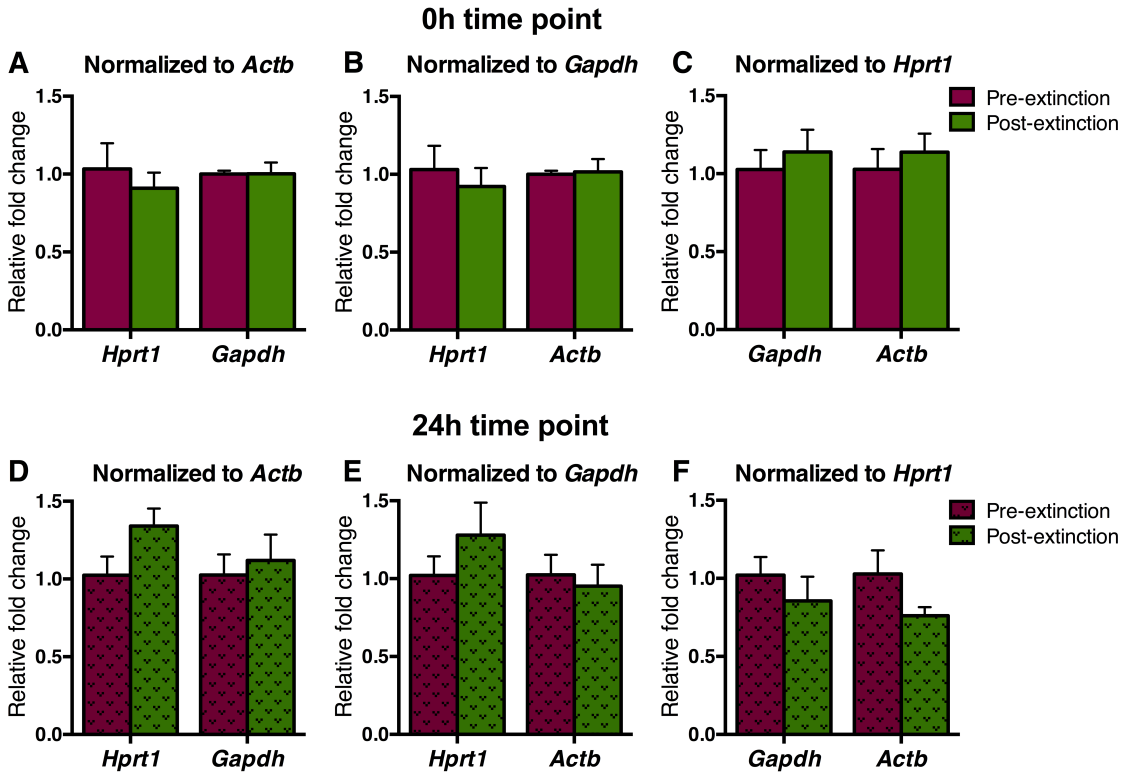

Figure 5.2 *Housekeeping gene validation. Each candidate housekeeping gene was assessed for stability as an internal control gene across experimental treatment groups (Pre-extinction versus Post-extinction) at different time points. There were no significant differences in gene expression when data was normalized to (A) Actb, (B) Gapdh or (C) Hprt1 at the 0h time point. There were also no differences when data was normalized to (D) Actb, (E) Gapdh or (E) Hprt1 at the 24h time point. Pre-extinction n = 33, post-extinction n = 32. Data represent mean +SEM.*

However, analyses of fold change differences between age groups (**Figure 5.3**) showed a difference between adolescents and adults for *Gapdh* normalized to *Actb* [t(14)=2.4, $p<0.05$], and for *Actb* when normalized to *Gapdh* [t(14)=2.6, $p<0.05$]. There were no differences between ages for *Actb* or *Gapdh* when normalized to *Hprt1*. Thus from this analysis, *Hprt1* appeared to be the most stable housekeeping gene, as the other two genes showed the smallest fold change away from 1 when normalized to *Hprt1*, with no significant differences between groups.

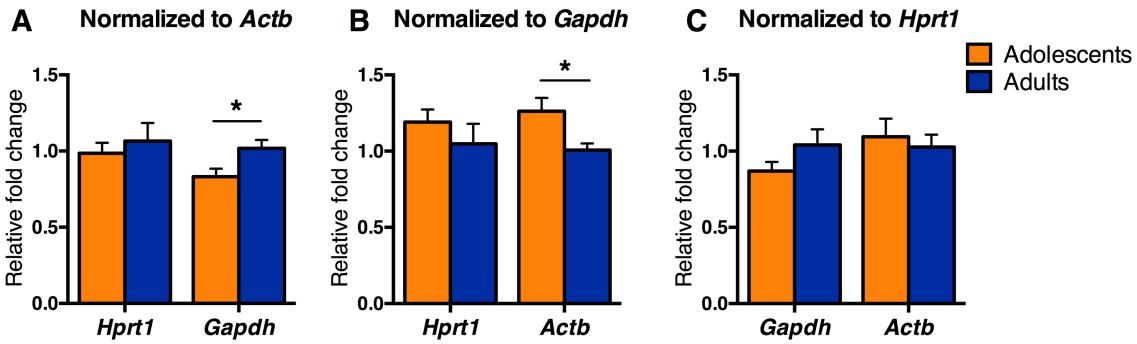

Figure 5.3 *Housekeeping gene validation. Each candidate housekeeping gene was assessed for stability as an internal control gene across Age groups. (A) There was a difference in fold change between adolescents and adults for Gapdh normalized to Actb, and for (B) Actb normalized to Gapdh. (C) There were no differences between ages for Actb or Gapdh when normalized to Hprt1. Adolescent n = 29, adult n = 36. Data represent mean +SEM. *p<0.05*

I confirmed the most appropriate housekeeping gene by using web-based algorithms specifically designed for this purpose. When C_T data from this experiment was entered into RefFinder, two of the four tests ($\Delta\Delta C_T$ method and NormFinder) selected *Hprt1* as the best candidate for an internal control gene, while GeNorm selected *Hprt1* and *Actb* as equal first. Only BestKeeper selected *Actb* as the first choice, with *Hprt1* second. Based on all analyses used, I determined *Hprt1* as the most suitable housekeeping gene to measure prefrontal D1R and D2R gene expression across age and treatment groups.

5.3.2 Efficiency of primers for mPFC internal control and target genes

To assess primer efficiency, qPCR results from a serial dilution of cDNA were plotted, then linear regression was performed and the r^2 and lines of best fit were determined for each gene (*Hprt1*, *Drd1*, *Drd2;* **Figure 5.4**). A primer with 100% efficiency will show a gradient of -3.3, as it takes 3.3 PCR cycles to double the number of amplicons for each 10-fold dilution of template. An r^2 value close to 1 indicates a close linear relationship between the amount of cDNA in reaction and the resultant C_T value for each set of primers. Values for r^2, gradient, and amplification efficiencies are provided in **Table 5.4.** All primer sets displayed efficiencies within the accepted range of 75-120% (Buh Gašparič et al. 2008), and the concentration of cDNA template at each dilution was within the range for reliable detection and quantification using qPCR i.e. $C_T<35$ (McCall et al. 2014). Thus all primers were deemed suitable for subsequent experimental RT-qPCR.

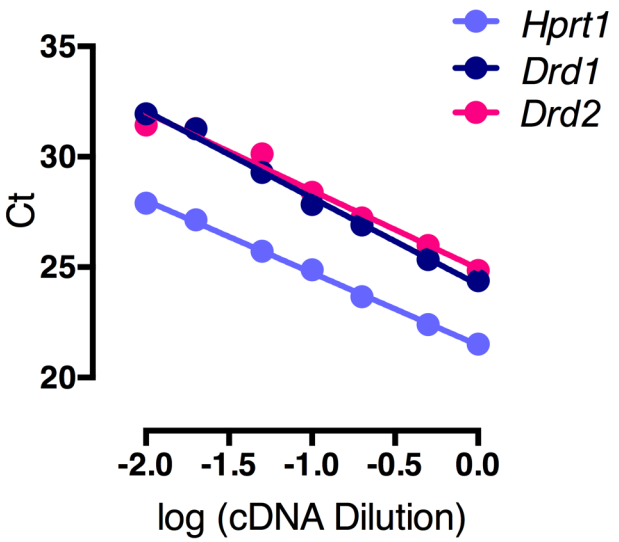

Figure 5.4 *Primer efficiency curves. Standard curves were generated for each primer set (Hprt1, Drd1, Drd2) by RT-qPCR using serial dilutions of cDNA. Average Ct values of triplicate reactions are plotted for each cDNA dilution. Lines of best fit were determined using linear regression analysis.*

Table 5.4 *Primer efficiency.*

Gene	r^2	Gradient	Amplification efficiency
Hprt1	0.9982	-3.274	102%
Drd1	0.9793	-3.535	92%
Drd2	0.9951	-3.933	80%

5.3.3 No age differences in mPFC dopamine receptor gene expression in naïve rats

In order to examine potential natural maturational differences in prefrontal dopamine receptor gene expression across adolescent development prior to any behavioral treatment, I compared gene expression for D1R and D2R in naïve rats aged P35, P53 and P88 (**Figure 5.5**). These age groups corresponded to the age at which rats received fear-cue extinction (adolescents: P35 and adults: P88) and cocaine-cue extinction (adolescents: P53 and adults: P88). For these analyses, qPCR was performed for the endogenous control gene (*Hprt1*) and target genes (*Drd1* and *Drd2*) for each sample. Analyses used the $2^{-\Delta C}{}_{T}$ method (Livak and Schmittgen 2001). Because cortical neural networks are governed by a balance of D1R versus D2R signaling (Seamans and Yang 2004), dopamine receptor gene expression was also analyzed as a ratio using $2^{-\Delta C}{}_{T\ D1R/D2R}$ where:

$$\Delta C_{T\ D1R/D2R} = C_{T\ D1R} - C_{T\ D2R}$$

Analyses of mPFC gene expression using one-way ANOVA revealed no difference between age groups for D1R [$F_{(2, 9)}$=1.54, p=0.26], D2R (F<1), or D1R/D2R ratio [$F_{(2, 9)}$=1.48, p=0.28].

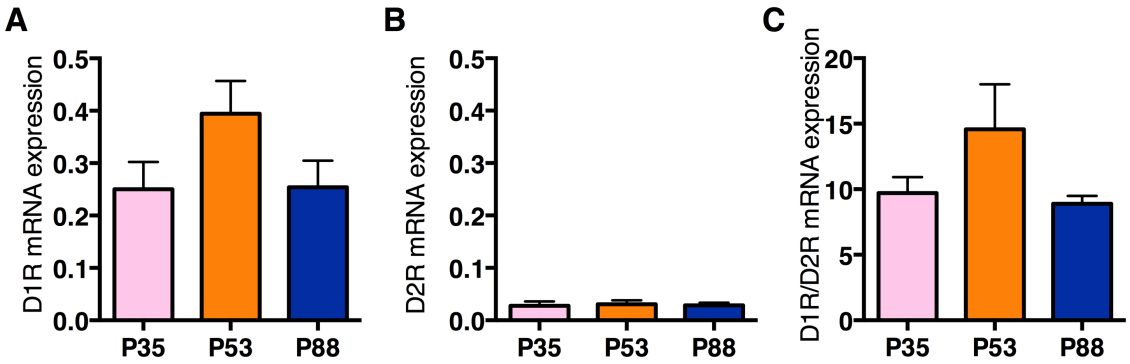

Figure 5.5 *Similar mPFC dopamine receptor gene expression in naïve animals across adolescent development. Gene expression for (A) D1R, (B) D2R, or (C) D1R/D2R ratio was similar for adolescents (P35), late adolescents (P53) and adults (P88). Results are inverted for display so that lower ΔCt values represent lower levels of gene expression. P35 n = 4, P53 n = 4, P88 n = 4. Data represent mean +SEM.*

5.3.4 Dopamine receptor gene expression in the mPFC following cocaine-cue extinction

In order to investigate the relationship between D1R versus D2R gene expression and cocaine-cue extinction, adolescent and adult rats first underwent cocaine self-administration and lever extinction. Firstly, there was no difference between adult and adolescent rats in cocaine self-administration, which replicates results described in chapter 3 of the present thesis. Analyses of active lever data using repeated measures ANOVA showed a significant effect of self-administration Day [$F_{(9, 567)}$=12.1, p<0.05], but no effect of Age [$F_{(1, 63)}$=2.6, p=0.1], and no interaction between Day and Age (F<1). Consistent with this, analyses of reward data revealed a significant effect of Day [$F_{(9, 567)}$=11.5, p<0.05] with no effect of Age [$F_{(1, 63)}$=2.4, p=0.1], and no interaction (F<1). Inactive lever response data showed no effect of Day or Age, and no interaction (Fs<1), indicating inactive lever pressing remained low over self-administration. RM ANOVA of active lever, inactive lever, and reward data for each age showed no pre-existing differences between Pre-extinction and Post-extinction groups prior to microdissection (ps>0.05), so the data are pooled within each age in **Figure 5.6.**

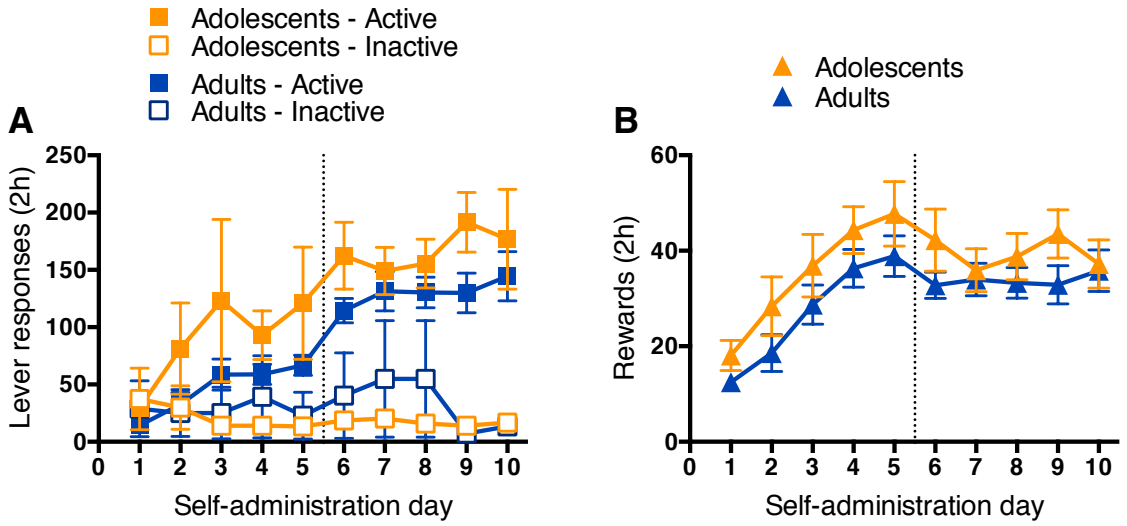

Figure 5.6 *Cocaine self-administration was similar for adolescent and adult rats. Self-administration responding occurred on a fixed ratio (FR) 1 for the first 5 days, and increased to FR3 for the final 5 days (broken line). (A) Responding on the active lever increased for both age groups over self-administration days, while responding on the inactive lever remained low. (B) Rewards i.e. cocaine infusions (0.3 mg/kg/infusion) earned increased for both age groups over self-administration days. Adolescent n = 29, adult n = 36. Data represent mean ±SEM.*

RM ANOVA of extinction lever data for each age showed no pre-existing differences between Pre-extinction and Post-extinction groups in lever extinction, therefore the data were pooled within each age in **Figure 5.7**. Analyses of active lever responses revealed a significant main effect of Day [$F_{(6, 378)}$=31.8, p<0.05], as well as a significant effect of Age [$F_{(1, 63)}$=6.1, p<0.05], but no significant interaction [$F_{(6, 378)}$=1.6, p=0.1]. Thus all rats showed a significant decrease in active lever pressing over lever extinction days, although overall active lever pressing was different between age groups. The age effect observed in active lever pressing was likely due to a general increase in lever pressing by adolescent compared to adult rats, as the same analyses of inactive lever response data revealed a significant main effect of Day [$F_{(6, 378)}$=8.3, p<0.05], a significant effect of Age [$F_{(1, 63)}$=27.6, p<0.05], and an interaction [$F_{(6, 378)}$=5.0, p<0.05]. Independent t-tests showed a significant difference between adolescents and adults for inactive lever pressing on Day 1 – 4, and 5 – 6.

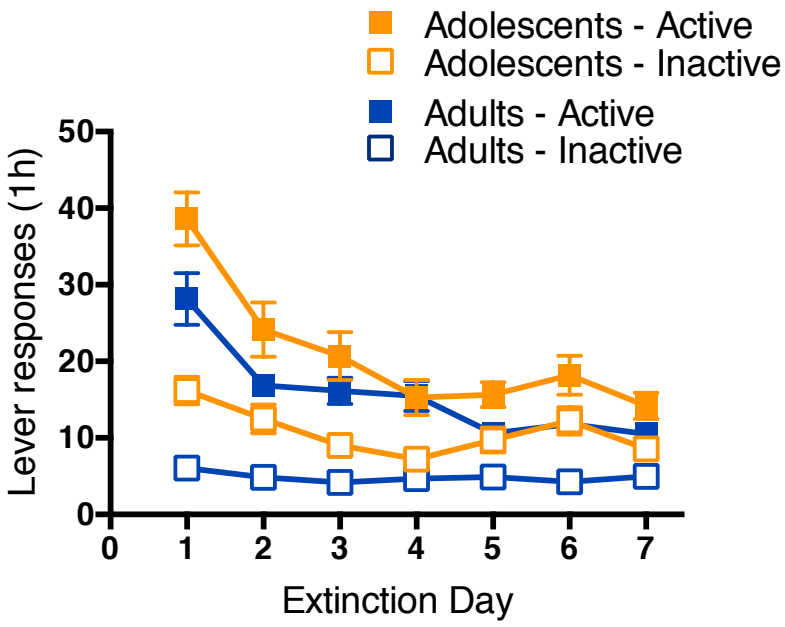

Figure 5.7 *Active lever responding decreased over lever extinction days, with adolescents pressing more on both the active and inactive lever overall, but no difference between age groups by final lever extinction day. Inactive lever responding remained low relative to active lever responding across days. Adolescent n = 28, adult n = 38. Data represent mean ±SEM.*

The day after final lever extinction, rats were assigned to one of two groups: Pre-extinction, which was handled but had no exposure to behavioral apparatus, or Post-extinction, which received cue extinction consisting of 120 cue presentations in the absence of cocaine or levers. The mPFC was collected for gene expression analyses by RT-qPCR immediately (0h), or 24 hours later (24h).

When tissue was collected immediately, there was no difference in prefrontal dopamine receptor gene expression for Pre-extinction rats (**Figure 5.8**). Independent t-tests showed no difference between adolescents and adults for D1R mRNA expression ($t<1$), D2R mRNA expression [$t_{(21)}=1.5$, $p=0.1$] or D1R/D2R ratio [$t_{(21)}=1.4$, $p=0.2$]. There was also no significant effect of cue extinction training. ANOVA for D1R gene expression revealed no effect of Age, Treatment, and no interaction ($Fs<1$). ANOVA for D2R gene expression similarly showed no effect of Age or Treatment ($Fs<1$), and no interaction [$F_{(1, 39)}=1.5$, $p=0.2$]. ANOVA for D1R/D2R ratio of gene expression also showed no effect of Age, Treatment, and no interaction ($Fs<1$).

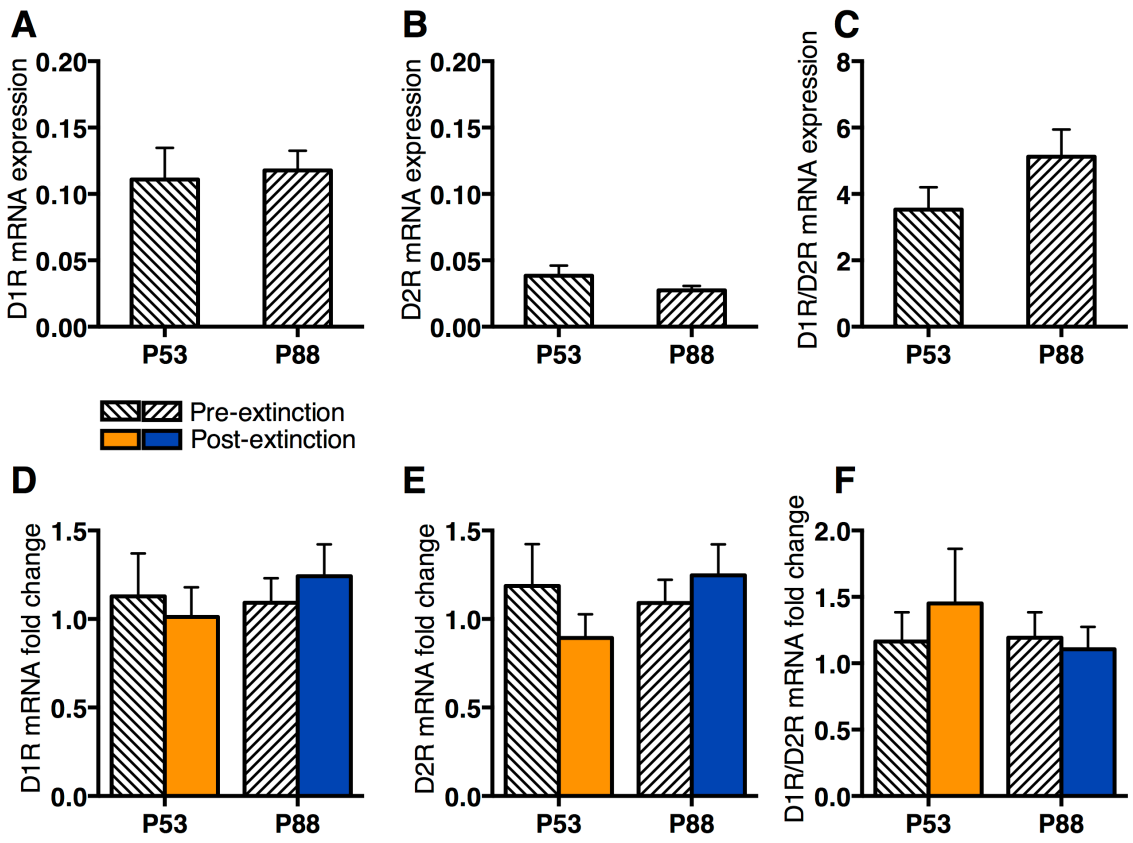

Figure 5.8 *Prefrontal dopamine receptor gene expression in adolescent (P53) and adult (P88) before or after cocaine-cue extinction at 0h. There were no significant age differences Pre-extinction for (A) D1R, (B) D2R, or (C) D1R/D2R ratio mRNA expression. There was also no observed age or treatment differences in gene expression as a result of cocaine-cue extinction for (D) D1R, (E) D2R. or (F) D1R/D2R ratio. P53 n = 16, P88 n = 27. Data represent mean +SEM.*

When tissue was collected 24 hours later, there was no difference in prefrontal dopamine receptor gene expression for the Pre-extinction group (**Figure 5.9**). T-tests showed no difference between adolescents and adults for D1R mRNA expression [t(8)=1.7, p=0.1], D2R mRNA expression [$t_{(8)}$=2.1, p=0.06] or D1R/D2R ratio [t(8)=1.8, p=0.1]. Interestingly, prefrontal D1R mRNA was significantly upregulated 24 hrs following cocaine-cue extinction in adults but not adolescents (**Figure 5.9D**). ANOVA for D1R gene expression showed no effect of Treatment [$F_{(1, 18)}$=2.8, p=0.1], but an overall effect of Age [$F_{(1, 18)}$=9.9, p<0.05], and a significant interaction [$F_{(1, 19)}$=9.7, p<0.05]. Post-hoc independent t-tests showed no significant difference between adolescent treatment groups [$t_{(10)}$=2.0, p=0.07], but significantly higher D1R mRNA expression in Post-extinction adults relative to Pre-extinction adults [$t_{(8)}$=2.7, p<0.05]. ANOVA for D2R gene expression showed no effect of Age [$F_{(1, 18)}$=1.8, p=0.2], no

effect of Treatment (F<1), and no interaction [$F_{(1, 19)}$=1.6, p=0.2]. ANOVA for D1R/D2R ratio of gene expression also showed no effect of Age, Treatment, and no interaction (Fs<1).

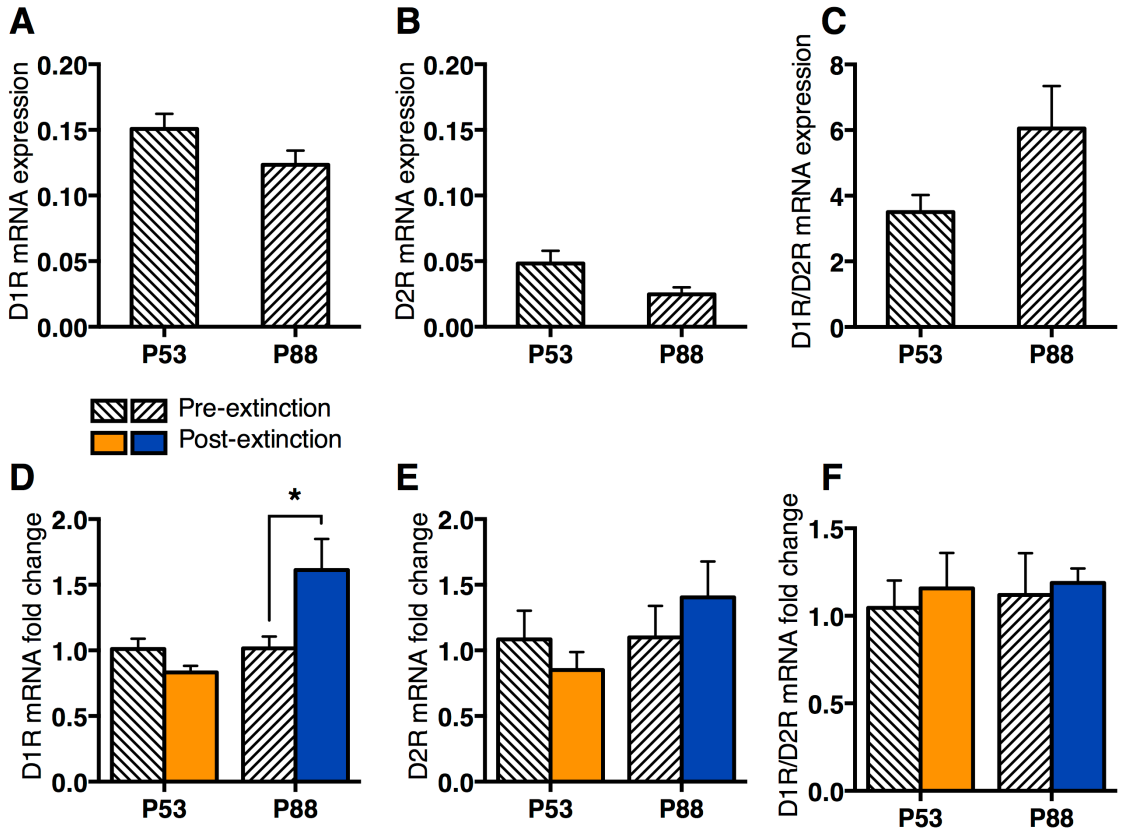

Figure 5.9 *Prefrontal dopamine receptor gene expression in adolescent (P53) and adult (P88) before or after cocaine-cue extinction when tissue was collected 24h later. There were no significant age differences Pre-extinction for (A) D1R, (B) D2R, or (C) D1R/D2R ratio mRNA expression. (D) Prefrontal D1R mRNA was significantly upregulated following cocaine-cue extinction in adults but not adolescents. There were no significant effects of cocaine-cue extinction for (E) D2R. or (F) D1R/D2R ratio. P53 n = 12, P88 n = 10. Data represent mean +SEM.*

5.3.5 Dopamine receptor gene expression in the mPFC following fear-cue extinction

To investigate potential age differences in prefrontal dopamine receptor gene expression relating to fear extinction, adolescent (P35) and adult (P88) rats underwent fear conditioning then extinction (**Figure 5.10**). Baseline freezing is summarized in **Table 5.5**. Independent t-tests showed no age difference at conditioning (t=1). Analyses of CS-elicited freezing during conditioning showed an overall effect of Conditioning trial [$F_{(2, 36)}$=34.4, p<0.05] and an overall effect of Age [$F_{(1, 18)}$=6.4, p<0.05], but no interaction [$F_{(2, 36)}$=2.1, p=0.1]. Thus all rats showed a significant increase in CS-elicited

freezing over conditioning, though freezing levels were different between age groups overall.

Table 5.5 *Mean ± SEM baseline freezing at conditioning and extinction. *p<0.05, significant effect of Age. P35 n = 8, P88 n = 12.*

Session	P35	P88
Conditioning	0.3 ± 0.3%	0.1 ± 0.1%
Extinction*	23 ± 10%	3% ± 1%

The day after fear conditioning, half the rats were handled for 2 mins (Pre-extinction), and remaining animals underwent fear cue extinction (Post-extinction). Tissue was collected 2 hours later. Independent t-tests revealed that adolescents froze more at baseline at extinction compared to adults [$t_{(8)}$=2.3, p<0.05]. Due to this effect, RM ANCOVA was used to analyze CS-elicited freezing during this session, in case the age differences at baseline affected subsequent behavior. Analyses revealed that when baseline freezing was controlled for, there was an overall effect of Extinction block [$F_{(5, 35)}$=3.4, p<0.05], with no effect of Age [$F_{(1, 7)}$=1.2, p=0.3] and no effect of baseline [$F_{(1, 7)}$=2.7, p=0.1].

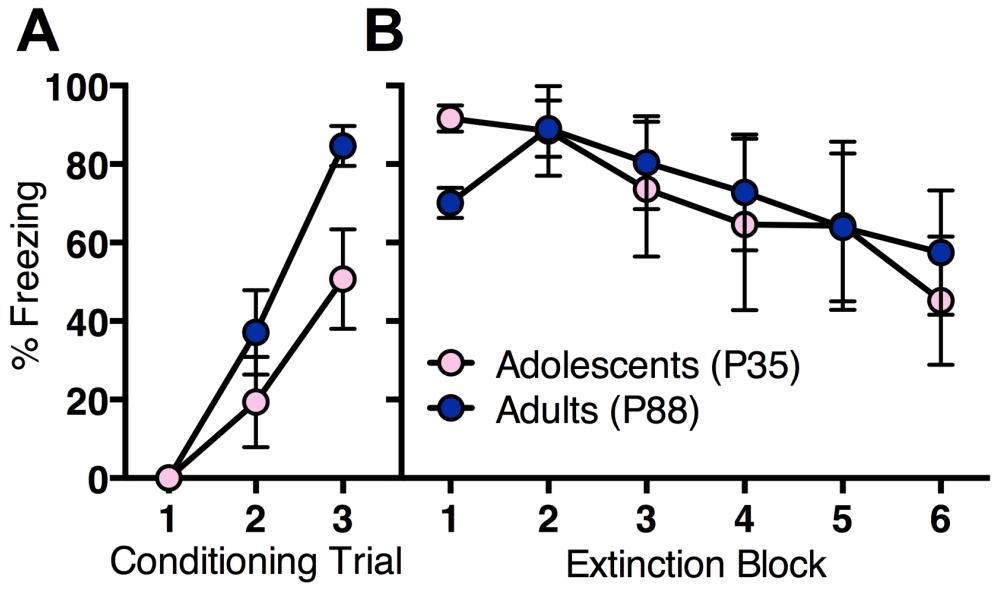

Figure 5.10 *Fear conditioning and within-session extinction for adolescent (P35) and adult (P88) rats. (A) Adolescents and adults showed different levels of freezing during conditioning, however both age groups showed an increase in CS-elicited freezing over repeated pairings of the CS and US. (B) Adolescents and adults that received extinction training showed comparable within-session CS-elicited freezing. For analyses of within-session extinction, data were collapsed into 6 blocks of 5 CS presentations per block (Extinction blocks). P35 n = 8, P88 n = 12. Data represent mean ±SEM.*

Prefrontal dopamine receptor gene expression before or after fear extinction showed age- and treatment-related differences (**Figure 5.11**). Adolescents showed higher prefrontal D1R mRNA expression than adults pre-extinction [$t_{(8)}$=3.1, p<0.05]. There was no age difference for D2R mRNA expression (t=1), however adolescents displayed a higher D1R/D2R ratio than adults [$t_{(8)}$=5.7, p<0.05]. These results suggest an age difference in prefrontal D1R/D2R mRNA ratio following fear conditioning, driven by increased D1R mRNA expression in adolescent rats.

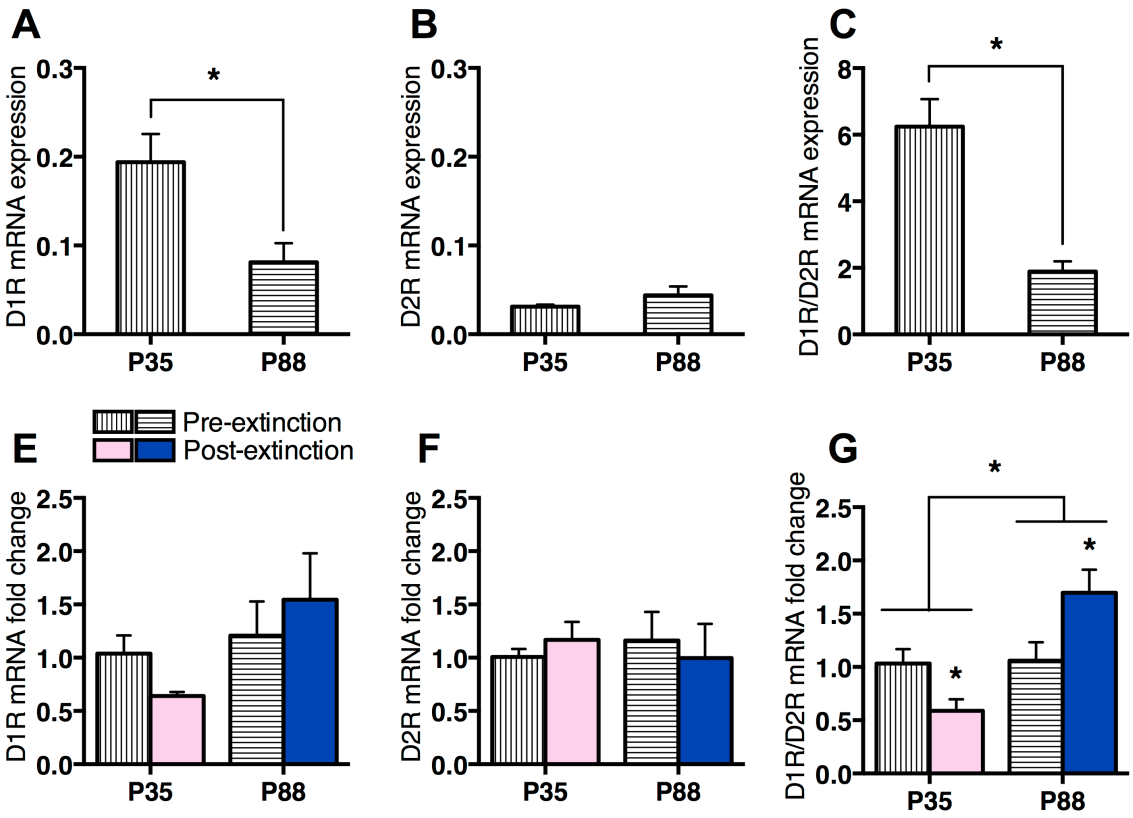

Figure 5.11 *Adolescent (P35) and adult (P88) mPFC dopamine receptor gene expression showed age- and treatment-related differences. (D) Pre-extinction prefrontal D1R gene expression was higher in adolescents compared to adults. (E) There were no differences in prefrontal D2R gene expression prior to extinction. (F) Pre-extinction prefrontal D1R/D2R ratio was higher in adolescents compared to adults. (G) There were no significant changes in prefrontal D1R or (H) D2R gene expression following extinction, however (I) D1R/D2R ratio was significantly downregulated in adolescents and upregulated in adults following extinction. P35 n = 8, P88 n = 12. Data represent mean +SEM. *p<0.05.*

There was no change in D1R mRNA expression at Post-extinction relative to Pre-extinction for either age, with ANOVA showing no effect of treatment (F<1), no effect of age [$F_{(1, 16)}$=2.4, p=0.1] and no interaction [$F_{(1, 16)}$=1.1, p=0.3]. There was also no change in D2R mRNA expression at Post-extinction compared to Pre-extinction for

either age, with ANOVA showing no effect of treatment, age, and no interaction (Fs<1). However, there was a significant difference in D1R/D2R mRNA ratio at Post-extinction relative to Pre-extinction for each age. While ANOVA revealed no effect of Treatment ($F < 1$), there was a significant effect of Age [$F_{(1, 16)}=9.4$, $p<0.05$] and a significant interaction between Treatment and Age [$F_{(1, 16)}=8.5$, $p<0.05$]. Post-hoc t-tests found that adolescent D1R/D2R mRNA ratio was significantly decreased following extinction [$t_{(6)}=2.6$, $p<0.05$], while adult D1R/D2R mRNA ratio was significantly increased [$t_{(10)}=2.3$, $p<0.05$]. These results show that D1R/D2R mRNA ratio changes in the opposite direction following extinction in adolescent or adult rats.

5.4 Discussion

In the present chapter I aimed to further investigate the role of prefrontal dopamine in adolescent versus adult extinction by examining gene expression for D1R and D2R in the mPFC. After optimizing RT-qPCR, I found that mRNA expression of prefrontal D1R, D2R, and D1R/D2R ratio showed no significant change across P35, P53 and P88 in naïve rats. In separate groups of rats that underwent cocaine self-administration and lever extinction, extinction of a cocaine-associated cue had no significant effects on prefrontal dopamine receptor gene expression in adolescent (P53) or adult rats (P88) when tissue was collected immediately. However, when tissue was collected 24 hours later, analyses revealed upregulation of prefrontal D1R mRNA following cocaine-cue extinction in adults but not adolescents. Finally, I found that prefrontal D1R gene expression was higher following fear conditioning in adolescent compared to adult rats, and that D1R relative to D2R gene expression (D1R/D2R ratio) was modulated in opposite directions following fear extinction learning during adolescence versus adulthood. Specifically, D1R/D2R ratio was decreased following extinction learning in adolescents and increased in adults.

5.4.1 First, some housekeeping…

My first study determined the most appropriate housekeeping gene for RT-qPCR. In order to serve as a valid internal control, expression of housekeeping gene must be stable across experimental groups. To select my housekeeping gene out of three candidate genes (*Actb*, *Gapdh*, *Hprt1*), I used mPFC tissue from rats that had undergone cocaine self-administration, lever extinction, and had then either received cue extinction training or been handled by the experimenter. Tissue was collected either immediately

after cue extinction or handling, or 24 hours later. Rats were late adolescent (P53) and adult (P88) on cue extinction day. According to relative gene expression analyses and the output from programs designed to measure candidate housekeeping gene stability, *Hprt1* exhibited the most stable expression across treatment groups, age groups, and time points. Thus, *Hprt1* was used as the housekeeping gene for subsequent RT-qPCR experiments. It should be noted that this housekeeping gene was used for subsequent analyses of adolescent (P35) and adult (P88) rats that underwent fear conditioning and extinction, although these treatment conditions and the P35 age group were not used in housekeeping gene selection. However, it is pertinent that the D1R/D2R ratio in particular was calculated without the need for a housekeeping gene. Rather, this value was determined by directly comparing mean C_T values for D1R versus D2R gene expression. Because D1R and D2R was compared within rats, normalizing to housekeeping gene produces the same value regardless. Thus:

$$D1R/D2R\ ratio = C_{T\,D1R} - C_{T\,D2R} = (C_{T\,D1R} - C_{T\,HKG}) - (C_{T\,D2R} - C_{T\,HKG})$$

e.g. D1R/D2R ratio $= 24.56 - 26.41 = -1.85$

$= (24.56 - 22.80) - (26.41 - 22.80) = -1.85$

5.4.2 Prefrontal dopamine receptor gene expression is similar in experimentally naïve rats age P35, P53, and P88

Following selection of an appropriate housekeeping gene, I then examined prefrontal dopamine receptor gene expression across adolescent development. For this study I measured gene expression for D1R, D2R, and D1R/D2R ratio in naïve rats age P35, P53, and P88, as these age groups corresponded to the age of cue extinction in fear conditioning and cocaine self-administration experiments in the previous two chapters. Analyses using RT-qPCR showed no significant difference in gene expression for D1R, D2R, or ratio across age, suggesting D1R and D2R reach adult expression levels by adolescence in naïve rats.

The present finding for D1R is consistent with a previous study that showed no difference in D1R mRNA expression in frontal cortex in P21 and P60 mice, which corresponded to no difference in protein expression between age groups (Araki et al. 2007). Others have also found no difference in prefrontal D1R binding between rats age P28 and P42 (Leslie et al. 1991). Investigations of mPFC D1R binding across a range of

adolescent age groups have likewise found no change across rats age P28, P35, P45, and P60 (Tarazi et al. 1999; Tarazi and Baldessarini 2000). Similarly, studies in non-human primates show that prefrontal D1R density stabilizes to adult-like levels around puberty (3 years of age), though levels show a slight and consistent decrease through puberty continuing into adulthood (Lidow and Rakic 1992; Lidow et al. 1991). A human qPCR study found no significant difference in PFC D1R gene expression between adolescents (14 – 17 years) and adults (35 – 50 years) (Rothmond et al. 2012). A study that used microarrays showed no changes relating to dopamine signaling, including D1R and D2R expression, across subjects aged 0 – 49 years (Harris et al. 2009). Data from that investigation was also used in a study by Shoval and colleagues (2014), who analyzed PFC transcriptome profiles from three human RNA sequencing studies (Kang et al. 2011; Somel et al. 2010; Harris et al. 2009), the Allen Brain Atlas (Sunkin et al. 2013), and a study of macaque monkeys (Somel et al. 2010). Analyses showed no significant changes in an any genes related to dopamine signaling across 16 brain regions in human or monkey data, including all five dopamine receptors, as well as proteins involved in dopamine metabolism and transport.

However, there is also evidence from rodent and human studies that dopamine receptor expression actually peaks during adolescence. A peak in D1R (Brenhouse et al. 2013; 2008) and D2R (Brenhouse et al. 2013) expression on glutamatergic projections from the PL to the NAc has been observed, while in the PFC more broadly, D1R and D2R density has been reported to be high at P40, then decline into adulthood across P60, P80, P100, and P120, with D1R declining more dramatically compared to D2R (Andersen et al. 2000). In fact, this is consistent with positron emission tomography (PET) findings in humans age 10 – 30 years, which show that D1R binding in the prefrontal cortex decreases from adolescence into adulthood (Jucaite et al. 2010). However, in that study, D1R binding was found to decrease non-linearly from adolescence to adulthood, with the most pronounced decline actually occurring *during* adolescence (age 10 – 16 years). Another human study reports a peak in D1R gene expression during adolescence compared to infancy and adulthood (Weickert et al. 2007). However, it is worth noting that cause of death for adolescents in that study was gunshot wound or stabbing, compared to aforementioned investigations where cause of death was most commonly heart failure or accident (though accident was not defined). It may be that a situation of high stress immediately prior to death and/or an associated

history of childhood stress may have confounded studies reporting high levels of prefrontal D1R gene expression. Indeed, early life adversity has been shown to exacerbate an adolescent peak in D1R expression on PFC projection neurons in adolescent rats (Brenhouse et al. 2013).

While findings of prefrontal D1R expression show some variability across the literature, data on PFC D2R expression is more consistent. In the present study, there was no difference in prefrontal D2R gene expression across age groups, consistent with previous literature reporting no difference in prefrontal D2R gene expression between rats age P21 and P60 (Araki et al. 2007). Studies of receptor binding in frontal cortex likewise report no difference between rats age P30 and P91 (Bruinink et al. 1983), as well as between P28 and P42 (Leslie et al. 1991), and P40 and P240-360 (8 – 12 months) (Noisin and Thomas 1988). Investigation of D2R binding across a range of adolescent age groups also showed no change across rats age P28, P35, P45, and P60 in the mPFC specifically (Tarazi et al. 1998; Tarazi and Baldessarini 2000). In monkeys, PFC D2R density has likewise been found to plateau around the time of puberty (Lidow and Rakic 1992; Lidow et al. 1991).

One known study to examine D1R/D2R mRNA ratio in the PFC found no difference between mice age P21 and P60 (Araki et al. 2007). Consistent with the present findings, in that study D1R gene expression was higher than D2R gene expression in both younger and older rats. While that study did not examine gene expression at any age *between* P21 and P60, a consistently higher level of D1R compared to D2R density has been reported in frontal cerebral cortex across rats age P28, P35, P45, and P60 (Tarazi and Baldessarini 2000).

5.4.3 Cocaine-cue extinction and prefrontal dopamine receptor gene expression

In Chapter 3, I showed that extinction of a cocaine-associated cue in late adolescent (P53) rats was impaired compared to adult (P88) rats, and that the observed adolescent deficit was rescued by enhancing D2R signaling in the IL of the mPFC. In the present chapter, I investigated whether cocaine-cue extinction corresponds to natural differences in prefrontal dopamine receptor gene expression.

Rats commenced cocaine self-administration in adolescence (P35) or adulthood (P70) and showed no age differences in lever pressing or infusions earned

over cocaine self-administration days. These findings are consistent with results from Chapter 3. For lever extinction in absence of the cocaine-associated cue, both age groups showed a decrease in lever pressing across extinction days. While analyses showed that adolescents pressed more across extinction days overall, post-hoc independent t-tests showed that responding was similar for each age group by the final day. Thus although there was an overall age difference in active lever pressing during extinction initially, both ages learned to inhibit active lever pressing to a similar level by the end of lever extinction. It should be noted that adolescents pressed significantly more than adults on the inactive lever across lever extinction. Interestingly, recent findings from our lab show that adolescent rats display decreased discrimination for the active lever over the inactive lever compared to adults during extended access (6-hour) daily cocaine self-administration sessions (Madsen et al. 2016). However, compared to inactive lever pressing during lever extinction in Chapter 3 (adult M=11, adolescent M=12), the age difference in the present chapter appears to be driven by a decrease in adult responding rather than an increase in adolescent responding. Precise reasons for this decrease are not clear, however it appears that though an age effect is statistically significant, it may not be biologically significant. Indeed, daily inactive pressing remains very low for both adolescents and adults (adult M=5, adolescent M=11) relative to active lever presses (adult M=16, adolescent M=21), indicating that both age groups discriminate between lever type.

The day after final lever extinction, rats were assigned to one of two groups: Pre-extinction group was handled by the experimenter but had no exposure to behavioral apparatus, whereas Post-extinction group received cocaine-cue extinction consisting of 120 cue-alone presentations. Medial PFC tissue was collected either immediately or 24 hours later. There were no age differences in prefrontal dopamine receptor gene expression in Pre-extinction groups at either time point. When tissue was collected immediately, there were no changes in gene expression related to cocaine-cue extinction. However, when tissue was collected 24 hours later, analyses revealed that prefrontal D1R mRNA expression was significantly upregulated in Post-extinction adult rats relative to Pre-extinction adult rats. There was no such effect for adolescent rats. There were also no effects of treatment on prefrontal D2R or D1R/D2R ratio gene expression for either age group.

Changes in adult D1R gene expression following cocaine-cue extinction observed in the present study are unprecedented as, to the best of our knowledge, there have been no studies of dopamine receptor gene expression in the mPFC following extinction of a drug-associated cue, or indeed any acute behavioral manipulation. Results from adult rats in Chapter 3 suggest that an upregulation of D1R mRNA in the mPFC may be beneficial for consolidation of cocaine-cue extinction, leading to enhanced retrieval of extinction learning when re-exposed to the drug-associated cue. Consistent with this idea, systemic treatment with the D1R agonist SKF-81297 has been shown to facilitate extinction of a cocaine-associated context in adult rats when tested drug-free the next day (Abraham et al. 2016). Conversely, systemic treatment with the D1R antagonist SCH-23390 impaired extinction of a cocaine-associated context in adult rats (Fricks-Gleason et al. 2012). The effect of manipulating D1R signaling on extinction of a cocaine-associated cue following self-administration has not previously been studied. Notably, D1R activation in the PFC is known to enhance the responsiveness of postsynaptic NMDA receptors implicated in long-term memory (Seamans et al. 2001a; Wang and O'Donnell 2001). In fact, D1Rs on output neurons from the PL of the mPFC are known to mediate the salience of drug-associated cues, as well as cue-induced reinstatement of drug-seeking (Kalivas and Duffy 1997; Everitt and Wolf 2002; Kalivas et al. 2005). Present findings add to previous extinction literature suggesting that prefrontal D1Rs may also modulate the salience of new cue-no drug associations in adults. Antagonizing D1R signaling in the PL at the time of cocaine-cue extinction following self-administration can test this idea.

In contrast to adults, I found no difference in prefrontal D1R mRNA following cocaine-cue extinction in adolescent rats. This suggests a marked difference in mPFC function related to cocaine-cue extinction in adolescents compared to adults. Since findings from Chapter 3 indicate that the prefrontal cortex mediates cocaine-cue extinction learning in adolescents, present findings may help explain adolescent impairments in long-term cocaine-cue extinction learning. Specifically, in Chapter 3 I found that enhancing prefrontal D2R signaling improved cocaine-cue extinction learning in adolescent rats. This and the present chapter's findings suggests possible divergent involvement of D1R versus D2R activity in cocaine-cue extinction learning across development. It will be exciting for future studies to examine the involvement of dopamine receptor signaling in both adolescent and adult drug-cue extinction learning

using the paradigm employed here. Notably, it has previously been shown that infusion of the D1R agonist SKF-38393 into the PL of the mPFC improved extinction of a cocaine-associated context in adolescent rats (Brenhouse et al. 2010). It would be pertinent to test the effect on a D1R agonist in the IL or PL of the adolescent and the adult mPFC using the current paradigm.

The lack of effects observed when tissue was collected immediately after extinction likely relates to timing. Changes in gene expression are widely-considered to constitute long-term modifications associated with learning and memory, as opposed to alterations in receptor conformation, surface expression, or G-protein coupling, which can occur more rapidly (Blitzer et al. 2005; Jaber et al. 1996). Findings on dopamine receptor turnover rates are difficult to interpret as the recovery rate of receptors is affected by initial receptor loss, however a review of the literature indicates extremely varying reports of minimum half-life of D1Rs of 22 hours (Fuxe et al. 1987), up to 56 hours (Giorgi et al. 1991), and 8 hours (Hall et al. 1983) up to 79 (Norman et al. 1987), or 119 hours in adult rats (Leff et al. 1984), with one report in adolescent rats of 45 hours (Leff et al. 1984) for D2Rs. These suggest changes relating to long-term receptor regulation are likely to occur in the order of several hours. Notably, one study of chronic agonist activation *in vitro* showed that mRNA levels of another GPCR, the β-adrenergic receptor, showed no significant changes until 4 hours after the start of incubation (Hadcock and Malbon 1988). While data on mRNA changes following acute behavioral manipulations is relatively scarce, one study showed changes in prefrontal ephrin type B receptor 2 (EphB2) mRNA levels in adolescent rats 5 hours following fear extinction (Cruz et al. 2015). Since the cocaine-cue extinction session in the present study was approximately 1 hour and 15 mins in length, and tissue was collected within 15 minutes following completion of the session, this may not have allowed enough time to capture changes in receptor mRNA levels.

5.4.4 Prefrontal dopamine receptor gene expression before or after extinction of conditioned fear

In Chapter 4, I showed that adolescent rats (P35) are impaired in extinction of conditioned fear compared to adult rats (P88), and that this deficit can be rescued by acutely enhancing prefrontal D2R signaling. In the present chapter, I determined

whether fear extinction is associated with differences in the prefrontal dopamine receptor gene expression in adolescent versus adult rats.

Prior to extinction, adolescents displayed increased D1R and D1R/D2R ratio mRNA compared to adults. This is consistent with those studies that report a peak in D1R gene expression (Garske et al. 2013; Rothmond et al. 2012) and receptor expression (Brenhouse et al. 2008; Andersen et al. 2000) in the PFC during adolescence compared to adulthood. In particular, as previously discussed, an adolescent peak in D1R expression on PFC projection neurons has been associated with early life adversity in rats (Brenhouse et al. 2013). Therefore, the increased adolescent D1R/D2R ratio observed in the present study may be a specific result of fear conditioning. By comparison, we found no age difference in D2R gene expression.

Since patterns of basal D1R and D2R mRNA expression in the cortex are found to correlate with receptor binding (Weiner et al. 1991), the current findings imply a markedly different prefrontal dopaminergic environment pre-extinction depending on age, with adolescent mPFC networks likely dominated by D1R activity relative to D2R activity compared to adults. Computational modeling shows that when the PFC is dominated by D1R relative to D2R signaling, this produces a state of net inhibition (Seamans and Yang 2004). Notably, the present findings in adolescents are similar to reports of rats with lesions of the mPFC, where fear conditioning and within-session extinction learning are intact but long-term extinction is impaired (Garcia 2006; Quirk et al. 2000b). Moreover, evidence from previous studies indicates that the mPFC is not recruited as efficiently during fear extinction in adolescence compared to adulthood (Kim et al. 2011; Baker and Richardson 2015; Pattwell et al. 2012). In humans, the intense emotionality characteristic of adolescence is thought be at least partly due to an under-recruitment of the PFC (Somerville et al. 2010). The present findings suggest that a unique dopaminergic profile in the mPFC during adolescence may contribute to adolescent mPFC dysfunction in relation to emotional learning.

To the best of my knowledge, the present study is the first to document changes in dopamine receptor gene expression following fear extinction in adolescent and adult rats. It is possible that present adolescent dopamine receptor mRNA changes represent aberrant prefrontal processing. However, the opposite effect observed in adolescent vs adult rats following the manipulation of prefrontal D2R activity suggest a fundamental

age difference in dopaminergic signaling in the mPFC in relation to extinction learning. It has previously been shown that increasing adolescent fear extinction training (by doubling the number of CS presentations) enhances long-term extinction learning (Kim et al. 2011; McCallum et al. 2010). Therefore, it may be more likely that the changes in prefrontal dopamine receptor gene expression observed in the present study represent beneficial but insufficient consolidation mechanisms. Notably, this is consistent with functional results from Chapter 4 that show enhancing prefrontal D2R signaling, which would decrease D1R/D2R signaling ratio functionally, improved adolescent extinction learning.

5.4.5 Conclusion

The previous chapters present novel findings on the functional involvement of prefrontal dopamine signalling in adolescent extinction learning across both appetitive (drug) and aversive (fear) domains. Data from D1R and D2R agonists suggest that the dopamine system is differentially involved in extinction learning during adolescence versus adulthood. In the present chapter, I extend these findings by providing molecular evidence of natural developmental differences in the prefrontal dopamine system following cocaine-cue extinction or fear extinction learning. Whereas cocaine-cue extinction was associated with upregulation of prefrontal D1R gene expression in adult rats, there was no such change observed for adolescents. Following fear extinction D1R/D2R ratio of gene expression upregulated in adult rats, but downregulated in adolescent rats. These findings show for the first time that underlying mechanisms of cue extinction learning across adolescent development includes dissociated involvement of the prefrontal dopamine system.

6 General discussion

Substance use disorder and anxiety disorders are the most commonly experienced mental health problems among young people worldwide. Critically, adolescents display poorer outcomes following extinction-based treatments for these disorders compared to other age groups. Extinction learning is mediated by plasticity in the PFC, a region undergoing dramatic reorganization during adolescence, which includes changes in the dopamine system. While developmental differences in prefrontal dopamine signaling have been implicated in adolescent drug-context extinction learning (Brenhouse et al. 2010), its role in extinction of a discrete drug-associated cue has never been previously investigated. In addition, the role of prefrontal dopamine signaling in extinction of conditioned fear during adolescence has never been explored to my knowledge. Therefore, I examined the role of prefrontal dopamine in adolescent cue extinction learning across both appetitive (drug) and aversive (fear) domains. The present findings not only extend understanding of extinction learning in general, but may provide potential therapeutic targets to facilitate extinction-based therapy in the clinic.

6.1 Summary of key findings

Key findings from the present thesis are summarized in **Table 6.1**. Chapter 3 and Chapter 4 characterized adolescent versus adult extinction learning and memory with behavioral tests and pharmacological manipulations. In the first series of experiments (Chapter 3), I investigated adolescent extinction in a cocaine self-administration paradigm. While cocaine intake and lever extinction were similar, I found that cue extinction reduced cue-induced reinstatement in adult but not adolescent rats. Infusion of the full D2R agonist quinpirole into the IL of the mPFC at the time of cue extinction significantly reduced cue-induced reinstatement in adolescents. This effect was replicated by acute systemic treatment with the atypical antipsychotic aripiprazole, a partial D2R-like agonist. In the next series of experiments (Chapter 4), I examined adolescent extinction in a fear conditioning paradigm. Testing adult (P88) rats during the dark phase of the light-dark cycle produced variable behavior during conditioning

and extinction, while late adolescent (P53) rats displayed highly variable behavior during both the dark and light phase. In contrast, I found that during the light phase younger adolescent rats (P35) displayed a robust deficit in long-term fear extinction compared to adults (P88). Intra-IL infusion of the D1R agonist SKF-81297 had no effects on within-session or long-term extinction for either adolescents (P35) or adults (P88). In contrast, intra-IL infusion of quinpirole improved long-term extinction in adolescent rats but delayed extinction acquisition in adult rats. Interestingly, systemic treatment with aripiprazole improved long-term fear extinction in adults, an effect previously observed in our lab for adolescent fear extinction (Ganella et al., under review).

Table 6.1 *Summary of key findings.*

		Adult	**Adolescent**
Behavioral	Short-term (working) extinction	Intact (fear)	Intact (fear)
	Long-term extinction	Intact (cocaine, fear)	Impaired (cocaine, fear)
Functional	D1R activity increase in the IL (full agonist, SKF-81297)	No effect (fear)	No effect (fear)
	D2R activity increase in the IL (full agonist, quinpirole)	Impaired short-term (fear)	Enhanced long-term (cocaine, fear)
	D2R activity "balance" (partial agonist, aripiprazole)	Enhanced long-term (fear)	Enhanced long-term (cocaine, fear; Ganella et al., under review)
Molecular	D1R gene expression	⬆ (cocaine) No change (fear)	No change (cocaine, fear)
	D2R gene expression	No change (cocaine, fear)	No change (cocaine, fear)
	D1R/D2R ratio gene expression	No change (cocaine) ⬆ (fear)	No change (cocaine) ⬇ (fear)

Chapter 5 extended findings from Chapter 3 and 4 in a series of molecular experiments using RT-qPCR. In these studies, I examined changes in dopamine receptor gene expression in the mPFC of naïve adolescent and adult rats, and in rats that received extinction of cocaine- or shock-associated cue. After optimizing RT-qPCR, I found that mRNA expression of prefrontal D1R, D2R, and D1R/D2R ratio showed no significant change across P35, P53, and P88 in naïve rats. Following cocaine self-administration and lever extinction, extinction of a cocaine-associated cue had no significant effects on prefrontal dopamine receptor gene expression in adolescent (P53) or adult rats (P88)

when tissue was collected immediately following extinction. However, when tissue was collected 24 hours later, I observed upregulation of prefrontal D1R mRNA following cocaine-cue extinction in adults only. Finally, I found that prefrontal D1R mRNA levels were higher following fear conditioning in adolescent versus adult rats, and that D1R/D2R ratio was modulated in opposite directions following fear extinction during adolescence versus adulthood.

6.2 Cue-associated learning across adolescent development

Findings show that adolescent extinction deficits relate to emotionally salient cues across both appetitive and aversive domains. Across cocaine self-administration experiments, adolescent and adult rats displayed comparable cocaine consumption across self-administration days. While previous studies include reports of increased (Anker and Carroll 2010) or decreased (Li and Frantz 2009) cocaine self-administration in adolescents compared to adults, the majority of studies have observed no difference between adolescents and adults in levels of cocaine self-administration (Belluzzi et al. 2005; Leslie et al. 2004; Kantak et al. 2007; Kerstetter and Kantak 2007; Frantz et al. 2006). This is consistent with descriptions of adolescent versus adult substance abuse that do not show age differences in overall rates of drug use in humans (Winters 2001; Segal and Stewart 1996). I also found that adolescent and adult rats displayed similar extinction of operant responding (lever pressing). While overall active and inactive responding during extinction was higher in adolescents compared to adults in Chapter 5, lack of interaction indicates that the rate of lever extinction was comparable between age groups. Importantly, in all cocaine experiments, responding during the last session of lever extinction was similar across age. Thus, differences in cue-induced reinstatement appear to be related to age differences in cocaine-cue extinction learning, rather than differences in cocaine consumption or operant extinction. Critically, this directly models clinical findings that adolescents show poorer outcomes to extinction-based behavioral therapies (Catalano et al. 1990; Perepletchikova et al. 2008; Ramo and Brown 2008; Winters and Arria 2011), and that adolescents are more likely to relapse to drug-associated cues compared to adults (Ramo and Brown 2008).

Across fear experiments during the light phase, both age groups consistently showed a significant increase in CS-elicited freezing across conditioning trials across all experiments. However, I observed a significant difference between adolescent (P35)

and adult rats in *overall* CS-elicited freezing during conditioning in two out of three experiments. Specifically, adolescents showed lower levels of freezing during CS-US presentations compared to adults. This result is different to previous studies comparing adolescent (P35) and adult (P70) rats (Kim et al. 2011; McCallum et al. 2010), which showed comparable freezing during conditioning across age groups. There is evidence that rats express learned fear in different ways across development, however freezing to an auditory cue generally emerges by P16 (Kim and Richardson 2007). The reasons for lower levels of freezing in adolescents in the present project are not clear. Since the effect was observed for the final RT-qPCR experiment as well as the intra-cranial experiment, it cannot be attributed to aberrant effects of surgery. Nevertheless, lower levels of freezing in adolescents did not appear to reflect weaker CS-US associations, since both age groups showed consistently high levels of CS-elicited freezing at the start of extinction the next day. Thus despite differences in the expression of fear during conditioning, extinction differences across age were never observed within-session. In addition, across fear experiments, age groups showed no difference in within-session extinction, suggesting comparable short-term (working) extinction learning and memory. Thus, spontaneous recovery in adolescents but not adults 24 hours after extinction suggests a deficit in long-term cued fear extinction compared to adults. Similar to cocaine cue extinction, this directly models clinical findings showing extinction-based therapy for anxiety is less effective in adolescents compared to other age groups (Southam-Gerow et al. 2001; Bodden et al. 2008).

Findings across paradigms suggest that adolescent deficits in cocaine-cue extinction are not due simply to increased severity of cognitive impairments related to cocaine exposure during adolescence compared to adulthood (Kantak et al. 2014; Pope et al. 2016; Black 2006). However, findings do not rule out the possibility that adolescents are specifically impaired in extinction of cues associated with pathological reward or fear learning during adolescence. In fact, adolescent and adult rats are reported to show comparable responding during contingent extinction of a cue associated with a natural reward (dextrose pellet) (Sturman et al. 2010). Another study found that adolescents were impaired in extinction of a cue that was continuously-reinforced by a natural reward (dextrose pellet), but showed more rapid extinction of a cue that was previously only partially-reinforced (Meyer and Bucci 2016). This suggests that adolescent extinction deficits may occur for cues with only particularly

high reward value. Lastly, adolescent rats have been shown to only be impaired in extinction of cued fear if both conditioning and extinction occurred during adolescence (Baker and Richardson 2015). If conditioning occurred earlier in life (P24) and extinction during adolescence (P35), rats maintained low levels of freezing at test the next day; if conditioning occurred during adolescence then extinction during adulthood (P70), extinction at test was again intact. However, consistent with present findings, when conditioning and extinction both occurred during adolescence, there was an impairment in extinction at test the next day. These findings suggest that adolescent extinction deficits occur only following highly salient cue learning during and not before or after this developmental period.

6.3 Quantitative versus qualitative mechanisms of cue extinction across adolescent development

Previous literature suggests that extinction learning involves similar prefrontal neural mechanisms across adolescence and adulthood. Evidence for this theory comes from fear studies showing that extinction involves excitation in the IL both during adolescence and adulthood. In the very first study that directly compared adolescent and adult rats in extinction-related IL activity, extinction was associated with increased phosphorylated MAPK (pMAPK) in IL in adults that showed successful long-term extinction, whereas adolescents showed impaired extinction and lower IL pMAPK (Kim et al. 2011). It was further shown that doubling the amount of extinction in adolescents increased IL MAPK phosphorylation and prevented the disrupted long-term extinction. Therefore, it was argued by Kim and colleagues that adolescent and adult extinction rely on similar mechanisms in the IL, except that adolescents were not as efficient as adults (Kim et al. 2011). Indeed, reducing IL excitability and burst firing with pre-extinction infusions of the M-type K channel agonist flupirtine delays within-session extinction and impairs expression of extinction memory in adolescent rats (Santini and Porter 2010). In another study, pre-extinction intra-IL infusion of the $GABA_A$ agonist muscimol had similar behavioral effects in adult rats (Sierra-Mercado et al. 2011). These findings highlight that extinction mechanisms in adolescence and adulthood overlap in the IL.

In addition, extinction of cued fear appears to specifically require glutamate signaling during both adolescence and adulthood. Pre-extinction infusion of the

metabotropic glutamate receptor 5 (mGlu5) negative allosteric modulator 2-methyl-6-(phenylethynyl)pyridine (MPEP) into IL blocks the recall of fear extinction in adolescent (Sepulveda-Orengo et al. 2013) and adult rats (Fontanez-Nuin et al. 2011). Pre-extinction intra-IL infusion of the NMDAR antagonist CPP (3-(2-carboxypiperazin-4-yl*)*propyl-1-phosphonic acid) impaired recall of extinction in adult rats (Burgos-Robles et al. 2007), while post-extinction systemic injections of the partial NMDAR agonist D-cycloserine (DCS) enhances extinction in adolescent rats (McCallum et al. 2010). Notably, while five CS-alone presentations was insufficient to produce a change in IL AMPA/NMDA ratio in adolescent mice (Pattwell et al. 2012), extinction involving 15 CS-alone presentations increased the AMPA/NMDA ratio in the IL consistent with AMPAR insertion and IL excitability in adolescent rats (Sepulveda-Orengo et al. 2013). These findings suggest that differences in extinction learning might relate to quantitative rather than qualitative differences in PFC function.

However, other studies suggest that at least some aspects of PFC involvement in adolescent cue extinction may be unique from other ages. For example, Baker and colleagues (2015) showed that rats conditioned as juveniles and extinguished as adolescents showed effective long-term fear extinction. However, extinction during adolescence led to decreased levels of IL pMAPK, irrespective of whether long-term extinction was impaired or not. Thus, even when long-term extinction was successful during adolescence, the prefrontal neural correlates were different at this age. Another study showed that reducing the synthesis of the tyrosine kinase receptor, ephrin type B receptor 2 (EphB2), in the IL facilitated fear extinction in adolescent (P30) rats but not adult (P60) rats (Cruz et al. 2015). Thus, it appears that at least some prefrontal mechanisms involved in adolescent extinction learning may be unique to this developmental period.

Results from pharmacological and molecular experiments in the present thesis provide direct evidence for a dissociation in prefrontal D1R and D2R involvement in extinction during adolescence compared to adulthood. While intra-IL infusion of the D1R agonist SKF-81297 had no effects on within session or long-term extinction for adolescents or adults, results from RT-qPCR suggest age differences in relative involvement of D1R signaling in extinction. Following cocaine-cue extinction, adult rats showed an upregulation of D1R gene expression that was absent in adolescent rats.

Following fear extinction, adults showed an increase while adolescents showed a decrease in D1R relative to D2R gene expression. While gene expression analyses revealed no treatment- or age-related differences in D2R expression by itself, the opposite change in ratio of D1R relative to D2R following fear extinction suggests a difference in D2R in relation to D1R function across age groups. It was striking that IL infusion of the D2R agonist quinpirole, which would functionally serve to further decrease a D1R/D2R ratio following extinction in adolescents facilitated extinction learning in adolescents across both appetitive and aversive learning domains. In contrast, intra-IL quinpirole, which would reverse the adult D1R/D2R ratio observed following fear extinction caused an impairment in fear extinction learning in adults.

Together, findings suggest that dopamine signaling via prefrontal D2R is beneficial for adolescent extinction consolidation, and that adolescents may be naturally harnessing this mechanism but to an insufficient extent during normal extinction learning. On the other hand, dopamine signaling via prefrontal D1R may be beneficial and naturally optimal for adult extinction learning.

6.4 Prefrontal dopamine signaling and extinction as a balance of strengthening and weakening of competing memories

The precise role of prefrontal dopamine in learning and memory is poorly understood despite extensive investigation. Because dopamine is a neuromodulator and not an excitatory or inhibitory neurotransmitter, it can have a range of effects depending not only on dopamine concentration but also the activity of other neurotransmitters, in addition to receptor expression and/or functionality (Seamans and Yang 2004). Prefrontal D1R or D2R signaling can also have effects on both working memory and long-term memory. Activation of D1Rs has been shown to follow an inverted U-shape function in working memory, such that either too little or too much activation can disrupt performance (Goldman-Rakic et al. 2000). D1R stimulation is also known to facilitate LTP via activation of cAMP, which triggers an intracellular signaling cascade that ultimately leads to long-term changes in expression of both ionotropic and metabotropic glutamate receptors (Seamans and Yang 2004; Sheynikhovich et al. 2013). The role of D2R in working memory is less clear (Jay 2003), though prefrontal D2R activation is generally associated with decreased cAMP synthesis and inactivation

of NMDA receptors (Seamans and Yang 2004). D2R activation has also been found to correspond with LTD (Sheynikhovich et al. 2013).

Present findings show that extinction learning in adults corresponds to increased prefrontal D1R gene expression. A role for prefrontal D1R in adult extinction learning is consistent with reports that D1R activity facilitates LTP in the PFC of adult rats *in vitro* (Gurden et al. 2000), and that LTP is involved in adult extinction across appetitive and aversive learning domains (Malenka and Bear 2004; Myers et al. 2011). In contrast, increased D1R gene expression was observed in adolescents only after fear conditioning, which may indicate stronger fear-cue associative learning at this age compared to adults. Although no age differences in dopamine gene expression were observed in pre-extinction groups in the cocaine experiment, it would be interesting to measure changes following cocaine-cue conditioning (self-administration) before extended lever extinction. Indeed, data from fear conditioning suggest a difference in initial associative learning between adolescents and adults involving prefrontal D1R, which is consistent with the hypothesis from Andersen and colleagues that increased prefrontal D1R expression in adolescents contributes to increased sensitivity to cocaine-associated cues at this age (Brenhouse and Andersen 2008; Brenhouse et al. 2010).

Present data also suggest that dopamine signaling via prefrontal D2R is beneficial for adolescent extinction consolidation. Given that D2R decreases cortical NMDA currents (Tseng and O'Donnell 2004), this may seem counterintuitive to evidence for NMDA-dependent plasticity involvement in both adolescents (McCallum et al. 2010; Pattwell et al. 2012) as well as adults (Burgos-Robles et al. 2007). However, there is evidence that suggests reducing NMDA activity specifically in the IL may be beneficial for adolescent extinction learning. In the study by Cruz and colleagues (2015), adolescent fear extinction was improved by decreasing expression of EphB2, a receptor shown to enhance NMDA-mediated plasticity. Authors argued that decreasing NMDA receptor activity might actually promote elimination of synapses not required for fear extinction, while protecting synapses that were potentiated during extinction learning from LTP reversal. In this way, increasing signal-to-noise ratio by a combination of LTP and LTD would strengthen IL outputs required for successful maintenance of extinction memory. Critically, the maturational change most consistently associated with adolescence is actually reduction of synaptic density, or,

synaptic pruning (Selemon 2013). It follows that changes relating to synaptic pruning might be more important in governing PFC plasticity mediating extinction learning during this period. Thus, the balance of LTP and LTD in learning and memory may be different in adolescence compared to adulthood.

Consistent with this idea, evidence from electrophysiology studies suggests that adolescence involves dysregulation in prefrontal excitation–inhibition balance, mediated by changes in dopamine signaling (O'Donnell 2010). A summary of similarities and differences in dopamine-mediated prefrontal signaling during adolescence versus adulthood is shown in **Table 6.2**. For instance, prefrontal fast-spiking GABAergic interneuron excitability is increased in both adolescent and adult rats by either the full D1R agonist SKF-81297 (Gorelova et al. 2002; Kroener and Lavin 2010) or the partial agonist SKF38393 (Tseng and O'Donnell 2007b). The activity of fast-spiking interneurons mediates prefrontal gamma oscillations implicated in information processing (Womelsdorf et al. 2007; Sohal et al. 2009), suggesting at least some overlap in D1R-mediated PFC network and information processing from adolescence into adulthood. However, D1R-mediated excitation of non-fast spiking interneurons does not emerge until after adolescence (Tseng and O'Donnell 2007b). Similarly, activation of prefrontal D2R signaling using quinpirole is shown to excite both fast spiking and non-fast spiking interneurons in adult rats, but not adolescent (P28-35) (Tseng and O'Donnell 2007a) or periadolescent (P14 – 35) rats (Seamans et al. 2001b; Gorelova et al. 2002). While the relative contributions of different interneuron subtypes to cortical inhibitory basal activity is still not well understood, recent evidence suggests that non-fast spiking interneurons may also contribute to cortical "up states" associated with information processing (Neske and Connors 2016). Critically, co-activation of prefrontal D1R and NMDARs produces depolarizing plateaus in PFC pyramidal neurons that resemble *in vivo* "up states" in adult but not adolescent rats (Tseng and O'Donnell 2005). By comparison, the ability of D2Rs to directly inhibit prefrontal pyramidal neurons is present during adolescence as well as adulthood (Tseng and O'Donnell 2004). Taken together, it appears that D1R-NMDA interactions are more robust during adulthood compared to adolescence, and that this is balanced by more efficient interneuron signaling by both D1R and D2R activation in adulthood (O'Donnell 2010). In other words, prefrontal excitation–inhibition balance is still maturing during adolescence (O'Donnell 2010).

Table 6.2 *Summary of D1R- and D2R-mediated activity of cortical interneuron and pyramidal neuron activity across adolescent development.*

		Adult	**Adolescent**	**Reference**
D1R	Fast spiking interneuron	Excite	Excite	(Gorelova et al. 2002; Kroener and Lavin 2010; Tseng and O'Donnell 2007b)
	Non-fast spiking interneuron	Excite	No effects	(Tseng and O'Donnell 2007b)
	Pyramidal neurons	Excite	No effects	(Tseng and O'Donnell 2005)
D2R	Fast spiking interneuron	Excite	No effects	(Tseng and O'Donnell 2007a; Seamans et al. 2001b; Gorelova et al. 2002)
	Non-fast spiking interneuron	Excite	No effects	(Tseng and O'Donnell 2007a; Seamans et al. 2001b; Gorelova et al. 2002)
	Pyramidal neurons	Inhibit	Inhibit	(Tseng and O'Donnell 2004)

Such balance of excitation and inhibition is important to understand in the context of LTD and LTP for the present thesis. Although extinction is most consistently associated with LTP, there is also evidence from fear studies that extinction also involves LTD. Specifically, extinction may involve a balance of LTD and LTP in the PFC. Although one study showed delays in fear extinction following low-frequency stimulation of the thalamus, known to produce LTD in the PFC (Herry and Garcia 2002), it is possible that the method they used also disrupted LTP, which can prevent the formation of the extinction memory. Indeed, low-frequency stimulation may completely prevent or even erase LTP (O'Dell and Kandel 1994). Critically, other findings directly suggest that extinction may require a combination of both LTP *and* LTD. For example, specific blockade of AMPA receptor endocytosis (that in turn prevents LTD) during extinction has been found to disrupt expression and recall of fear extinction (Dalton et al. 2007). This is consistent with another study that found impaired acquisition of fear extinction following inhibition of the NMDA receptor subunit GluN2B (Sotres-Bayon et al. 2007), which is required for the induction of LTD under some circumstances (Yang et al. 2005). Moreover, fear extinction corresponds to lasting reductions in excitatory synaptic efficacy in the mPFC-BLA pathway, which are similar to specific induction of LTD in PFC-BLA projecting neurons using optogenetic stimulation (Cho et al. 2013). Although behavioral effects of induced LTD were not

examined in that study, findings showed that excitatory postsynaptic currents (EPSCs) on BLA neurons were similarly depressed in slice preparations following fear extinction or following induction of LTD using optogenetic photostimulation of mPFC fibers (Cho et al. 2013). Extinction-related induction of LTD in BLA projecting neurons from the PFC is consistent with findings that activation of the BLA induced by electrical stimulation of the mPFC in freely moving rats is attenuated following fear extinction (Vouimba and Maroun 2011).

Overall, it appears that extinction involves both LTP and LTD, which may correspond to new learning of a CS-No US association as well as weakening of the original CS-US memory. Given that D1R and D2R signaling are associated with LTP and LTD respectively, it follows that extinction learning may benefit from a balance of dopaminergic signaling via D1R and D2R (**Figure 6.1**). Thus, a potential imbalance in D1R vs D2R expression following cued conditioning, and/or a difference in D1R and D2R functionality during adolescence compared to adulthood may help to explain deficits in extinction learning during this period. During adolescence, activation at D1Rs during emotional learning contributes to particularly strong cue associations. At the same time, underdeveloped signaling at D2Rs may disrupt weakening of the original CS-US memory during extinction, making this memory more likely to relapse when the cue is re-encountered. Enhancing prefrontal D2R signaling using quinpirole may facilitate LTD-mediated weakening of the CS-US association, allowing endogenous dopamine signaling via D1Rs to stabilize the new CS-No US association via LTP. By comparison, during adulthood, enhancing prefrontal dopamine signaling at D2Rs using quinpirole may delay extinction acquisition by disrupting a natural balance in D1R versus D2R signaling that is optimal for simultaneously learning a new CS-No US association and weakening the original CS-US association. Balancing D2R signaling using aripiprazole may be beneficial for cue extinction learning in both ages, by optimizing dopamine signaling at D2Rs while allowing dopamine to signal at D1Rs.

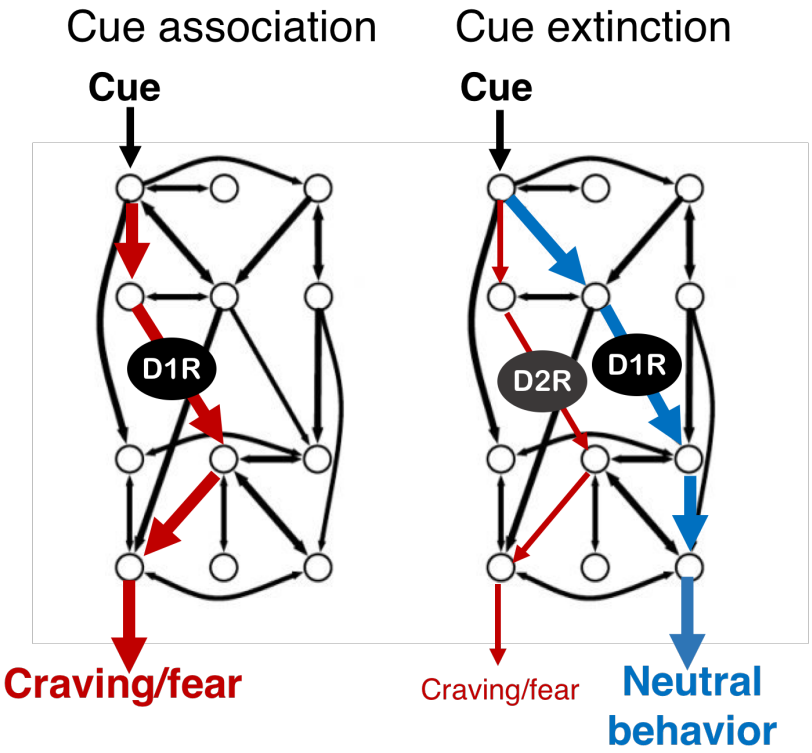

Figure 6.1 *Proposed network model for the role of prefrontal dopamine signaling via D1Rs and D2Rs in cue associative learning (left) and cue extinction learning (right). D1R activation is associated with LTP, which may favor new associative learning. D2R activation is associated with LTD, which may facilitate weakening of associations. Cue associative learning is strongly mediated by D1R signaling, which can drive pathological behaviors. Cue extinction learning may involve a balance of D1R and D2R signaling to simultaneously weaken the original cue association while strengthening a new cue extinction association that drives neutral cue-associated behavior.*

6.5 Balancing prefrontal dopamine receptor signaling: aripiprazole

Interestingly, pre-extinction systemic treatment with the partial D2R agonist aripiprazole was able to facilitate cocaine-cue extinction in adolescents as well as fear extinction in adults. The discrepancy between intra-IL and systemic injection results in adults is likely to be due to quinpirole being a selective D2R agonist working postsynaptically, whereas aripiprazole is a *partial* D2R agonist. In adult rodents, aripiprazole treatment actually produces behavioral effects consistent with D2R antagonist effects at the postsynaptic cell and agonist activity at the presynaptic cell (Kikuchi et al. 1995; Momiyama et al. 1996; Semba et al. 1995). Aripiprazole does not induce contralateral rotation in striatal-lesioned adult rats or hyperlocomotion in striatum-lesioned adult mice, consistent with antagonist activity at the postsynaptic cell (Kikuchi et al. 1995). Consistent with presynaptic agonist action, aripiprazole is reported to inhibit dopamine neuron firing in the VTA (Momiyama et al. 1996) and

reduce dopamine concentration in the striatum and frontal cortex (Semba et al. 1995). However, aripiprazole has also been shown to increase dopamine release in the frontal cortex in previously stressed adult rats (Ratajczak et al. 2016). Most importantly, since aripiprazole is a *partial* D2R agonist, it is able to act as an agonist when dopamine levels are too low, and an antagonist when levels are too high (Burris 2002). In fact, the beneficial effects of aripiprazole on cognitive symptoms of psychosis are consistently attributed to its ability to optimize the balance of dopamine signaling at both presynaptic and postsynaptic cells. A capacity to "balance" D2R signaling in the PFC may explain why aripiprazole was able benefit both adolescents and adults, despite quinpirole impairing adult fear extinction learning.

It should be noted aripiprazole also has effects on receptors apart from the D2R. Out of the D2R-like receptors (D2R, D34, and D4R), aripiprazole shows the highest affinity for the D2R (both pre- and post-synaptic). By comparison, reported affinity for D3R is 1/10 that of D2R, and D4R is 1/400 less (Sibley et al. 1994). While behavioral effects of aripiprazole are primarily characterized by partial agonist activity at both postsynaptic and presynaptic D2Rs (Lawler et al. 1999), it is also known to act on serotonin (5HT) receptors (Davies et al. 2004). Specifically, aripiprazole is a partial agonist at the $5HT_{1A}$ receptor (Jordan et al. 2002; Bortolozzi et al. 2007) and an antagonist at the $5HT_{2A}$ receptor (Davies et al. 2004), as well as $5HT_6$ and $5HT_7$ receptors (Lawler et al. 1999). Since mPFC dopamine signaling is known to be strongly mediated by the serotonin system (Benes et al. 2000) (Taylor and Benes 1996), it is possible that aripiprazole's effects relate to its actions at serotonin as well as at dopamine receptors. Notably, aripiprazole has been shown to reverse cognitive impairments in adult rats by reducing excessive glutamate release in the mPFC (Carli et al. 2010), an effect recapitulated by systemic treatment with a $5\text{-}HT_{2A}$ receptor antagonist (Gozzi et al. 2010). Thus, especially in adults, it may also be that aripiprazole is improving extinction learning by improving cognitive function in the mPFC via actions on serotonin/glutamate systems. In fact, the serotonin system also been shown to interact with dopamine afferents and GABAergic interneurons in the PFC (Taylor and Benes 1996), as well as to mediate dopamine fiber infiltration of the PFC (Taylor et al. 1998). This is of particular relevance during adolescence, as dopaminergic and serotonergic inputs to the PFC peak during this period compared to other developmental stages (Kalsbeek et al. 1988). It is also possible that aripiprazole produces behavioral

changes by altering dopamine signaling in other brain regions. However, recent findings from my lab using immunohistochemistry for c-Fos and the dopamine- and cAMP-regulated neuronal phosphoprotein DARPP-32 strongly suggest that aripiprazole's effects, at least for adolescent fear extinction, relate to activation of neurons with dopaminergic innervation in the mPFC and not the amygdala (Ganella et al., under review). It will be interesting for future studies to examine the effect of aripiprazole on neural activation in adult rodents, to help elucidate precise mechanisms of its effects across development.

Importantly, present aripiprazole data have strong translational potential. Aripiprazole is already FDA-approved for use in clinical populations for the treatment of psychosis, including adolescents (Davies et al. 2004; Burris 2002). Aripiprazole is widely used not only for its efficacy, but for its favourable safety profile and low side effect profile (DeLeon et al. 2004). Findings in late adolescent rats suggest that aripiprazole can enhance extinction learning following drug use, while findings in adult rats add to recent data in adolescents that aripiprazole can improve extinction learning following fear learning (Ganella et al., under review). Exposure-based therapies can be effective for both substance use disorder and anxiety, however treatment often requires multiple therapy sessions that can take months or years. Furthermore, anxiety disorders, followed by substance use disorders, show the lowest treatment rates compared to prevalence in all types of mental disorders (Carli et al. 2010). This was identified in part due to financial costs and accessibility of behavioral therapy, which is often more expensive and time consuming compared to medication (Gozzi et al. 2010). An effective pharmacological adjunct that acutely accompanies behavioral therapy could significantly reduce the amount of treatment necessary during this vulnerable period, and reduce chronic use of medication. Present findings suggest that aripiprazole may be beneficial as an acute adjunct to exposure-based therapy for addition or anxiety.

6.6 Limitations

While the current project presents several intriguing findings, caveats must be considered. For instance, it should be noted that in cocaine experiments, self-administration, cue extinction, and test all occurred in the same context. If adults showed enhanced extinction of context compared to adolescents, the compound extinction of both context and cue might be expected to produce lower cue-induced

reinstatement, compared to extinction of the cue alone (Kearns et al. 2012). Consistent with this, one previous study investigating extinction of a discrete cocaine-associated cue reported higher cue-induced reinstatement in adult rats when extinction was received in a different context to self-administration and test compared to the same context (Torregrossa et al. 2010). My lab has also shown that exposure to drug-taking context can be as effective as lever extinction in reducing cocaine-primed reinstatement compared to abstinence in adult rats (Kim et al. 2015). While adolescent rats (P38) have been reported to show more days to extinguish a cocaine-associated context (Brenhouse and Andersen 2008), it is as yet unknown whether adolescents and adults differ in extinction of a context associated with self-administered drugs. Notably, evidence from a study of adolescent fear extinction suggests that cue extinction in the same context as conditioning is more effective than cue extinction in a different context for reducing cue-induced relapse-like behavior (Pattwell et al. 2016). Together with results from lever extinction, this strongly suggests that a difference in context extinction is not likely to explain an observed age difference in cue-induced reinstatement. Indeed, adolescent and adult rats both decreased active lever pressing over consecutive lever extinction days at the same rate in the same context as drug self-administration, suggesting comparable inhibition of both operant behavior and/or context in adolescents and adults. Extinction and test in the fear conditioning paradigm were conducted in a different context to conditioning, eliminating conditioned context as a potential confound in these experiments.

In my pharmacological studies, it may be possible that different doses of dopamine receptor agonists may produce different results. Doses used in the present thesis are based on previous reports of behavioral effects in adult rats following intra-IL infusions for SKF-81297 (0.1 μg/hemisphere; Zahrt et al. 1997; Floresco and Phillips 2001) and quinpirole (1.0 μg/hemisphere; Floresco et al. 2006). As previously discussed, prefrontal D1R activation is known to follow an inverted-U-shape function in terms of working memory (Goldman-Rakic et al. 2000). While the dose-related impact of D2R activation is less clear (Jay 2003), investigating a dose-response for prefrontal D1R or D2R activation for extinction learning in adolescents versus adults may reveal differences in terms of optimum range to affect performance across age. The chosen dose for acute systemic treatment with aripiprazole was also based on observed behavioral effects in rats from previous investigations (5 mg/kg; Feltenstein et al.,

2007). At this dose, aripiprazole is likely to induce a D2R occupancy of >80%, which is also the level required for behavioral effects in humans (Natesan et al., 2006; Sørensen et al., 2008). As discussed previously, I hypothesize that aripiprazole is acting postsynaptically at the present dose. It would still be interesting to investigate possible differences in dose-dependent responding to aripiprazole across age groups, as the effect of aripiprazole has not previously been examined in adolescent rodents as far as I am aware.

Importantly, the effects of quinpirole and aripiprazole in the present thesis are not likely due to non-specific effects. The plasma half-life of orally administered quinpirole (2 mg/kg) in rats is only 9.5 hours (Whitaker and Lindstrom 1987). Since the half-life of quinpirole when administered intracranially is not expected to be longer than when administered orally, it is not likely that quinpirole is affecting recall in present experiments. Likewise, aripiprazole at a dose of 10 mg/kg has been shown to reach 80% of peak level in brain of rats 1 hour after oral administration, and decline at a rate of $t_{1/2}$=1.8 hours (Shimokawa et al. 2005). Thus, the present effects were not likely due to residual drug affecting recall. Furthermore, my laboratory has observed that pre-extinction aripiprazole facilitates long-term extinction when extinction-test interval was 1 week (Kim, unpublished observations). In addition, acute systemic aripiprazole administration has shown no effect on general locomotor activity up to a dose of 10 mg/kg (Viana et al. 2013), while acute intra-mPFC quinpirole (1.0 μg/side) has shown no effects on locomotion (St Onge et al. 2011) or anxiety-like behavior (Wall et al. 2003). Finally, rats will not self-administer quinpirole (Collins and Woods 2007), and aripiprazole fails to produce CPP in adult mice (Shibasaki et al. 2012).

The extent to which the rodent PFC contains structural and functional homologs of areas in human prefrontal regions remains somewhat contentious (Wise 2008). Some argue that rats lack a cortical “granular” layer, characteristic of primate PFC (Wallis 2011). However, projections of the mPFC are highly conserved across species, as is the general arrangement of cortical layers. Furthermore, a number of studies point to shared functional roles for the mPFC across rodents and humans. This includes human imaging studies that strongly support a role for the mPFC in extinction learning (Phelps et al. 2004) (Gottfried and Dolan 2004) and recall (Mueller et al. 2014), consistent with findings from rodent literature.

It is yet unclear from current experiments precisely how D1R and/or D2R gene expression is modulated, however data from fear RT-qPCR suggest a potential interaction between the regulation of D1R relative to D2R. It is possible that observed changes in dopamine receptor gene expression relate to activity of miRNA, which are known to modulate post-transcriptional regulation of dopamine receptor mRNA, including mRNA turnover and translation (Kuzhikandathil 2014). It should also be noted that the timing of tissue collection was different in each paradigm. Thus, treatment-related differences were observed 24 hours after cocaine-cue extinction, and 2 hours after fear extinction. It is possible that similar differences to fear extinction might be observed 2 hours after cocaine-cue extinction. Different genes can show vastly different regulation in response to the same stimulus (Hong et al. 2004), and sampling at different time points following acute behavioral manipulation can reveal differences in temporal expression patterns in the same gene (Lamprecht et al. 2009; Ressler et al. 2002). Since I measured differences in gene expression at the same time following behavioral manipulations for each age and treatment group within each experiment, it is unlikely that differences in temporal expression account for observed differences between age or treatment groups for the different studies. However, it would be informative to add more time points for tissue collection to paint a more detailed picture of precise transcriptional mechanisms associated with extinction learning during adolescence versus adulthood. Finally, it is possible that differences in prefrontal gene expression for D1R, D2R or D1R/D2R in naïve rats may be revealed by increasing the number of samples, although the relatively small variability in current data suggest that increasing *n* may not change group averages. What's more, the same number of samples (*n*=4/group) was sufficient to reveal group differences in subsequent fear experiments. It would however be interesting for future investigations to measure differences in dopamine receptor gene expression in the IL and PL of the mPFC separately, under the condition that samples include all cortical layers, due to the relative distribution of D1R versus D2R discussed previously.

It is also important to acknowledge that mRNA levels do not necessarily correspond to protein levels. Some previous literature indicate a strong correlation between cortical D1R and D2R mRNA expression and receptor binding (Vijayraghavan et al. 2007), including one study of D1R gene and protein expression across development (Araki et al. 2007). Others have found a lack of correspondence for

cortical D2R mRNA levels versus receptor binding (Mansour et al. 1990). However, it is likely that those results are confounded by enhanced sensitivity of *in situ* hybridization used for mRNA visualization compared to receptor autoradiography used for protein visualization. Considering the relatively low expression of D2R in the cortex, it follows that autoradiography may not detect proteins to the same level as labeled riboprobes might detect mRNA. Critically, autoradiography relies on the specificity of the labeled ligands for visualization, which often show overlap between dopamine receptor subtypes.

6.7 Future directions

Future studies will further elucidate the precise mechanisms of extinction learning and memory across adolescent development. The development of reliable antibodies specific to the D1R and D2R will allow future studies to more conclusively examine changes in receptor expression following behavioral manipulations. However, as discussed in Chapter 4, D1R and D2R show important similarities, which have implications for the development of selective antibodies. Indeed, antibodies against GPCRs are notoriously unreliable, owing at least in part to high levels of homology even across broad GPCR groups (Floresco et al. 2006). A number of studies have used immunostaining to visualize D1R in the PFC, including differentiation between the D1-like subtypes D1R and D5R (Lidow et al. 2003), and staining for D1R in the PFC of adolescent rats (Brenhouse et al. 2008). However, staining of D2R has shown conflicting results across previous literature, with some reporting extensive labeling throughout all layers of cortex (Lawler et al. 1999), and others have showing little to no staining (Davies et al. 2004). Very recent findings suggest that newer D2R antibodies may show improved specificity (Soares-Cunha et al. 2016).

Several informative experiments could also be achieved using the present behavioral paradigms. Here I showed that long-term adolescent cue extinction was improved by enhancing IL D2R signaling. It has previously been shown that adolescent fear extinction can be improved by increasing exposure (by doubling the number of CS presentations). It would be interesting to test whether the same effect is seen for drug-cue extinction. In both fear and drug cue extinction it would be worth attempting to naturally enhance adolescent extinction learning in order to compare changes in the PFC associated with successful extinction learning in adolescents versus adults. Given

that adolescence is defined by increased prefrontal plasticity associated with long-term behavioral changes (Selemon 2013), it would also be fascinating to uncover whether successful cue extinction learning during adolescence may be more long-lasting compared to adult cue extinction learning. This could have profound implications for school and government policy on the targeted treatment of mental health during adolescence.

It would also be valuable for future studies to examine appetitive and aversive cue extinction learning in combination. This might involve investigating whether extinction of a drug-associated cue affects subsequent extinction of a fear conditioned cue, or *vice versa*. Since substance abuse and anxiety disorders are highly comorbid (Merikangas et al. 1998), it would be especially interesting to examine how adolescent and adult rats respond to the same cues across appetitive and aversive learning domains.

There are several other models that could be utilized to examine the contribution of D1R and/or D2R signaling in adolescents versus adult cue extinction learning. Transgenic reporter mice, with endogenously labeled D1R or D2R, could allow for visualization using immunohistochemistry for a marker of neuronal activity such as c-Fos in the mPFC following cue extinction learning (Thibault et al. 2013). Transgenic D1R- or D2R-Cre mice could be used in an optogenetic paradigm, where prefrontal D1R or D2R could be selectively activated or silenced during cue extinction training (Soares-Cunha et al. 2016; Riga et al. 2014). The development of transgenic rats represents an exciting next step for behavioral neuroscience to uncover molecular mechanisms involved in a range of learning and memory tasks, including cue extinction. Since D1R and D2R are co-expressed on neurons in the PFC, these methods may not provide a full picture. However, the existence of separate D1R- and D2R-expressing populations of neurons in the PFC have also been identified (Gee et al. 2012). Interestingly, optogenetic activation of D1R- or D2R-expressing neurons in the striatum has been shown to induce different types of learning behavior (Kravitz et al. 2012; Lobo et al. 2010). However, to the best of my knowledge, this has not yet been determined for PFC.

6.8 Conclusions

The present thesis adds to growing evidence that adolescents are impaired in fear cue extinction, and shows for the first time adolescent impairment in cocaine cue extinction. An evolutionary hypothesis has been proposed for adolescent suppression of fear extinction, (Pattwell et al. 2013). Specifically, adolescence is associated with leaving the safety of the nest in search of reproductive success (Spear 2000). Specific danger cues are particularly relevant for guiding behavior while exploring new environments, therefore we may benefit from cued fear learning being resistant to degradation to optimize chances for survival. An evolutionary argument could also be made for suppression of reward-related extinction. Indeed, adolescence is associated with one new and extremely powerful reward: sex. It makes sense that adolescents might continue to seek this reward even after previous attempts have failed. Finally, I believe that suppressed extinction of emotionally salient cues could also be evolutionarily beneficial for social learning. Our teen years are perhaps our most important for establishing meaningful relationships with peers beyond immediate family and/or carers; our closest lifelong friends are often those we met during secondary school or early university. It is not hard to conceive that certain people in a young person's life can become their most influential cues, guiding behavior to build these critical social networks.

Of course, deficits in cue extinction are not always beneficial in modern society. Epidemiological data indicate that persistence of mental health problems such as addiction and anxiety in adolescents relates more to recurrence rather than chronicity of youth-onset disorders (Kessler et al. 2012). Present data suggest that this may be explained at least in part to extinction impairments during adolescence, and highlight a role for prefrontal dopamine signaling in cue extinction learning across adolescent development. Findings provide evidence for a novel theory of extinction learning involving simultaneous strengthening of new learning via D1R activation in combination with weakening of pathological cue associations via D2R activation. Given that neural correlates of adolescent behavior are highly conserved across species, results of this thesis are an important step not only for understanding the mechanisms of extinction learning across adolescent development, but also for developing more effective treatments for people living with substance abuse and/or anxiety disorders.

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