Fear extinction during adolescence: neural mechanisms and implications for treating anxiety disorders

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Fear extinction during adolescence: neural mechanisms and implications for treating anxiety disorders

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Adolescent-onset anxiety disorders are more common and costly than those that emerge later in life. Unfortunately, approximately half of adolescents who receive cognitive behaviour therapy experience significant symptom relapse. This finding is consistent with adolescents' performance on the preclinical model of fear extinction, such that they show impaired extinction retention. To understand why exposure-based treatments may be less effective during adolescence, I investigated the neurotransmitter systems involved in adolescent extinction.

In my first series of experiments, described in Chapter 2, I examined whether the cannabinoid receptor 1 agonist WIN55212-2 ameliorates impaired extinction retention in adolescent rats. Unexpectedly, WIN55212-2 increased fear expression during extinction training and had no effect on extinction retention in both juvenile and adolescent rats. This finding was accompanied by an age-related decrease in the expression of cannabinoid receptor 1 protein in the medial prefrontal cortex and amygdala.

In Chapter 3, I explored the role of NMDA receptors in extinction during adolescence. While NMDA receptors were required for extinction in rats conditioned and extinguished as adolescents, this was not the case for rats conditioned as juveniles and extinguished as adolescents. NMDA receptor-independent extinction in this latter group was not due to the interval between conditioning and extinction training, or the experience of a developmental transition.

In Chapter 4, I investigated the role of opioid receptor mediated prediction error in extinction learning. Opioid receptors were utilised by juvenile rats, adult rats, and adolescent rats that were conditioned as juveniles during a single session of extinction training. In contrast, rats conditioned and extinguished as adolescents did not use opioid receptors until the second session of extinction training.

Taken together, these experiments highlight the complex neurobiology of extinction during adolescence. Impaired extinction retention in rats conditioned and extinguished as adolescents is likely due to an under-recruitment of cannabinoid receptor 1, NMDA receptors, and opioid receptors on the first day of extinction training. The mechanisms by which altering the age at which adolescent rats acquire fear leads to engagement of opioid and NMDA receptors, and subsequently good extinction, remains to be understood. The clinical and theoretical implications of these findings are discussed.

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Table of Contents

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The experiments in this publication form the basis of **Chapter 4**.

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The experiments in this publication form the basis of **Chapter 2**.

King, G., Baker, K.D., **Bisby, M.A.**, Chan, D., Cowan, C.S.M., Stylianakis, A.A., Zimmerman, K.S., & Richardson, R. (2019). A precision medicine approach to pharmacological adjuncts to extinction: a call to broaden research. *Psychopharmacology, 236, 143-161.*

Bisby, M.A., Baker, K.D., & Richardson, R. (2018). Elucidating the mechanisms of fear extinction in developing animals: a special case of NMDA receptor-independent extinction in adolescent rats. *Learning and Memory, 25,* 158-164.

The experiments in this publication form the basis of **Chapter 3**.

Baker, K.D., **Bisby, M.A.**, & Richardson, R. (2016). Impaired fear extinction in adolescent rodents: Behavioural and neural analyses. *Neuroscience and Biobehavioural Reviews, 70,* 59-73.

Other publications

Moulds, M.L., **Bisby, M.A.**, Wild, J., & Bryant, R.A. (in prep). Rumination in Posttraumatic Stress Disorder: A Systematic Review.

Bisby, M.A., Kimonis, E.R., & Goulter, N. (2017). Low Maternal Warmth Mediates the Relationship Between Emotional Neglect and Callous-Unemotional Traits Among Male Juvenile Offenders. *Journal of Child and Family Studies, 26,* 1790-1798.

Presentations

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Bisby, M.A., Baker, K.D., & Richardson, R. (2019). The importance of considering age when designing interventions: differential effects of the pharmacological adjunct WIN55212-2 on fear extinction across development. *Poster presented at the World Congress of Behavioural and Cognitive Therapies.* Berlin, Germany.

Bisby, M.A. (2018). Adolescents are not little adults: the importance of considering the developing brain in designing treatment interventions. *Oral presentation at the Sydney Postgraduate Psychology Conference.* Sydney, Australia.

Bisby, M.A., Baker, K.D., & Richardson, R. (2018). Adolescents are not little adults: the importance of considering the developing brain when designing treatment interventions. *Poster and oral presentation at the UNSW Science Postgraduate Research Competition.* Sydney, Australia.

Bisby, M.A., Baker, K.D., & Richardson, R. (2017). Elucidating the mechanisms of fear extinction in developing animals: a special case of NMDA receptor-independent extinction in adolescent rats. *Poster presented at the Society for Neuroscience.* Washington, D.C., U.S.A.

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Bisby, M.A., Baker, K.D., & Richardson, R. (2017). Improving fear extinction in adolescence: are endocannabinoids the answer? *Poster and oral presentation at the UNSW Science Postgraduate Research Competition.* Sydney, Australia.

List of Abbreviations

AdolesCond-Ext	rats conditioned and extinguished as adolescents
AdolesCond-AdultExt	rats conditioned as adolescents and extinguished as adults
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BLA	basolateral amygdala
CBR	cannabinoid receptor
CeA	central amygdala
CS	conditioned stimulus
FAAH	fatty acid amide hydrolase
IL	infralimbic cortex
ITC	intercalated cells
JuvCond-Ext	rats conditioned and extinguished as juveniles
JuvCond-AdolesExt	rats conditioned as juveniles and extinguished as adolescents
GABA	gamma-aminobutyric acid
mGluR	metabotropic glutamate receptor
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
NMDAR	N-methyl-D-aspartate receptor
NS	novel stimulus
pMAPK/ERK	phosphorylated mitogen activated kinase/extracellular signal-regulated kinase
PL	prelimbic cortex
SEM	standard error of the mean
US	unconditioned stimulus
vlPAG	ventrolateral periaqueductal gray

Chapter 1: General Introduction

Anxiety disorders are the most prevalent mental health condition in Australian adolescents, affecting 7% of 12-17 year olds each year (Lawrence et al., 2015). Anxiety in adolescence often persists into later life (Kessler et al., 2007; Lee et al., 2014) due to a variety of factors, including adolescents not seeking treatment, not receiving evidence-based treatment from health practitioners, or receiving treatment that is not sufficiently effective (Australian Institute of Health and Welfare, 2014; Harris et al., 2015; Merikangas et al., 2011). Indeed, approximately half of adolescents who do receive the gold-standard treatment for anxiety disorders – cognitive behaviour therapy – report significant symptom relapse at follow-up (Ginsburg et al., 2014; Kodal et al., 2018). Therefore, there is not only a need to encourage and facilitate access to appropriate mental health services, but also to improve long-term outcomes for those who receive evidence-based treatment.

One approach to improving treatment outcomes is to examine the mechanisms by which the symptoms of anxiety disorders can reduce across treatment. Exposure therapy, one of the critical elements of cognitive behaviour therapy, is based on the process of fear extinction (Craske, Hermans, & Vervliet, 2018; Paredes & Morilak, 2019). As fear extinction learning predicts treatment responding in anxious youth (Geller et al., 2019), finding approaches to facilitate extinction would be a step towards improving treatment outcomes. Despite substantial progress in characterising the neural mechanisms of extinction in *adult* rodents and humans, the translation of this work to clinical settings has been difficult (Griebel & Holmes, 2013). Such difficulties may be due to a lack of attention to individual differences based on developmental stage (King et al., 2019). Far less is known about the neurobiological processes which underlie

extinction in adolescents, and there is little doubt that there are important developmental differences which would impact extinction performance.

In light of recent randomised controlled trials highlighting that ineffectiveness of gold-standard psychological treatments for anxiety during adolescence (e.g., Waite, Marshall, & Creswell, 2019), there is substantial merit in discovering pharmacological adjuncts which may "boost" treatment outcomes by enhancing the underlying neural mechanisms of extinction. Specific, targeted interventions for anxiety during adolescence must be designed by identifying how the adolescent brain differs from the adult brain. As pharmacological adjuncts which facilitate extinction in adults do not always translate to adolescence (e.g., fluoxetine; Chan et al., unpublished), it is critical to gain a developmentally appropriate understanding of the underlying neural mechanisms. To this end, my thesis used a rodent model to examine the role of three neurotransmitter systems that are known to be involved in extinction, at least in adults, in adolescent rodents. This research not only contributes to our understanding of developmental differences in the process of extinction but may also help inform future research in clinical populations with the goal of improving treatment outcomes for anxious adolescents by identifying candidate pharmacological adjuncts.

The cost of anxiety disorders

The experience of mental illness takes a toll on both personal and economic levels. On an individual level, mental health disorders can lead to a variety of adverse outcomes, including relationship difficulties, physical health disorders, and financial instability (Kessler et al., 2009). At a broader level, there is also a significant economic burden due to lost productivity and health care costs that can be attributed to mental illness. Alarmingly, the economic impact of mental illness has now surpassed the

impacts of both cardiovascular disease and cancer, illustrating how pervasive the problem is (Bloom et al., 2011). In examining mental health conditions more specifically, anxiety disorders emerge as the most common and one of the most economically disabling mental illnesses in Australia and around the world (Baxter, Vos, Scott, Ferrari, & Whiteford, 2014; Harris et al., 2015; Slade et al., 2009).

Anxiety in young people is of particular concern given the high prevalence and associated costs (Lawrence et al., 2015; Lee et al., 2014; Suhrcke, Pillas, & Selai, 2008). Some of the costs associated with early-onset anxiety disorders arise from the later-life impairments in psychosocial functioning, such as reduced educational attainment, unemployment, maladaptive coping, and chronic stress (Essau, Lewinsohn, Olaya, & Seeley, 2014). Moreover, some evidence suggests that adolescent anxiety leads to more functional impairment later in life than childhood anxiety (e.g., Essau et al., 2014). Therefore, adolescence is a window of vulnerability to persistent anxiety as well as a window of opportunity for intervening to minimise that burden.

Cognitive Behaviour Therapy

Currently, the gold-standard treatment for anxiety disorders is cognitive behaviour therapy (David, Cristea, & Hofmann, 2018). One component of cognitive behaviour therapy is exposure, in which patients gradually confront previously feared situations (e.g., spiders, contaminated objects) and learn that they can cope (Abramowitz, 2013; Craske, Treanor, Conway, Zbozinek, & Vervliet, 2014). Although cognitive behaviour therapy successfully reduces anxiety symptoms across the course of treatment in most adult patients (Carpenter et al., 2018; Olatunji, Cisler, & Deacon, 2010), meta-analyses indicate that only half of all adult patients reach symptom remission at both short-term (6 months, 53.9-55%) and long-term (22-84 months,

56.1%) follow-up time points (Loerinc et al., 2015; Springer, Levy, & Tolin, 2018). As the other half of patients experience significant symptom relapse, we need to focus on how to maximise the retention of treatment gains. In particular, it is critical to identify the mechanisms that underlie the return of fear following treatment cessation.

A similar picture emerges during adolescence. Across the course of treatment, anxiety symptoms typically reduce for adolescent patients, however, the rate of symptom reduction is significantly slower in younger patients relative to adult patients (Barry, Yeung, & Lau, 2018). Unfortunately, in more recent trials, cognitive behaviour therapy has been associated with no improvements in anxiety symptoms compared to a wait list control (Waite et al., 2019). Furthermore, there is a significant return of symptoms in approximately half of adolescent patients who received cognitive behaviour therapy for an anxiety disorder when assessed at long-term follow-up time points (Ginsburg et al., 2014; Kodal et al., 2018). In fact, symptom remission has been reported to be as low as 23% in adolescent patients at 1-year follow-up (Wergeland et al., 2014). These findings indicate that the provision of cognitive behaviour therapy, our gold-standard treatment, does not result in long-term symptom reduction for the majority of adolescents with anxiety disorders, as is also the case for adults. In order to modify exposure-based treatments and facilitate treatment outcomes, we need to understand the basic mechanisms which underlie successful reductions in fear across the long-term, and whether these differ across development.

A preclinical model of exposure therapy: fear extinction

Individuals learn to inhibit fear through the process of extinction, a well-established pre-clinical model of exposure therapy (Craske et al., 2014; Paredes & Morilak, 2019). The degree to which participants learn to inhibit fear during

experimental laboratory extinction tasks has been shown to predict treatment responding for cognitive behaviour therapy (Geller et al., 2019), suggesting that finding approaches to improve fear extinction could eventually translate to improved treatment outcomes for anxiety or fear-related disorders. It is particularly useful to investigate the mechanisms of extinction using a preclinical model as the learning processes and neurobiological circuitry underlying extinction learning are conserved across rodents and humans (Flores, Fullana, Soriano-Mas, & Andero, 2018; Milad & Quirk, 2012). Specifically, rodent models provide the opportunity for testing the involvement of potential neurotransmitter systems using pharmacological approaches that may not yet be approved for use in humans and allow more experimental control than is possible using human participants. Typically, preclinical models assess both the acquisition and inhibition of fear using Pavlovian fear conditioning and extinction procedures.

Fear conditioning. During fear conditioning, a neutral stimulus (e.g., a tone) is paired with an aversive unconditioned stimulus (US; e.g., shock). As a result, the tone becomes a conditioned stimulus (CS) and can elicit fear responses (e.g., increased skin conductance in humans, freezing in rodents) even without the shock (LeDoux, 2000).

Acquisition of fear extinction. Once learned, conditioned fear to the CS can be reduced by repeatedly presenting the tone in the absence of the shock, a procedure termed *fear extinction*. The reduction in learned fear responses during extinction suggests that the animal has learnt that the previously aversive CS is now safe. Several theoretical accounts have been put forward to explain extinction learning. One of the first theories suggested that extinction was "unlearning", that is, a reduction in the strength of the CS-US association that was originally learned (Rescorla & Wagner, 1972). This account, known as the Rescorla-Wagner model, is based on changes in *prediction error*, which is the difference between the predicted and actual outcome of a

given event (e.g., a CS presentation). This model asserts that the CS-US association is acquired through positive prediction error during fear conditioning and, subsequently, the strength of the CS-US association can be decreased through negative prediction error. In extinction training, negative prediction error is generated as the actual outcome (i.e., the CS = no US) is less than the expected outcome (i.e., the CS = US). This assertion is supported by a wealth of evidence indicating that pharmacologically disrupting negative prediction error prevents inhibitory learning, including fear extinction (Li & McNally, 2014; McNally, Johansen, & Blair, 2011; Sengupta, Winters, Bagley, & McNally, 2016). However, there are several circumstances when fear is prone to recover following extinction (see below, p. 6), suggesting that some of the original association survives extinction and/or that other learning processes may also be occurring. Therefore, later theories suggested that extinction learning involves two processes: reductions in the strength of the original conditioning memory, and also the acquisition of a new extinction memory that competes for expression with the original fear memory (Bouton, 2002; Dunsmoor, Niv, Daw, & Phelps, 2015; Myers & Davis, 2007).

Consolidation of fear extinction. Following extinction training, the new extinction memory is typically then consolidated (Quirk & Mueller, 2008). The term "consolidation" refers to the time-dependent stabilisation of the temporary memory into a long-term memory that is subsequently less vulnerable to interference (Herry et al., 2010; McGaugh, 2000). Memory consolidation is required for later retrieval of the extinction memory, as blocking this process (e.g., via administration of agents that block neuronal activity and cellular processes shortly after an extinction session; Furini, Myskiw, & Izquierdo, 2014; Santini, Ge, Ren, Pena de Ortiz, & Quirk, 2004) impairs later recall.

Retention of fear extinction. When animals are tested with an extinguished CS at a later time point, fear responding to the CS is significantly reduced. However, this reduction in fear is gated by temporal, spatial, and interoceptive contexts (Bouton, 1993; Myers & Davis, 2007). That is, fear of the CS can return following extinction training in several ways, including renewal, reinstatement, and spontaneous recovery (Bouton, 2002). In *renewal*, fear returns when the extinguished cue is presented in a different context from the one in which extinction training occurred, such as the fear conditioning context or a novel context (Bouton & Bolles, 1979; Bouton & King, 1983). For *reinstatement*, exposure to the initial aversive stimulus or a stressful experience elicits the recovery of extinguished fear (Rescorla & Heth, 1975). Lastly, *spontaneous recovery* refers to the return of fear following the passage of time in the absence of any further extinction training (Quirk, 2002; Rescorla, 2004).

The recovery of fear following extinction training in preclinical models is paralleled by relapse following completion of exposure therapy. A particular challenge for clinicians and clients is that fear returns to previously aversive cues that were targeted in exposure therapy despite reductions in fear across treatment (Vervliet, Craske, & Hermans, 2013). For instance, relapse can be observed when a cue is encountered outside the therapy room (renewal), following a life stressor (reinstatement), or one year following treatment completion in the absence of booster sessions (spontaneous recovery). Therefore, there is significant translational value in identifying and manipulating the neurobiological processes involved in extinction using pharmacological adjuncts to strengthen memory consolidation and promote long-term protection against relapse (Craske et al., 2018).

A curious case of impaired fear extinction

Although relapse does occur in adults following successful extinction, reductions in fear are typically retained when animals are tested in the extinction context shortly after extinction training. The reductions in conditioned fear responses elicited by the CS across extinction training are maintained when the CS is presented the next day (i.e., at extinction retention test). However, during adolescence, animals often exhibit a return of conditioned fear to the CS at the extinction retention test the next day despite reductions across extinction training (for review, see Baker, Bisby, & Richardson, 2016).

In the first study to examine *extinction retention* performance during adolescence, McCallum et al. (2010) compared extinction learning and retention in juvenile (the period prior to adolescence), adolescent, and adult rats. While all age groups showed equivalent acquisition of fear on the first day, and extinction of fear responses on the second day, adolescent rats showed significantly higher conditioned responding (as measured by freezing) to the CS when tested on the third day. That is, adolescent rats showed impaired extinction retention relative to both younger and older rats. This finding has since been replicated across several research groups using both rat and mouse models (e.g., Baker & Richardson, 2015; Ganella, Lee-Kardashyan, et al., 2017; Pattwell et al., 2012), indicating that the adolescent impairment in extinction retention is robust and replicable.

Subsequent studies have revealed that human adolescents also exhibit impairments in extinction retention relative to younger and older ages. These studies with human participants typically have different procedures for fear conditioning, extinction, and fear relapse, as well as different measures of fear responses than the studies with rodents. Despite these differences, it is still possible to conclude that there

are alterations in extinction across development. A common procedure used in research with human participants is differential conditioning, in which one neutral CS (the CS+) is paired with an aversive US, while another neutral CS (the CS-) is never paired with an aversive US. Conditioned fear is then measured by comparing responses to the CS+ relative to responses to the CS- (Ryan, Zimmer-Gembeck, Neumann, & Waters, 2019). Compared to children and adults, adolescents show heightened responding to the CS+ relative to the CS- at extinction recall (Ganella, Drummond, Ganella, Whittle, & Kim, 2018).

In some cases, deficits in *extinction learning* in adolescent rodent and humans have also been observed. For instance, Pattwell et al. (2012) found that adolescent mice continued to exhibit high levels of CS-elicited freezing across extinction training sessions, indicative of diminished extinction learning. Moreover, this impairment was replicated in human adolescents, who showed impaired acquisition of extinction relative to children and adults (Pattwell et al., 2012). Consistent with the above findings, deficits in both extinction learning and retention have been found using CS-revaluation learning. During extinction learning, it is expected that individuals will re-evaluate the CS+ in a more positive manner as the cue no longer predicts an aversive outcome (Ryan et al., 2019). Interestingly, when compared to both children and adults, adolescents show less positive re-evaluations of the CS+ during extinction training, and this developmental difference persisted at extinction retention (Waters, Theresiana, Neumann, & Craske, 2017). This suggests that adolescents are poor at learning new information about the CS and updating their behavioural responding accordingly, and adds to the growing literature demonstrating impaired inhibitory learning during adolescence.

Given the sustained responding to previously dangerous cues observed in healthy adolescents, it is not surprising that adolescents diagnosed with anxiety disorders relapse following cognitive behaviour therapy. As previously mentioned, a key component of cognitive behaviour therapy is learning that feared stimuli are not as dangerous as they are perceived to be, and this is achieved by repeatedly presenting clients with feared stimuli in the absence of the feared outcome. It is therefore important that clients not only acquire extinction during the sessions, but also that the extinction learning is retained between sessions to further treatment gains. Mirroring the extinction deficits found in preclinical research in animal and human subjects, adolescent patients show a slower rate of symptom reduction across cognitive behaviour therapy, as well as a return of symptoms following treatment completion (Barry et al., 2018; Ginsburg et al., 2014; Kodal et al., 2018). Although the rates of relapse following cognitive behaviour therapy are similar in adolescents and adults, the stark differences between the developing and adult brain, as well as the liability for long-term adverse outcomes following adolescent anxiety, warrant a developmentally nuanced approach to improving treatment outcomes. Due to the similarities in fear extinction and exposure therapy, and the findings indicating the predictive value of fear extinction for treatment outcomes (Geller et al., 2019; Waters & Pine, 2016), interventions which ameliorate impaired extinction could then be translated to clinical trials, and perhaps eventually to clinical interventions for anxious adolescents.

My thesis took a pharmacological approach to improving extinction in adolescence, as manipulating neurotransmission during extinction learning is a viable pathway to long-term reductions in fear (Bandelow et al., 2015; Sartori & Singewald, 2019). I utilised a pharmacological approach, as opposed to a behavioural modification approach, to augment extinction for two reasons. First, such an approach will contribute

to remedying the significant gap in our understanding of the neural mechanisms of extinction during adolescence (Baker et al., 2016). Second, as current behavioural strategies (i.e., cognitive behaviour therapy) do not lead to long-term reductions in fear/anxiety during adolescence, the addition of a pharmacological adjunct may confer more benefit. Using a rodent model, I pharmacologically manipulated three neurotransmitter systems using agonists/antagonists and observed subsequent effects on extinction learning and retention. Across my thesis, I investigated how the administration of specific pharmacological agents alongside extinction training can impact extinction performance during adolescence. As neurotransmitters impact fear behaviour through their actions in specific brain regions, I will briefly review the neural circuit of fear extinction in adult animals and compare this to the neural circuit of fear extinction during adolescence. First, a brief overview of the neural circuit of fear expression will be provided to enable a comparison between the neural mechanisms of fear expression versus inhibition.

The neural circuit of fear expression and extinction during adulthood

Fear expression. During fear conditioning, information about the CS and US converges in a specific brain region, resulting in *long-term potentiation* at the neuronal junction (Ressler & Maren, 2019). Long term potentiation refers to the persistent strengthening of the synapse, and this increase in synaptic plasticity is required for behavioural change to occur (Johansen, Hamanaka, et al., 2010; Kwon et al., 2014; Nabavi et al., 2014). The site of this convergence is theorised to be in the basolateral amygdala (BLA), a region of the temporal lobe. The BLA also regulates fear expression by moderating the activity of fear output neurons in an adjacent nucleus, the central amygdala (CeA; Asede, Bosch, Lüthi, Ferraguti, & Ehrlich, 2015; Janak & Tye, 2015; Tovote, Fadok, & Lüthi, 2015). There are two pathways from the BLA to the CeA

involved in fear expression: direct excitatory projections, and indirect inhibitory projections via a cluster of inhibitory interneurons termed 'the intercalated cells' of the amygdala (ITC; reviewed in Duvarci & Paré, 2014).

At the microcircuit level, the direct and indirect pathways from the BLA innervate different divisions of the CeA (i.e., medial and lateral; McDonald, 1982). For instance, excitatory projections from the BLA project directly to the medial CeA, the division of the CeA which is the site of "fear output" neurons (i.e., those neurons projecting to the downstream structures that mediate fear responding; Rizvi, Ennis, Behbehani, & Shipley, 1991). In the case of the indirect pathway, the BLA has excitatory projections to the intercalated cells, which then have inhibitory projections to the lateral CeA. The inhibitory projections from the ITC to the lateral CeA effectively disinhibit activity of the medial CeA (by CeA lateral → CeM projections) thereby increasing fear responses (for reviews, see Ehrlich et al., 2009; Tovote et al., 2015).

The fear output neurons of the medial CeA project to downstream structures, one of which is the ventrolateral PAG (vlPAG), a region which mediates freezing in rodents (Haubensak et al., 2010; Herry & Johansen, 2014; Koutsikou et al., 2014). The projections from the medial CeA to the vlPAG are inhibitory and yet increased activity of this projection results in increased freezing, suggesting that disinhibition of neurons within the vlPAG is involved in heightened fear expression (Oka, Tsumori, Yokota, & Yasui, 2008; Tovote et al., 2016).

Cortical regions play a critical role in modulating fear expression pathways involving the amygdala and vlPAG. Two sub-regions of the medial prefrontal cortex (mPFC), the prelimbic (PL) and infralimbic (IL) cortices are particularly relevant for understanding fear regulation in rodents. The prevailing view of the mPFC in fear

regulation proposes is that there is often a dichotomy of function between the IL and PL in both physiological and anatomical terms (for reviews, see Giustino & Maren, 2015; Milad & Quirk, 2012). Broadly, activation of the PL is thought to promote fear expression to conditioned cues while activation of the IL is thought to reduce fear expression to such cues (Senn et al., 2014). Both roles involve recruitment of pathways to, and from, the amygdala. Strong reciprocal connectivity has been identified between the mPFC and BLA (Little & Carter, 2013), such that there are neurons in the BLA which both receive input from, and project back to, the mPFC, indicating a communication loop (Hübner, Bosch, Gall, Lüthi, & Ehrlich, 2014; McGarry & Carter, 2017). As summarised in **Figure 1.1**, communication between the PL and BLA leads to increased activation of medial CeA neurons, reduced inhibition of vlPAG neurons, and ultimately increased fear expression. Understanding the neural circuitry which underlies increased fear expression is important, particularly for cases of maladaptive, persistent fear (e.g., impaired extinction in adolescence).

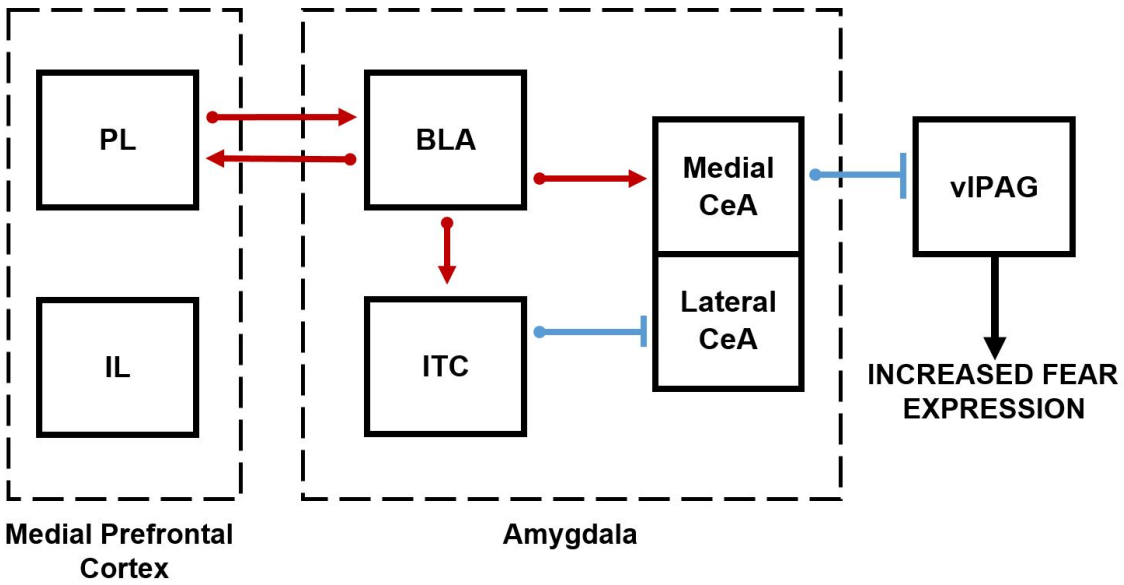

Figure 1.1. Neural circuit of fear expression. Red lines indicate excitatory projections and blue lines indicate inhibitory projections.

PL = prelimbic cortex, IL = infralimbic cortex, BLA = basolateral amygdala, ITC = intercalated cells, CeA = central amygdala, vlPAG = ventrolateral periaqueductal gray.

Fear extinction. Three of the same structures involved in fear expression, the mPFC, amygdala, and vlPAG, are also involved in fear extinction. During extinction training, information about the CS and the absence of US converge in the BLA to initiate the formation of a new inhibitory memory (Amano, Duvarci, Popa, & Paré, 2011; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011). The acquisition of an extinction memory is dependent on the generation of negative prediction error, which is coded in the vlPAG and indirectly conveyed to the BLA (Arico, Bagley, Carrive, Assareh, & McNally, 2017; Parsons, Gafford, & Helmstetter, 2010). The essential role of the vlPAG in generating prediction error for extinction has been shown by findings that silencing the vlPAG blocks within-session extinction (Arico et al., 2017) and disrupts synaptic plasticity within the BLA which is associated with encoding the extinction memory (Parsons et al., 2010). Nevertheless, the exact nature of the negative prediction error signal underlying extinction and the path by which this signal reaches the BLA are unknown in adult animals (Li & McNally, 2014), let alone during development.

The prediction error signalled by the vlPAG is encoded during extinction by the BLA. The essential role of the BLA in extinction learning is demonstrated by findings that reducing neuronal activity within this region prior to extinction training impairs extinction retention the following day (Davis & Bauer, 2012; Zimmerman & Maren, 2010). In addition to the BLA, long-term retention of fear extinction also relies on the IL, as inhibition of this region with pharmacological or optogenetic approaches during extinction training impairs extinction retention performance whilst sparing within-session extinction (Do-Monte, Manzano-Nieves, Quinones-Laracuente, Ramos-Medina, & Quirk, 2015), presumably by interfering with the consolidation of the extinction memory.

It is not only the activation of the IL and BLA that is critical for successful extinction, but also the communication between them (Bukalo et al., 2015; Marek, Strobel, Bredy, & Sah, 2013). This role has been demonstrated by findings that extinction retention is compromised following chemogenetic/optogenetic inhibition of either BLA-projecting IL neurons or IL-projecting BLA neurons whilst within-session extinction is spared in both instances (Bloodgood, Sugam, Holmes, & Kash, 2018; Senn et al., 2014). Although an understanding of the information that is relayed by each projection pathway is still developing, there is some evidence that the BLA encodes the saliency of the CS during extinction training and then sends this information to the mPFC (Davis, Zaki, Maguire, & Reijmers, 2017; Klavir, Prigge, Sarel, Paz, & Yizhar, 2017). Once in receipt of this information, the mPFC modulates BLA activity (Yizhar & Klavir, 2018) and ultimately leads to a reduction of fear expression by inhibiting neuronal activity within the medial CeA across extinction training.

The exact pathway/s by which neuronal activity within the medial CeA is inhibited by the BLA and IL are not clearly delineated. Three paths have been suggested that originate from the IL: via the BLA, via the ITC, and via the lateral CeA (Amano, Unal, & Paré, 2010; Amir, Amano, & Paré, 2011; Duvarci & Paré, 2014; Strobel, Marek, Gooch, Sullivan, & Sah, 2015; Wood et al., 2019). As each proposed path is supported by evidence, all pathways have been included in the neural circuit of extinction as summarised in **Figure 1.2**.

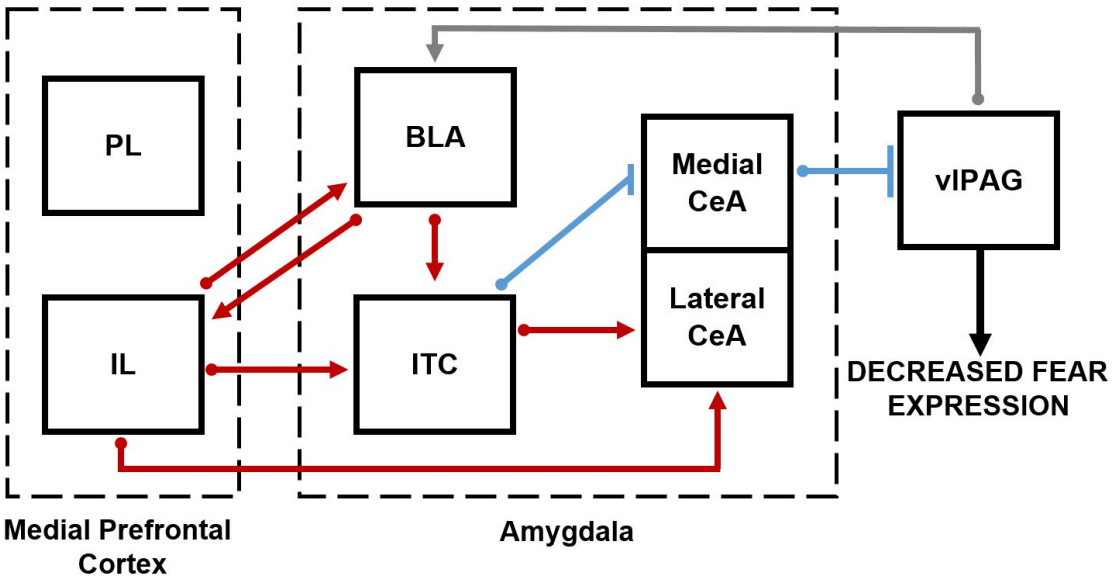

Figure 1.2. Neural circuit of fear extinction. Red lines indicate excitatory projections and blue lines indicate inhibitory projections. Grey lines indicate projections of an unknown nature.

PL = prelimbic cortex, IL = infralimbic cortex, BLA = basolateral amygdala, ITC = intercalated cells, CeA = central amygdala, vlPAG = ventrolateral periaqueductal gray.

Neural circuit of fear extinction during adolescence

During adolescence, effective communication between the amygdala and mPFC is compromised. Due to the continued growth and neuronal remodelling that occurs in these regions across adolescence, functional connectivity between the two regions is transiently reduced (as reviewed in Baker et al., 2016; Casey, Glatt, & Lee, 2015). In terms of anatomical changes within the amygdala, grey matter *volume* decreases while grey matter *density* increases, likely due to a combination of increased myelination and continued synaptic pruning (Gennatas et al., 2017). These changes coincide with significant alterations in the prefrontal cortex. Specifically, structural changes within this region include reductions in grey matter growth due to synaptic pruning, as well as increases in white matter growth due to a surge in myelination (Koss, Belden, Hristov, & Juraska, 2014; Spear, 2013; Tau & Peterson, 2010). Furthermore, effectiveness of mPFC projections in modulating BLA activity is substantially reduced during

adolescence. This has been demonstrated in electrophysiological studies reporting that stimulation of mPFC neurons results in significantly weaker inhibition over BLA neurons during adolescence relative to adulthood (note that no distinction was made between neurons in the PL versus IL; Selleck, Zhang, Samberg, Padival, & Rosenkranz, 2018). This reduced inhibition of BLA activity would likely be due to a reduced efficiency of the excitatory projections from the mPFC which target inhibitory neurons within the BLA to reduce the activity of excitatory BLA neurons (Cho, Deisseroth, & Bolshakov, 2013). Taken together, these results clearly show that the amygdala and mPFC, two regions critically involved in extinction of learned fear (at least in adults) undergo significant structural and functional maturation during adolescence.

These changes in the mPFC and amygdala during adolescence likely result in altered synaptic plasticity and neuronal activation within these regions during extinction relative to other ages. In juvenile and adult rats, synaptic plasticity (as measured by phosphorylated mitogen activated protein kinase/ extracellular signal-regulated kinase [termed pMAPK/ERK]) is increased in the IL following extinction training. However, this pattern of synaptic plasticity is not observed in adolescents. In fact, levels of synaptic plasticity in the IL, as well as the BLA, are much lower in adolescent rats following extinction training, such that they match the levels of synaptic plasticity in rats that did not receive extinction training (Baker & Richardson, 2015; Kim, Li, & Richardson, 2011). These findings indicate that the IL and BLA are not being recruited during extinction training in adolescence, perhaps contributing to the deficits in extinction retention the following day.

Similar deficits in recruiting the mPFC and amygdala have been observed in human adolescents. The presence of strong negative coupling between these two regions (i.e., increased mPFC activity that coincides with decreased amygdala activity, as

identified using functional magnetic resonance imaging) is thought to indicate efficient 'top-down regulation' of the amygdala (Lee, Heller, van Reekum, Nelson, & Davidson, 2012). Compared to young adults (aged 18-22 years), adolescents (aged 10-17 years) show reduced negative coupling between the two regions during an emotional faces task (Gee et al., 2013). This indicates reduced prefrontal regulation of amygdalar responding during adolescence relative to adulthood, and is consistent with other findings examining functional connectivity between the regions during fear extinction recall. Indeed, human adolescents show a lack of functional connectivity between the ventromedial prefrontal cortex and amygdala, which is correlated to concurrent poor extinction retention performance (Ganella, Barendse, Kim, & Whittle, 2017). On the basis of the work described, it appears that impaired extinction retention during adolescence is, at least in part, a consequence of insufficient BLA- and IL-mediated regulation of conditioned fear responses from the CeA. However, as yet, there is no evidence supporting a causal link between over- or under-activation of regions within the mPFC and/or the amygdala and extinction learning and/or retention during adolescence in either rodent or human species.

Neurotransmitters involved in fear extinction during adulthood

The main focus of the current thesis was to investigate how pharmacological manipulations impact extinction learning and retention during adolescence. Although many neurotransmitter systems have been implicated in extinction (for review, see Sartori & Singewald, 2019), I will focus here on discussing the three neurotransmitter systems that were pharmacologically targeted in my experimental chapters: the opioid system, the glutamatergic system, and the endocannabinoid system. It should be noted that some pharmacological agents (e.g., D-cycloserine) have already progressed to the stage of investigation alongside cognitive behaviour therapy for adolescents in

randomised controlled trials. However, as discussed later (p. 26), the limited efficacy of available adjuncts in this age group warrants an investigation into other candidates. A brief overview of each system in adult animals will be provided in the immediate sections below and further detail will be described in the subsequent chapters.

Opioids. As discussed earlier, new learning involves the generation of prediction error between the expected outcome and the actual outcome. In the case of fear extinction, negative prediction error is generated as the actual outcome (CS = no shock US) is less than the expected outcome (CS = shock US). The use of prediction error to update behavioural responding to a cue (i.e., to no longer fear the CS during extinction) is modulated by the endogenous opioid system. Whereas other receptor types improve memory by acting on the product of prediction error (i.e., by enhancing extinction consolidation), endogenous opioids have a crucial role in generating the prediction error signal which is required for new learning (Cole & McNally, 2007b). In the first study to demonstrate the role of opioids in fear extinction, McNally and Westbrook (2003) found that administration of the opioid antagonist naloxone prior to extinction training impaired within-session acquisition (i.e., rats did not show a decrease in fear responding to the CS). This impairment was sustained at extinction retention test the following day, indicating that the systemic blockade of opioid release prevented the formation of an extinction memory. Furthermore, the impairing effect of naloxone was not found when it was administered post-extinction training, supporting the role for opioid transmission during extinction *acquisition* rather than consolidation (McNally, Pigg, & Weidemann, 2004; McNally & Westbrook, 2003). It has been suggested that opioid release contributes to the detection of the discrepancy between the expected and actual outcome (i.e., prediction error), which is the inhibitory signal for extinction learning that is encoded by the amygdala (McNally et al., 2004).

According to the model of opioid involvement in extinction proposed by Parsons et al. (2010), opioids are released from the vlPAG in the beginning of extinction training as rats are exhibiting high levels of freezing. As opioid release accumulates, vlPAG output neurons are disinhibited, including those neurons that project to the mPFC. To date, the exact nature of how information reaches the mPFC from the vlPAG (i.e., direct versus indirect projections) has not been elucidated. This issue is considered in more detail in Chapter 4. In any case, as a result of the disinhibition of vlPAG output neurons, the activity of the mPFC is increased, leading to feed-forward inhibition of the fear output neurons in the medial CeA via the BLA and intercalated cells. Without the initial opioid release from the vlPAG during extinction, extinction training is impaired and synaptic plasticity in the mPFC and BLA is reduced (as assessed using pMAPK/ERK; Parsons et al., 2010). Therefore, opioid release from the vlPAG regulates synaptic plasticity in the mPFC and BLA during extinction learning, a process known to be critical for successful extinction.

Glutamate. Once prediction error has been generated and the extinction memory has been acquired, memory consolidation is then required in order to transform the short-term, labile memory trace in to a stable long-term memory (Dudai, 2004). The glutamatergic system plays a large part in memory consolidation, for instance, by contributing to long-term potentiation (Luscher & Malenka, 2012; Riedel, Platt, & Micheau, 2003). There are three types of glutamatergic receptors: N-methyl-D-aspartate (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPARs), and metabotropic glutamate receptor types (mGluRs; Riedel et al., 2003). Most, if not all, neurons within the brain express at least one type of glutamate receptor, implicating glutamatergic signalling as critical for most neuronal processes (for review, see Zhou & Danbolt, 2014). Glutamatergic activation of each of the aforementioned receptor types

appears necessary for fear extinction processes. For example, reducing glutamatergic transmission via NMDAR antagonists (e.g., ifenprodil, MK801) during the extinction consolidation period impairs extinction retention (Sotres-Bayon, Bush, & LeDoux, 2007; Sotres-Bayon, Diaz-Mataix, Bush, & LeDoux, 2009; Zimmerman & Maren, 2010). Similarly, pharmacological blockade of either AMPARs (using the antagonist CNQX), mGluR1 (using the antagonist CPCCOEt), or mGluR5 (using the antagonist MPEP) results in poor extinction retention (Fontanez-Nuin, Santini, Quirk, & Porter, 2011; Kim et al., 2007; Trent, Barnes, Hall, & Thomas, 2017).

On the other hand, increasing glutamatergic transmission facilitates extinction consolidation and subsequent retention. For instance, increasing the activation of AMPAR or mGlu5R glutamatergic receptor types (e.g., AMPARs via the drug PEPA, mGlu5Rs via the drug CDPPB) during extinction training also improves extinction retention in adult rodents (Sethna & Wang, 2014; Zushida, Sakurai, Wada, & Sekiguchi, 2007). Consistent with these findings, administration of the NMDAR partial agonist D-cycloserine prior to extinction training improves extinction retention the following day (Ledgerwood, Richardson, & Cranney, 2003; Walker, Ressler, Lu, & Davis, 2002). As the facilitating effects of D-cycloserine on extinction retention are seen regardless of whether the agent is administered before or after extinction training (Ledgerwood et al., 2003), it is likely that the agent works by strengthening the consolidation, rather than the acquisition, of extinction learning. The enhancing effects of D-cycloserine are also evident in humans, such that the agent improves extinction retention in healthy adults (Klass, Glaubitz, Tegenthoff, & Lissek, 2017; but see Guastella, Lovibond, Dadds, Mitchell, & Richardson, 2007). Furthermore, the augmenting effects of D-cycloserine on fear extinction have been extended to clinical trials examining the agent's impact on exposure therapy outcomes with some success (Hofmeijer-Sevink et al., 2017; Mataix-

Cols et al., 2017; Rosenfield et al., 2019). Beyond D-cycloserine, negative allosteric modulators of mGlu1/5Rs (e.g., RGH-618) and other NMDAR antagonists (e.g., ketamine, NBTX-001) are in clinical phase development as pharmacological treatments for anxiety disorders; however, these agents appear to act primarily through reducing overall anxiety levels rather than facilitating extinction processes (Sartori & Singewald, 2019).

Endocannabinoids. Another neurotransmitter system involved in extinction consolidation is the endocannabinoid system. While there are several receptors and endogenous ligands in this system, in this thesis I focus on the contribution of the predominant receptor, cannabinoid receptor 1 (CB1R). CB1Rs are abundant in the BLA and mPFC, and regulate the acquisition and extinction of learned fear through retrograde signalling pathways within these regions (Castillo, Younts, Chávez, & Hashimotodani, 2012; Hu & Mackie, 2015). More specifically, the activation of CB1Rs modulates the release of several important neurotransmitters that are required for extinction consolidation, including glutamate (Fitzgerald, Seemann, & Maren, 2014; Papagianni & Stevenson, 2019).

It is well-established that CB1Rs are critically involved in fear extinction in adult animals. This has been demonstrated by findings that blocking the activation of CB1Rs via systemic or intracranial administration of the CB1R antagonist SR141716A impairs extinction learning and retention (Chhatwal, Davis, Maguschak, & Ressler, 2005; Marsicano et al., 2002). Conversely, increasing the activation of CB1Rs improves fear extinction. For example, systemic administration of the CB1/2R agonist WIN55212-2 improves extinction in both non-stressed and stressed adult rodents (Ghasemi, Abrari, Goudarzi, & Rashidy-Pour, 2017; Pamplona, Prediger, Pandolfo, & Takahashi, 2006). Furthermore, intra-amygdala infusion of WIN55212-2 prior to

extinction training protects against reinstatement and spontaneous recovery in adult rats, two forms of fear relapse following extinction training (Lin, Mao, & Gean, 2006). These findings indicate that increasing the activation of CB1Rs can enhance extinction and reduce relapse of extinguished fear, at least in adults.

Neurotransmitters involved in fear extinction during adolescence

As investigations of how different neurotransmitters contribute to extinction in developing animals are still preliminary, there is a substantial dearth of available research into this issue (for review, see Kim, Perry, Ganella, & Madsen, 2017). Consequently, research examining the effects of novel pharmacological adjuncts on extinction during adolescence is lacking. To the best of my knowledge, no research has been conducted on the effects of pharmacologically manipulating the opioid system on fear extinction during adolescence. Given this gap in knowledge, I investigated the contribution of opioid receptor activation to extinction learning and retention in adolescent animals and these studies are described in Chapter 4. The involvement of the glutamatergic and endocannabinoid systems in extinction in adolescents have, however, been examined in a few studies and will be reviewed here. As both systems will be discussed in more detail in later chapters, only a brief overview will be provided in this chapter.

Glutamate. The involvement of glutamate, and particularly NMDARs, in extinction during adolescence is perhaps the best researched of all the neurotransmitter systems. As in adults, administration of the partial NMDAR agonist D-cycloserine immediately following extinction training improves extinction retention the following day in adolescent rats (Baker, McNally, & Richardson, 2018; McCallum et al., 2010). D-cycloserine is thought to improve fear extinction in this age group by facilitating

recruitment of the mPFC, a region which is typically under-engaged during extinction training and extinction retention test. To illustrate, rats treated with D-cycloserine immediately following extinction training show an increase in synaptic plasticity (as measured by pMAPK/ERK) in the mPFC at the extinction retention test relative to rats treated with saline (Baker et al., 2018).

There is also evidence that NMDARs are required when adolescents are extinguished with conditions that result in good levels of extinction retention (i.e., low levels of CS-elicited fear at test). One such condition is when double the number of extinction trials are given (Kim et al., 2011). When the NMDAR antagonist MK801 is administered to adolescent rats prior to a second day of extinction training, they show impaired extinction retention the next day relative to adolescents administered saline (which exhibit low levels of freezing; Baker & Richardson, 2017). The role of NMDARs during extinction in adolescence is discussed in more detail in Chapter 3.

Endocannabinoids. Significant fluctuations in levels of cannabinoid receptors and their endogenous ligands have been reported across adolescence, suggesting that the endocannabinoid system is still undergoing refinement during this developmental stage (for review, see Meyer, Lee, & Gee, 2018a). Some researchers have suggested that dysregulated fear during adolescence is underpinned by aberrant endocannabinoid signalling (see Lee, Hill, & Lee, 2016) but direct evidence for this hypothesis is limited. The strength of this suggestion is also tempered by inconsistencies in reports of age-related expression of CB1Rs in the mPFC and BLA (two regions heavily implicated in extinction), although, these inconsistencies could be dependent on the method for measuring endocannabinoid receptor expression. In Chapter 2, I will describe these findings in detail and suggest that clear inferences about the developmental trajectory of

CB1Rs, as well as their influence on extinction performance, across age cannot yet be made based on the literature to date.

Hope is not lost: improving fear extinction in adolescence

In light of the impaired extinction retention phenotype during adolescence, preclinical research has focused on identifying methods which improve extinction performance during this developmental stage (Baker et al., 2016). This is clinically relevant, as current exposure-based treatments for anxiety disorders do not provide long-term symptom remission for adolescents (Barry et al., 2018), and extinction learning predicts treatment responding (Geller et al., 2019). Therefore, developing new and time-limited pharmacological adjuncts to psychotherapy is especially important. The advantage of combining pharmacological adjuncts which target specific neurotransmitter systems with exposure-based therapies is that such agents could facilitate extinction learning and/or consolidation processes underpinning this therapy and maximise treatment outcomes (Graham, Callaghan, & Richardson, 2014; Sartori & Singewald, 2019).

One particularly promising pharmacological candidate is the NMDAR partial agonist D-cycloserine. Indeed, a recent meta-analysis found that the administration of D-cycloserine alongside exposure-based cognitive behaviour therapy leads to small, but significant, improvement in treatment outcomes with no moderating effect of age (Mataix-Cols et al., 2017). As discussed previously, improved extinction retention in adolescent rats has been observed following administration of D-cycloserine (McCallum et al., 2010). The success of D-cycloserine as a treatment adjunct has, however, been mitigated by the agent's limited success for some anxious youth. In the first randomised controlled trial of D-cycloserine in paediatric obsessive compulsive

disorder, Farrell et al. (2013) found that including the agent alongside five sessions of exposure-based therapy resulted in greater improvements relative to placebo. Such a result was promising, given that the study recruited patients who were considered "difficult to treat", and a large proportion of the sample presented with secondary comorbid psychiatric diagnoses. Although the study did not examine whether age influenced the effects of D-cycloserine (participants were 8-18 years old), a subsequent randomised controlled trial did compare the effects of D-cycloserine in children versus adolescents. Unexpectedly, Farrell et al. (2018) found that D-cycloserine did not augment treatment outcomes when administered after a single, prolonged session of exposure-based therapy. Furthermore, adolescent participants (11-14 years old) who received D-cycloserine had significantly poorer global functioning at 3-month follow-up relative to same-age peers who had received placebo, as well as younger participants (7-10 years old) in both drug conditions. While both of these trials had small sample sizes (*N*s = 17-35), limiting the strength of their conclusions, the evidence supporting D-cycloserine for anxious adolescents is limited.

In light of these findings, in this thesis I investigated another pharmacological candidate for augmenting exposure-based therapies during adolescence. Specifically, cannabinoid-based agents have been shown to improve fear extinction in both rodent and human studies using adult populations (e.g., Das et al., 2013; Pamplona et al., 2006), and randomised controlled trials for their use in adults with anxiety disorders are currently underway (e.g., van der Flier et al., 2019). Considering the promising results of this pharmacological adjunct in adult rodents, it may (or may not) also enhance extinction in adolescent rodents. To investigate this possibility, I examined whether a cannabinoid-based agent improved extinction retention in adolescent rats (Chapter 2). Furthermore, considering the mixed findings of the NMDAR partial agonist D-

cycloserine in augmenting extinction during adolescence, I examined the requirement of NMDARs in successful fear extinction during this developmental period (Chapter 3). Finally, I investigated the contribution of the opioid system to extinction during adolescence (Chapter 4), a neurotransmitter system which had not previously been explored. The overarching aim of my thesis is to further our understanding of the neurotransmitters involved in extinction during adolescence using a rodent model. Eventually, the preclinical work discussed in my thesis could help inform clinical decisions about which pharmacological adjuncts may be more or less effective alongside exposure therapy for anxious adolescents.

Chapter 2: Developmental differences in the effects of CB1/2R agonist WIN55212-2 on fear extinction

The first-line treatment for anxiety disorders, psychotherapy, does not result in persistent symptom reduction for approximately half of all adolescents who receive the treatment (Ginsburg et al., 2014; Kodal et al., 2018). One modification to psychotherapy which might improve outcomes is to facilitate the consolidation and retention of the learning that takes place by adding cognitive enhancing drugs alongside active treatment sessions (Hofmann, Fang, & Gutner, 2014; Singewald, Schmuckermair, Whittle, Holmes, & Ressler, 2015). This suggestion is tempered by evidence that some pharmacological agents can lead to adverse effects during adolescence (e.g., some young people report increased suicidal ideation when prescribed selective serotonin reuptake inhibitors; Garland & Ons, 2016; Hammad, Laughren, & Racoosin, 2006). Therefore, extensive preclinical testing in both rodent and human studies is warranted prior to clinical dissemination.

Historically, research has focused on adjuncts which target the glutamatergic system, such as D-cycloserine (Hofmann, Otto, Pollack, & Smits, 2015). Although D-cycloserine does improve overall treatment outcomes in most clinical trials, the effects are variable and dependent on the individual's age (Farrell et al., 2018), as well as their within-session reductions in fear (Mataix-Cols et al., 2017; Smits et al., 2013). Therefore, there are many individuals for whom D-cycloserine is ineffective, or may even worsen symptoms. One source of potential alternative pharmacological adjuncts would be those that are already approved, or are in the process of being approved, for therapeutic use in treating other health conditions. One such candidate, cannabis, is being increasingly approved for medicinal purposes around the world (Sznitman & Bretteville-Jensen, 2015). For instance, cannabis can be accessed in Australia for cancer

pain, refractory paediatric epilepsy, and chemotherapy-induced nausea (Australian Government, 2017).

The potential effectiveness of cannabinoid agents in alleviating the symptoms of mental health conditions are beginning to be reported. Short-term use of such agents in conjunction with psychological and/or pharmacological treatment has been associated with symptom improvement – but not necessarily symptom remission (Hoch et al., 2019). While there are several reviews speaking to the potential of cannabinoid adjuncts to improve psychological treatment outcomes for mental health disorders (e.g., Blessing, Steenkamp, Manzanares, & Marmar, 2015; Korem, Zer-Aviv, Ganon-Elazar, Abush, & Akirav, 2016), there is a lack of clinical research experimentally evaluating their effectiveness. In any case, work in healthy rodents and humans suggests that cannabinoid adjuncts improve fear extinction by acting on the endocannabinoid system (Mayo et al., 2019), and considerable mechanistic insights remain to be gained using preclinical models.

The endocannabinoid system has been implicated in learning and memory processes in both rodents and humans, and particularly fear and extinction learning (Drumond, Madeira, & Fonseca, 2017; Mechoulam & Parker, 2013; Ney, Matthews, Bruno, & Felmingham, 2018; Ohno-Shosaku & Kano, 2014). However, the following overview of the endocannabinoid system will focus on rodents, in line with the experiments reported in this chapter. Within the endocannabinoid system, there are two primary receptors, cannabinoid receptor 1 (CB1R) and 2 (CB2R), as well as the ligands that bind to these receptors (Lu & Mackie, 2016). As noted in Chapter 1 (p. 21), CB1Rs are abundant in the mPFC and amygdala, and have a well-established role in aversive learning. Therefore, I will focus on the contribution of CB1Rs to the extinction of learned fear during adolescence.

Increasing CB1R activity improves extinction in adult rodents

As CB1Rs are expressed in the mPFC and amygdala in adult rodents, they are well-placed to influence the neurotransmitter systems involved in fear extinction (Hu & Mackie, 2015; Katona et al., 2001; Wędzony & Chocyk, 2009). CB1Rs modulate several neurotransmitter pathways by acting on both excitatory glutamatergic pyramidal neurons and inhibitory GABAergic interneurons (Papagianni & Stevenson, 2019). The activation of CB1Rs mediates not only the post-synaptic activation of different neuronal subtypes, but also circulating levels of various neurotransmitters which mediate memory consolidation (e.g., GABA, glutamate, serotonin, dopamine; Fitzgerald et al., 2014; Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009). For example, pharmacological manipulation of CB1R activation impacts serotonin release, and blocking the activation of CB1Rs prevents the facilitating effects of the selective serotonin reuptake inhibitor fluoxetine on fear extinction in adult mice (Gunduz-Cinar et al., 2016; Mendiguren, Aostri, & Pineda, 2018).

A role of CB1Rs in extinction was first demonstrated in a study that compared genetically modified CB1R adult knock-out mice to wild-type mice (Marsicano et al., 2002). While there were no differences between the groups during fear conditioning or fear recall the following day, CB1R knock-out mice were impaired in extinction learning. That is, CS-elicited freezing did not decrease across the 30 non-reinforced CS presentations in CB1R knock-out mice. This impairment was maintained when knock-out mice were tested for extinction retention 5 days later, and was then replicated in wild-type mice that were administered the CB1R antagonist SR141716A prior to extinction training. Furthermore, the same impairment in extinction learning and retention has been observed using the CB1R inverse agonist AM251 in adult rodents (de Oliveira Alvares, Pasqualini Genro, Diehl, Molina, & Quillfeldt, 2008; Kuhnert, Meyer,

& Koch, 2013). Together, these findings indicate that both genetic knock-out and pharmacological antagonism of CB1Rs impairs extinction, illustrating the importance of the receptor in inhibitory learning.

Conceptually extending the findings described above, pharmacologically increasing activation of CB1Rs facilitates extinction learning and retention. Using contextual fear conditioning, Pamplona et al. (2006) examined the effects of systemic administration of the non-selective CB1/2R agonist WIN55212-2 on context extinction. During the first extinction session (9 min of shock-free exposure to the conditioned context), freezing in WIN-treated rats was significantly less than freezing in vehicle-treated rats, suggesting enhanced extinction learning. WIN-treated rats continued to show lower levels of freezing when tested drug-free 24-h, 48-h, or 7 days later, indicating enhanced extinction retention (Pamplona, Bitencourt, & Takahashi, 2008; Pamplona et al., 2006). Subsequent experiments conducted by Pamplona et al. (2006) ruled out possible locomotor effects of WIN55212-2 administration that could have contributed to reductions in freezing. The extinction-enhancing effects of WIN55212-2 administration have also been extended to stressed adult rats that typically demonstrate impaired performance, illustrating the benefits of increased CB1R activity on extinction (Ghasemi et al., 2017).

While the role of the endocannabinoid system in fear extinction during adulthood has been examined, research into the endocannabinoid system, and specifically CB1Rs, during adolescence is limited. In the only potentially relevant study to date, Reich et al. (2013) examined the effect of the CB1R agonist ACEA in modulating extinction of trace fear conditioning in what were characterised as 'adolescent' rats. The rats received either chronic mild stress or handling for 3 weeks (~ P40-45 to ~ P61-66), and then received trace fear conditioning and extinction. Trace

fear conditioning differs slightly from delay fear conditioning as an interval is introduced between the offset of the conditioned stimulus and onset of the unconditioned stimulus. As a result, the two procedures involve overlapping but distinct brain regions and neurotransmitters (Hunt & Richardson, 2007; Raybuck & Lattal, 2014). Prior to trace fear extinction, all rats received an injection of either the CB1R agonist ACEA or vehicle. While there were no effects of CB1R agonism on extinction learning, ACEA-treated rats showed enhanced extinction retention when tested drug-free the following day. However, the 'adolescent' subjects were at least 60 days old at the time of extinction training. As the adolescent period only spans from 28-52 days in rodents (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013; Spear, 2000), the subjects used by Reich et al. (2013) were actually adults. Therefore, conclusions made concerning the effects of CB1R agonism on extinction *during adolescence* cannot be made from this study. Instead, the results of Reich et al. (2013) are more consistent with subsequent reports that ACEA administration improved extinction learning in *adult* rats (extinction retention was not assessed; Simone, Green, Hodges, & McCormick, 2015).

Taken together, the aforementioned studies clearly demonstrate the extinction-facilitating effects of CB1R agonism for context fear (Pamplona et al., 2008, 2006) and trace fear (Reich et al., 2013; Simone et al., 2015) in adult rats. However, given that the extinction retention deficit in adolescence has been observed using delay fear conditioning (as reviewed in Chapter 1; Baker et al., 2016), it was important to first investigate whether the extinction-enhancing effects of CB1R agonism would extend to delay fear conditioning in adult rats prior to examining its effects on extinction in adolescent rats. Based on the work examining context and trace fear extinction in adult rats, I expected that administration of the CB1/2R agonist WIN55212-2 would also

improve delay fear extinction learning and retention in adult rats. If so, then the critical question was whether it would facilitate extinction in adolescent rats, which typically exhibit an impairment in extinction retention.

In addition to extinction retention, I also examined the effect of WIN55212-2 on fear renewal (a form of fear relapse) in adolescents. This additional test of fear relapse was included to extend the findings observed outside of the extinction context. It has been argued that fear renewal is a more clinically relevant illustration of relapse, as anxious patients are more likely to encounter feared stimuli (e.g., a spider) either in the place where they acquired their fear (e.g., their home) or a novel context (e.g., at school) rather than the extinction context (e.g., the therapist's office; Vervliet et al., 2013). As the current preclinical investigation was designed to examine whether WIN55212-2 could augment extinction, and eventually exposure-based therapy if extended from laboratory studies to clinical application, fear renewal testing was included to increase the clinical relevance of my findings.

Methods

Subjects

All animals used in this thesis were maintained on a 12 h light/dark cycle (lights on at 0700) in a humidity- and temperature-controlled colony room with food and water available *ad libitum*. Animals were treated in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013), and all procedures were approved by the Animal Care and Ethics Committee at UNSW Sydney.

Experimentally naïve Sprague-Dawley male rats were used. Most of the animals used in the experiments reported in this chapter (237 out of 253) were derived from the

breeding colony maintained by the School of Psychology at UNSW Sydney. Some adult rats in Experiment 2.1 (16 out of 28) were obtained from the Animal Resources Centre (Perth, Australia), the supplier of the breeders used to produce the other animals used in the experiments reported in this chapter.

Apparatus

In fear conditioning and extinction experiments, four MED Associates chambers [24cm (length) x 30cm (width) x 21cm (height)] were used, with two designated as Context A and the other two as Context B. Fear conditioning and renewal testing occurred in Context A while extinction training and extinction retention testing occurred in Context B.

Each chamber was enclosed in a sound- and light-attenuating cabinet where ventilation fans provided constant low level (approx. 58dB) background noise. All chambers were constructed primarily of Perspex with two stainless steel sidewalls. In Context A chambers, the floor consisted of stainless steel rods (4 mm wide), spaced 16 mm apart, just above a stainless steel tray filled with corncob bedding. A clear Perspex dividing wall diagonally bisected the chamber, creating a triangle-shaped space. A small panel of 8 red LEDs mounted to the inside of the sound attenuating cabinet was the only light source in Context A. The two Context B chambers differed in size, flooring, lighting, and visual features to those in Context A. The Context B chambers did not have a dividing wall but did have a clear Perspex insert covering the grid floor. Sheets of paper with vertical black and white stripes (2.5 cm width) were attached to the ceiling and front wall of the chamber. In addition to red LEDs, a white light above the chamber provided low-level illumination inside the chamber (~ 4 lux, Deglitch light meter QM1587). All chambers were cleaned with tap water after each experimental session.

In addition to examining the effect of WIN55212-2 on fear extinction, I also examined whether this drug altered locomotor and anxiety-like behaviour in an open-field arena. Four identical Med Associates arenas, each individually housed in ventilated, light- and sound-attenuating cabinets, were used for these tests. Each arena ([43.2cm (length) x 43.2cm (width) x 30.5cm (height)]) was constructed of a clear Perspex floor and walls. Sixteen infrared detectors were set 3cm above the floor on two opposing walls to detect locomotor activity.

Procedure

The animals bred at UNSW Sydney were weaned on P21-23. The behavioural procedures commenced with fear conditioning at P24-25 for juveniles, P34-36 for adolescents, and beyond P70 for adults (Cowan & Richardson, 2018). Typically, rats were handled for 4-5 min and pre-exposed to the conditioning context for 10 min each day for two days prior to undergoing fear conditioning, with the following exceptions: in Experiment 2.3, rats received context pre-exposure only for the second day of handling; rats in Experiment 2.5 received the same handling procedures without any context pre-exposure; and rats in Experiment 2.6 were not handled prior to euthanasia.

The following procedures were used for all experiments involving fear extinction. Rats received Pavlovian fear conditioning, extinction training, and an extinction retention test on consecutive days. In Experiment 2.3 only, a test of fear renewal was conducted the day after extinction retention testing. All sessions began with a 2 min baseline period. Presentations of the CS and US were controlled by a computer running Med-PC IV software (Med Associates). The animals' behaviour was recorded via a camera mounted on the rear wall of each cabinet.

During conditioning, there were 3 pairings of a white noise conditioned stimulus (CS; 8dB above background, 10 sec duration) which co-terminated with a scrambled footshock unconditioned stimulus (US; 1 sec duration) delivered through a grid floor. The intertrial intervals were 135 and 85 sec. Two footshock intensities (0.4mA and 0.6mA) were used in both Experiments 2.1 and 2.2. As no interactions between shock intensity and drug condition were observed for CS-elicited freezing at fear conditioning, extinction, or the extinction retention test (largest $F_{1, 30} = 2.27$, $p = .14$), results from the two shock intensities were combined for both experiments. The shock intensity was set to 0.4mA in subsequent experiments, as well as in the experiments reported in Chapters 3 and 4.

Extinction training consisted of 30 non-reinforced presentations of the CS (10 sec each; 10 sec inter-trial interval). Across extinction training, 6 trials were averaged to represent 1 block of extinction. The next day, animals were tested with a single CS presented for 2 min in the extinction context (extinction retention test). In Experiment 2.3, animals were also tested with a single CS presented for 2 min in the conditioning context (renewal test).

During the experimental sessions, rat behaviour was scored as freezing or not freezing every 3 sec during the baseline period and the CS presentations. Freezing was scored for any observation where there was the absence of all movement other than that required for respiration (Fanselow, 1980). A random sample of ~30% of the test data was cross-scored by an observer unaware of the experimental conditions. Inter-rater reliability was high for all experiments reported in this Chapter (*r*s =. 95 -. 99).

In Experiments 2.5 and 4.4, the following procedures were used for the open field test to assess locomotor and anxiety-like behaviour (Kraeuter, Guest, & Sarnyai,

2019). Animals were placed in the centre of the chamber, and movement was recorded across a 10 min period. Locomotor behaviour was assessed by tracking the total distance travelled (cm) and speed (cm/sec). Concurrently, anxiety-like behaviour was measured as the percentage of time spent in the inner zone of the chamber (14.29cm^2 of 43.2cm^2). As movement was recorded using infrared beam breaks and automatically scored using Activity Monitor software (MED Associates), the open field test data was not recorded for cross-scoring. A summary of the procedures used across experiments in Chapter 2 is presented in Table 2.1.

Drugs

WIN55212-2 (Tocris Bioscience; #1038) was dissolved in saline (0.9% wt/vol) with 10% dimethyl sulfoxide and 0.1% Tween80. The vehicle solution consisted of saline, 10% dimethyl sulfoxide, and 0.1% Tween80. Injections were given in a volume of 2ml/kg 20 min before the extinction training session, based on the study by Pamplona et al. (2006) with adults. WIN55212-2 was administered at a dose of 0.25mg/kg in Experiments 2.1, 2.2, 2.3, and 2.5, and at 0.125mg/kg, 0.25mg/kg, or 2.50mg/kg in Experiment 2.4.

Data Analysis

All statistical analysis reported in this thesis was performed with IBM Statistical Package for the Social Sciences (SPSS, version 25). The statistical output for these experiments, and all subsequent experiments, is presented in the Appendices. The data reported in this thesis are presented as mean values ± standard error of the mean (SEM).

Freezing during the baseline periods, extinction retention test, and renewal test, as well as open field test data were analysed using two-way between-subjects analyses of variance (ANOVAs). Effect sizes for test data were calculated using Cohen's d (*d*, in

Table 2.1. Summary of procedures across Experiments 2.1-2.7 in Chapter 2.

	Age group/s	Handling	Pre-exposure	Conditioning	Injection	Extinction Training	Retention	Renewal	Open Field	Neural
2.1	Adult	■	■	■	■	■	■			
2.2	Adolescent	■	■	■	■	■	■			
2.3	Juvenile, Adolescent	■	■	■	■	■	■	■		
2.4	Adolescent	■	■	■	■	■	■			
2.5	Adolescent	■		■	■				■	
2.6	Juvenile, Adolescent, Adult									■
2.7	Juvenile, Adolescent, Adult	■	■	■		■				■

Note. Grey shading indicates the inclusion of the procedures in each experiment.

experimental designs with two groups) or partial Eta squared (η_p^2, in experimental designs with more than two groups). Conditioning and extinction training data were analysed using a mixed-design ANOVA with drug and/or age as a between-group factor and block of trials as a within-subject factor. Whenever a mixed-design ANOVA was used, violations of the assumption of sphericity (as determined by Mauchly's test) led to the use of Greenhouse-Geisser corrections. Two-group test data were analysed with independent samples *t*-tests, in which the assumption of homogeneity of variances was tested using Levene's test. In cases where either assumption was violated, the corrected statistic, degrees of freedom, and *p* values are reported.

Exclusion Criteria. For all experiments in the thesis, rats were considered statistical outliers and excluded from analyses if their CS-elicited freezing at test was ≥2.5 standard deviations away from the group mean. The number of rats excluded from each experiment, if any, is stated at the beginning of each experiment.

Experiment 2.1

WIN55212-2 improves extinction retention in adult rats

Prior to investigating the effects of WIN55212-2 administration on extinction retention in adolescent rats, Experiment 2.1 was designed to extend previous findings using CB1R agonists to augment context fear extinction (Pamplona et al., 2008, 2006) and trace fear extinction (Reich et al., 2013; Simone et al., 2015) to studies of delay fear extinction in adult rats. In this experiment, adult rats received fear conditioning, extinction training, and an extinction retention test on consecutive days. Animals were administered either WIN55212-2 (0.25mg/kg, i.p.) or vehicle 20 min prior to extinction training. Six rats were excluded from Experiment 2.1. Two animals were excluded for being statistical outliers (WIN55212-2 group), and an additional four rats (Vehicle

group) were excluded because they failed to extinguish (as indicated by CS-elicited freezing above 90% on the last block of extinction training).

Baseline. Levels of freezing during baseline sessions were low in both groups, and there were no differences between groups depending on future allocation to drug type in baseline freezing levels prior to conditioning ($F_{1, 26} = 2.64$, $p = .12$), extinction training ($F_{1, 26} = 1.05$, $p = .32$), or the extinction retention test ($F_{1, 26} = 2.93$, $p = .10$; **Table 2.2**).

Table 2.2

Mean (±SEM) percent baseline freezing before conditioning, extinction training, and extinction retention test in Experiment 2.1.

	Vehicle (*n* = 13)	**WIN55212-2 (*n* = 15)**
Conditioning	1.15 (0.46)	2.83 (0.88)
Extinction	2.50 (1.90)	0.67 (0.30)
Test	6.92 (2.53)	2.67 (0.75)

Conditioning. CS-elicited freezing increased across conditioning trials ($F_{2, 52} = 74.93$, $p < .001$; **Figure 2.1A**) with no differences between subsequent-drug groups ($F_{1, 26} = 1.13$, $p = .30$) or a drug × trial interaction ($F < 1$).

Extinction. During extinction training, CS-elicited freezing decreased across blocks in both groups (block main effect: $F_{2.47, 64.14} = 23.29$, $p < .001$; **Figure 2.1B**). There was no effect of drug or drug × block interaction (Fs < 1), indicating equivalent fear extinction across both groups. Unlike previous reports which indicated a facilitating effect of WIN55212-2 administration on extinction learning in adult rats (e.g., Pamplona et al., 2006), there was no effect of WIN55212-2 administration on CS-elicited freezing during extinction training in similar aged animals.

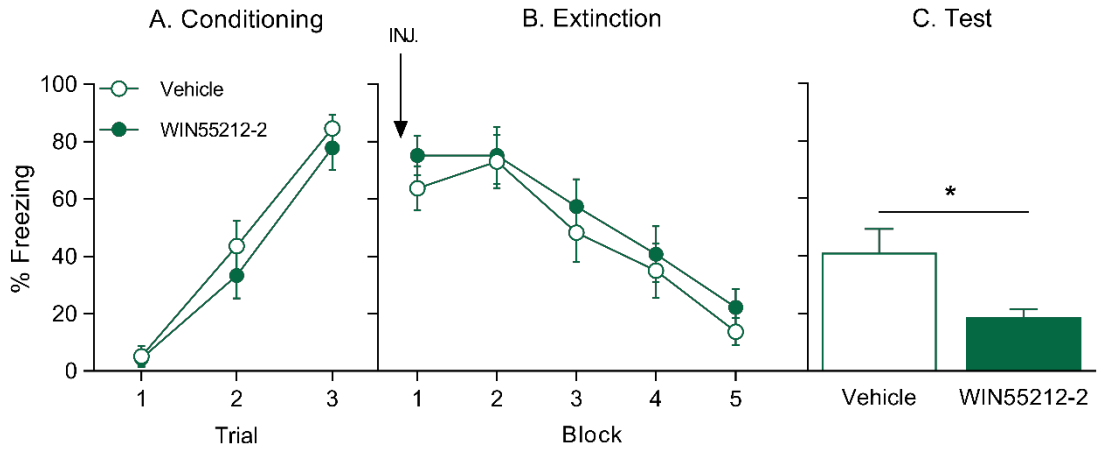

Figure 2.1. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) of adult rats in groups Vehicle and WIN55212-2. * indicates a significant group difference ($p < .05$).

Test. The next day, WIN-treated rats had lower levels of CS-elicited freezing at test than VEH-treated rats ($t_{14.58} = 2.60$, $p = .02$, $d = 1.01$; **Figure 2.1C**), indicating better extinction retention. This finding extends previous work using WIN55212-2 to enhance extinction of context fear (e.g., Pamplona et al., 2006, 2008) and trace fear (Reich et al., 2013) by showing a similar effect in a delay fear conditioning procedure with an auditory CS.

Experiment 2.2

WIN55212-2 does not improve extinction retention in adolescent rats

As WIN55212-2 administration improved fear extinction to a discrete auditory CS in adult rodents (Experiment 2.1), I then tested if WIN55212-2 had a similar effect in adolescent rats. I predicted that WIN55212-2-treated adolescent rats would show improved extinction retention relative to vehicle-treated adolescent rats, based on the findings of Experiment 2.1. Three rats were excluded from Experiment 2.2 as they did not extinguish successfully (above 90% freezing on the last block of extinction training: two from the WIN group and one from the VEH group).

Baseline. There were no differences between subsequent drug groups in baseline freezing levels prior to conditioning ($F < 1$), extinction training ($F < 1$), or the extinction retention test ($F < 1$; **Table 2.3**).

Table 2.3

Mean (±SEM) percent baseline freezing before conditioning, extinction training, and extinction retention test in Experiment 2.2.

	Vehicle (*n* = 17)	**WIN55212-2 (*n* = 17)**
Conditioning	1.62 (0.74)	1.76 (0.63)
Extinction	9.12 (4.35)	9.12 (2.44)
Test	8.38 (3.33)	11.32 (3.22)

Conditioning. Conditioning proceeded as expected. Across conditioning trials, there was an increase in CS-elicited freezing ($F_{2, 64} = 56.72$, $p < .001$; **Figure 2.2A**). There was no effect of subsequent drug ($F < 1$) or drug × trial interaction ($F < 1$).

Extinction. During extinction training, CS-elicited freezing decreased across trial blocks ($F_{2.93, 93.66} = 35.44$, $p < .001$; **Figure 2.2B**). There was a main effect of drug ($F_{1, 32} = 9.78$, $p = .004$) and no drug × block interaction ($F_{2.93, 93.66} = 1.31$, $p = .28$), indicating that WIN55212-2 treated adolescent rats had higher levels of CS-elicited freezing across extinction training compared to vehicle-treated adolescent rats. This finding is in contrast to the lack of drug effect on CS-elicited freezing in adult rats observed in Experiment 2.1.

Test. When tested for extinction retention the following day, if anything the WIN-treated animals had higher levels of CS-elicited freezing, but the groups did not statistically differ ($t_{32} = 1.89$, $p = .07$, $d = .65$; **Figure 2.2C**). Therefore, WIN55212-2

administration affected CS-elicited freezing during extinction training in adolescent rats, but did not affect extinction retention the following day.

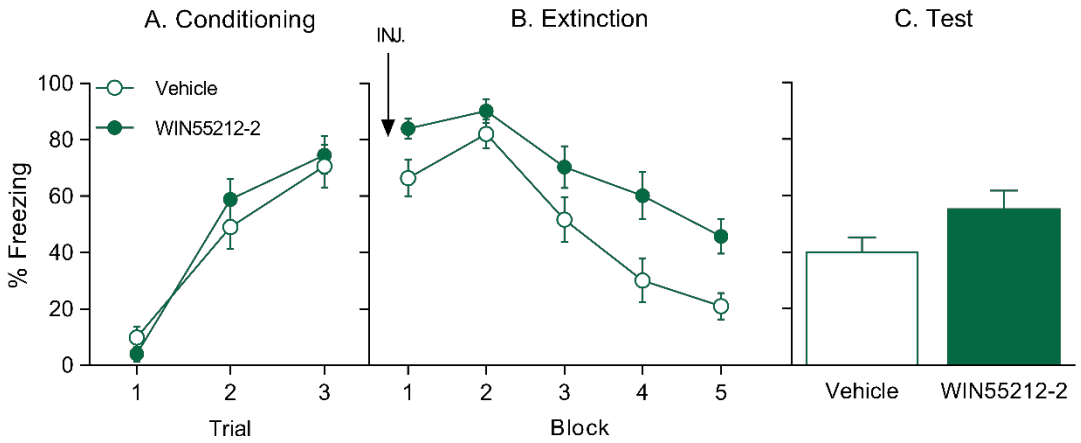

Figure 2.2. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) of adolescent rats in groups Vehicle and WIN55212-2.

Experiment 2.3

WIN55212-2 does not improve extinction retention in juvenile or adolescent rats

The results of Experiment 2.2 suggest that extinction retention in adolescent rats is not augmented by WIN55212-2 administration, unlike what is observed in adult rats. Relative to same-age controls injected with vehicle, adolescent rats treated with WIN55212-2 showed increased CS-elicited freezing across extinction training and no differences in freezing at the extinction retention test. This was in contrast to adult rats, per Experiment 2.1. Specifically, levels of CS-elicited freezing in adults treated with WIN55212-2 versus vehicle did not differ during extinction training, but WIN55212-2 did result in improved extinction retention.

I sought to further investigate the behavioural effects of WIN55212-2 in developing animals in Experiment 2.3, which had three broad aims. The first aim was to replicate the null effect of WIN55212-2 on extinction retention in adolescent rats. The second aim was to examine whether a similar outcome would be observed in juvenile

rats. The juvenile period is immediately prior to adolescence and is characterised by good extinction retention, despite the immaturity of the amygdala and mPFC (Kim & Richardson, 2010). If WIN55212-2 administration does not result in improved extinction due to adolescent-specific mechanisms, extinction retention in juvenile rats should be improved by this agonist.

The third aim of this experiment was to investigate whether WIN55212-2 would also have no effect on fear renewal in developing animals. Past work (Lin et al., 2006) has shown that WIN55212-2 protects against fear relapse in adult rats, as assessed by either reinstatement (i.e., relapse precipitated by pre-test administration of the US, or some stressful event) or spontaneous recovery (i.e., relapse following the passage of time; Bouton, 2002). It should be noted that Lin et al. (2006) administered WIN55212-2 *after* an extended CS retrieval session (which can be considered an extinction session), and argued that the effects of the drug on later fear recovery were due to disrupted reconsolidation of the fear memory rather than facilitated extinction. In any case, I investigated the effects of WIN55212-2 on fear relapse in developing rats by including a test of fear renewal the day after the test of extinction retention. I did not include a group of adult rats as a direct contrast in Experiment 2.3 for two reasons: 1. To minimise the number of animals used in this thesis, and 2. Experiment 2.1 already demonstrated the replication of previous effects of WIN55212-2 on extinction in adult rats. Three rats were excluded from Experiment 2.3: one was a statistical outlier (Juvenile Vehicle group) and two did not extinguish successfully (above 90% freezing on the last block of extinction training: Juvenile Vehicle group).

Baseline. There were no differences in baseline freezing levels between age or future drug allocation groups prior to conditioning (largest $F_{1, 43} = 1.11$, $p = .30$),

extinction training (Fs < 1), extinction retention test (Fs < 1), or renewal test (largest $F_{1, 43} = 2.28$, $p = .14$; **Table 2.4**).

Table 2.4

Mean (±SEM) percent baseline freezing before conditioning, extinction training, extinction retention test, and renewal test in Experiment 2.3.

	Juvenile Vehicle (*n* = 11)	**Juvenile WIN55212-2 (*n* = 13)**	**Adolescent Vehicle (*n* = 11)**	**Adolescent WIN55212-2 (*n* = 12)**
Conditioning	2.05 (0.74)	0.96 (0.45)	1.59 (0.51)	2.08 (1.10)
Extinction	6.14 (1.95)	5.77 (2.05)	6.82 (3.41)	5.00 (1.85)
Retention Test	7.95 (2.59)	10.58 (2.42)	14.55 (5.24)	10.21 (4.60)
Renewal Test	12.50 (4.00)	8.08 (3.62)	21.59 (7.43)	11.46 (3.67)

Conditioning. During conditioning, CS-elicited freezing increased across trials ($F_{2, 86} = 75.05$, $p < .001$; **Figure 2.3A**). There was no effect of age, subsequent drug group, or interactions (all Fs < 1).

Extinction. The next day, CS-elicited freezing decreased across extinction blocks ($F_{2.91, 124.97} = 24.35$, $p < .001$; **Figure 2.3B**). There was no effect of age ($F_{1, 43} = 2.07$, $p = .16$), indicating equivalent fear inhibition in both juvenile and adolescent rats. However, reductions in CS-elicited freezing across extinction training differed between drug conditions (drug main effect: $F_{1, 43} = 4.39$, $p = .04$; drug × block interaction: $F_{2.91, 124.97} = 2.94$, $p = .04$). Specifically, vehicle-treated rats reached a lower level of CS-elicited freezing by the end of extinction training than did WIN55212-2-treated rats (Block 5 main effect of drug: $F_{1, 43} = 12.00$, $p = .001$). These results suggest that pre-extinction WIN55212-2 administration had a similar effect (i.e., increased levels of CS-elicited freezing) in both juvenile and adolescent rats, replicating, and extending the results reported in Experiment 2.2 with adolescent rats.

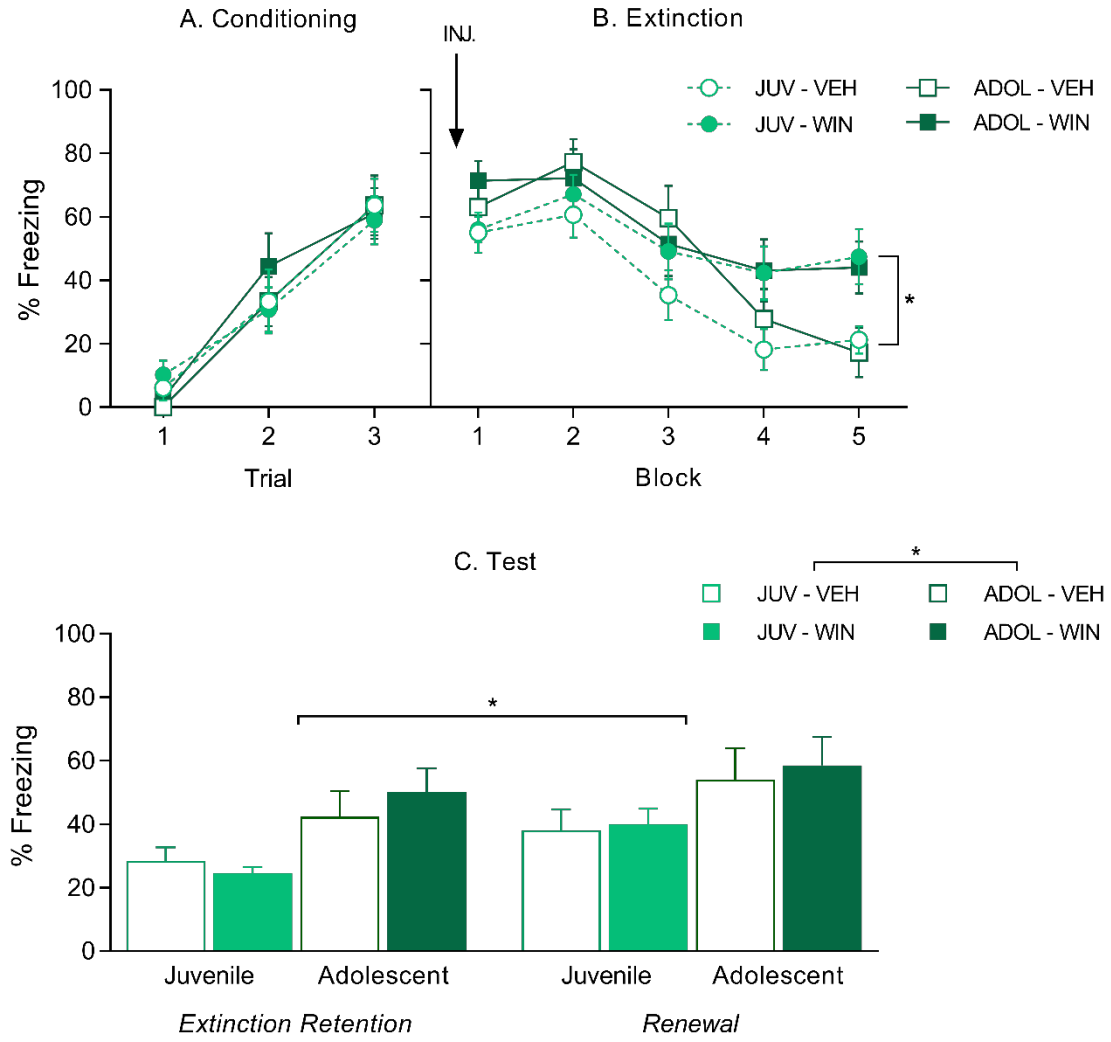

Figure 2.3. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and test (**C**) for groups Juvenile Vehicle, Juvenile WIN55212-2, Adolescent Vehicle, and Adolescent WIN55212-2.
***** indicates a significant group (**B**), age (**C**), or context (**C**) difference ($p < .05$).

Test. Across two consecutive days, rats were tested for freezing to the CS in two contexts: the extinction training context (i.e., extinction retention test) and the conditioning context (i.e., renewal test). These data were analysed in a 2 (test) x 2 (age) x 2 (drug) ANOVA, with the first factor being a repeated measure.

Adolescents had higher levels of CS-elicited freezing than the juvenile rats across the two tests ($F_{1, 43} = 9.38$, $p = .004$, $\eta_p^2 = .18$; **Figure 2.3C**), such that adolescent rats showed a higher return of fear regardless of test context. Further, there was a main

effect of test context ($F_{1, 43} = 12.22$, $p = .001$, $\eta_p^2 = .22$), such that higher levels of CS-elicited freezing were observed in Context A than in Context B, indicative of renewal. There was no main effect of drug ($F < 1$), or interactions between factors (all Fs < 1), indicating that WIN55212-2 administration did not influence either extinction retention or renewal in juvenile or adolescent rats. Therefore, in contrast to expectations, the attenuation of relapse following WIN55212-2 administration reported in adult rats (Lin et al., 2006) was not observed in either juvenile or adolescent rats using renewal.

Experiment 2.4

Effects of WIN55212-2 dose on extinction in adolescent rats

The findings of Experiments 2.1-2.3 indicate that although WIN55212 -2 improves fear extinction in adult rats, the same dose has no effect on extinction retention in juvenile or adolescent rats. One possible explanation for this discrepancy is that pharmacological agents are often metabolised differently in developing animals compared to adult animals (Levant, Zarcone, Davis, Ozias, & Fowler, 2011; O'Hara, 2016). Thus, dose response functions can be shifted, in either direction, in the developing animal (Spear & Brake, 1983).

This suggestion is supported by my observations of divergent effects of the same dose of WIN55212-2 (0.25mg/kg) in adolescent and adult rats. Specifically, WIN-treated adult rats showed unaffected extinction learning and improved extinction retention, while WIN-treated adolescent rats showed impaired extinction learning and unaffected extinction retention. While CS-elicited freezing in adult rats was unaffected by a 0.25mg/kg dose of WIN55212-2, much higher doses (e.g., 2.5mg/kg; Pamplona et al., 2006) have been reported to increase freezing responses to a context. Therefore, adolescent rats may be more sensitive to the effects of WIN55212-2 administration than

adult rats, and a different dose may be effective in facilitating extinction retention in adolescents. To test this possibility, I examined the effects of three doses of WIN55212-2: (WIN: 0.125, 0.25, and 2.5mg/kg) on extinction retention in adolescent rats. The lowest dose (0.125mg/kg) was half the dose tested in Experiments 2.1-2.3, while the highest dose (2.5mg/kg) was ten times higher than the original dose.

Baseline. There were no differences in baseline freezing between groups prior to conditioning ($F_{3, 29} = 1.07$, $p = .38$), extinction training ($F < 1$), or extinction retention ($F < 1$; **Table 2.5**).

Table 2.5

Mean (±SEM) percent baseline freezing before conditioning, extinction training, and extinction retention test in Experiment 2.4.

	Vehicle (*n* = 8)	**WIN - 0.125 (*n* = 8)**	**WIN - 0.25 (*n* = 8)**	**WIN - 2.50 (*n* = 9)**
Conditioning	2.19 (1.20)	1.25 (0.67)	7.50 (4.46)	3.06 (2.49)
Extinction	4.38 (2.74)	3.13 (1.48)	4.69 (2.34)	8.33 (4.04)
Test	13.75 (7.54)	8.13 (3.40)	22.81 (8.18)	16.67 (8.25)

Conditioning. Levels of CS-elicited freezing increased across conditioning trials ($F_{2, 58} = 51.97$, $p < .001$; **Figure 2.4A**). There was no difference between groups ($F < 1$) or a group × trial interaction ($F < 1$).

Extinction. The next day, rats treated with vehicle exhibited a decrease in freezing across extinction training whereas rats treated with the two highest doses of WIN55212-2 showed higher levels of freezing across extinction training, indicative of a slower rate of extinction learning. While CS-elicited freezing overall decreased across blocks ($F_{2.85, 82.61} = 21.36$, $p < .001$; **Figure 2.4B**), a significant effect of group ($F_{3, 29} = 7.07$, $p = .001$) and a group × block interaction ($F_{8.55, 82.61} = 2.38$, $p = .02$) were found.

To explore the group differences, post-hoc comparisons were made using Dunnett's test. There was no difference in CS-elicited freezing during extinction training between rats treated with vehicle and the 0.125mg/kg dose of WIN55212-2 (p = .62). However, animals treated with the 0.25mg/kg or 2.5mg/kg dose of WIN55212-2 showed significantly higher CS-elicited freezing during extinction training than vehicle-treated animals (p = .003 and p = .002, respectively).

The interaction was followed up by one-way ANOVAs to examine group differences at the start (Block 1) and end (Block 5) of extinction training. All groups had similar, high levels of freezing at the start of extinction training (Block 1 group effect: $F_{3, 29} = 1.18$, p = .34). However, rats administered the two highest doses of WIN55212-2 had significantly higher freezing at the end of extinction training (Block 5 group effect: $F_{3, 29} = 7.07$, p = .001) relative to rats administered vehicle (post-hoc comparisons both $p \leq .001$).

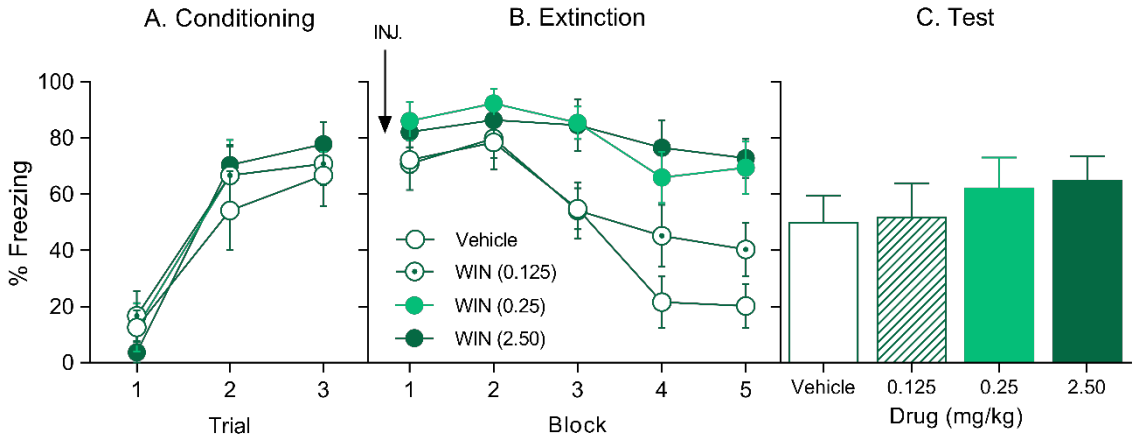

Figure 2.4. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) of adolescent rats in groups Vehicle, WIN55212-2 (0.125mg/kg), WIN55212-2 (0.25mg/kg), and WIN55212-2 (2.50mg/kg).

Test. When tested for extinction retention, there were no differences in CS-elicited freezing between groups ($F < 1$; **Figure 2.3C**). Therefore, although the two highest doses of WIN55212-2 impaired extinction learning relative to vehicle, this difference did not persist to the extinction retention test. Further, the finding that the lower or higher doses did not enhance extinction retention provides convincing evidence that the different effects of WIN55212-2 on juvenile and adolescent versus adult rats in the previous experiments in this Chapter were not a result of a shifted dose-response function.

Experiment 2.5

Effects of WIN55212-2 on locomotor and anxiety-like behaviour in adolescent rats

Across Experiments 2.2-2.4, I consistently observed increased CS-elicited freezing during extinction training in juvenile and adolescent rats when treated with doses of WIN55212-2 of at least 0.25mg/kg. In contrast, no such effects were detected in adult rats given 0.25mg/kg WIN55212-2 (Experiment 2.1). In terms of potential explanations, an increase in freezing during extinction training may reflect increased anxiety-like behaviour, decreased locomotor activity, or impaired extinction learning.

The existing literature on the effects of acute WIN55212-2 administration on anxiety-like behaviour is restricted to adult rodents and the effects appear to be dose-dependent. Lower doses of WIN55212-2 (between 0.6-2.5mg/kg) have been found to reduce anxiety-like behaviour (i.e., have *anxiolytic* effects) on two commonly used measures: the elevated plus maze and open field test (Drews, Schneider, & Koch, 2005; Faragi, Komaki, Firouzi, Salehi, & Shahidi, 2017; Naderi et al., 2008). As these doses were higher than that used in the experiments reported in this chapter, it is not surprising that anxiolytic effects weren't observed in adult rats in Experiment 2.1. In

contrast, higher doses of WIN55212-2 (between 3.0-5.0mg/kg) induce increased anxiety-like behaviour (i.e., have *anxiogenic* effects) on two other measures: the elevated zero maze and light/dark box test (Carvalho, Reyes, Arith-Ruth, Sterling, Unterwald, & Van Bockstaele, 2010; Rutkowska, Jamontt, & Gliniak, 2006). These findings indicate that there is a threshold at which acute cannabinoid administration switches from being anxiolytic to anxiogenic in adult rats. Based on my results, it is possible that the threshold for the anxiolytic/anxiogenic switch is lower in developing animals, resulting in anxiogenic effects with a dose of 0.25mg/kg during extinction training (i.e., the dose-response function has been shifted to the left).

There appears to be a dose-response curve for the effects of cannabinoid agents on locomotion; however, these results are less consistent than for anxiety-like behaviour. On the open field test, several studies have observed *hyper*-locomotive or null effects of lower doses of WIN55212-2 (0.1-0.6mg/kg), while *hypo*-locomotive or null effects of higher doses of WIN55212-2 (1.0-2.50mg/kg) have been observed (Cosenza et al., 2000; Drews et al., 2005; Pamplona et al., 2006; Polissidis et al., 2013). These findings indicate a dose-response function for the effects of WIN55212-2 on locomotor behaviour, at least in adult rats, with a narrower range than for anxiety-like behaviour.

The effect of WIN55212-2 on locomotor behaviour in adolescent rats seems to be the opposite of what has been observed in adult rats. Specifically, Acheson et al. (2011) observed increased locomotion on the Morris Water Maze in adolescent rats treated with WIN55212-2 (1mg/kg) relative to vehicle, a dose which reduces locomotion in adult rats (Cosenza et al., 2000). To reconcile these findings with the reduced movement observed in WIN55212-2-treated adolescent rats during extinction

training, I examined locomotor and anxiety-like behaviour in adolescents following WIN55212-2 (0.25mg/kg) on the open field test.

Locomotor Activity. Two measures of locomotion were calculated: total distance travelled and average speed of locomotion. WIN55212-2 treatment had no effect when either total distance travelled ($t_{9.12} = .72$, $p = .49$; **Figure 2.5A**) or average locomotor speed was compared ($t_{14} = 1.39$, $p = .19$; **Figure 2.5B**).

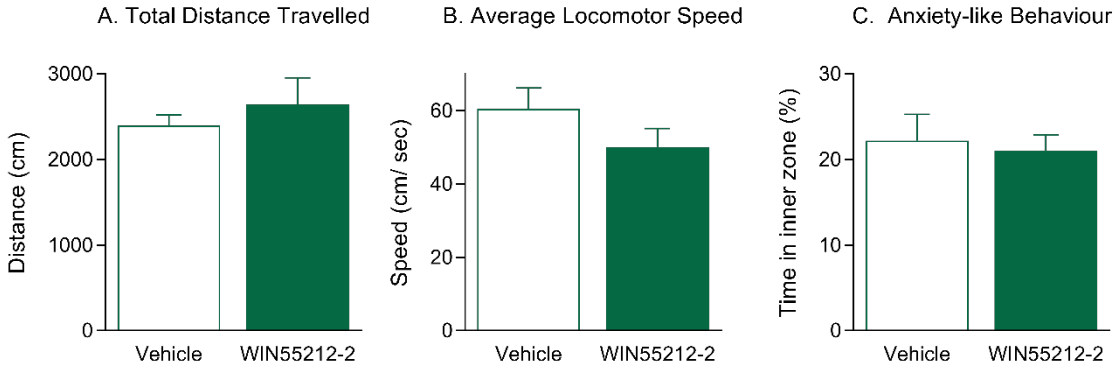

Figure 2.5. Mean (±SEM) total distance travelled (**A**), average locomotor speed (**B**), and anxiety-like behaviour (**C**) of adolescent rats in groups Vehicle and WIN55212-2.

Anxiety-like Behaviour. The percentage of time spent in the inner zone of the open field (relative to total time) was calculated as an index of anxiety-like behaviour. There was no difference between vehicle- and WIN55212-2-treated rats in time spent in the inner zone ($t_{14} = .33$, $p = .75$; **Figure 2.5C**), demonstrating that anxiety-like behaviour was unaffected by WIN55212-2 administration. Therefore, the findings of Experiments 2.2-2.4 that WIN55212-2 increased CS-elicited freezing during extinction training (i.e., when the drug was on-board) in adolescent rats are unlikely to be due to either decreased locomotion or increased anxiety-like behaviour induced by this agent.

Experiment 2.6

Developmental differences in CB1R expression in the medial prefrontal cortex and amygdala

Across Experiments 2.1-2.4, it is clear that extinction retention in developing rats was not improved by WIN55212-2 administration, unlike in adult rats. In addition, I found that WIN55212-2 (0.25mg/kg) increased levels of CS-elicited freezing during extinction training in adolescent and juvenile rats, but not in adult rats. Furthermore, the findings of Experiment 2.5 indicate that increased freezing (i.e., fear expression) to the CS presentations during extinction training in WIN55212-2-treated adolescent rats was not a function of altered locomotor or anxiety-like behaviour, suggesting a more specific effect on fear responding. This finding also fits with the observation that WIN55212-2 treatment did not increase baseline levels of freezing at extinction training when the drug was on board (Experiments 2.2-2.4) as any general locomotor or anxiety-like effects would be expected to alter this behaviour as well. As WIN55212-2 did not affect extinction retention in juvenile or adolescent rats, it may then be the case that CB1R expression in the regions which underpin extinction (i.e., mPFC and amygdala) is different across development. This suggestion is also supported by a previous review of work on the endocannabinoid system across development (Lee et al., 2016).

Prior to outlining developmental alterations in this system, is worth noting that different measurement techniques have been used across published experiments. A common approach has been to measure the amount of receptor protein in a particular sample. Proteins can be quantified using methods such as Western blotting or immunohistochemistry (Bauman, Ricke, Drew, Huang, & Ricke, 2016; Mahmood & Yang, 2012). Secondly, expression of CB1R messenger RNA (mRNA) can be measured through techniques such as Reverse Transcription – Polymerase Chain Reaction or

RNAScope (Wang et al., 2012; Wong & Medrano, 2005). Levels of mRNA are considered a precursor to protein expression, such that the mRNA will eventually be transcribed into protein (Vogel & Marcotte, 2012). However, due to a variety of biological factors, mRNA levels cannot be used to reliably predict receptor protein levels (for review, see Liu, Beyer, & Aebersold, 2016). Lastly, functionality of the receptors can be assessed through radioligand binding studies by applying a CB1R receptor agonist directly to tissue samples (Hillard, Edgemond, & Campbell, 1995). As each of these measurements address a different facet of the receptor, it is unsurprising that discrepancies arise when making comparisons.

CB1Rs are abundantly expressed in the adult rodent mPFC and amygdala (Busquets-Garcia et al., 2016; Hu & Mackie, 2015). Although developing animals also have CB1Rs in both regions, reports of age-related changes in CB1R expression within the mPFC and amygdala, are somewhat inconsistent, and dependent on the measure used. In terms of the mPFC, expression of CB1R protein has been shown to decrease from adolescence to adulthood (Amancio-Belmont, Romano-López, Ruiz-Contreras, Méndez-Díaz, & Prospéro-García, 2017), and this pattern was also observed within the adolescent period (from P29-P50; Ellgren et al., 2008). When mRNA and binding techniques have been used to measure CB1Rs, less consistent findings have been reported. For instance, CB1R mRNA has been reported to both increase and decrease in the mPFC from adolescence to adulthood (Heng, Beverley, Steiner, & Tseng, 2011; Vangopoulou et al., 2018). Similarly, CB1R binding has been reported as either the same, or reduced, in adolescence relative to adulthood (Lee & Hill, 2013; Vangopoulou et al., 2018). Only one study has compared CB1R levels between juveniles and adolescents, and found a reduction in CB1R mRNA expression with age (Heng et al., 2011). Although the evidence is inconsistent, there appears to be a decrease in CB1R

protein in the mPFC from adolescence to adulthood (a summary of reported findings is presented in **Table 2.6**). However, it is unknown whether CB1R protein levels in the mPFC of adolescents are comparable or different to younger animals as no studies to date have compared CB1R protein across the juvenile, adolescent, and adult periods in the same experiment.

Table 2.6

Summary of reported findings of CB1Rs in the prefrontal cortex across age.

	Region	**Measure**	**Findings**
Amancio-Belmont et al. (2017)	mPFC	Protein	Decrease from adolescence to adulthood
Ellgren et al. (2008)	mPFC	Protein	Decrease from early- to mid- to late-adolescence
Heng et al. (2011)	PL	mRNA	Decrease from juvenile to adolescence to adulthood
	IL	mRNA	Decrease from juvenile to adolescence to adulthood
Vangopoulou et al. (2018)	PL	mRNA	Increase from adolescence to adulthood
	IL	mRNA	Increase from adolescence to adulthood
	PL	Binding	Increase from adolescence to adulthood
	IL	Binding	No change from adolescence to adulthood
Lee & Hill (2013)	mPFC	Binding	No difference between adolescence and adulthood

mPFC = medial prefrontal cortex, PL = prelimbic cortex, IL = infralimbic cortex

There are fewer examinations of CB1R mRNA expression and binding capacity across age in the amygdala than in the mPFC, and there have been no developmental comparisons of CB1R protein. In the CeA, equivalent CB1R mRNA expression and binding levels have been observed between adolescence and adulthood (Vangopoulou et al., 2018). In the BLA, there is an opposite pattern of mRNA expression and binding capacity with age, such that CB1R mRNA decreases and binding capacity increases from adolescence to adulthood (Vangopoulou et al., 2018). When CB1Rs are measured in the amygdala more broadly (i.e., without any distinction between subregions), binding capacity has been reported to increase, decrease, or remain stable between adolescence and adulthood (Hill, Eiland, Lee, Hillard, & McEwen, 2019; Lee & Hill, 2013; Rodríguez de Fonseca, Ramos, Bonnin, & Fernández-Ruiz, 1993). Overall, reports of CB1R mRNA and binding in the amygdala across age have been remarkably inconsistent (a summary of reported findings is presented in **Table 2.7**).

Across the two regions, discrepancies between reports are likely due to different measurement approaches. Unfortunately, only one study (Heng et al., 2011) has compared CB1R mRNA expression across juveniles, adolescents, and adults within the one experiment, and there have been no investigations comparing CB1R expression (using any method) in the amygdala using each of these age groups. Therefore, the developmental trajectory of CB1Rs in the regions which underpin successful extinction is unclear, and inferences regarding how the development of the endocannabinoid system may be related to fear extinction performance cannot be made with confidence.

Table 2.7

Summary of reported findings of CB1Rs in the amygdala across age.

	Region	Measure	Findings
Vangopoulou et al. (2018)	BLA	mRNA	Decrease from adolescence to adulthood
	CeA	mRNA	No change from adolescence to adulthood
	BLA	Binding	Increase from adolescence to adulthood
	CeA	Binding	No change from adolescence to adulthood
Rodriguez de Fonseca et al. (1993)	Limbic forebrain	Binding	Increased in adolescence relative to juvenile period and adulthood
Lee & Hill (2013)	Amygdala	Binding	Increase from adolescence to adulthood
Hill et al. (2019)	Amygdala	Binding	No change from adolescence to adulthood

BLA = basolateral amygdala, CeA = central amygdala

In light of my earlier behavioural findings (Experiments 2.1-2.4) and the inconsistent reports described above, I investigated whether there were developmental differences in CB1R density in the mPFC or amygdala, as assessed using protein expression. In Experiment 2.6, I examined protein levels of CB1Rs in the mPFC and amygdala of experimentally naïve juvenile, adolescent, and adult animals. As WIN55212-2 did not affect extinction retention in either juvenile or adolescent rats, I hypothesised that CB1R expression may be different in these two age groups relative to adult rats. Rats were sacrificed on P25-27 (juvenile), P36-37 (adolescent), or >P70 (adult). Brain tissue was then processed according to a standard Western blotting protocol. During the Western blotting process, the brain sample is first homogenised and the total amount of protein in the sample is calculated. Following this, specific

antibodies are applied to the sample that bind to the protein of interest – in this case, cannabinoid receptor 1. Once the protein of interest has been labelled with antibodies, the sample is imaged to determine the amount of the target protein that is present in the sample (Mahmood & Yang, 2012). In each sample, the level of CB1R protein was normalised to the total protein and expressed as a proportion relative to adults (i.e., the control group). One rat was excluded from Experiment 2.6 (Adult group) due to errors in tissue sample processing.

Western Blot Analysis. Rats were euthanised using carbon dioxide, and then their brains were removed, rapidly frozen, and stored at -30°C prior to dissection. Frozen brains were sectioned into 2mm sections using a brain matrix and sectioning blades. Tissue was collected from the mPFC (including the PL and IL) and amygdala (including the BLA, CeA, and ITC) using a tissue punch with a diameter of 1mm. Brain tissue was homogenised in lysis buffer containing a protease inhibitor (Roche Diagnostics). Protein concentrations of samples were then determined using a Bradford Assay and equal amounts of protein (30-40µg, dependent on concentration of individual samples per gel) were separated by electrophoresis on 4-15% mini-Protean TGX (Tri-Glycine eXtended) stain-free gels (Bio-Rad). Once proteins were transferred to PVDF membranes, non-specific immunoreactivity was blocked with 5% milk in Tris-buffered saline with Tween20 for 90 min. Membranes were incubated overnight at 4°C in Tris-buffered saline with Tween20 with anti-CB1 (1:1000, Cell Signalling #93815), repeatedly washed in Tris-buffered saline with Tween20, and then incubated in anti-rabbit (1:5000, Bio-Rad #172-1019) in 5% milk in Tris-buffered saline with Tween20 for 60 min before being washed again. Protein visualisation was determined using the enhanced luminol-based chemiluminescent detection method with a ChemiDoc XRS+ imaging system (Bio-Rad) and ImageLab 6.0.1 software. The intensity of CB1R protein

in each sample was normalised to the total protein using stain-free images to control for variance in loading differences between samples and then expressed relative to the mean of samples from the adult control group from that blot to control for inter-blot differences.

CB1R protein levels. There was a clear decrease in CB1R protein expression across development in both the mPFC and amygdala. In the mPFC, CB1R expression decreased from the juvenile period to adulthood ($F_{2, 34} = 3.71$, $p = .04$), and this was a large effect size ($\eta_p^2 = .18$; **Figure 2.6A**), consistent with the majority of the literature examining the developmental trajectory of CB1Rs in this region using protein, mRNA, and binding techniques (e.g., Amancio-Belmont et al., 2017; Ellgren et al., 2008; Heng et al., 2011; see **Table 2.5**).

As was observed in the mPFC, expression of CB1R protein also decreased from the juvenile to adult period in the amygdala ($F_{2, 35} = 9.11$, $p = .001$), and this was also a large effect size ($\eta_p^2 = .34$; **Figure 2.6B**). This developmental decrease in the amygdala was consistent with findings reported by Vangopoulou et al. (2018) using mRNA expression (see Table 2.6). The full Western blots used as representative images are included in Appendix I.

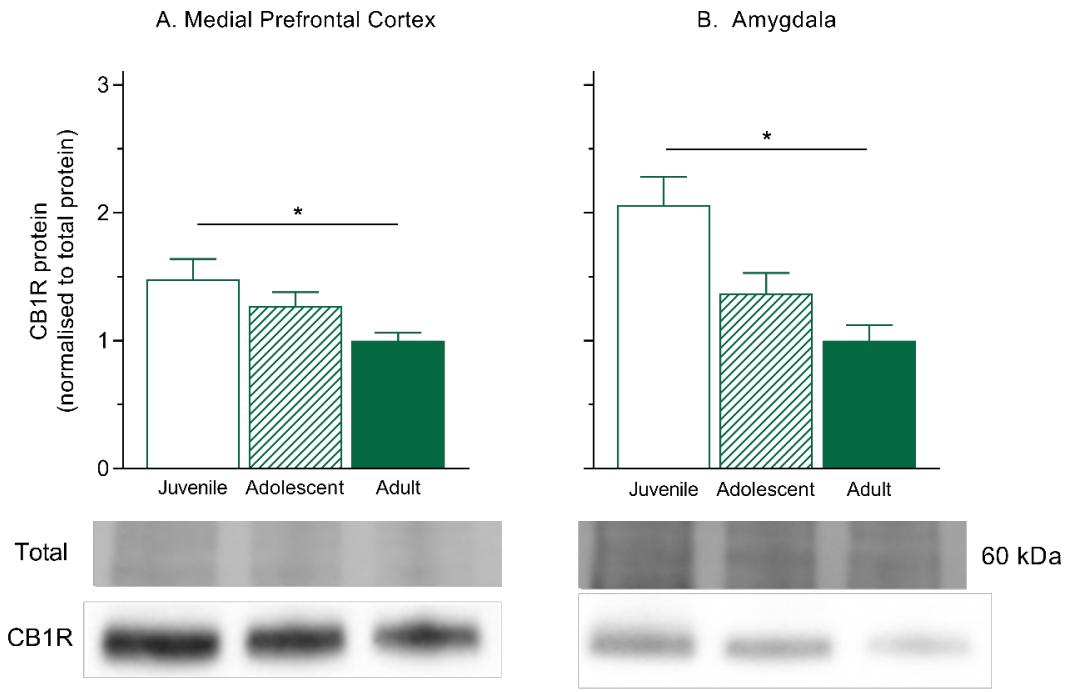

Figure 2.6. Mean (±SEM) levels of CB1R protein in juvenile (n = 14), adolescent (n = 11), and adult (n = 12-13) rats in the medial prefrontal cortex (**A**) and amygdala (**B**). Representative Western blots included below.
***** indicates a significant group difference ($p < .05$).

Experiment 2.7

Changes in CB1R expression following extinction training across development

The results of Experiment 2.6 indicate a decrease in the expression of CB1R protein across age occurs in both the mPFC and amygdala. In addition to age-related differences in the endocannabinoid system at the basal level, there may also be differences in how CB1Rs are expressed following extinction training. Further, the changes in the density of CB1Rs in extinction-related regions such as the BLA after extinction training may even occur in a neuronally-specific manner. Using TetTag transgenic mice, Trouche et al. (2013) 'tagged' neurons which were active during fear conditioning (i.e., fear neurons), and then identified how these neurons were altered by extinction training the next day. The authors assessed two types of fear neurons: 'active' neurons which were subsequently reactivated during extinction training, and 'silent'

neurons which were not reactivated. This enabled an investigation of how the neural circuit within the BLA is remodelled during extinction training.

Of relevance to the present thesis, Trouche et al. (2013) found that the density of CB1Rs around 'active' fear neurons in the BLA increased following extinction training. No changes in the density of CB1Rs around 'silent' fear neurons were observed. As CB1Rs increased around 'active' fear neurons, Trouche et al. suggested that this increase in CB1Rs causes these fear neurons to remain active despite extinction training and may contribute to relapse following extinction training. In this way, increased CB1Rs following extinction training may contribute to persistent fear. However, as Trouche et al. assessed changes in the *number,* rather than the *function*, of active neurons within the BLA, the role of CB1Rs in increased relapse remains speculative. In any case, I examined whether a similar change in receptor density could be detected after extinction training using Western blotting.

Furthermore, I investigated whether there were any age-dependent extinction-induced changes in CB1R expression in the amygdala that may be associated with persistent fear following extinction training. To investigate this question, I examined CB1R protein levels in the amygdala of juvenile (P25-27), adolescent (P36-39), and adult (>P70) rats that had received fear conditioning and either extinction training or equivalent exposure to the extinction context (i.e., no extinction). Experiment 2.7 tested three predictions. First, I predicted that an increase in CB1R density in the amygdala would be seen in all rats that received extinction training relative to their no-extinction counterparts (Trouche et al., 2013). Second, I expected a decrease in CB1R expression across age in rats that did not receive extinction training, consistent with the findings reported in Experiment 2.6 using home cage animals. Third, I hypothesised that adolescent rats may show greater levels of CB1R expression following extinction

training compared to juvenile and adult rats. As juvenile rats typically show good extinction retention, I expected to see an adult-like change in CB1R density in this age group.

In line with Trouche et al. (2013), rats were sacrificed 24-h after extinction training or context exposure. Brain tissue was processed as in Experiment 2.6 and CB1R protein level was normalised to the total protein for each sample and expressed as a proportion relative to adult rats that did not receive extinction training (i.e., the control group). Two rats from Experiment 2.7 (Juvenile No Extinction group, Adolescent No Extinction group) were excluded from analysis due to errors in tissue sample processing.

Baseline. There were no differences in baseline freezing between groups prior to conditioning (largest $F_{1, 56} = 2.70$, $p = .11$) or extinction training/context exposure (largest $F_{1, 56} = 2.13$, $p = .13$; **Table 2.8**).

Table 2.8

Mean (±SEM) percent baseline freezing before conditioning and extinction training in Experiment 2.7.

	Juvenile - Extinction (*n* = 10)	**Juvenile – No Extinction (*n* = 10)**	**Adolescent – Extinction (*n* = 11)**	**Adolescent – No Extinction (*n* = 11)**
Conditioning	1.00 (0.55)	1.50 (0.67)	1.36 (0.62)	6.25 (4.94)
Extinction/ No Extinction	10.25 (4.12)	20.50 (5.70)	12.73 (3.76)	5.23 (2.66)
	Adult - Extinction (*n* = 11)	**Adult – No Extinction (*n* = 9)**		
Conditioning	0.91 (0.51)	5.83 (3.51)		
Extinction/ No Extinction	6.82 (4.41)	7.50 (5.14)		

Conditioning. During conditioning, CS-elicited freezing increased across trials ($F_{2, 112} = 69.07$, $p < .001$; **Figure 2.7A**). There was no main effect of age ($F_{2, 56} = 3.13$, $p = .05$), subsequent extinction group ($F_{1, 56} = 2.21$, $p = .14$), or interactions (largest $F_{4, 112} = 1.43$, $p = .23$). There was, however, a significant age × trial interaction ($F_{4, 112} = 3.07$, $p = .02$), indicating that freezing increased differently across the age groups. The age × trial interaction was followed up by ANOVAs to examine group differences at each of the conditioning trials. Although there were no group differences at Trial 1 (largest $F_{2, 56} = 3.19$, $p = .05$) or Trial 3 (largest $F_{2, 56} = 2.71$, $p = .08$), there was a main effect of age at Trial 2 (largest $F_{2, 56} = 3.43$, $p = .04$). This main effect of age at Trial 2 was followed up by post-hoc comparisons using Tukey's Honestly Significant Difference test, and revealed no significant differences between juvenile, adolescent, and adult rats (all pair-wise comparisons $p > .05$).

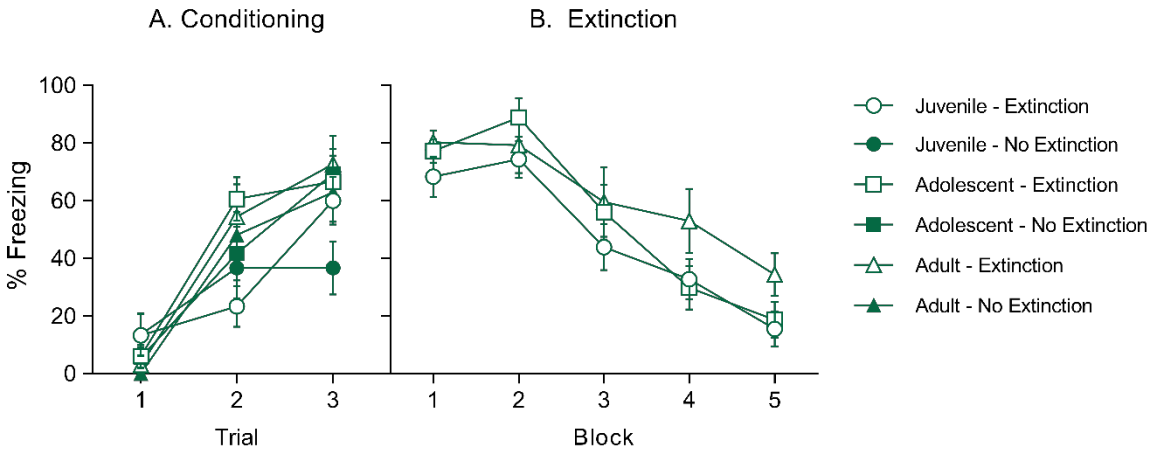

Figure 2.7. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**) and extinction training (**B**) in groups Juvenile – Extinction, Juvenile – No Extinction, Adolescent – Extinction, Adolescent – No Extinction, Adult – Extinction, and Adult – No Extinction.

Extinction. As there was a significant main effect of age at Trial 2 of conditioning, this was used as a co-variate in the subsequent analysis of extinction training. Including this covariate did not affect the statistical outcomes. During extinction training, CS-elicited freezing decreased across blocks (block main effect: $F_{4,}$

$_{112}$ = 21.30, p < .001; **Figure 2.7B**). There was no age × block interaction ($F_{8, 112}$ = 1.59, p = .14) or main effect of age (F < 1), indicating equivalent extinction learning across groups.

CB1R protein. **Figure 2.8** illustrates that CB1R levels appeared to decrease from the juvenile period to adulthood, regardless of extinction condition; however, ANOVA did not detect any significant effects of age ($F_{2, 56}$ = 2.54, p = .09) or extinction training (F < 1), nor an age × extinction interaction (F < 1). Therefore, the age-related decrease in levels of CB1R protein in rats that did not receive extinction training did not replicate basal levels of CB1R protein as reported in Experiment 2.6, and no extinction-induced changes in CB1R expression in the amygdala were found.

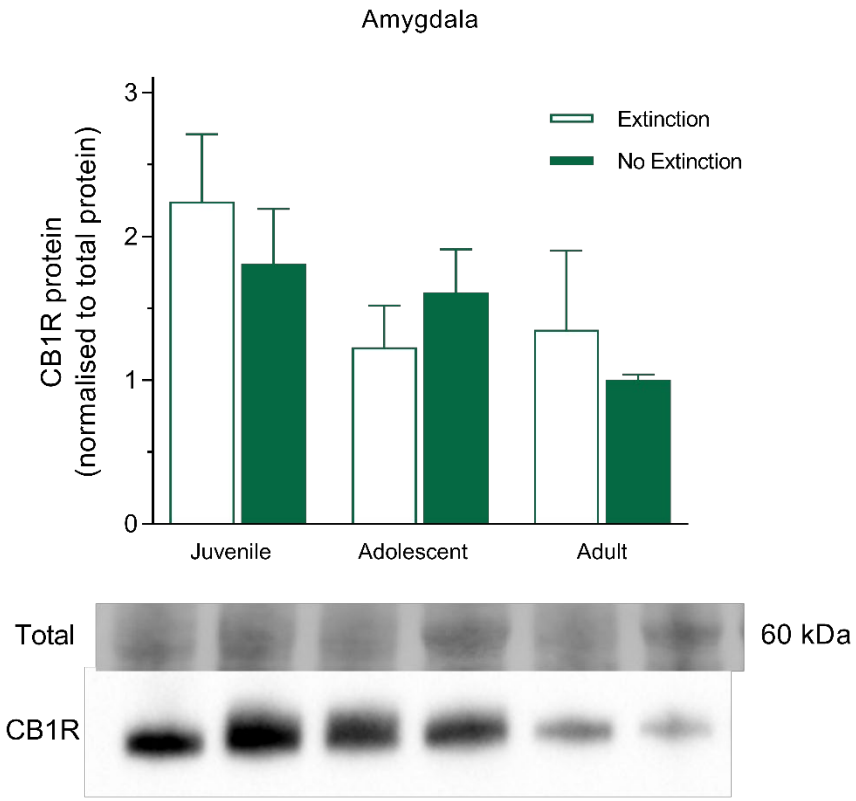

Figure 2.8. Mean (±SEM) levels of CB1R protein in the amygdala of juvenile, adolescent, and adult rats that received either extinction training or no extinction training. Representative Western blot images included below.

It is important to acknowledge that the age-related decrease seen in CB1R expression in the amygdala (Experiment 2.6) were not observed in Experiment 2.7. However, upon visual inspection of Figure 2.8, there appeared to be a similar pattern of an age-related decrease in CB1R expression across both the extinction and non-extinguished conditions in Experiment 2.7. In regards to the non-extinguished controls, I expected to replicate a decrease in CB1R expression in the amygdala of these animals like that found in home-cage animals in Experiment 2.6 despite the differences in that the No Extinction group did receive fear conditioning but no extinction training. Although unexpected, there are alternative accounts for this result (aside from a failed replication). First, receptors can be altered after learning experiences such as fear conditioning (e.g. NMDARs; Baez, Cercato, & Jerusalinsky, 2018). Therefore, CB1R density within the amygdala may have been altered as a result of fear conditioning in the No Extinction group in Experiment 2.7 relative to the home cage controls in Experiment 2.6. Moreover, fear conditioning may have altered CB1R density differently across age, further confounding group comparisons across the two experiments. Although Trouche et al. (2013) did not observe changes in CB1Rs in the adult BLA following fear conditioning relative to home cage animals, to the best of my knowledge, there is no research which has examined age-related changes in CB1R density following fear conditioning. Therefore, the reported CB1R density of rats that did not receive extinction training in Experiment 2.7 cannot be taken as a failure to replicate the CB1R density of home cage controls in Experiment 2.6.

Second, the lack of extinction-induced changes in CB1R density within the amygdala may be due to a methodological limitation. Specifically, the rationale for this experiment was based on an experiment that observed changes in CB1Rs around a specific population of neurons in the BLA that had been tagged as ‘active’ during both

fear conditioning and extinction. As the technique used in this experiment, Western blot analysis, processes the whole amygdala without distinction between regions or neuronal subtypes, any age-related changes in CB1Rs may have been obscured. For instance, there may be age- or extinction-related changes in CB1Rs around 'active' neurons, or changes in CB1R expression within other nuclei which have been implicated in fear learning, such as the CeA (Kamprath et al., 2011; note that this suggestion is speculative). Therefore, future work should examine age- and extinction-related changes in CB1Rs using techniques which allow neuronal and regional specificity, such as immunohistochemistry.

Discussion

The experiments reported in this chapter identified several developmental differences in the effects of a CB1/2R agonist on fear expression and extinction in rats. Based on past work examining the effects of CB1R agonism on extinction retention in adult rats (e.g., Pamplona et al., 2006; Reich et al., 2013), I predicted that increasing the activation of CB1Rs via administration of the CB1/2R agonist WIN55212-2 would ameliorate the impaired extinction retention usually observed in adolescent rodents. Prior to examining the effects of WIN55212-2 on adolescent rats, I extended previous work demonstrating the augmenting effects of CB1R activation on extinction of context and trace fear to delay fear extinction (Experiment 2.1). However, when the same dose of WIN55212-2 was administered prior to extinction training in adolescent rats, there was no improvement in extinction retention (Experiment 2.2). This lack of an effect in adolescents was replicated and also extended to juveniles (Experiment 2.3), and was not due to the dose (0.25mg/kg) used as both lower and higher doses (0.125mg/kg and 2.5mg/kg) were also ineffective in adolescent rats (Experiment 2.4). In addition, WIN55212-2 had no effect on relapse following extinction training, as measured by

renewal, in either juveniles or adolescents (Experiment 2.3), in contrast to what has been reported in adults using reinstatement and spontaneous recovery procedures (Lin et al., 2006).

Nevertheless, there was clear evidence that adolescent rats responded to WIN55212-2 because I consistently detected higher levels of CS-elicited freezing during extinction training in WIN55212-2-treated adolescent rats (Experiments 2.2-2.4). An increase in CS-elicited freezing during extinction was also observed in WIN55212-2-treated juvenile rats (Experiment 2.3). Higher CS-elicited freezing during extinction training in adolescents was not a function of decreased locomotor activity or increased anxiety-like behaviour, as assessed by the open field test (Experiment 2.5). The observed developmental differences in the effects of WIN55212-2 on behaviour were paralleled by a linear decrease in the expression of CB1Rs in the mPFC and amygdala across age from the juvenile period to adulthood (Experiment 2.6). A subsequent Western blotting experiment explored possible extinction-induced changes in CB1R protein in the amygdala. Although the analyses did not find any age- or extinction-related differences, visual observation of the data indicated a similar pattern of findings to Experiment 2.6, in which CB1R protein expression decreased across age (Experiment 2.7). Together, the results of the current experiments indicate that the endocannabinoid system is different during adolescence compared to adulthood, at both behavioural and neural levels.

Typically, adolescent rats show a deficit in extinction retention relative to juvenile and adult rats (Baker et al., 2016; McCallum et al., 2010). Consistent with this finding, I observed increased fear expression when adolescent rats were tested for extinction retention and renewal relative to juvenile rats (Experiment 2.3). However, a developmental difference in extinction retention between adolescents and older animals

was not found across experiments; the mean level of CS-elicited freezing at the extinction retention test in the adult control group in Experiment 2.1 (Vehicle: 41%) was almost identical to that of adolescents in Experiment 2.2 (Vehicle: 40%). Although unexpected, the equivalent levels of CS-elicited freezing at test observed in the adolescent and adult control groups across my first two experiments enabled a comparison of the effects of WIN55212-2 on extinction retention when both controls are performing similarly. This comparison clearly demonstrated that WIN55212-2 administration before extinction training enhanced extinction retention the following day in adults but not in adolescents.

Across Experiments 2.2-2.4, juvenile and adolescent rats clearly had higher levels of CS-elicited freezing during extinction training when treated with WIN55212-2 at doses of 0.25mg/kg or higher. As the increase in freezing was not observed during baseline sessions in any experiment, and there were no differences on the open field test, administration of WIN55212-2 is likely to have selectively affected the animal's response to the CS during extinction training. In adult rats, previous work has demonstrated that administration of high doses of WIN55212-2 (2.5mg/kg) increase freezing across extinction training relative to lower doses (0.25mg/kg, 1.25mg/kg). As I observed increased freezing in developing rats that were administered doses equal to or above 0.25mg/kg, WIN55212-2 may increase CS-elicited freezing during extinction training in the same manner across age with the dose-response curve shifted towards lower doses in developing animals.

Administration of WIN55212-2 may increase freezing during extinction training by affecting non-associative processes, such as habituation. Reductions in CS-elicited freezing during extinction training are a function of both associative learning (i.e., acquiring the CS-noUS association) and non-associative learning (i.e., habituation due

to repeated presentations of the CS; Jordan, Todd, Bucci, & Leaton, 2015). CB1Rs can influence freezing during extinction training by modulating habituation rather than extinction learning, as demonstrated using CB1R knock-out mice (Kamprath et al., 2006). An impairment in habituation processes has also been demonstrated following administration of high doses of WIN55212-2. In zebra finches, repeated playback of the same song causes habituation of neuronal responses, such as the induction of the immediate early gene *zenk*. The habituation of the *zenk* response to repeated song playback was blocked by administration of high (1mg/kg – 3mg/kg), but not low (0.01mg/kg – 0.3mg/kg), doses of WIN55212-2 (Whitney, Soderstrom, & Johnson, 2003). A high dose (3mg/kg) of WIN55212-2 has also been shown to prevent the habituation of *Arc*, another immediate early gene, following repeated song playback in zebra finches (Gilbert & Soderstrom, 2013). Based on these findings, administration of high doses of WIN55212-2 (in this case, 0.25mg/kg or higher) may prevent habituation to the non-reinforced CS presentations in developing rats as well, resulting in persistently high freezing across extinction. Therefore, future research should test whether WIN55212-2 administration impacts habituation to a white noise in adolescent rats, and if so, whether the neural markers of habituation are also affected.

In this chapter, I demonstrated that administration of the CB1/2R agonist WIN55212-2 had different effects on extinction retention in developing animals compared to adult animals. It is possible that this agent has different effects at CB1Rs across development; however, the current experiments do not directly provide evidence for, or against, this possibility. In any case, the findings reported in this chapter are consistent with other research demonstrating that this adjunct does not impact learning and memory processes in adolescents. For example, Carvalho et al. (2016) found that chronic systemic administration of WIN55212-2 induced conditioned place aversion in

adult rats but not in adolescent rats. What is most interesting about that study is that the behavioural effects were accompanied by structural changes in the mPFC, such as the number of dendritic branches, dendritic spine density, and dendritic branch length, in the adults but not in the adolescents. One possible direction for future research would be to investigate whether acute WIN55212-2 administration has different effects on dendritic spine plasticity in the mPFC (or other brain regions) in adolescent and adult rats, considering that plasticity in this region is associated with improved extinction consolidation (for review see, Zimmermann, Richardson, & Baker, 2019).

It may be the case that WIN55212-2 administration did not enhance extinction consolidation and lead to improved extinction retention in juvenile or adolescents due to a relatively immature endocannabinoid system. In support of this idea, I observed a decrease in basal expression of CB1R protein across age in Experiment 2.6 (however this was not replicated in Experiment 2.7 under different experimental parameters). This is consistent with past reports of changes in CB1R protein in the mPFC from adolescence to adulthood (Amancio-Belmont et al., 2017) and within adolescence (Ellgren et al., 2008), as well as a decrease in CB1R mRNA from the juvenile period to adulthood (Heng et al., 2011). Moreover, the developmental decrease in CB1R protein in the amygdala is consistent with reports of CB1R mRNA decreasing from adolescence to adulthood (Vangopoulou et al., 2018). While increased CB1R density may be developmentally appropriate in young animals, it may also reflect receptor inefficiency or receptor compensation in the case of reduced endocannabinoid availability.

With respect to the first suggestion, a number of possible mechanisms could underlie reduced efficiency, if it were the case. First, alterations in the binding capacity of CB1Rs could compromise receptor efficiency and subsequently, the extent to which increasing receptor activation would impact behaviour. However, there are conflicting

reports of CB1R binding capacity across age (Hill et al., 2019; Lee & Hill, 2013; Vangopoulou et al., 2018) and so claims about alterations in CB1R binding capacity cannot be made with confidence. Second, CB1Rs may not facilitate extinction in developing animals due to alterations in co-localisation with other receptor types across age. Indeed, the actions of CB1Rs on extinction depend, at least in part, on the expression of CB1Rs at inhibitory neurons, such as cholecystokinin-expressing neurons (Bowers & Ressler, 2015; Rovira-Esteban et al., 2019; Ruehle et al., 2013). Alterations in the co-localisation of CB1Rs with cholecystokinin would therefore affect the capacity of CB1Rs to modulate inhibitory neurotransmission within the BLA, and subsequently fear extinction. Clearly, future research is needed to identify whether CB1Rs are less efficient during development, in addition to the co-localisation between CB1Rs and other receptor types (e.g., cholecystokinin) across age.

On the other hand, increased CB1R receptor density may instead reflect low availability of endocannabinoids, which are the ligands that bind to the receptor. The actions of CB1Rs on neurotransmission and synaptic plasticity are dependent on the binding of endocannabinoids to CB1Rs, such that reduced circulating endocannabinoids limits the capacity of CB1Rs to influence learning and memory tasks. Further, receptor density increases when ligand availability is low to ensure that all ligands will be taken up by receptors. This suggestion is supported by clinical research findings, in which individuals with PTSD (a clinical population that exhibits impaired extinction) show an increased availability of CB1Rs (Neumeister et al., 2013; Zuj, Palmer, Lommen, & Felmingham, 2016), suggested to be a compensatory mechanism in light of reduced availability of circulating endocannabinoids (Hauer et al., 2013). However, increased CB1Rs as compensation against low endocannabinoid availability may not apply to impaired extinction during adolescence. Rather, levels of the endocannabinoid

anandamide have been reported to either peak or reach adult-like levels during adolescence (Lee, Hill, Hillard, & Gorzalka, 2013; Meyer et al., 2018). In any case, the difference in CB1R expression in the mPFC and amygdala during development may interfere with the mechanisms by which WIN55212-2 augments extinction in adults.

The findings from the present chapter should be considered in light of two important limitations. First, WIN55212-2 is a mixed CB1/2R agonist – therefore, the effects of this drug on extinction learning and retention are a function of its action at both CB1Rs and CB2Rs (Felder, Joyce, & Briley, 1995; Pertwee, 2010). While there is limited expression of CB2Rs in the prefrontal cortex and amygdala relative to CB1Rs (Atwood & Mackie, 2010; Navarro et al., 2016), it is important to recognise the possibility that age-related differences in CB2R density and/or function could have contributed to the current findings. Future work should endeavour to replicate the current findings with a more selective CB1R agonist, such as ACEA (Pertwee, 2010). Furthermore, the conclusions drawn regarding developmental differences in CB1R expression are tempered by the chosen technique. As discussed earlier, Western blotting processes whole brain regions without regional specificity. This limitation is further compounded by the use of a brain punch with a specified diameter that exceeds the size of the target brain regions. Consequently, it is likely that the samples processed in the current thesis contained adjacent regions – for instance, it is possible that a small portion of the anterior cingulate cortex was captured in the medial prefrontal cortex analysis.

In extending this work to clinical application, the current findings suggest that pharmacological agents which work through CB1/2R agonism may not be beneficial in augmenting exposure-based therapies in adolescents. While WIN55212-2 is not suitable for human use, a similar compound: Δ9 – tetrahydrocannabinol, has been proposed as a

possible therapeutic adjunct (Black et al., 2019). This proposal is despite the fact that Δ9 – tetrahydrocannabinol is responsible for both the anxiogenic and psychoactive effects of cannabis consumption (Rong et al., 2017). In addition, adolescent exposure to THC can lead to impaired social behaviours, impaired spatial learning, increased anxiety, and excessive locomotor activation (Fantegrossi, Wilson, & Berquist, 2018; Rubino & Parolaro, 2015). Therefore, it may be safer to investigate the therapeutic potential of other cannabinoid compounds which work via different pathways. One such compound, cannabidiol, works primarily through modulating endocannabinoid transmission rather than CB1/2R activity, and does not lead to anxiogenic or pro-psychotic effects (Bitencourt & Takahashi, 2018; Todd & Arnold, 2016). Clearly, it is important to distinguish between the different cannabinoid compounds when identifying possible treatment adjuncts to avoid undesired side effects, especially early in development.

Based on the results of the current experiments, it is clear that enhancing the endocannabinoid system using the CB1/2R agonist WIN55212-2 does not improve extinction retention in adolescent rats. Rather, I observed markedly different effects of the drug across development, in that WIN55212-2 increased fear expression during extinction training in juvenile and adolescent rats at a lower dose than has been reported in adult rats (Pamplona et al., 2006). These differences may be, at least in part, associated with the observed age-related decrease in CB1R protein expression in the mPFC and amygdala, two regions underlying fear extinction. Together with other reports of developmental differences in responding to extinction-enhancing pharmacological adjuncts (e.g., fluoxetine: Chan et al., 2018) that will be discussed in Chapter 5, the current findings highlight the importance of considering the adolescent brain when selecting adjuncts to improve treatment outcomes for anxious adolescents.

Furthermore, my findings raise the question of whether other neurotransmitter systems are also differentially involved in extinction during this developmental window.

Chapter 3: NMDA receptor-independent fear extinction in adolescent rats

As mentioned in Chapter 1 (p. 20), glutamate is the primary excitatory neurotransmitter in the mammalian brain, and regulates synaptic plasticity and neuronal activation (Riedel et al., 2003). There are several types of glutamate receptor that mediate fast synaptic transmission, including the NMDA receptor (NMDAR; Miller & Yeh, 2016). In the experiments reported in this chapter I explored the role of NMDARs in fear extinction during adolescence.

NMDARs are typically involved in fear extinction

Most of the work examining the roles of NMDARs in fear extinction comes from studies using adult rodents and has led to the conclusion that NMDARs are necessary for both the learning and consolidation of extinction (for review, see Davis, 2011; Singewald et al., 2015). In the first study to examine the role of NMDARs in fear extinction, Falls et al. (1992) found that infusion of the NMDAR antagonist AP5 into the basolateral amygdala immediately prior to extinction training dose-dependently impaired extinction, as indicated by a smaller decrease in fear potentiated startle to the CS from the pre-extinction test to the post-extinction test. This finding has since been replicated numerous times using both systemic and intra-amygdala administration of NMDAR antagonists (e.g., Santini, Muller, & Quirk, 2001; Zimmerman & Maren, 2010).

For instance, Sotres-Bayon et al. (2007) demonstrated that blocking NMDARs using systemic administration of the NMDAR antagonist ifenprodil immediately prior to extinction training impaired within-session extinction learning. That is, conditioned responding in ifenprodil-treated rats was higher during extinction training than in saline-treated rats. The following day, rats given ifenprodil prior to extinction training showed

impaired extinction retention relative to those given a saline injection. Together, this work demonstrates that NMDARs need to be engaged during extinction, otherwise extinction learning (and subsequent retention) is impaired.

In some cases, systemic administration of an NMDAR antagonist (e.g., MK801) can lead to a temporary increase in locomotor activity while the drug is "on board". For example, Langton and Richardson (2009) reported that animals given an injection of MK801 (i.p.) 30 min prior to an extinction session exhibited ~15% freezing to a conditioned white noise, whereas those given a saline injection exhibited ~55% freezing at the start of extinction training (i.e., conditioned responding in MK801-treated animals was essentially absent). Similar to the findings reported by Sotres-Bayon et al. (2007) using ifenprodil, Langton and Richardson (2009) observed that MK801-treated rats showed impaired extinction retention relative to saline-treated rats at test the following day. Therefore, the effects of NMDAR antagonism persist at extinction retention even when using drugs that impair expression of conditioned responding during extinction training.

Using targeted intracranial administration of NMDAR antagonists, past work has identified the brain regions which recruit NMDARs during different phases of extinction. To assess extinction learning, Sotres-Bayon et al. (2007, 2009) infused either the NMDAR antagonist ifenprodil or a vehicle solution into the mPFC or BLA immediately prior to extinction training. Only rats that received ifenprodil in the BLA showed impaired extinction learning and retention, suggesting that a specific subregion of the *amygdala* is the site of NMDAR-dependent synaptic plasticity during extinction training. Interestingly, a double dissociation emerged when the role of NMDARs in extinction consolidation was tested. When Sotres-Bayon et al. (2009) infused either ifenprodil or vehicle in the mPFC or BLA immediately following extinction training,

only rats that received ifenprodil in the mPFC showed impaired extinction retention the following day. This finding demonstrated that NMDARs in the mPFC are involved in extinction consolidation.

NMDARs are also typically required for fear extinction in juvenile and adolescent animals. Langton et al. (2007) tested the involvement of NMDARs in juvenile rats by administering MK801 or saline 10 min prior to extinction training. While saline-treated rats showed good extinction retention at test, MK801-treated rats showed impaired extinction retention. Similar findings were later observed in adolescent rats using an adapted procedure. As two days of extinction are required for adolescent rats to show good extinction retention, Baker and Richardson (2017) gave adolescent rats an injection of MK801 or saline 10 min prior to the *second* day of extinction training. As predicted, MK801-treated rats showed impaired extinction retention relative to saline-treated rats. Taken together, these findings appear to indicate that NMDAR activation during extinction is necessary for successful extinction retention in juvenile, adolescent, and adult rodents.

A different means to an end: NMDAR-independent fear extinction

Although the view that NMDARs are required for successful fear extinction has been long-held, there are cases of successful fear extinction in the absence of NMDAR activation – for both developing and adult rodents. The infant period, in rats, is considered to be up to P21, immediately prior to the juvenile period (Cowan & Richardson, 2018). In contrast to older animals, infant rats given MK801 (0.1mg/kg, s.c.) 10 min prior to extinction training exhibit good extinction retention the following day (Langton et al., 2007). The absence of an impairment in extinction retention in infants was not due to a lack of sensitivity to MK801 administration, as MK801 impaired fear learning in this age group. Further, Langton et al. (2007) tested a range of

doses of MK801 in the infant (0.5, 0.1, and 0.2mg/kg) and did not observe an impairment in extinction retention with any dose. These findings suggest that infants do not use NMDARs to acquire and retain extinction.

Evidence from neural studies investigating the brain regions recruited during extinction training in infants also supports the idea that infants use NMDAR-independent mechanisms during extinction. The MAPK pathway is one activity-dependent signalling cascade which leads to long-term potentiation and synaptic strengthening associated with the formation of long-term memories. This cascade begins with the activation of NMDARs and involves the phosphorylation of MAPK (i.e., pMAPK/ERK; Wang & Peng, 2016). Unlike juveniles and adults, infant rats do not show increased levels of this synaptic plasticity marker in the mPFC following extinction training (Kim, Hamlin, & Richardson, 2009). Therefore, there appears to be an early developmental period when NMDARs or their downstream plasticity-related signalling cascades are not involved in extinction.

NMDAR-independent fear inhibition has also been reported in adults undergoing *re-extinction*. The process of re-extinction involves animals being trained to fear a discrete cue/context, being exposed to extinction training for that cue/context, and then being retrained to the same cue/context and having the fear to that stimulus extinguished for a second time. Unlike the extinction the first time, re-extinction is unaffected by administration of MK801 prior to training (Langton & Richardson, 2009). That is, although MK801 administration reduces freezing during training sessions for both extinction and re-extinction, impaired extinction retention in MK801-treated rats is only observed when extinction happens for the first time. Similarly, intra-amygdala infusions of the NMDAR antagonist DL-APV prior to re-extinction training do not impair re-extinction of contextual fear (Laurent, Marchand, & Westbrook, 2008). These

findings indicate that while extinction relies on NMDARs, the process of re-extinction is NMDAR-independent.

Recent work suggests that there may be a third instance of NMDAR-independent extinction which occurs during adolescence. In contrast to the typical adolescent impairment in extinction retention observed when animals are both conditioned and extinguished within adolescence (e.g., Baker & Richardson, 2015; McCallum et al., 2010), adolescent rats exhibit surprisingly good extinction retention when the fear was acquired before adolescence. As discussed in Chapter 1, adolescents typically exhibit impaired extinction retention relative to both younger and older age groups (Baker et al., 2016). This impairment was initially hypothesised as being due to the animal's age at the time of extinction training with no consideration of the animal's age at fear conditioning. Baker and Richardson (2015) tested this assumption by manipulating the age at which animals undergo fear conditioning and extinction. In this study, adolescent rats that acquired their fear as juveniles were compared to adolescent rats that acquired their fear as adolescents, as well as rats conditioned and extinguished as juveniles (refer to **Figure 3.1**). Baker and Richardson (2015) predicted that all adolescent rats, regardless of their age at fear learning, would demonstrate impaired extinction retention relative to juvenile rats.

Across fear conditioning and extinction training, there were no differences in fear learning or recall between juvenile rats, rats conditioned as juveniles and extinguished as adolescents, and rats conditioned and extinguished as adolescents. Further, extinction learning was equivalent between groups. If it were the case that the timing of extinction training was critical for impaired extinction retention, as was expected, then rats conditioned as juveniles and extinguished as adolescents should

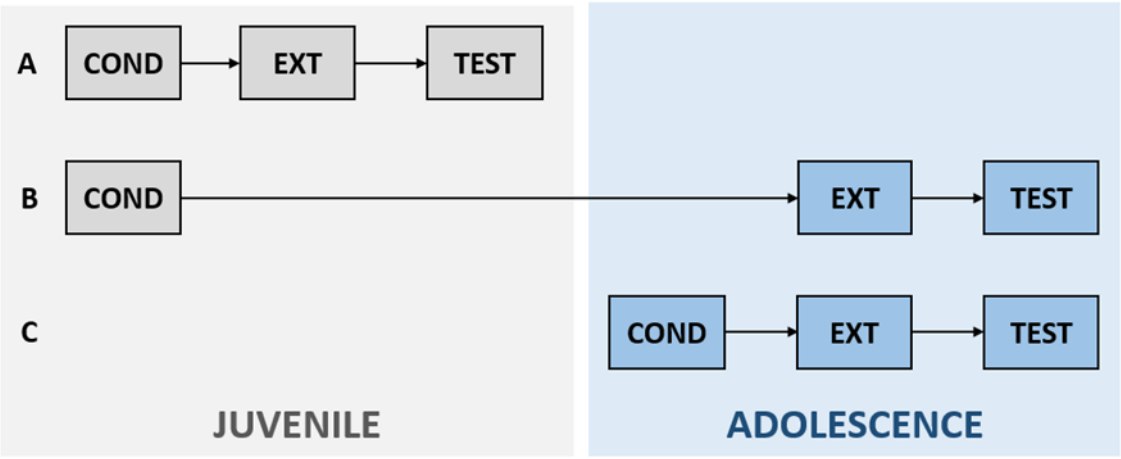

Figure 3.1. Schematic representation of experimental timeline for rats conditioned and extinguished as juveniles (**A**), rats conditioned as juveniles and extinguished as adolescents (**B**), and rats conditioned and extinguished as adolescents (**C**).
COND = fear conditioning, EXT = extinction training, TEST = extinction retention test.

exhibit such an impairment. However, these animals displayed good extinction retention, in contrast to rats conditioned and extinguished as adolescents. To investigate the neural mechanisms underlying impaired and successful extinction in adolescence, pMAPK/ERK expression was measured in the mPFC and amygdala. These analyses revealed similar levels of pMAPK/ERK expression in all adolescent rats, regardless of their age at conditioning and subsequent extinction retention performance. That is, both of the adolescent groups showed less pMAPK/ERK in the mPFC and amygdala compared to juvenile and adult rats, a profile which typically associated with poor extinction retention. This work demonstrated an inconsistency between extinction retention performance and typically observed patterns of NMDAR-dependent synaptic plasticity in adolescent rats that acquired fear as juveniles.

As pMAPK/ERK is downstream of NMDAR activation, the results of Baker and Richardson (2015) suggest that perhaps NMDARs are not being activated during extinction in rats conditioned as juveniles and extinguished as adolescents. However, this is fairly indirect evidence as pMAPK/ERK activation can be a result of several

different pathways other than NMDARs, including voltage-dependent calcium channels, growth factors acting at tropomyosin receptor kinase B, and endocannabinoids binding to CB1R (e.g., Davis & Bauer, 2012; Grewal, York, & Stork, 1999; Karanian, Brown, Makriyannis, & Bahr, 2005; Revest et al., 2014). The experiments reported in this chapter further explored extinction in adolescents that acquired fear as juveniles using the NMDAR antagonist MK801. I expected that unlike rats conditioned and extinguished within the same developmental period, rats conditioned as juveniles and extinguished as adolescents would show NMDAR-independent fear extinction.

Methods

Subjects

Of the 176 Sprague-Dawley rats used in the experiments reported in this chapter, 144 (used in Experiments 3.1, 3.2, 3.3, and 3.5) were derived from the breeding colony maintained by the School of Psychology at UNSW and 32 (used in Experiment 3.4) were obtained from the Animal Resources Centre (Perth, Australia; which was the supplier of the breeders used to produce the other animals).

Procedure

These experiments used the same apparatus as was used in the experiments described in Chapter 2. Briefly, rats received pre-exposure and fear conditioning in Context A chambers whereas extinction and test occurred in Context B chambers. The intensity of the footshock was set to 0.4mA for all experiments except Experiment 3 (set to 0.5mA for reasons described in the introduction to that experiment). A summary of the procedures used across experiments in Chapter 3 is presented in Table 3.1.

Table 3.1. Summary of procedures across Experiments 3.1-3.5 in Chapter 3.

	Age group/s	Handling	Pre-exposure	Conditioning	Delay (days)	Injection	Extinction Day 1	Extinction Day 2	Retention
3.1	Juvenile JuvCond-AdolesExt				Juvenile: 1-d JuvCond-AdolesExt: 10				
3.2	JuvCond-AdolesExt				JuvCond-AdolesExt: 6				
3.3	JuvCond-AdolesExt Adolescent				JuvCond-AdolesExt: 10 Adolescent: 1			Adolescent only	
3.4	Adult				1 or 10				
3.5	AdolesCond-AdultExt				8 weeks				

Note. Grey shading indicates the inclusion of the procedures in each experiment.

Drugs

(+)-MK 801 maleate (Tocris Bioscience; 0.1mg/kg diluted in saline) or saline (0.9% wt/vol) was administered subcutaneously (in the nape of the neck) in a volume of 1 ml/kg, 10 min before extinction, as in past studies (Baker & Richardson, 2015; Langton & Richardson, 2010). MK801 reaches maximal brain concentrations 10-30 min after systemic injection in the rat (Vezzani et al., 1989).

Data Analysis

Statistical analyses were conducted as in Chapter 2. As the comparisons in Experiment 3.3 were replications of previous findings, planned contrasts were used to analyse test performance. The decision-wise error rate was controlled at the 0.05 level for each contrast tested. A random sample of ~30% of the test data in this chapter was cross-scored by an observer unaware of the experimental conditions. Inter-rater reliability was high for all experiments (*r*s = .93 - .98).

Experiment 3.1

NMDAR-independent extinction in rats conditioned as juveniles and extinguished as adolescents

In Experiment 3.1, I tested whether the extinction of fear acquired as a juvenile and extinguished as an adolescent was dependent on NMDARs. As these rats did not show increased expression of the synaptic plasticity pMAPK/ERK (typically downstream of NMDAR activation; Baker & Richardson, 2015), even though they exhibited good extinction retention, I predicted that they would demonstrate NMDAR-independent extinction. These rats were compared to juvenile rats, a group that does require NMDARs to acquire and retain extinction (Langton et al., 2007).

All rats received fear conditioning as juveniles on P24. Rats in the first condition, JuvCond-Ext, received conditioning, extinction, and test on consecutive days (from P24-26). Approximately 10 days following fear conditioning, rats in the second condition, JuvCond-AdolesExt, were given extinction training and test as adolescents (from P34-36). Animals in each condition were injected with either MK801 (0.1mg/kg, s.c.) or saline prior to extinction training.

Results

Baseline. Levels of freezing during baseline sessions were low (i.e., all means < 10%) in all groups across all three phases of the experiment (i.e., fear acquisition, extinction training, and extinction retention test). There were no differences between groups depending on future allocation to age at extinction or drug type in baseline freezing levels prior to fear conditioning (Fs < 1), extinction training (largest $F_{1, 45}$ = 2.91, p = .10: drug main effect), or extinction recall (Fs < 1; **Table 3.1**).

Table 3.2

Mean (±SEM) percent baseline freezing before conditioning, extinction, and test in Experiment 3.2.

	JuvCond-Ext Saline (*n* = 12)	**JuvCond-Ext MK801 (*n* = 12)**	**JuvCond-AdolesExt Saline (*n* = 11)**	**JuvCond-AdolesExt MK801 (*n* = 11)**
Conditioning	0.0 (0.0)	0.5 (0.5)	0.2 (0.2)	0.2 (0.2)
Extinction	7.3 (2.7)	3.4 (1.3)	9.0 (3.0)	4.8 (2.0)
Test	3.6 (1.5)	3.4 (1.6)	3.8 (1.5)	2.1 (2.1)

Conditioning. Across CS-US pairings, levels of CS-elicited freezing increased ($F_{1.60, 67.06}$ = 31.47, p < .001), and there were no effects of group or drug (Fs < 1). However, there was a trial × group × drug interaction ($F_{1.60, 67.06}$ = 3.91, p =. 03; **Figure**

3.2A), as a result of a group × drug interaction at Trial 2 ($F_{1, 42} = 4.80$, $p = .03$) and no differences in freezing between groups by the third trial of fear conditioning (largest $F_{1, 42} = 1.30$, $p = .26$). As such, there was some evidence that animals in conditions MK801 JuvCond-Ext and Saline JuvCond-AdolesExt acquired fear more rapidly at Trial 2 but by the end of conditioning, overall levels of fear were equivalent across groups.

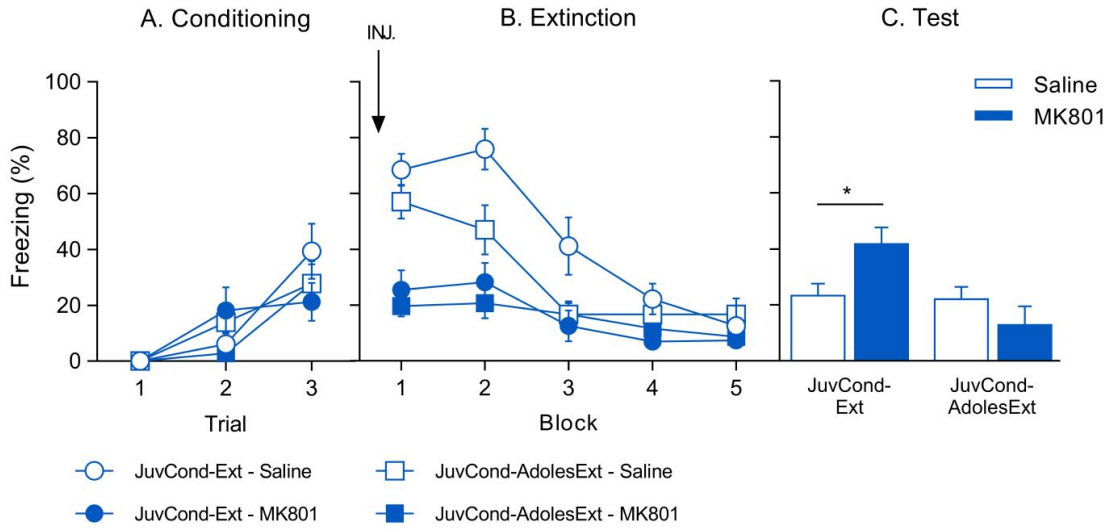

Figure 3.2. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) in groups JuvCond-Ext – Saline, JuvCond-Ext – MK801, JuvCond-AdolesExt – Saline, and JuvCond-AdolesExt – MK801.
***** indicates a significant effect of post-hoc comparison ($p < .05$).

Extinction. Given the group × drug interaction on Trial 2 of fear conditioning, this variable was used as a co-variate in the analysis of CS-elicited freezing during extinction training. During extinction training, there was a significant effect of block ($F_{2.84, 119.46} = 40.50$, $p < .001$; **Figure 3.2B**). Further, there were two significant interactions: block × group ($F_{2.84, 119.46} = 2.99$, $p = .04$) and block × drug ($F_{2.84, 119.46} = 10.75$, $p < .001$). The former interaction reveals that while freezing decreased significantly over extinction blocks, saline-treated JuvCond-AdolesExt rats extinguished at a faster rate than saline-treated JuvCond-Ext rats. The latter interaction

indicates that the difference in freezing between the saline and MK801 groups was largest at the start of extinction training.

Due to the locomotor effects of the drug, MK801-treated rats had lower levels of freezing during extinction training than saline-treated rats, as demonstrated by the significant effect of drug ($F_{1, 42} = 28.31$, $p < .001$). The effect of MK801 on freezing during extinction training did not differ between JuvCond-Ext and JuvCond-AdolesExt groups (drug × group interaction $F_{1, 42} = 2.40$, $p = .13$), indicating that MK801 had similar motoric effects in both groups.

Test. As illustrated in **Figure 3.2C**, there were significant differences in CS-elicited freezing across groups at test ($F_{1, 42} = 8.54$, $p = .006$, $\eta_p^2 = .17$). The group × drug interaction was also significant ($F_{1, 42} = 7.09$, $p = .01$, $\eta_p^2 = .14$). Post-hoc comparisons revealed significant differences across drug treatments in rats trained and extinguished as juveniles (MK801 vs. SAL: $t_{20} = 2.67$, $p = .02$), indicating that MK801 impaired extinction retention in juvenile rats and replicating previous findings (Langton et al., 2007). However, there was no difference between drug treatments in rats trained as juveniles and extinguished as adolescents (MK801 vs. SAL: $t_{22} = 1.20$, $p = .24$). Both groups that were conditioned as juveniles and extinguished as adolescents showed low levels of freezing at test, consistent with previous findings that rats conditioned as juveniles and extinguished as adolescents do not recruit signalling cascades downstream of NMDAR activation in the BLA or IL during extinction training (Baker & Richardson, 2015).

Experiment 3.2

NMDAR-independent extinction is not altered by a shorter interval between conditioning and extinction

The results of Experiment 3.1 demonstrated that rats conditioned as juveniles and extinguished as adolescents do not use NMDARs in extinction when a delay of about ten days is introduced between these procedures. I predicted that NMDAR-independent extinction in these animals was determined by their transition to adolescence, rather than the interval of around ten days. Therefore, changing the duration of the interval between conditioning and extinction (i.e., either shortening or lengthening it) should not cause extinction to be NMDAR-dependent in these animals.

In Experiment 3.2, I tested whether MK801 impaired extinction retention in rats with a shorter delay of 6 days between conditioning at P26 and extinction at P32. By moving the conditioning age from P24 to P26, and extinction age from P34-36 to P32, I tested the boundary conditions of this procedure (i.e., that of conditioning as juveniles and extinguished as adolescents). Half the animals were injected with MK801 prior to extinction training while the other half were injected with saline. I predicted that MK801 administration would not have an effect on extinction retention in rats conditioned as juveniles and extinguished as adolescents, even with a shorter delay between the two procedures.

Results

Baseline. There were no differences in baseline freezing levels between groups prior to fear conditioning ($t_7 = 1.00$, $p = .35$), extinction training ($t_{8.45} = 1.55$, $p = .16$), or test ($t_{13.89} = .00$, $p = 1.00$; **Table 3.3**).

Table 3.3

Mean (±SEM) percent baseline freezing before conditioning, extinction, and test in Experiment 3.2.

	Saline JuvCond-AdolesExt (*n* = 8)	**MK801 JuvCond-AdolesExt (*n* = 8)**
Conditioning	0.0 (0.0)	0.31 (0.31)
Extinction	15.94 (6.41)	5.52 (2.07)
Test	6.56 (3.81)	6.56 (4.17)

Conditioning. Across conditioning trials, levels of CS-elicited freezing increased ($F_{1.35, 18.87} = 31.50$, $p < .001$). There was no effect of the subsequent drug or block × drug interaction, indicating equivalent fear learning between groups (Fs < 1; **Figure 3.3A**).

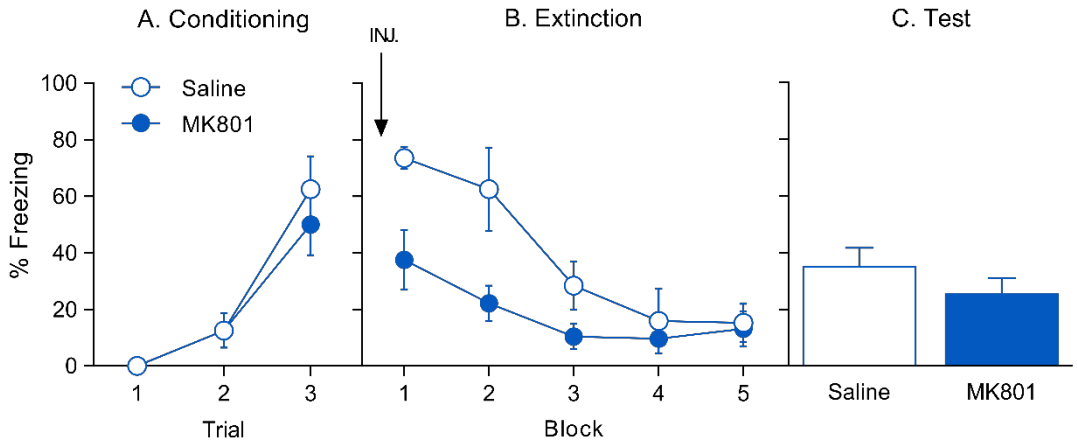

Figure 3.3. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) in groups JuvCond-AdolesExt – Saline and JuvCond-AdolesExt – MK801.

Extinction. During extinction training, there was a main effect of block ($F_{2.55, 35.72} = 19.80$, $p < .001$), drug ($F_{1, 14} = 5.16$, $p = .04$), and a block × drug interaction ($F_{2.55, 35.72} = 4.03$, $p = .02$; **Figure 3.3B**). These results demonstrate that overall, CS-elicited freezing was higher in saline-treated animals than MK801-treated animals during

extinction training. At the end of extinction, CS-elicited freezing was comparable in saline- and MK801-treated rats.

Test. At test, both groups showed low, and comparable, levels of freezing to the CS ($t_{14} = 1.16$, $p = .27$, $d = 0.58$; **Figure 3.3C**). As predicted, MK801-treated rats did not demonstrate impaired extinction retention relative to saline-treated rats at test. These results once again show that animals trained as juveniles and then extinguished as adolescents do not use NMDARs to inhibit fear during extinction, replicating the findings reported in Experiment 3.1. The results of the present experiment also show that the earlier findings were not due to the longer duration between conditioning and extinction. The results of the present experiment also show that the earlier findings were not due to the long duration (i.e., 10 days) between conditioning and extinction as the same effect was observed with a shorter delay (i.e., 6 days) that still spanned the transition between the juvenile and adolescent periods.

Experiment 3.3

NMDAR involvement in extinction during adolescence depends on age at conditioning

The results of Experiments 3.1 and 3.2 demonstrated that NMDARs are not required for extinction in adolescent rats that were conditioned as juveniles. However, neither of the two experiments reported allow me to make a direct comparison between the two groups of interest in my thesis: adolescent rats that show impaired extinction retention and adolescent rats that show good extinction retention. Therefore, it was important to compare rats conditioned as juveniles and extinguished as adolescents to a group of rats conditioned and extinguished as adolescents in Experiment 3.3.

To allow for impairing effects of MK801 to be seen in rats conditioned and extinguished as adolescents, two days of extinction training were required (Baker & Richardson, 2015; McCallum et al., 2010). With these extended extinction procedures adolescent rats have impaired extinction retention when MK801 is administered prior to the second extinction session (Baker & Richardson, 2017).

In Experiment 3.3, I compared the effects of MK801 administration on extinction in two groups of adolescent rats: those that acquired fear as juveniles (JuvCond-AdolesExt*)* and those that acquired fear as adolescents (AdolesCond-Ext*)*. To ensure saline-treated rats in both groups would show good extinction retention, rats conditioned and extinguished as adolescents received two days of extinction training. In contrast, rats conditioned as juveniles and extinguished as adolescents only required one extinction training session. MK801 administration occurred on the day prior to test in both groups: the second day of extinction training in AdolesCond-Ext rats, and the only day of extinction training in JuvCond-AdolesExt rats.

Based on past work (e.g., Baker & Richardson, 2017) and the results of Experiments 3.1 and 3.2 in this chapter, it was expected that extinction retention in rats conditioned as juveniles and extinguished as adolescents would not be affected by MK801 administration. In contrast, MK801 administration was expected to impair extinction retention in rats conditioned and extinguished as adolescents.

The procedures followed those used in Experiment 3.1 with the exception that the shock intensity during conditioning was increased from 0.4mA to 0.5mA to test the generality of the findings while also reducing the likelihood of floor effects at test. As expected, freezing in both groups during CS-US acquisition at conditioning was higher than in Experiment 3.1 (compare **Figure 3.4**, panel A to **Figure 3.2**, panel A). One rat

was excluded from Experiment 3.3 due to being a statistical outlier at test (group Saline AdolesCond-Ext).

Results.

I analysed the first day of extinction training for AdolesCond-Ext rats separately, and then compared the second day of extinction training for AdolesCond-Ext rats to the first day of extinction training for JuvCond-AdolesExt rats. This allowed a direct comparison of the effects of MK801 administration across conditions.

Baseline. Levels of baseline freezing were similar across groups during all stages of the experiment (conditioning: $Fs < 1$; extinction day 1: $t_{21} = .29$, $p = .78$; extinction day 2: largest $F_{1, 51} = 2.36$, $p = .13$: test: largest $F_{1, 51} = 1.10$, $p = .30$; **Table 3.4**).

Table 3.4

Mean (±SEM) percent baseline freezing before conditioning, extinction, and test in Experiment 3.3.

	JuvCond-AdolesExt Saline (*n* = 16)	**JuvCond-AdolesExt MK801 (*n* = 16)**	**AdolesCond-Ext Saline (*n* = 11)**	**AdolesCond-Ext MK801 (*n* = 12)**
Conditioning	1.3 (1.1)	0.5 (0.3)	0.2 (0.2)	0.4 (0.3)
Extinction 1	-	-	3.0 (1.4)	3.6 (1.6)
Extinction 2	12.0 (3.6)	5.6 (3.0)	4.3 (1.8)	4.4 (1.4)
Test	4.8 (3.5)	5.2 (1.9)	3.4 (1.9)	1.3 (0.9)

Conditioning. CS-elicited freezing increased across conditioning trials ($F_{1.53, 78.01} = 75.53$, $p < .001$; **Figure 3.4A**). There was no significant effect of condition ($F_{1, 51} = 3.45$, $p = .07$) or drug ($F < 1$), or interactions between condition and drug ($F < 1$) or condition and trial ($F_{1.53, 78.01} = 1.95$, $p = .16$), indicating that all groups had similar

overall levels of CS-elicited freezing during conditioning, and comparable rates of acquisition.

Extinction 1. The first day of extinction training (when both groups that had been conditioned as adolescents were drug-free) proceeded as expected (**Figure 3.4B**). Both groups showed evidence of within-session extinction ($F_{2.87, 60.28} = 36.13$, $p < .001$). There were no differences between groups that were to be injected with saline or MK801 the following day (drug main effect $F_{1, 21} = 1.70$, $p = .21$; drug × trial interaction $F < 1$).

Extinction 2. When all four groups received extinction training, there was a decrease of levels of CS-elicited freezing over blocks ($F_{3.12, 159.08} = 68.70$, $p < .001$; **Figure 3.4C**). As in the previous experiment, MK801-treated animals had lower levels of CS-elicited freezing (drug main effect $F_{1, 51} = 33.36$, $p < .001$; drug × block interaction $F_{3.12, 159.08} = 18.29$, $p < .001$). In addition, there was a significant effect of condition ($F_{1, 51} = 5.70$, $p = .021$), a condition × drug interaction ($F_{1, 51} = 5.42$, $p = .024$), a condition × block interaction ($F_{3.12, 159.08} = 4.77$, $p = .003$), and a condition × drug × block interaction ($F_{3.12, 159.08} = 5.69$, $p = .001$). Not surprisingly, these analyses confirmed that there was a "savings effect". That is, AdolesCond-Ext rats, in the saline group, had overall lower levels of freezing and extinguished faster during their second day of extinction training compared to the JuvCond-AdolesExt rats, in the saline group, on their first day of extinction training. Importantly, both groups of saline-treated rats showed similar levels of freezing on their first day of extinction (group difference: $F < 1$, $p = .90$). Further, MK801 administration had the same effect for both groups when "on board", as can be seen in Figure 3.4C.

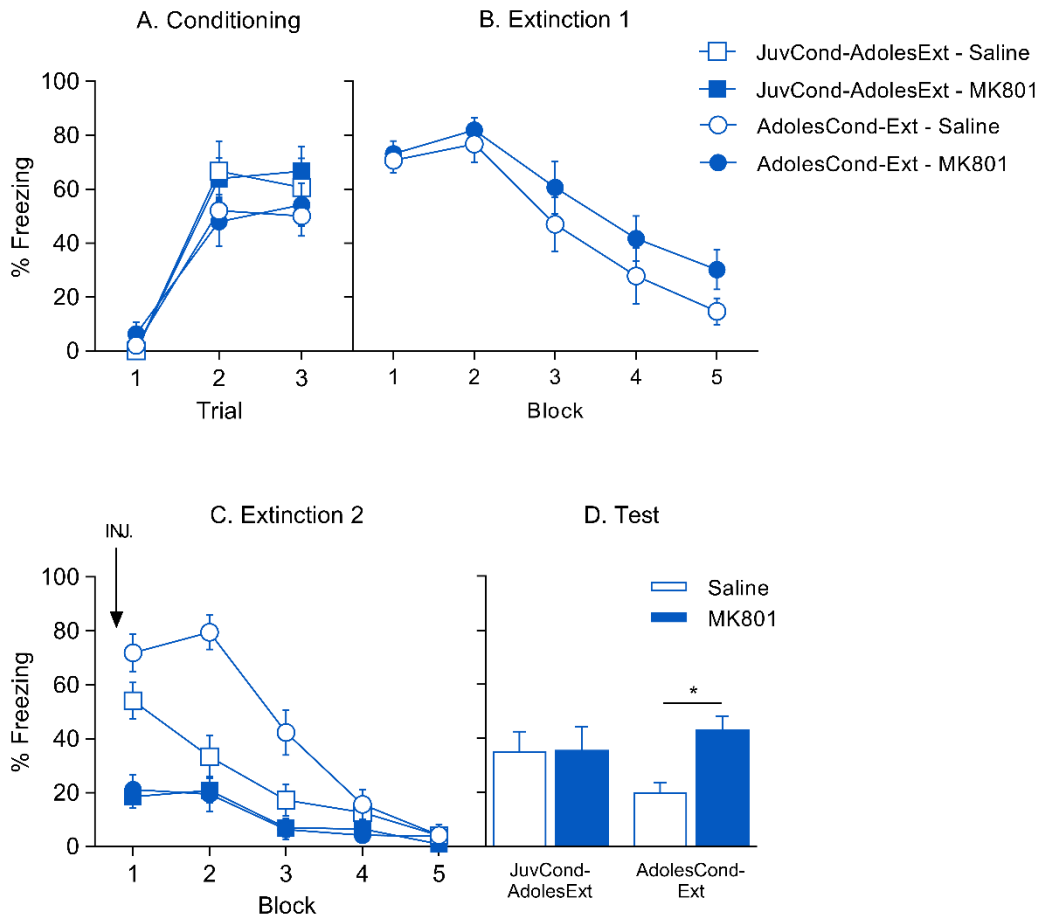

Figure 3.4. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training day one (**B**), extinction training day two (**C**), and extinction retention test (**D**) in groups JuvCond-AdolesExt – Saline, JuvCond-AdolesExt – MK801, AdolesCond-Ext – Saline, and AdolesCond-Ext – MK801.
***** indicates a significant *a priori* comparison ($p < .05$).

Test. Both groups given saline had low levels of CS-elicited freezing at test (see **Figure 3.4D**), which was as expected given that those conditioned in adolescence were given two days of extinction and those trained as juveniles normally show good extinction retention following even one day of extinction. Further, rats conditioned and extinguished as adolescents that were given MK801 prior to extinction training showed higher CS-elicited freezing at test relative to their saline counterparts. However, MK801 administration did not affect CS-elicited freezing in rats conditioned as juveniles and extinguished as adolescents.

This description of the data was confirmed by statistical analyses. Specifically, replicating the results of Experiment 3.1, but with a higher shock intensity, MK801 did not impair extinction retention in rats conditioned as juveniles and extinguished as adolescents ($t_{29.22} = 0.04$, $p = .97$, $d = 0.01$). In contrast, MK801-treated rats conditioned and extinguished as adolescents showed a greater return of fear at test relative to their saline-treated counterparts ($t_{19.97} = 3.90$, $p = .001$, $d = 1.62$). Further, saline-treated rats that were conditioned and extinguished in adolescents showed good extinction retention, replicating previous studies (e.g., Baker & Richardson, 2017; McCallum et al., 2010) demonstrating the benefits of extended extinction training for this age group.

Experiment 3.4

A delay between conditioning and extinction does not lead to NMDAR-independent in extinction in adult rats

My findings so far suggest that adolescent rats conditioned as juveniles recruit an NMDAR-independent process to extinguish fear, in contrast to animals trained and extinguished as juveniles (Experiments 3.1, 3.2) or those trained and extinguished as adolescents (Experiment 3.3). Across these three experiments, a delay of 6-11 days occurred between fear learning and extinction in rats conditioned as juveniles and extinguished as adolescents. Therefore, I next examined another explanation for my findings – that the introduction of a delay converts extinction into a NMDAR-independent process.

In Experiment 3.2, I attempted to address this issue by reducing the delay between conditioning and extinction to 6 days. However, that is still considerably longer than when conditioning and extinction occur on consecutive days. It is not possible to have a shorter delay though and still have conditioning occur in the juvenile

period and extinction in adolescence. Therefore, I took a different approach to this issue in this experiment. Specifically, Experiment 3.4 was designed to test this possibility by inserting either a 1 or 10 day delay between conditioning and extinction in adult rats. Although it would have been desirable to conduct this experiment in adolescent rats, it would introduce an additional variable – specifically, that two days of extinction are required in adolescence for NMDAR involvement. In order to test the effects of a delay on the involvement of NMDARs on the *first* day of extinction training, it was necessary to use adult rats in Experiment 3.4. As in Experiments 3.1 and 3.2, the shock intensity used at conditioning was 0.4 mA. Half of the animals at each interval were injected with MK801 prior to extinction training while the others were injected with saline.

Results.

Baseline. Although levels of baseline freezing were very low for all groups in all phases of this experiment (i.e., all < 5%; **Table 3.5**) there was a significant effect of interval in the conditioning phase ($F_{1, 28} = 7.93$, $p = .009$). That is, those animals to be extinguished 1 day after conditioning had higher levels of baseline freezing during the adaptation period in the conditioning phase relative to those animals to be extinguished 10 days after conditioning. There was also a significant effect of interval for the extinction phase ($F_{1, 28} = 4.46$, $p = .04$), such that animals extinguished 1 day after conditioning had higher levels of baseline freezing than animals extinguished 10 days after conditioning.

Table 3.5

Mean (±SEM) percent baseline freezing before conditioning, extinction, and test in Experiment 3.4.

	1 day Saline (*n* = 8)	**1 day MK801 (*n* = 8)**	**10 day Saline (*n* = 8)**	**10 day MK801 (*n* = 8)**
Conditioning *	3.4 (1.3)	2.5 (1.1)	0.3 (0.3)	0.6 (0.4)
Extinction *	4.1 (1.2)	2.8 (1.5)	1.6 (0.9)	0.6 (0.4)
Test	4.7 (1.5)	2.5 (0.9)	3.4 (2.4)	2.2 (1.3)

* indicates a significant difference ($p < .05$)

Conditioning. Due to the significant effect of interval on baseline freezing levels at the time of conditioning (see above), CS-elicited freezing during conditioning was analysed with ANCOVA using baseline freezing as a covariate. Groups showed equivalent rates of learning across trials ($F_{2, 54} = 40.67$, $p < .001$), with a trial × subsequent drug interaction ($F_{2, 54} = 3.35$, $p = .04$; **Figure 3.5A**). Despite a significant interaction, no effects of drug or group emerged in follow-up analyses of conditioning trials (largest $F_{1, 27} = 2.51$, $p = .13$, Trial 2).

Extinction. Given group differences in baseline freezing levels at extinction, baseline freezing was used as a co-variate in the analysis of CS-elicited freezing during extinction training. There was a significant effect of block ($F_{2.43, 65.68} = 15.44$, $p < .001$; **Figure 3.5B**), as CS-elicited freezing decreased over the course of extinction training. In addition, there was a significant effect of drug ($F_{1, 27} = 39.15$, $p < .001$), due to the MK801-treated rats exhibiting less freezing than saline-treated rats, as in the previous experiments. The drug × block interaction was also significant ($F_{2.43,65.68} = 13.28$, $p < .001$), due to the MK801- and saline-treated rats exhibiting different levels of freezing at the start of extinction training but similar levels by the end. A delay between

conditioning and extinction sessions did not affect within-session extinction (main effect of interval and the interval × drug interaction *Fs* < 1).

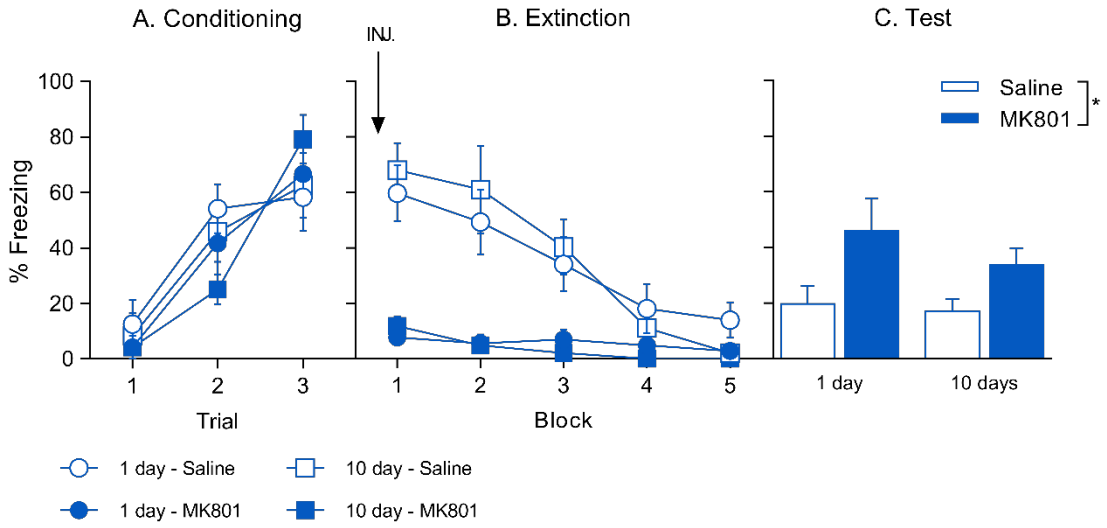

Figure 3.5. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) in groups 1 day – Saline, 1 day – MK801, 10 day – Saline, and 10 day MK801.
***** indicates a significant effect of drug ($p < .05$).

Test. **Figure 3.5C** shows that at test, MK801-treated rats had higher levels of CS-elicited freezing than saline-treated controls, regardless of the interval between conditioning and extinction. This description of the data was supported by the statistical analysis which showed a significant effect of drug treatment ($F_{1, 28} = 8.47$, $p = .007$, $\eta_p^2 = .23$). The effect of delay between conditioning and extinction, as well as the interval × drug interaction, were not significant (*Fs* < 1). Therefore, introducing a 10 day delay between conditioning and extinction sessions does not lead to a switch to an NMDAR-independent form of extinction, at least in adult rats.

Experiment 3.5

The transition from adolescence to adulthood does not lead to NMDAR-independent extinction

As the introduction of a 10 day delay between conditioning and extinction did not lead to NMDAR-independent extinction in adult rats (Experiment 3.4), there is likely a different explanation for why rats conditioned as juveniles and extinguished as adolescents demonstrate NMDAR-independent extinction (Experiments 3.1, 3.2, 3.3). Perhaps instead, the transition from one developmental stage into another (i.e., from the juvenile to the adolescent period) leads to a switch from NMDAR-dependent to NMDAR-independent extinction. If so, extinction may be NMDAR-independent when animals undergo conditioning and extinction in different stages of development, even if extinction does not occur in adolescence. To test this possibility, in Experiment 3.5 rats were conditioned as adolescents on P34-35 but extinction occurred approximately 5 weeks later when the animals were adults (i.e., on ~P70). One rat was excluded from Experiment 3.5 due to being a statistical outlier at test (group Saline AdolesCond-AdultExt).

Results.

Baseline. There were no significant group differences in baseline freezing prior to conditioning (t_{23} = .01, p = .99), extinction training ($t_{11.90}$ = 2.01, p = .07), or test (t_{23} = .70, p = .49; **Table 3.6**).

Table 3.6

Mean (±SEM) percent baseline freezing before conditioning, extinction, and test in Experiment 3.5.

	AdolesCond-AdultExt Saline (*n* = 12)	**AdolesCond-AdultExt MK801 (*n* = 13)**
Conditioning	2.3 (0.9)	2.31 (0.9)
Extinction	15.6 (6.0)	3.3 (1.2)
Test	8.5 (3.0)	11.9 (3.7)

Conditioning. Both groups exhibited an equivalent increase in levels of CS-elicited freezing across training trials ($F_{1.59, 36.59} = 19.02$, $p < .001$), and neither the effect of subsequent drug nor the block × drug interaction was significant ($Fs < 1$; **Figure 3.6A**).

Extinction. During extinction training, there was a main effect of block ($F_{2.81, 64.64} = 19.75$, $p < .001$), drug ($F_{1, 23} = 22.92$, $p < .001$), and a block × drug interaction ($F_{2.81, 64.64} = 19.75$, $p < .001$; **Figure 3.6B**). The drug effect was a result of the rats given MK801 having lower overall levels of CS-elicited freezing than rats that received saline and the interaction was due to the two groups performing differently at the start of extinction training but relatively similarly by the end. It is worth noting that the addition of a 5 week delay between conditioning and extinction did not affect CS-elicited freezing relative to a 1 day delay (compare **Figure 3.6**, panel B to **Figure 3.5**, panel B).

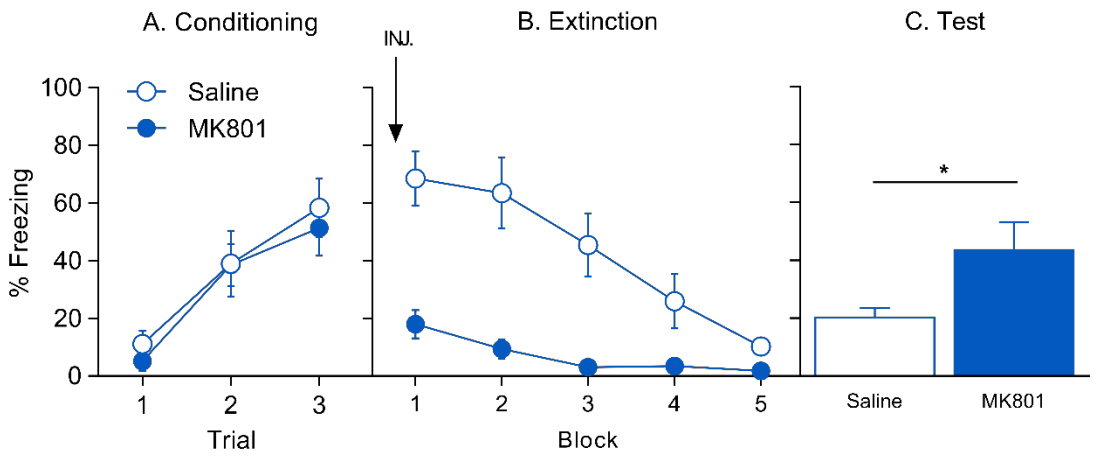

Figure 3.6. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) in groups AdolesCond-AdultExt Saline and AdolesCond-AdultExt MK801.
* indicates a significant effect of drug ($p < .05$).

Test. At test, rats given MK801 the day before during extinction training had higher levels of CS-elicited freezing than rats that received saline prior to extinction ($t_{14.61} = 2.41$, $p = .03$, $d = 0.95$; **Figure 3.6C**). Therefore, the transition from adolescence to adulthood did not change the involvement of NMDARs in extinction, suggesting that it is not the experience of *any* developmental transition that causes extinction to be NMDAR-independent in adolescent rats that acquired fear as juveniles. The findings of Experiment 3.5 also lend further support to the assertion that NMDAR-independent extinction is not due to the mere passage of time between conditioning and extinction (i.e., Experiment 3.4).

Discussion

Although adolescent rodents are typically impaired in extinction retention, Baker and Richardson (2015) reported a circumstance in which they exhibit good extinction retention – when fear is acquired as a juvenile and then extinguished in adolescence. However, expression of pMAPK/ERK in the mPFC and BLA did not increase following extinction training, a pattern of neural activity expected in rats that

demonstrate good extinction retention. The experiments reported in this chapter illustrated another facet of fear inhibition in this group – namely, that NMDARs are not required for successful extinction.

The NMDAR-independent extinction of rats conditioned as juveniles and extinguished as adolescents (Experiments 3.1, 3.2, and 3.3) is in stark contrast to the NMDA-dependent extinction of rats conditioned and extinguished as juveniles (Experiments 3.1), adolescents that received two days of extinction training (Experiment 3.3), and adults (Experiment 3.4 and 3.5). The lack of involvement of NMDARs in extinction in adolescent rats that had acquired fear as juveniles was not a consequence of the delay between conditioning and extinction, as blocking NMDARs prior to extinction resulted in poor extinction retention in adult rats despite the introduction of a similar delay (Experiment 3.4), or an even longer delay (Experiment 3.5). Lastly, antagonising NMDARs prior to extinction training in rats conditioned as adolescents and extinguished as adults impaired extinction retention (Experiment 3.5), indicating that the transition between any developmental phases was not responsible for the NMDAR-independent extinction observed in adolescent rats that acquired fear as juveniles.

Although I predicted that NMDARs would not be involved in extinction in rats conditioned as juveniles and extinguished as adolescents, there is a body of work examining how memories are acquired and then expressed in different developmental stages that would have supported finding that these rats utilise an NMDAR-dependent extinction process. These studies demonstrated that an animal's age at learning determines how the learned fear is later expressed. The first set of studies to investigate this question examined developmental differences in measures of fear expression. In the infant rat, fear is expressed by freezing (Hunt, Richardson, & Campbell, 1994), whereas

fear can be assessed in the juvenile rat by both freezing and fear-potentiated startle (Richardson, Paxinos, & Lee, 2000). Infants cannot express fear via fear-potentiated startle as the neural structures supporting potentiated startle are not yet mature (Barnet & Hunt, 2006; Weber & Richardson, 2001). When rats are conditioned as infants and later tested as juveniles, they do not express fear via fear-potentiated startle. That is, the rats express fear in a manner appropriate to their age at training rather than their age at test (Barnet & Hunt, 2006; Richardson & Fan, 2002). In addition, juvenile rats that were conditioned as infants do not use the neural structures that have matured since the time of conditioning to express fear. The PL region of the mPFC is not required for fear expression in infancy, unlike later in life (Chan et al., 2011; Li, Kim, & Richardson, 2012a). Li et al. (2012b) compared the involvement of the PL in the expression of fear memories in juvenile animals that acquired fear during that developmental stage or earlier during infancy. Only rats conditioned and tested as juveniles showed impaired expression of fear following temporary inactivation of the PL using muscimol; animals that acquired fear in an earlier developmental stage did not require the later maturing PL to express fear. These findings provide supporting evidence that developing animals do not express fear using neural regions that have matured after fear learning.

On the other hand, rats conditioned as juveniles and extinguished as adolescents do not use the neural circuit that was functional when they acquired their fear. Typically, blocking NMDARs impairs extinction retention in juvenile rats, and extinction during this age usually leads to increased pMAPK/ERK expression in the mPFC and amygdala (Baker & Richardson, 2015; Langton et al., 2007). That being said, the brain undergoes quite different neurodevelopmental changes during the juvenile to adolescent transition compared to the infant to juvenile transition (for review, see Semple et al., 2013). Whereas the infant to juvenile transition is

characterised by increased synaptic density and myelination brain-wide, the juvenile to adolescent transition is characterised by experience-dependent synaptic pruning (e.g., of mPFC inputs to the BLA) and cell death (Cressman et al., 2010; Spear, 2013; Yu et al., 2013; see Zimmermann et al., 2019 for review). Consequently, the infant to juvenile transition is fundamentally different to the juvenile to adolescent transition. As a result, there are significant limitations in generalising the molecular mechanisms of extinction across the transition from the infant to juvenile period to that of the juvenile to adolescent period.

Further highlighting the unique neurobiology of extinction in rats conditioned as juveniles and extinguished as adolescents is the fact that the mechanisms underlying other circumstances of NMDAR-independent extinction do not easily explain why extinction is NMDAR-independent in this circumstance. In the case of re-extinction in adult rats, it has been proposed that the neurobiology of re-extinction is different to extinction for the first time as it involves the recall and subsequent reconsolidation of an existing extinction memory (Langton & Richardson, 2009; Lingawi, Laurent, Westbrook, & Holmes, 2018). However, rats conditioned as juveniles and extinguished as adolescents cannot be retrieving a prior memory of extinction as this was the first instance of extinction training.

In a second instance of NMDAR-independent extinction, it was proposed that the immaturity of the mPFC during infancy contributes to NMDAR-independent extinction at that age (for review, see Kim & Richardson, 2010). This conclusion was based on the finding that temporary inactivation of the mPFC (using bupivacaine, a sodium channel modulator) during extinction training did not affect extinction retention in infant rats, unlike juvenile and adult rats (Kim et al., 2009; Sierra-Mercado, Corcoran, Lebrón-Milad, & Quirk, 2006). This explanation cannot account for

NMDAR-independent extinction in adolescent rats that acquired fear as juveniles for two reasons: first, the mPFC is involved in extinction in juvenile rats, and second, adolescent rats can recruit NMDARs on the second day of extended extinction training.

Although infant rats do not require NMDARs during extinction, activation of the BLA is required. Indeed, temporary inactivation of this region (using bupivacaine) during extinction training results in extinction memory deficits in infant rats (Kim & Richardson, 2008). This finding is consistent with the developmental trajectory of the mammalian brain, as the amygdala is sufficiently mature to be involved in the aversive fear circuit by P10 (Tallot, Doyère, & Sullivan, 2016). As the amygdala is mature enough to support extinction in adolescence, extinction in rats conditioned as juveniles and extinguished as adolescents may also rely on activation of the BLA during extinction in the absence of NMDARs (as it does in infants). However, as Baker and Richardson (2015) did not find increased expression of pMAPK/ERK in the BLA following extinction training in these animals, the BLA may not be involved in extinction in this group of adolescent rats. Indeed, the BLA is not required for the second instance of NMDAR-independent inhibitory learning, re-extinction (for a review, see Lingawi et al., 2018). It should be noted that to date, no studies have confirmed that the BLA is involved in extinction in adolescent rodents under any circumstances, let alone in adolescent rats that acquired fear as juveniles.

In the absence of NMDAR activation, it is possible that another glutamate receptor subtype was involved during extinction in rats conditioned as juveniles and extinguished as adolescents. One candidate is metabotropic glutamate receptor type 5 (mGluR5), as their activation can induce long-term potentiation, the synaptic process required for the formation of new inhibitory memories (Bortolotto & Collingridge, 1993; Maren, 2015). In adult rodents, genetic deletion or pharmacological blockade of

mGluR5 prior to extinction impairs extinction recall in adult rodents, and this has been isolated to the actions of mGluR5 in the IL (Fontanez-Nuin et al., 2011; Xu, Zhu, Contractor, & Heinemann, 2009). In adolescent rats, administration of the mGluR5 antagonist MPEP 30 min prior to extinction training prevents extinction-induced synaptic plasticity in the IL, and impairs extinction retention (Sepulveda-Orengo, Lopez, Soler-Cedeno, & Porter, 2013). Therefore, activation of mGluR5 in the IL may perhaps be one neural mechanism involved in fear extinction in rats conditioned as juveniles and extinguished as adolescents.

It is important to acknowledge a limitation of the use of MK801 as an NMDAR antagonist in the current thesis. Although MK801 acts primarily as an NMDAR antagonist, it has some actions as a nicotinic acetylcholine receptor antagonist (Amador & Dani, 1991; Ramoa et al., 1990). However, MK801 has much more potency (around 100 times) at NMDARs than nicotine receptors (Briggs & McKenna, 1996; Löscher, Potschka, Wlaź, Danysz, & Parsons, 2003) and has little effect on nicotine responses when used alongside behavioural tasks (Zakharova, Danysz, & Bespalov, 2005). Therefore, it is possible, but unlikely, that some of the observed effects of MK801 are due to antagonistic actions on acetylcholine receptors.

Taken together, the experiments in this chapter suggest that different pharmacological adjuncts may be appropriate for those adolescents who acquired fear during childhood versus those adolescents who acquired fear during adolescence. As rats conditioned as juveniles and extinguished as adolescents do not require NMDARs to extinguish fear, it is also possible that extinction in these animals would not benefit from pharmacological adjuncts which work to increase NMDAR activation, such as D-cycloserine. Indeed, if it is the case that NMDARs are not involved in extinction in any capacity in adolescents who acquired fear as juveniles, this may explain why clinical

trials examining the benefits of D-cycloserine alongside exposure therapy in adolescents have been unsuccessful or inconsistent (e.g., Farrell et al., 2013, 2018). Specifically, it may be the case that only those adolescents who acquired fear as adolescents (and not earlier) benefit from the use of D-cycloserine as an adjunct. In any case, this work highlights the importance of taking a thorough history of the presenting problem during clinical assessment, and formulating treatment plans and possible pharmacological adjuncts accordingly.

The findings reported in this chapter clearly demonstrated the NMDAR-independent extinction of rats conditioned as juveniles and extinguished as adolescents, and explored possible explanations for this finding. Across several experiments, I found that neither the delay between conditioning and extinction, nor the experience of a developmental transition, altered the typical requirement for NMDARs during extinction. Although the neurobiological mechanisms involved in extinction in rats conditioned as juveniles and extinguished as adolescents remain to be determined, the current findings highlight that the neural basis of extinction consolidation is qualitatively different in this group. Indeed, these findings bring into question whether there are also differences in the neural mechanisms which underlie extinction learning during adolescence, particularly for those adolescent rats that acquired fear as juveniles.

Chapter 4: Involvement of opioid receptors in extinction in adolescent rats

Together, the findings of Chapters 2 and 3 indicate that the neurotransmitter systems involved in extinction consolidation (i.e., endocannabinoid, glutamatergic) during adolescence are different to those involved during adulthood. Perhaps, there are also developmental differences in the neurotransmitters involved in extinction *learning*, given that this process is essential for consolidation. Therefore, in this chapter I investigated the involvement of the opioid system in extinction learning during adolescence, as opioid neurotransmission is critical for extinction learning in adulthood (McNally, 2009).

There are several processes by which reductions in conditioned fear responses are achieved across extinction training (for reviews, see Cooper, Clifton, & Feeny, 2017; Craske et al., 2018; Weisman & Rodebaugh, 2018). As discussed in Chapter 1 (p. 4), extinction is considered an experimental model of exposure therapy, and involves the acquisition of a new inhibitory association of the feared stimulus and the absence of the expected aversive outcome (Bouton, 2002). The acquisition of extinction learning is dependent on the recognition of the discrepancy between the expected outcome (e.g., in animal models, a US such as a footshock) and the actual outcome (e.g., no US such as a footshock). The identification of this discrepancy, termed *prediction error*, is critical for new associative learning (Fanselow, 1998; Rescorla & Wagner, 1972). Learning theory posits that the larger the prediction error signal, the greater the learning. In rodent models, this has been shown to be the case for both positive prediction error (i.e., when the actual outcome is *greater* than the expected outcome) and negative prediction error (i.e., when the actual outcome is less than the expected outcome; Delamater & Westbrook, 2014). As presentations of the feared stimulus are *less* aversive than

expected during fear extinction, extinction learning involves the generation of negative prediction error.

Adolescent rodents and humans show age-specific deficits in extinction learning and/or retention, and this impairment may be due, at least in part, to impaired prediction error signalling. There is preliminary evidence to support this suggestion. Specifically, Waters et al. (2017) examined differences in extinction using a differential aversive conditioning procedure in healthy children, adolescents, and adults. In this procedure, individuals rated the valence of reinforced (CS+) and non-reinforced (CS-) conditioned stimuli across conditioning, extinction training, and testing. Although all age groups similarly rated the CS+ as more negative than the CS- during conditioning, adolescents reported less positive re-evaluations of the CS+ during extinction training than children and adults, suggesting that adolescents did not use the new information about the stimulus to update their responding. This developmental difference in re-evaluations also persisted to test the following day. While the aforementioned findings are not a direct indication of a deficit in prediction error in adolescents, they do suggest age-related differences in extinction learning, a process which is underpinned by prediction error.

Opioid receptor-mediated prediction error instructs fear extinction

The role of prediction error in extinction learning has been studied more closely in preclinical models using pharmacological adjuncts which disrupt the neural signalling underlying prediction error. Opioid neurotransmission has been identified as a neural signal of prediction error, as blocking opioid receptor activation impairs an animal's ability to learn new information about aversive stimuli (for review, see McNally et al., 2011). For example, the opioid receptor antagonist naloxone prevents one-trial blocking of fear learning, indicating that opioids reduce prediction error during

fear learning (Cole & McNally, 2007a). Furthermore, Cole and McNally (2007b) used a blocking-unblocking design to specifically examine negative prediction error. In this design, the role of opioid receptors in both positive and negative prediction error could be evaluated. Briefly, in the third stage of the blocking-unblocking design, animals are expected to decrease their fear to a CS due to the generation of negative prediction error. However, rats treated with naloxone prior to the third stage continued to show high fear responding to the CS, suggesting that negative prediction error was impeded by opioid receptor blockade. Simultaneously, the experiment also revealed that positive prediction error was increased with naloxone (as demonstrated by an increase in fear to a previously blocked CS – "unblocking"), suggesting that opioid receptor activation also mediates positive prediction error.

With respect to extinction specifically, the role of opioids in regulating negative prediction error was first demonstrated using systemic administrations of naloxone prior to extinction training in adult rats (McNally & Westbrook, 2003). In contrast to saline-treated animals, those that were treated with naloxone showed significantly higher CS-elicited freezing across extinction training sessions, indicative of impaired extinction learning. This impairment in extinction learning persisted to test the following day, as rats treated with naloxone the day before showed greater CS-elicited freezing relative to rats treated with saline. Subsequent experiments determined that blocking opioid receptor activation prior to extinction training had impaired extinction *learning*, rather than consolidation, as post-extinction administration of naloxone had no effect on extinction retention the following day.

The actions of opioid receptors during extinction training have been isolated to the vlPAG, a structure in the midbrain (Herry & Johansen, 2014). This has been demonstrated by findings that targeted pharmacological blockade of opioid receptors

within the vlPAG impairs extinction learning and subsequent retention in adult rats (McNally et al., 2004). Later work extended these findings, and explored whether opioid receptor activation in the vlPAG was linked to synaptic plasticity (as assessed by pMAPK/ERK expression) in two other structures involved in fear extinction: the mPFC and amygdala. To do so, Parsons et al. (2010) blocked opioid receptor activation during extinction training in the vlPAG using naloxone infusions, and then assessed pMAPK/ERK expression in the mPFC and amygdala 45 min following extinction training. Some animals were not sacrificed for histology and underwent an extinction retention test the following day. As reported previously, naloxone-treated adult rats showed impaired extinction retention relative to saline-treated adult rats. Interestingly, pMAPK/ERK expression in both the mPFC and amygdala of naloxone-treated rats after extinction training was significantly lower than that of saline-treated rats (to the extent that it was equivalent to pMAPK/ERK expression observed in home-cage control rats). This finding indicates that opioid receptor activation in the vlPAG at the time of extinction training regulates activity-dependent synaptic plasticity in the mPFC and amygdala, which is critical for extinction retention.

As extinction relies on opioid receptor-mediated prediction error signalling within the vlPAG (Parsons et al., 2010), deficits in prediction error signalling during adolescence may result in a lack of extinction-induced synaptic plasticity. In support of this suggestion, rats conditioned and extinguished as adolescents show dysregulated synaptic plasticity within the mPFC and amygdala following one session of extinction training. Specifically, adolescent rats show lower pMAPK/ERK expression in these regions compared to juvenile and adult rats, and equivalent pMAPK/ERK expression to rats that did not undergo extinction training (Baker & Richardson, 2015; Kim, Li, & Richardson, 2011). This pattern is similar to what Parsons et al. (2010) observed in

naloxone-treated adult rats following extinction training. Although correlational, a lack of extinction-induced synaptic plasticity within the mPFC and amygdala of adolescent rats after a single session of extinction training may be a function of impaired opioid receptor signalling in the vlPAG during extinction learning.

In contrast to the work conducted in adult rodents, the role of opioid receptor-mediated prediction error signalling during adolescence has not been investigated. Given that preclinical research has demonstrated that deficits in prediction error can result in individuals not using new information about a feared stimulus to update their behavioural responding (Cole & McNally, 2007a), deficits in prediction error may account for impairments in extinction learning and/or retention during adolescence that have been reported across both rodent and human species (Bisby, Richardson, & Baker, 2020; Ganella et al., 2018; McCallum et al., 2010; Pattwell et al., 2012). Furthermore, deficits in prediction error may possibly contribute to poor exposure therapy outcomes in this age group. Therefore, in Chapter 4 I explored the use of opioid receptor-mediated prediction error signalling in extinction learning and retention in adolescent rats. I first replicated the extinction-impairing effects of systemically blocking opioid receptor activation using the opioid receptor antagonist naloxone in adult rats, prior to examining adolescent rats. In addition, I examined the involvement of opioid receptors in two cases of good extinction during adolescence: adolescent rats that receive two days of extinction training, and rats conditioned as juveniles and extinguished as adolescents.

Methods

Subjects

One hundred and ten experimentally naïve Sprague-Dawley male rats were used in the experiments reported in this chapter.

Procedure

These experiments used the same apparatus as was used in the experiments described in Chapter 2. Briefly, rats received pre-exposure and fear conditioning in Context A chambers whereas extinction and test occurred in Context B chambers. The intensity of the footshock was set to 0.4mA for all experiments. In Experiment 4.4, locomotor and anxiety-like behaviour was assessed using the open field test. A summary of the procedures used across experiments in Chapter 4 is presented in Table 4.1.

Drugs

Naloxone hydrochloride (Sigma-Aldrich; N7758) was dissolved in saline (0.9% wt/vol). Animals were injected subcutaneously in the nape of the neck with naloxone or saline (both in a volume of 1ml/kg) 10 min before the extinction training session, based on doses and time of administration procedures in previous publications (Kim & Richardson, 2009; McNally & Westbrook, 2003). Naloxone was administered at a dose of 5mg/kg in all experiments, as well as at 2.5mg/kg and 10mg/kg in a dose-response study in Experiment 4.3.

Data Analysis

Statistical analyses were conducted as in Chapter 2. A random sample of ~30% of the test data in this chapter was cross-scored by an observer unaware of the experimental conditions. Inter-rater reliability was high for all experiments (*r*s = .92 - .99).

Table 4.1. Summary of procedures across Experiments 4.1-4.6 in Chapter 4.

	Age group/s	Handling	Pre-exposure	Conditioning	Injection	Extinction Day 1	Extinction Day 2	Retention	Open Field
4.1	Adult	■	■	■	■	■		■	
4.2	Adolescent	■	■	■	■	■		■	
4.3	Adolescent	■	■	■	■	■	■	■	
4.4	Adolescent	■			■				■
4.5	Juvenile JuvCond-AdolesExt	■	■	■	■	■		■	
4.6	JuvCond-AdolesExt	■	■	■	■	■		■	

Note. Grey shading indicates the inclusion of the procedures in each experiment.

Experiment 4.1

Opioid receptors are required for extinction learning in adult rats

Prior to investigating the role of prediction error during extinction in adolescents, Experiment 4.1 aimed to replicate previous findings in adult rats demonstrating impaired extinction learning and retention following pre-extinction administration of naloxone (McNally & Westbrook, 2003). To examine this question, adult rats were systemically administered either naloxone (5 mg/kg) or saline prior to extinction training and extinction retention was tested the following day.

Baseline. Levels of freezing during baseline sessions were low in both groups, and there were no differences between groups depending on future allocation to drug type in baseline freezing levels prior to conditioning ($t_{13} = .53$, $p = .60$), extinction training ($t_{13} = .54$, $p = .60$), or extinction retention test ($t_{13} = 1.21$, $p = .25$; **Table 4.2**).

Table 4.2

Mean (±SEM) percent baseline freezing before conditioning, extinction training, and extinction retention test in Experiment 4.1.

	Saline (*n* = 8)	**Naloxone (*n* = 7)**
Conditioning	3.75 (2.63)	6.43 (4.46)
Extinction	0.31 (0.31)	0.71 (0.71)
Test	7.50 (2.63)	17.50 (8.38)

Conditioning. Across conditioning, CS-elicited freezing increased across trials ($F_{2, 26} = 73.11$, $p < .001$; **Figure 4.1A**) with no differences between subsequent drug groups or drug × trial interaction (largest $F_{2, 26} = 2.22$, $p = .13$).

Extinction. Adult rats injected with naloxone prior to extinction training had persistently high levels of freezing across extinction training, whereas saline-treated

adult rats showed a decrease in freezing across this session (**Figure 4.1B**). A two-way ANOVA detected a significant effect of block ($F_{4, 52} = 3.41$, $p = .02$) and drug ($F_{1, 13} = 20.15$, $p = .001$) but no block × drug interaction ($F_{4, 52} = 1.96$, $p = .12$). These results suggest that naloxone impaired the acquisition of extinction in adult rats.

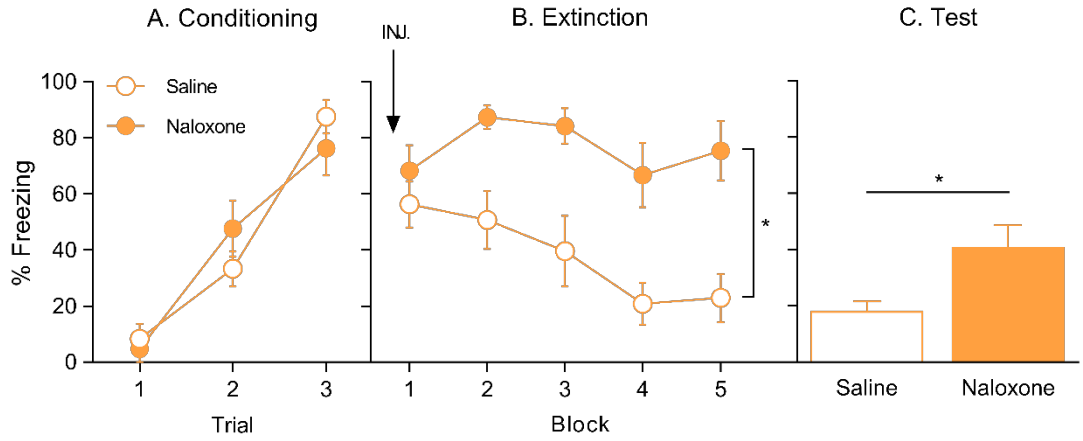

Figure 4.1. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) in adult rats in groups Saline and Naloxone.
* indicates a significant effect of drug ($p < .05$).

Test. When tested the next day, naloxone-treated adult rats showed significantly higher levels of CS-elicited freezing than saline-treated rats ($t_{13} = 2.84$, $p = .01$, $d = 1.43$; **Figure 4.1C**). This suggests that blocking opioid receptor activation during extinction training impaired extinction learning and resulted in impaired extinction retention the following day, consistent with the extinction-impairing effects of opioid receptor blockade reported by McNally and Westbrook (2003).

Experiment 4.2

Opioid receptors are not required for extinction learning in adolescent rats

After replicating that extinction in adult rats is dependent upon opioid receptors, I next sought to investigate whether opioid receptor activation during extinction is impaired in adolescent rats. If the impairment in extinction retention in adolescent rodents and humans is due to a complete failure to engage opioid receptor-dependent prediction error mechanisms during extinction training, blockade of opioid receptors via naloxone should have no effect on CS-elicited freezing during extinction training and test. Alternatively, it is also possible that adolescents may partially use opioid receptor-mediated prediction error to extinguish fear because conditioned responses typically decrease across extinction training and return to a lower level (albeit still at high levels) the following day compared to non-extinguished controls in adolescent rats and humans (see Baker et al., 2016). In that case, naloxone administration would be expected to increase CS-elicited freezing in adolescent rats across extinction training and at test. Therefore, Experiment 4.2 investigated the role of opioid receptor-mediated prediction error signalling in adolescence by administering adolescent rats either naloxone or saline prior to extinction training using similar procedures as in Experiment 4.1 with adults.

Baseline. There were no differences in freezing between future drug groups prior to conditioning ($t_{23} = .69$, $p = .50$), extinction training ($t_{12.29} = 1.77$, $p = .10$), or the extinction retention test ($t_{23} = .14$, $p = .89$; **Table 4.3**).

Table 4.3

Mean (±SEM) percent baseline freezing before conditioning, extinction training, and extinction retention test in Experiment 4.2.

	Saline (*n* = 12)	**Naloxone (*n* = 13)**
Conditioning	4.17 (2.00)	2.50 (1.44)
Extinction	8.52 (3.31)	2.50 (0.80)
Test	6.88 (2.04)	7.31 (2.31)

Conditioning. CS-elicited freezing increased across conditioning trials ($F_{2, 46}$ = 58.09, p < .001), with no difference between future drug groups or drug × trial interaction being detected (largest $F_{2, 46}$ = 1.59, p = .21; **Figure 4.2A**). Therefore, similar acquisition of fear conditioning was observed in both groups.

Extinction. CS-elicited freezing decreased across extinction training ($F_{4, 92}$ = 26.32, p < .001) with no difference between naloxone- and saline-treated adolescent rats ($F_{1, 23}$ = 3.73, p = .07; **Figure 4.2B**). The drug × block interaction did not reach significance ($F_{4, 92}$ = 1.91, p = .12). Therefore, naloxone did not prevent reductions in conditioned fear during extinction training in adolescent rats.

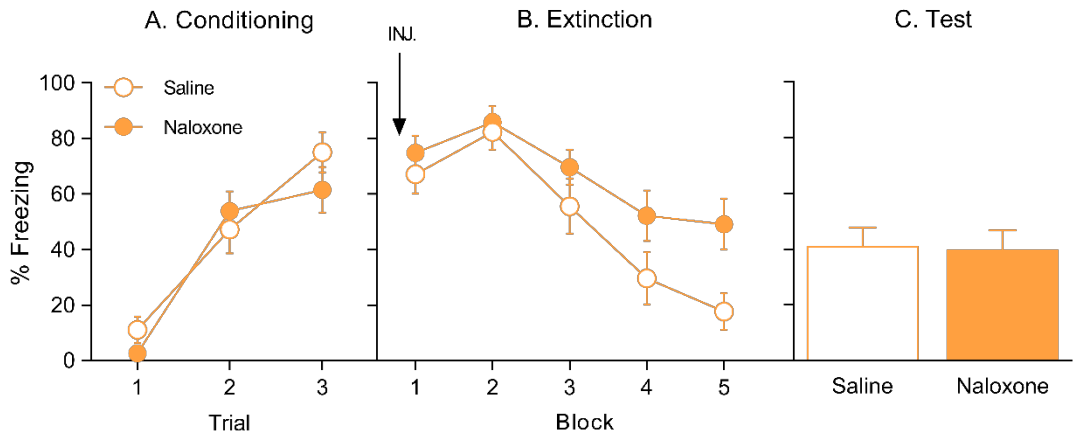

Figure 4.2. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) of adolescent rats in groups Saline and Naloxone.

Test. As was the case for extinction training, there was no difference in levels of CS-elicited freezing between naloxone- and saline-treated adolescent rats at test (t_{23} = .11, p = .91, d = 0.04; **Figure 4.2C**). Clearly, opioid receptor antagonism did not impair extinction learning or retention when administered prior to a single extinction session, suggesting that prediction error is not recruited in adolescents during extinction training.

Experiment 4.3

Opioid receptors are required for the second day of extinction training in adolescent rats

As illustrated in Experiment 4.2, antagonism of opioid receptors did not impair reductions in CS-elicited freezing expression during either extinction training or test in adolescent rats, suggesting that prediction error may not be the mechanism driving decrements in conditioned responses across extinction training in adolescents. This raises the question of whether reductions in CS-elicited freezing across extinction training in adolescents are due to an alternative process, such as habituation.

I next tested whether adolescents recruit opioid receptor-mediated prediction error signalling under circumstances that result in good extinction retention. For example, adolescent rats demonstrate good extinction retention, akin to levels in adults, when given an additional session of extinction training (Baker & Richardson, 2017; McCallum et al., 2010). As I expected that opioid receptor-mediated prediction error signalling would be recruited during the additional session, naloxone was administered prior to the second day of extinction training. In addition, three doses of the opioid receptor antagonist naloxone were used (2.5mg/kg, 5mg/kg, and 10mg/kg) in order to determine whether the effects of naloxone were dose-dependent.

Baseline. There were no differences in freezing between future drug groups prior to conditioning ($F_{3, 37} = 1.04$, $p = .39$), extinction training session one ($F_{3, 37} = .51$, $p = .68$), extinction training session two ($F_{3, 37} = .85$, $p = .48$), or the extinction retention test ($F_{3, 37} = 1.03$, $p = .39$; **Table 4.4**).

Table 4.4

Mean (±SEM) percent baseline freezing before conditioning, extinction training, and extinction retention test in Experiment 4.3.

	Saline (*n* = 10)	**NAL – 2.5mg (*n* = 10)**	**NAL – 5mg (*n* = 11)**	**NAL – 10mg (*n* = 10)**
Conditioning	4.00 (1.67)	1.00 (0.55)	3.86 (2.06)	1.50 (1.25)
Extinction 1	4.50 (2.03)	4.75 (3.19)	8.64 (4.70)	3.50 (1.67)
Extinction 2	3.50 (1.55)	7.25 (4.63)	11.14 (4.02)	6.25 (2.74)
Test	3.75 (1.41)	9.75 (3.38)	12.27 (5.30)	8.25 (2.24)

Conditioning. CS-elicited freezing increased across trials ($F_{2, 74} = 106.56$, $p < .001$) with no effect of future drug allocation ($F < 1$; **Figure 4.3A**). There was, however, a significant drug × trial interaction ($F_{6, 74} = 2.29$, $p = .04$). Follow-up post-hoc comparisons at each trial did not reveal any significant group differences though (largest $F_{3, 37} = 1.19$, $p = .33$), suggesting equivalent acquisition of fear conditioning across groups.

Extinction 1. On the first day of extinction training, all groups showed a reduction in CS-elicited freezing across blocks ($F_{4, 148} = 46.65$, $p < .001$) with no effect of subsequent drug group or interaction (Fs < 1; **Figure 4.3B**).

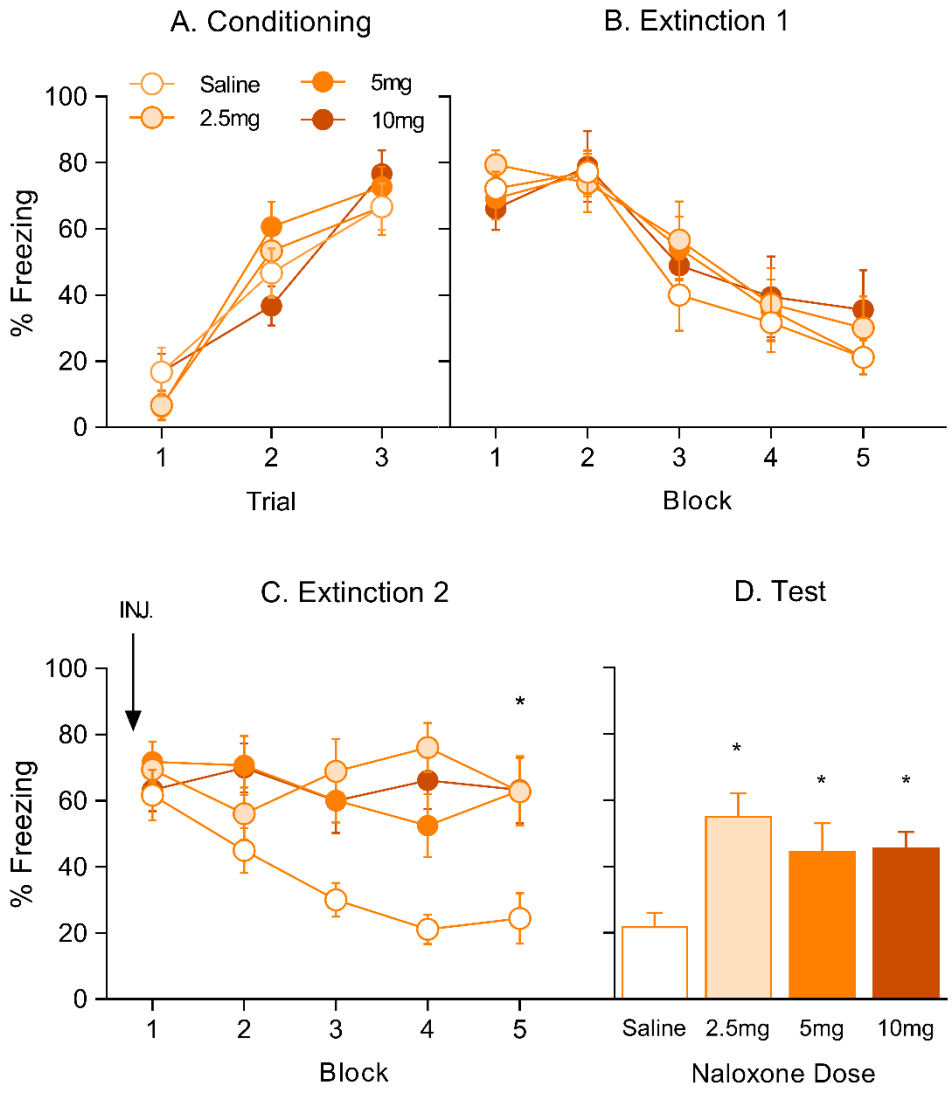

Figure 4.3. Mean (±SEM) levels of CS-elicited freezing at conditioning (**A**), extinction training day one (**B**), extinction training day two (**C**), and extinction retention test (**D**) of adolescent rats in groups Saline, Naloxone – 2.5mg/kg, Naloxone – 5mg/kg, and Naloxone – 10mg/kg.
***** indicates a significant post-hoc comparison ($p < .05$).

Extinction 2. Significant differences in levels of CS-elicited freezing were observed on the second day of extinction training, 10 min following the injection of naloxone (**Figure 4.3C**). Although CS-elicited freezing reduced in all groups across extinction training (block main effect: $F_{4, 148} = 2.72$, $p = .03$), levels of freezing differed between drug groups (drug main effect: $F_{3, 37} = 7.30$, $p = .001$). Furthermore, there was a significant drug × block interaction ($F_{12, 148} = 2.04$, $p = .03$). To explore the group

differences in overall levels of CS-elicited freezing, post-hoc comparisons were made using Dunnett's test. Across extinction training, rats treated with all doses of naloxone expressed significantly higher levels of CS-elicited freezing than rats treated with saline (all *p*s ≤ .02). The interaction was followed up by one-way ANOVAs to examine group differences at the start (Block 1) and end (Block 5) of extinction training. All groups had similar levels of CS-elicited freezing at the start of extinction training (Block 1 group effect: $F < 1$). In contrast, groups differed markedly at the end of extinction training (Block 5 group effect: $F_{3, 37} = 3.88$, $p = .02$), with rats administered naloxone having significantly higher CS-elicited freezing compared to rats administered saline at the end of extinction training (Dunnett's test post-hoc comparisons of all naloxone groups to saline were $p \leq 02$). Therefore, naloxone (at all doses tested) impaired reductions in CS-elicited freezing across the second day of extinction training in adolescent rats, consistent with the effects of naloxone administration in adult rats, as reported in Experiment 4.1.

Test. The difference in CS-elicited freezing between naloxone- and saline-treated adolescent rats observed at the end of the second extinction session persisted at the extinction retention test the next day ($F_{3, 37} = 4.87$, $p = .006$, $\eta_p^2 = .28$; **Figure 4.3D**). Compared to rats administered saline, rats given naloxone had significantly higher levels of CS-elicited freezing (2.5mg/kg: $p = .002$, 5mg/kg: $p = .04$, 10mg/kg: $p = .03$), indicative of impaired extinction retention.

The results of Experiment 4.3 suggest that on the second day of extinction training, adolescent rats use prediction error to acquire extinction learning, mirroring the results in adult rats (Experiment 4.1) and directly contrasting with the lack of opioid involvement observed on the first day of extinction training in adolescent rats (Experiment 4.2). This is consistent with previous work observing recruitment of adult-

like neural mechanisms on the second day of extinction in adolescents. For example, adolescent rats recruit NMDA receptors on the second, but not the first, day of extinction training (Baker & Richardson, 2017), and additional extinction trials are also associated with adult-like upregulations in pMAPK/ERK, a marker of synaptic plasticity, in the mPFC unlike when adolescents are given fewer extinction trials (Kim et al., 2011).

Experiment 4.4

Effects of naloxone on locomotor and anxiety-like behaviour

As naloxone administration led to increased CS-elicited freezing during the second session of extinction training in the previous experiment, a subsequent experiment was designed to determine if such increases were due to altered anxiety-like or locomotor behaviour. I expected that, similar to WIN55212-2 administration (see Experiment 2.5), naloxone administration would have no impact on either behaviour in naïve adolescent rats. The open field test procedures were as described in Chapter 2 (p. 35) and naloxone (5mg/kg) was administered to adolescent rats 10 min prior to open field testing.

Locomotor Activity. Two measures of locomotion were calculated: total distance travelled and average speed of locomotion. Naloxone treatment had no effect when either total distance travelled ($t_{10} = .71$, $p = .50$; **Figure 4.4A**) or average locomotor speed was compared ($t_{10} = .01$, $p = .99$; **Figure 4.4B**).

Anxiety-like Behaviour. The percentage of time spent in the inner zone of the open field (relative to total time) was calculated as an index of anxiety-like behaviour. There was no difference between saline- and naloxone-treated rats in time spent in the inner zone ($t_{10} = 1.34$, $p = .21$; **Figure 4.4C**). Therefore, naloxone administration did not

affect locomotor- nor anxiety-like behaviour in adolescent rats, indicating that the effects of naloxone on within-session learning were likely due to specific effects on fear expression, as measured using CS-elicited freezing.

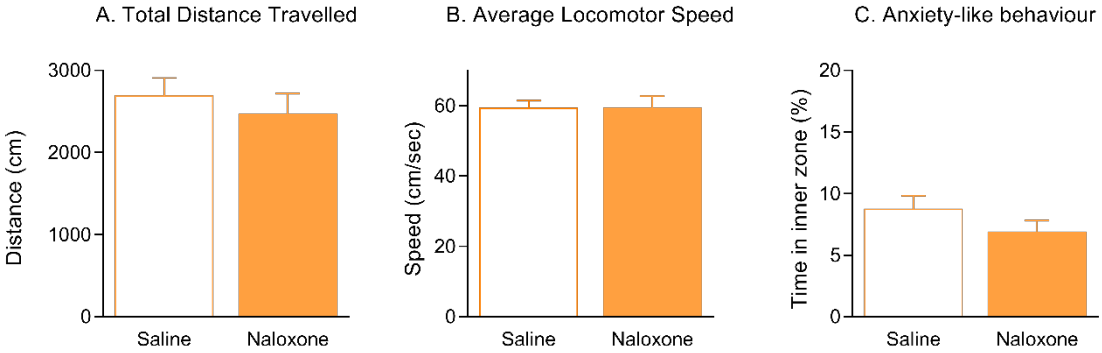

Figure 4.4. Mean (±SEM) total distance travelled (**A**), average locomotor speed (**B**), and anxiety-like behaviour (**C**) of adolescent rats in groups Saline and Naloxone.

Experiment 4.5

Opioid receptors are required for extinction learning in juvenile rats

In contrast to the lack of any effects of naloxone administration prior to the first session of extinction training in adolescent rats (Experiment 4.2), naloxone administration prior to the second session of extinction training impaired extinction learning and retention (Experiment 4.3). That is, naloxone-treated rats expressed higher levels of CS-elicited freezing across extinction training and testing than saline-treated rats. These findings suggest that prediction error mechanisms are recruited during adolescence under conditions that promote good extinction retention (i.e., extended extinction training).

There is, as noted in Chapter 3, an instance in which adolescent rats demonstrate good extinction retention in the absence of an additional extinction training session. That is, adolescent rats that acquire fear during the juvenile period and then receive one extinction session approximately 11-days later demonstrate good extinction retention

(see Chapter 3). Therefore, Experiment 4.5 aimed to examine whether adolescents that acquired fear as juveniles (*JuvCond-AdolesExt*) used opioid-mediated prediction error signalling during extinction training. This group of adolescent rats were compared to rats conditioned and extinguished as juveniles (*JuvCond-Ext*) in order to equate age at fear conditioning. Previous work has shown that juvenile rats use opioid receptors during extinction, as naloxone given before extinction training impairs extinction learning and retention in this age group (Kim & Richardson, 2009). All animals received fear conditioning as juveniles, and then received an injection of naloxone or saline prior to extinction training either 1 or 11 days later.

Baseline. There were no differences in baseline freezing between future age at extinction, future drug groups, or interaction prior to conditioning (largest $F_{1, 39} = 1.20$, $p = .28$), extinction training (largest $F_{1, 39} = 1.87$, $p = .18$), or the extinction retention test (largest $F < 1$, $p = .52$; **Table 4.5**).

Table 4.5

Mean (±SEM) percent baseline freezing before conditioning, extinction training, and extinction retention test in Experiment 4.5.

	JuvCond-Ext Saline (*n* = 8)	**JuvCond-Ext Naloxone (*n* = 9)**	**JuvCond-AdolesExt Saline (*n* = 13)**	**JuvCond-AdolesExt Naloxone (*n* = 13)**
Conditioning	1.56 (0.94)	1.94 (1.00)	1.15 (0.67)	0.77 (0.33)
Extinction	3.13 (1.55)	13.33 (3.93)	10.19 (4.03)	12.31 (6.29)
Test	12.50 (3.98)	17.22 (5.70)	11.73 (4.82)	15.38 (5.67)

Conditioning. Across conditioning, CS-elicited freezing increased ($F_{2, 78} = 55.07$, $p < .001$; **Figure 4.5A**) with no effect of future age at extinction, future drug allocation, or age x drug interaction being detected (largest $F_{1, 39} = 1.93$, $p = .17$).

Extinction. CS-elicited freezing decreased across extinction training ($F_{3.14,\ 122.53}$ = 11.26, p < .001; **Figure 4.5B**) with no difference between age groups (age main effect: F < 1). There was, however, a main effect of drug ($F_{1,\ 39}$ = 17.46, p < .001) and a drug x block interaction ($F_{3.14,\ 122.53}$ = 5.12, p = .001). The interaction was followed up by two-way ANOVAs to examine group differences at the start (Block 1) and end (Block 5) of extinction training. All groups showed similar levels of CS-elicited freezing at the start of extinction training (Block 1 age effect: F < 1, drug effect: F < 1). However, there was a main effect of drug at the end of the extinction training (Block 5 age effect: F < 1, drug effect: $F_{1,\ 39}$ = 15.66, p < .001), suggesting that naloxone administration impaired reductions in CS-elicited freezing across extinction training regardless of the animal's age at extinction.

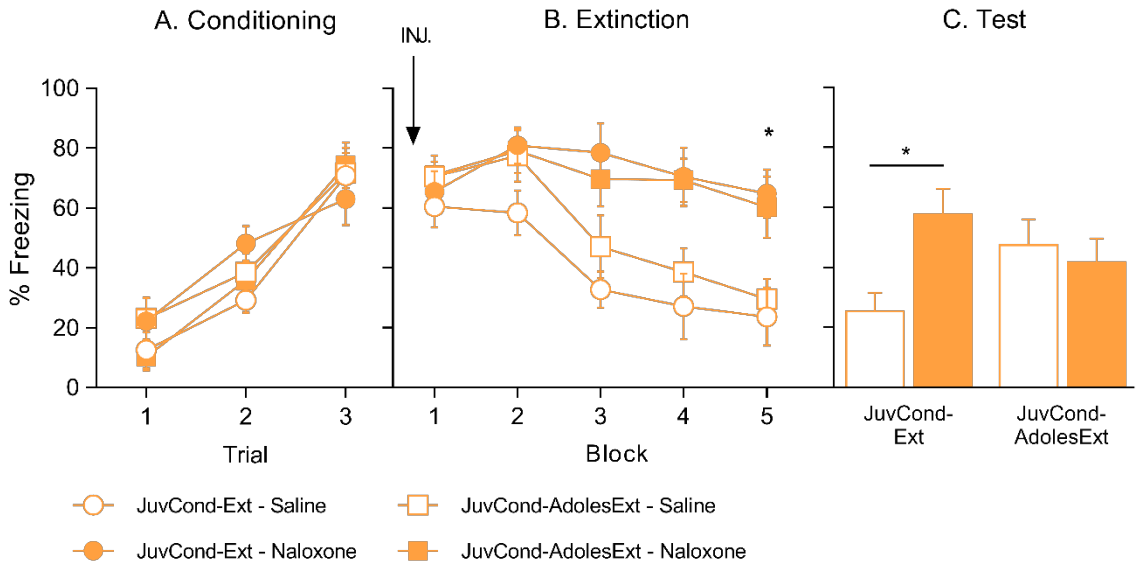

Figure 4.5. Mean (±SEM) CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) in groups JuvCond-Ext – Saline, JuvCond-Ext – Naloxone, JuvCond-AdolesExt – Saline, and JuvCond-AdolesExt - Naloxone.
* indicates a significant post-hoc comparison.

Test. The overall impairing effect of naloxone treatment did not persist to test the following day ($F_{1,\ 39}$ = 2.79, p = .10), and no differences in CS-elicited freezing were found between age groups (F < 1; **Figure 4.5C**). However, there was a drug x age

interaction ($F_{1, 39} = 5.59$, $p = .02$), so follow-up post-hoc comparisons were conducted between naloxone- and saline-treated rats within each allocated age at extinction group. Whereas naloxone-treated JuvCond-Ext rats showed significantly higher CS-elicited freezing than saline-treated juvenile rats ($t_{15} = 3.16$, $p = .006$, $d = 1.55$), there was no difference between the drug treatments in JuvCond-AdolesExt rats ($t_{24} = .50$, $p = .62$, $d = 0.20$).

Therefore, the findings of Experiment 4.5 replicated previous reports that opioid receptors are involved in extinction during the juvenile period, as blocking opioid receptor activation using naloxone impaired extinction learning and retention (Kim & Richardson, 2009). In contrast, an impairing effect of naloxone treatment was not observed in rats conditioned as juveniles and extinguished as adolescents. While this may suggest opioid receptor-independent extinction, it cannot immediately be interpreted in this manner upon visual inspection of the group means. That is, levels of CS-elicited freezing in the saline-treated rats conditioned as juveniles and extinguished as adolescents was almost equivalent to naloxone-treated rats conditioned and extinguished as juveniles at test (i.e., those showing "impaired" extinction retention) and is slightly higher than previously reported (e.g., Chapter 3: p. 79, 82, 86; Baker & Richardson, 2015). It is worth noting, however, that an effect of naloxone administration was seen on CS-elicited freezing during extinction training in rats conditioned as juveniles and extinguished as adolescents. Specifically, naloxone-treated rats in this condition showed higher levels of CS-elicited freezing similar to what was observed in adult rats (Experiment 4.1), adolescent rats given two sessions of extinction training (Experiment 4.2), and in contrast to adolescent rats given one session of extinction training (Experiment 4.3).

Experiment 4.6

Opioid receptors involvement in extinction learning during adolescence depends on age at conditioning

Unexpectedly, Experiment 4.5 did not replicate the well-established finding that rats conditioned as juveniles and extinguished as adolescents show good extinction retention. As impaired extinction retention has not previously been reported in this group of adolescent rats, the finding of impaired extinction retention in this group may be a Type I error. Therefore, I conducted Experiment 2.6 using the same parameters as Experiment 2.5, but did not include a comparison group of juvenile rats. This decision was made for two reasons: first, to minimise the number of animals used in this thesis and second, examining the effect of opioid receptor blockade in juvenile rats would be a replication of previously reported findings (Experiment 4.5; Kim & Richardson, 2009). In Experiment 2.6, I expected that rats conditioned as juveniles and extinguished as adolescents would show low levels of CS-elicited freezing at test when administered saline compared to those administered naloxone.

Baseline. There were no differences in freezing between future drug groups prior to fear conditioning ($t_{10} = 1.53$, $p = .16$), extinction training ($t_{12.39} = .63$, $p = .54$), or the extinction retention test ($t_{15} = 1.58$, $p = .14$; **Table 4.6**).

Table 4.6

Mean (±SEM) percent baseline freezing before conditioning, extinction training, and extinction retention test in Experiment 4.6.

	Saline (*n* = 8)	**Naloxone (*n* = 9)**
Conditioning	1.88 (0.78)	0.56 (0.37)
Extinction	15.63 (4.25)	21.11 (7.60)
Test	5.63 (2.25)	11.67 (3.00)

Conditioning. As expected, CS-elicited freezing increased across conditioning trials ($F_{2, 30} = 16.48$, $p < .001$), with no difference between future drug groups or a drug x trial interaction being detected (largest $F_{2, 30} = 1.13$, $p = .34$; **Figure 4.6A**).

Extinction. Although CS-elicited freezing decreased across extinction training (block main effect: $F_{4, 60} = 11.90$, $p < .001$), there was a significant difference in overall levels of CS-elicited freezing between naloxone- and saline-treated adolescent rats (drug main effect: $F_{1, 15} = 8.27$, $p = .01$; **Figure 4.6B**). A significant block x drug interaction was also found ($F_{4, 60} = 3.69$, $p = .01$), which was followed up by post-hoc independent *t*-tests. While there was no difference in CS-elicited freezing at the start of extinction training (Block 1: $t_{15} = .77$, $p = .45$), naloxone-treated rats expressed significantly higher CS-elicited freezing at the end of extinction training (Block 5: $t_{15} = 3.74$, $p = .002$). Therefore, naloxone prevented reductions in CS-elicited freezing across extinction training in rats conditioned as juveniles and extinguished as adolescents.

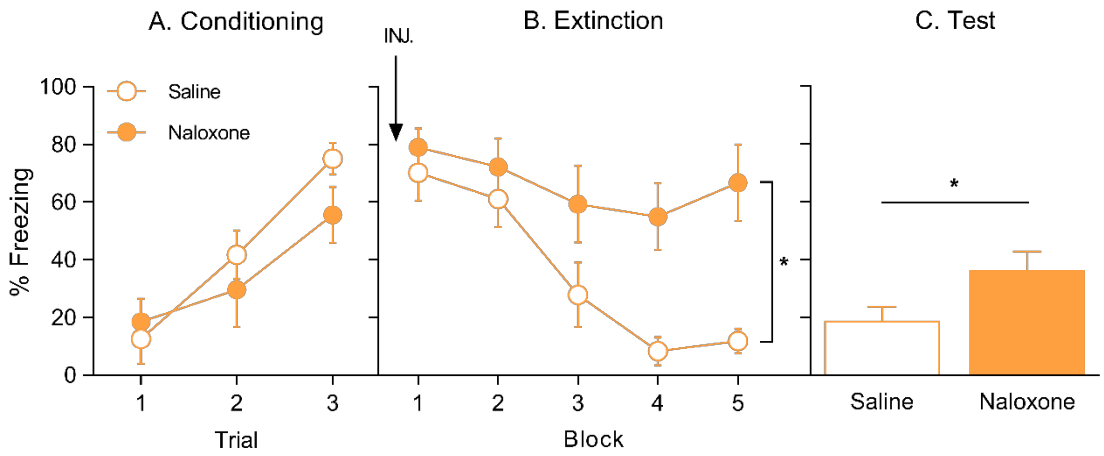

Figure 4.6. Mean (±SEM) CS-elicited freezing at conditioning (**A**), extinction training (**B**), and extinction retention test (**C**) of JuvCond-AdolesExt rats in groups Saline and Naloxone. * indicates a significant effect of drug ($p < .05$).

Test. As was observed during extinction training, naloxone-treated rats expressed significantly higher levels of CS-elicited freezing than saline-treated rats ($t_{15} = 2.21$, $p = .04$, $d = 1.08$; **Figure 4.6C**), suggesting that adolescent rats that acquire fear as juveniles recruit opioid-mediated prediction error signalling during the first day of extinction training, unlike adolescents that acquire fear as adolescents.

Discussion

The acquisition of extinction learning is critical for successful exposure therapy, and this process is underpinned by prediction error. The experiments reported in this chapter suggest the presence of a developmental deficit in generating and/or utilising prediction error signalling that emerges during adolescence based on opioid receptor involvement during extinction training. In juvenile and adult rats, opioid receptor antagonism impaired extinction learning, as indicated by persistently high levels of CS-elicited freezing across extinction training (Experiments 4.1 and 4.5). While this effect was not seen when naloxone was administered prior to the first day of extinction training in adolescent rats (Experiment 4.2), it was observed during a second day of

extinction training (Experiment 4.3). Furthermore, when examining a second instance of good extinction retention during adolescence, naloxone administration impaired extinction learning in adolescent rats that were conditioned as juveniles (Experiments 4.5 and 4.6). This latter finding indicates that the experience of conditioning during the juvenile period somehow altered the involvement of opioid receptors in extinction learning during adolescence. An additional experiment determined that increases in CS-elicited freezing observed in naloxone-treated adolescent rats could not be accounted for by altered locomotor or anxiety-like behaviour, and instead reflected specific effects of opioid receptor antagonism on learned fear expression (Experiment 4.4). Taken together, these findings indicate that the recruitment of prediction error signalling mechanisms promotes extinction during adolescence.

As was the case for extinction learning, naloxone administration impaired extinction retention in adult rats (Experiment 4.1), juvenile rats (Experiment 4.5), and in adolescent rats when administered on the second day of extinction training (Experiment 4.3) or when fear had been acquired as juveniles (Experiment 4.6; but see Experiment 4.5). As rats were tested drug-free (naloxone half life: 90 min; Lynn & Galinkin, 2018; Ngai, Berkowitz, Yang, Hempstead, & Spector, 1976), impairments in extinction retention were likely to be a direct result of disrupting extinction learning via naloxone administration the previous day. Indeed, increases in fear expression across extinction training appeared to predict impaired extinction retention in naloxone-treated rats (with the exception of Experiment 4.5). Conversely, blocking opioid receptors during the first extinction session did not lead to increases in fear expression during extinction training, and had no effect on extinction retention the following day in adolescent rats (Experiment 4.2). Therefore, I conclude that opioid receptors are involved during the second, but not the first, day of extinction training in adolescence, and that there is a

quantitative, rather than qualitative, difference in extinction during this developmental period (at least in regards to the involvement of the opioid system).

Neural circuit of prediction error

This deficit in recruiting opioid receptors in rats conditioned and extinguished as adolescents during the first extinction training session may be due to changes in opioid receptor density and/or function. There are three classes of opioid receptors: μ , δ, and κ opioid receptors (Mansour, Khachaturian, Lewis, Akil, & Watson, 1988). To determine the relative contribution of each of the different subtypes to extinction learning, McNally et al. (2005) administered selective μ, δ, and κ receptor antagonists into the vlPAG of adult rats prior to extinction training. Only the μ opioid receptor antagonist CTAP impaired extinction learning, as indicated by increased CS-elicited freezing across both extinction training and test. Therefore, changes in the density and/or function of μ opioid receptors within the vlPAG during adolescence could detrimentally impact extinction learning.

While there has been limited investigations into the developmental trajectory of μ opioid receptors in the vlPAG, the same level of μ opioid receptor expression in this subregion has been reported in juvenile and adult rodents; these studies, however, did not include an adolescent group for comparison (Recht, Kent, & Pasternak, 1985; Smith, Ratnaseelan, & Veenema, 2018). Therefore, the trajectory of μ opioid receptor expression in the vlPAG during adolescence remains unknown. While it may be the case that the expression of these receptors is stable across development, including in adolescence, it is also possible that the expression of these receptors develops in a non-linear manner. That is, although receptor expression is similar in juveniles and adults, it could be different in adolescence. This latter pattern has been reported with other systems, such as muscarinic receptors in the prefrontal cortex (Aubert, Cécyre,

Gauthier, & Quirion, 1996; Kim et al., 2017). If age-related changes in μ opioid receptor density do contribute to impaired extinction in rats conditioned and extinguished as adolescents, such changes should have also resulted in impaired extinction learning in rats conditioned as juveniles and extinguished as adolescents. As this was not the case, perhaps slower extinction learning in rats conditioned and extinguished as adolescents was not due to developmental changes in the opioid system.

Second, there may be deficits in the neural circuit involved in transmitting prediction error information from the vlPAG to the mPFC and amygdala for extinction learning and consolidation in rats conditioned and extinguished as adolescents. Although it is known that information is conveyed to these structures, the pathways by which this occurs are currently under investigation (Parsons et al., 2010; Yeh, Watanabe, Sulkes-Cuevas, & Johansen, 2018). There are direct projections from the vlPAG to the mPFC and CeA (Lu, Jhou, & Saper, 2006; Rizvi et al., 1991), but information may also pass indirectly through other structures, such as the nucleus reuniens. The nucleus reuniens has been implicated in the acquisition and consolidation of fear learning (Eleore, López-Ramos, Guerra-Narbona, & Delgado-García, 2011; Troyner, Bicca, & Bertoglio, 2018), as well as extinction learning/consolidation. Specifically, Ramanathan et al. (2018) found that temporary pharmacological inactivation of the nucleus reuniens during extinction training impaired the acquisition and retention of cued fear extinction in adult rats. In other words, inactivation of the nucleus reuniens led to heightened fear expression to the CS across both extinction training and a drug-free test. In addition to the newly identified role of the nucleus reuniens in extinction, the structure is well-placed to relay prediction error information from the vlPAG and mPFC. Specifically, there are projections from the vlPAG to the nucleus reuniens, and bidirectional projections between the nucleus reuniens and the

mPFC (Dolleman-Van Der Weel et al., 2019). The functionality of the nucleus reuniens during adolescence is yet to be investigated and therefore, any claims about the role of this region in extinction during this developmental period are speculative. Even so, if the vlPAG is still developing and is slower to be engaged during adolescence, this could contribute to a slower rate of extinction learning in adolescent rats.

As adolescents do use opioid receptor-mediated prediction error signalling to acquire extinction when given additional extinction training, this suggests that extinction (and by extension, exposure therapy) could be facilitated by maximising opioid receptor activation. This could be achieved by administering opiates, however this approach raises important issues of efficacy and safety in paediatric populations. Indeed, acute opiate administration may not be appropriate in clinical settings due to pain-modulating effects and propensity for addiction (Corder, Castro, Bruchas, & Scherrer, 2018). Another way to temporarily increase opioid neurotransmission at the time of extinction training would be to prevent the breakdown of enkephalin catabolising enzymes. One agent, RB101(S), increases the endogenous opioid signal through such actions and thus, potentiates the behavioural effects of endogenous opioids (Jutkiewicz, 2007; Roques, 2000). As intra-vlPAG infusions of RB101(S) prior to extinction training facilitates extinction learning and retention in adult rats (McNally, 2005), this pharmacological adjunct may ameliorate impaired extinction during adolescence. However, as RB101(S) does not cross the blood-brain-barrier and needs to be administered centrally (Thanawala, Kadam, & Ghosh, 2008), the translational potential of this agent is limited. Perhaps in the future, an opioid agonist will be designed to cross the blood-brain-barrier and bind to opioid receptors without inducing undesirable side effects, such as sedation and respiratory depression (Ghelardini, Di

Cesare Mannelli, & Bianchi, 2015). If so, administration of such an agonist alongside exposure therapy may lead to improved treatment outcomes.

The current findings have a number of important clinical implications. Perhaps most strikingly, the slower involvement of opioid receptors during extinction learning in adolescent rats compared to juvenile and adult rats suggests that exposure-based therapies may take longer to recruit the neural mechanisms required for successful inhibitory learning in adolescent patients. Consequently, symptom reductions may not be observed in anxious adolescents until later in treatment – for instance, at the third or fourth exposure session. On a similar note, my findings suggest that the metric by which exposure is judged as successful should centre on disconfirming the patient's predicted outcome of an exposure session (i.e., a measure of prediction error) rather than reductions in self-reported distress (i.e., a measure of habituation). While these suggestions are speculative in nature, they do warrant further examination in clinical settings to realise the implications of my preclinical findings. Lastly, the importance of assessing the age at which a patient acquired their fear is reiterated in the current chapter. Indeed, the pace at which an individual progresses through cognitive behaviour therapy may rest on their age at fear learning – such that those who acquired the fear as children demonstrate faster, and more persistent, symptom reductions than those who acquired their fear as adolescents.

The research presented in this chapter highlights the differential involvement of opioid receptors during extinction training across development. Whereas opioid receptors are utilised by juvenile and adult rats during the first session of extinction training, rats conditioned and extinguished as adolescents demonstrated reductions in fear expression across extinction training that was independent of opioid receptor activation. Adolescent rats did, however, use opioid receptors during extinction training

under two conditions that led to good extinction retention at test: during a second extinction training session, and when fear was acquired as a juvenile. My findings highlight a developmental difference in the neural mechanisms underlying extinction learning, and add to the growing literature identifying differences in inhibitory learning mechanisms between adolescents and adults. Furthermore, an adolescent-specific disruption in opioid receptor-mediated prediction error signalling, if also observed in analogue and clinical adolescent human samples, may explain why exposure-based therapies are often less effective during adolescence.

Chapter 5: General Discussion

Taken together, the experiments presented in this thesis illustrate the complex and nuanced nature of extinction during adolescence. Past work had reported an impairment in extinction learning and/or retention during adolescence in both rodents and humans, such that adolescents show a higher return of fear following extinction training compared to younger and older groups (Kim et al., 2011; McCallum et al., 2010; Pattwell et al., 2012). In an attempt to elucidate why adolescents show such an impairment, the research reported in this thesis examined the involvement of three neurotransmitter systems in extinction during adolescence. In my first series of experiments, I explored whether increasing CB1R activation could augment extinction during adolescence (Chapter 2). Next, I examined whether NMDA receptors were required for extinction learning and consolidation during adolescence (Chapter 3). Finally, my last series of experiments investigated the role of opioid receptor-mediated prediction error signalling in extinction learning during adolescence (Chapter 4).

In Chapter 2, I examined whether increasing endocannabinoid signalling through agonism of CB1Rs could improve extinction in adolescent rats. Unlike in adult rats, the CB1/2R agonist WIN55212-2 failed to facilitate extinction retention in adolescent rats and instead, selectively increased fear expression during extinction training. A similar pattern of results was observed in juvenile rats, such that WIN55212-2 led to increased fear expression during both extinction training and test. This latter finding indicated that WIN55212-2 impacted extinction differently in developing rats, and these effects were not restricted to adolescence. To follow up on these behavioural findings, I examined the developmental trajectory of CB1R expression, as assessed by Western blotting, in the mPFC and amygdala, two regions within which CB1Rs contribute to extinction consolidation. In both regions, a linear decrease in CB1R

expression was seen across age (i.e., from the juvenile period to adulthood). In light of such a decrease, perhaps an immature endocannabinoid system contributed to the different effects of systemic CB1R agonism on extinction in developing rats.

My second series of experiments, as described in Chapter 3, explored the role of NMDARs during extinction consolidation in adolescent rats. This series of experiments was based on previous work arguing that NMDARs were needed for extinction consolidation (for review, see Davis 2011; Singewald et al., 2015). In contrast to such an assertion, rats conditioned as juveniles and extinguished as adolescents demonstrated good extinction retention despite blockade of NMDARs. That is, administration of the NMDAR antagonist MK801 prior to extinction training had no effect on extinction retention in this group of adolescent rats. The involvement of NMDARs in extinction was not determined by the animal's age at conditioning or extinction training, the interval between the two procedures, nor the experience of a developmental transition. Rather, the experience of fear conditioning as a juvenile altered the requirement for NMDARs during extinction training in adolescence.

In Chapter 4, I investigated whether opioid receptors were necessary for extinction learning during adolescence. As was the case for adult rats, opioid receptors were necessary for extinction learning in adolescents under conditions that led to good extinction retention the next day. Specifically, opioid receptors were necessary during the second extinction training session in rats conditioned and extinguished as adolescents, as well as during the first extinction training session when rats were conditioned as juveniles and extinguished as adolescents. In stark contrast, opioid receptors were not recruited during the first session of extinction training when rats were conditioned and extinguished as adolescents. As opioid receptors mediate prediction error signalling to direct extinction learning, a lack of opioid receptor

involvement may contribute to impaired extinction retention in this group of adolescent rats.

In the next section I review the behavioural performance of adolescent rats during extinction training and retention across my thesis and highlight several methodological considerations relevant to my work. In the immediately following section I then consider potential mechanisms which might underlie the observed developmental differences in the involvement of the three neurotransmitter systems in extinction learning and consolidation. In particular, I highlight how the involvement of each system changes depending on the amount of extinction training given (i.e., one versus two sessions of extinction training), as well as the animals' age at fear conditioning (i.e., juvenile versus adolescent). Lastly, I consider the importance of acknowledging sex differences in extinction and finish with a discussion of behavioural and pharmacological options for facilitating extinction/exposure therapy in adolescents.

A review of behavioural findings

As the acquisition of fear extinction is typically measured as the extent to which fear responding declines across repeated presentations of the CS, extinction learning deficits might be inferred when an animal shows a slower decrease (or no decrease at all) in their fear expression across trials. While a few past publications have identified a deficit in extinction learning in rodents and humans during adolescence according to fear expression across extinction training (e.g., Hefner & Holmes, 2007; Pattwell et al., 2012), a number of others have reported intact extinction acquisition but impaired extinction retention the following day (e.g., Baker & Richardson, 2017; Kim et al., 2011; McCallum et al., 2010), and have attributed the latter to issues with extinction consolidation.

Extinction training

Across experiments, I consistently observed substantial decreases in fear responding in saline and vehicle-treated adolescents during the first session of extinction training. For example, in a typical experiment the level of CS-elicited freezing on the first block of six CS-only trials would be 70-80%, which decreased to 10-20% by the fifth block of extinction training. At the second extinction training session, the following day, a 'savings effect' was observed. That is, the rate of reduction in fear expression across the second extinction training session was faster when compared to the first extinction training session (note that this savings effect was much more prominent in Experiment 3.3 compared to Experiment 4.3). This savings effect has also been observed in other papers which involved two days of extinction training in adolescent rats (e.g., Baker & Richardson, 2017). This pattern of fear expression across the two sessions of extinction training would suggest that there is at least some small amount of extinction learning that takes place during the first extinction training session which is subsequently retrieved at the second extinction training session.

If adolescent rats did indeed acquire some level of extinction learning during the first session of extinction training, one would expect that blocking the associative learning mechanisms that underlie extinction learning (e.g., negative prediction error) would increase fear expression at a subsequent extinction retention test. In my third series of experiments (i.e., Chapter 4), I examined whether this was the case. In contrast to what was observed in juvenile and adult rats, blocking opioid receptors during the first session of extinction training in adolescent rats had no impact on fear expression at test, suggesting minimal extinction learning had taken place. These results suggest that in rats conditioned and extinguished as adolescents, extinction learning does not solely

drive reductions in fear expression during the first extinction training session and perhaps, a non-associative learning process – such as habituation – is also involved.

Habituation

In stark contrast to adult rats, naloxone administration did not alter CS-elicited freezing during the first day of extinction training in adolescent rats that were conditioned during adolescence. This would suggest that decrements in fear expression in the rats conditioned and extinguished as adolescents across the course of the first session of extinction training occurred due to a process independent of prediction error signalling. One such alternative process is habituation, a non-associative type of learning which leads to reduced behavioural responding due simply to repeated presentations of a stimulus (Groves & Thompson, 1970). Although classical models of habituation have focused on behavioural changes to stimuli such as a loud noise or a puff air, habituation has also been conceptualised as occurring to feared stimuli across exposure therapy in clinical settings (Foa & Kozak, 1986; Myers & Davis, 2007). While habituation has been suggested to contribute to short-term treatment gains within exposure therapy, there is a substantial body of work indicating that within-session habituation (as assessed by reductions in fear/anxiety across the session) fails to predict symptom improvements at the next session (Baker et al., 2010; Kircanski et al., 2012; Sripada & Rauch, 2015). This is likely because habituation only temporarily weakens fear responses within exposure sessions and does not involve learning new associations of safety (Craske et al., 2008; Plendl & Wotjak, 2010).

Even in an animal experimental paradigm, determining the relative contributions of habituation versus extinction learning to decrements in fear expression across extinction training is difficult (and perhaps impossible). However, if habituation was indeed responsible for the reductions in fear expression seen in adolescent rats across

the first session of extinction training, this would be consistent with clinical outcomes in anxious youth. For instance, recent outcomes from a randomised controlled trial in children and adolescents diagnosed with an anxiety disorder found that across the course of cognitive behaviour therapy, within-session habituation was not associated with reductions in anxiety symptoms at post-treatment or 1-year follow-up (Peterman, Carper, & Kendall, 2019). Instead, initial fear activation was the only variable to predict later anxiety symptoms, and so the authors suggested that habituation has limited clinical utility for young people. Together with my findings, this work suggests that moving forward, the clinical focus should be on facilitating extinction learning, rather than within-session reductions in fear/anxiety, during exposure therapy sessions. In particular, extinction learning could be facilitated by the clinician actively checking in with the client's predicted outcome of an exposure session, and ensuring that the exposure continues until the predicted outcome has been disconfirmed (and this has been recognised by the patient).

Extinction retention

Consistent with past work, I found that adolescent rats demonstrated impaired extinction retention relative to other age groups. Although McCallum et al. (2010) reported an 80% return of CS-elicited freezing in adolescent rats following a single day of extinction training (as determined by CS-elicited freezing at test divided by CS-elicited freezing on Block 1 of extinction training), I consistently only observed a 40-50% return of CS-elicited freezing at test in my experiments. However, this was a greater level of fear relapse than what I observed in juveniles (Experiments 2.3, 3.2, and 4.5) and adults (Experiments 3.5 and 4.1; with the exception of Experiment 2.1). The difference in fear recovery between my experiments and those published previously is likely due to the shock intensity used. Specifically, I used a 0.4mA shock compared to a

0.6mA shock in the earlier studies (e.g., Baker et al., 2018; Kim et al., 2011; McCallum et al., 2010).

Although somewhat less than has been reported in some earlier experiments, the level of fear recovery in my experiments meant that there was room to observe an impairing effect of drug treatment on extinction retention. In other words, the consistent null results I observed with the NMDAR antagonist MK801 and opioid receptor antagonist naloxone on extinction retention in rats conditioned and extinguished as adolescents is unlikely due to a ceiling effect. Specifically, there was room for the animals to express up to 60-70% CS-elicited freezing at test, in line with the level if CS-elicited freezing observed in Block 1 of extinction training.

Neurotransmitters: what, where, and when?

Across my experiments, I found that the neural mechanisms involved in extinction learning and consolidation on the first day of extinction training in adolescents differ from those on the second day, as well as when the fear was acquired as a juvenile. It is critical to understand the conditions under which the adolescent brain accurately discriminates between threat and safety, as well as those in which it does not, in order to modify extinction-based therapies appropriately. Therefore, in the next section I discuss how the involvement of the opioid, glutamatergic, and endocannabinoid systems differ across three instances of extinction in adolescence.

Opioids

During associative learning, the difference between the expected and actual outcome of a stimulus presentation is encoded as a prediction error signal within the vlPAG (Yau & McNally, 2019). It is thought that the encoding of this signal is regulated by the opioid system, as blockade of opioid receptors impairs performance on

learning tasks that require prediction error (Cole & McNally, 2007a; McNally & Westbrook, 2003). After replicating that administration of the opioid receptor antagonist naloxone impaired extinction learning and retention in adult rats (Experiment 4.1), I found that naloxone administration did not alter fear expression during extinction training or test in adolescent rats when administered on the first day of extinction training (Experiment 4.2), suggesting that in this circumstance, adolescents were not engaging opioid receptor-dependent prediction error mechanisms at the time. As naloxone did impair extinction retention in adolescents when administered on the second day of extinction training (Experiment 4.3), prediction error mechanisms are likely engaged with additional training.

In considering the mechanisms underlying disrupted extinction learning in rats conditioned and extinguished as adolescents, there may be a disruption in the neural circuit involved in encoding of the prediction error signal during the first day of extinction training. Although there is no direct evidence for this, there is some indirect evidence which supports this suggestion. However, as all of this indirect evidence comes from studies on fear conditioning, rather than extinction, this suggestion is admittedly speculative. With that caveat in mind, when an animal encounters an unexpected shock and as a result, the prediction error is large, there is a large shock-evoked activity spike in a population of "prediction error coding" neurons within the vlPAG. That is, the shock-evoked response mirrors the level of *positive* prediction error generated. Similarly, the shock-evoked response is small when an animal encounters an expected shock (i.e., the prediction error is small; Johansen, Tarpley, LeDoux, & Blair, 2010). A population of neurons within the CeA projects to the vlPAG to modulate the activity of these prediction error coding neurons. To illustrate, optogenetic inactivation of vlPAG-projecting neurons within the CeA prevents the decrease in shock-evoked

responding to expected shocks in prediction error coding neurons within the vlPAG, such that the shock-evoked responding resembled that of unexpected shocks. In other words, inhibition of the CeA-vlPAG pathway led to excessive neural responding to expected threat (Ozawa et al., 2017).

In addition, vlPAG-projecting neurons within the CeA activate μ opioid receptors in the vlPAG to modulate learning tasks based on positive prediction error (Ozawa & Johansen, 2018). As μ opioid receptors are the opioid receptor subtype within the vlPAG which has been primarily implicated in extinction learning (McNally et al., 2005; Yau & McNally, 2019), vlPAG-projecting neurons within the CeA likely also play a critical role in regulating negative prediction error. However, the role of the CeA-vlPAG pathway in extinction learning has yet to be elucidated in adult animals. Therefore, although I can speculate that adolescent-specific alterations in the density or function of the vlPAG-projecting CeA neurons, or the vlPAG neurons which express μ opioid receptors, could lead to impaired prediction error signalling and consequently, compromise extinction learning, an understanding of whether this circuit is involved in negative prediction error in adult animals is required.

Glutamate

In the several hours following extinction training, the short-term, labile extinction memory trace is "consolidated" into a long-term extinction memory (Squire, Genzel, Wixted, & Morris, 2015). This process was thought to typically require NMDAR-mediated glutamatergic signalling (Davis, 2011), however, there are now several reports of consolidation of extinction being NMDAR-independent. In particular, my findings indicate that rats that were conditioned as juveniles and extinguished as adolescents demonstrate NMDAR-independent extinction consolidation. This is in direct contrast to juvenile rats, adolescent rats that receive two days of extinction

training, and adult rats, suggesting qualitatively different glutamatergic signalling in this group of adolescent rats.

As NMDARs were not required for extinction consolidation in rats conditioned as juveniles and extinguished as adolescents (Experiments 3.1-3.3), it raises the question as to whether pharmacological adjuncts which act through NMDARs to facilitate extinction would work in this group. For instance, D-cycloserine is a partial NMDAR agonist that improves extinction retention and exposure therapy outcomes in some, but not all, individuals (Guastella et al., 2007; Rosenfield et al., 2019; Walker et al., 2002). Interestingly, recent work has identified a group of rats which show NMDAR-independent extinction and do not respond to D-cycloserine treatment (Tang & Graham, 2019a, 2019b). Specifically, female rats that are reproductively experienced (i.e., mothers) show good extinction retention that is unaffected by either blocking or increasing NMDAR activation (using MK801 and D-cycloserine, respectively). This suggests that NMDARs are simply not involved during extinction training in this group. To further elucidate the involvement of NMDARs during extinction training in rats conditioned as juveniles and extinguished as adolescents, future research could administer D-cycloserine following a weak extinction protocol (e.g., 15 CS presentations). A weak extinction protocol would produce an impairment in extinction retention (i.e., higher levels of CS-elicited freezing at test) and avoid a floor effect at test, allowing the potential facilitating effect of D-cycloserine administration to be detected. As rats conditioned as juveniles and extinguished as adolescents show good extinction in the absence of NMDARs, I hypothesise that this group of adolescent rats would be similar to mothers and not benefit from D-cycloserine administration.

If glutamate signalling is still involved in extinction consolidation in rats conditioned as juveniles and extinguished as adolescents, a different receptor type than

NMDARs may drive glutamatergic signalling. One candidate is mGluR5, as administration of the mGluR5 antagonist MPEP prior to extinction training has been shown to impair extinction retention in adolescent rats, indicating a role for mGluR5 signalling during adolescence (Sepulveda-Orengo et al., 2013). Nevertheless, this suggestion is tempered by some evidence that NMDARs are necessary for mediating the enhancement of some forms of extinction. For example, using a conditioned place preference procedure, Gass and Olive (2009) examined the effects of increasing mGluR5 signalling on extinction using the positive allosteric modulator CDPPB. While administration of CDPPB facilitated the extinction of conditioned place preference, co-administration of the NMDAR antagonist MK801 prevented this CDPPB-induced improvement in extinction. Therefore, it is unlikely that mGluR5 signalling could underlie extinction consolidation independently of NMDAR activation in rats conditioned as juveniles and extinguished as adolescents. In any case, possible interactions between NMDAR and mGluR5 signalling during extinction in rats conditioned as juveniles and extinguished as adolescents could be examined by co-administration of an agent which increases mGluR5 signalling (e.g., CDPPB) and the NMDAR antagonist MK801 prior to extinction training.

Endocannabinoids

In Chapter 2, I observed decreases in CB1R protein expression in the mPFC and amygdala from the juvenile period to adulthood (Experiment 2.5), consistent with the occurrence of synaptic and axonal pruning across development (Tau & Peterson, 2010). While my findings were indicative of changes in gross receptor expression within these two regions, significant scope remains in identifying the localisation of CB1Rs within each region. Indeed, as neuronal activity in the BLA during extinction training is regulated by the actions of CB1Rs on both glutamatergic and GABAergic neurons

(Krabbe, Gründemann, & Lüthi, 2018; Ruehle et al., 2013), it is critical to identify whether the expression of CB1Rs on these two neuronal subtypes changes across development. Changes in the localisation patterns of CB1Rs across development could have cascading effects on neuronal activity within the BLA and consequently, impact fear extinction. To identify whether there are developmental alterations in the localisation of CB1Rs on glutamatergic/GABAergic neurons, techniques such as in-situ hybridisation or immunohistochemistry could be employed. Whereas in-situ hybridisation can be used to quantify the degree of co-localisation of CB1R and glutamatergic/GABAergic receptor *mRNA* within cells, immunohistochemistry can be used to locate CB1R and glutamatergic/GABAergic receptor *protein* (Seidman, 2008; Wang et al., 2012). The use of either technique, separately or in combination[1], could identify changes in neuronal activation within specific neuronal subtypes (e.g., by examining gene or protein expression of immediate early genes such as c-Fos).

In addition to identifying the neuronal subpopulation in which CB1Rs are located within brain regions such as the BLA, it is also important to identify whether these receptors are required for in fear extinction during adolescence. Specifically, the requirement for CB1Rs in extinction can be determined by administration of the CB1R antagonist SR141716A prior to extinction training, as SR141716A impairs extinction learning and retention in adult rodents (e.g., Bowers & Ressler, 2015; Marsicano et al., 2002). In adolescent rats specifically, future work could compare the requirement for CB1Rs in extinction during the first versus the second session of extinction training, similar to the experimental methods used in Chapter 4 to examine opioid receptor involvement using naloxone. I hypothesise that CB1Rs would be required during the

[1] Recent advances have led to the development of a novel approach which combines in-situ hybridisation and immunohistochemistry to enable localisation of both receptor mRNA and protein (Grabinski, Kneynsberg, Manfredsson, & Kanaan, 2015).

second, but not the first, session of extinction training in adolescent rats, similar to the cases of NMDARs and opioids. This finding would be consistent with an overall slowing of neurotransmitter involvement in extinction during adolescence.

Fear extinction in adult rodents has been shown to also be mediated by the endocannabinoid anandamide, an endogenous ligand which binds to receptors to produce long-term changes in synaptic strength (Ohno-Shosaku & Kano, 2014). Anandamide acts at several receptor types, including but not restricted to CB1Rs (e.g., transient receptor potential vanilloid type 1 receptors; Zlebnik & Cheer, 2016). In the first study to examine the role of endocannabinoids in extinction, Marsicano et al. (2002) found that anandamide levels in the BLA (but not the mPFC) were increased following extinction of cued fear in adult mice. It would be interesting to investigate whether an extinction-induced increase in anandamide within the BLA is also observed in adolescent rats. As anandamide signalling has been implicated in successful extinction in adult rodents, increases may not be seen after only one extinction training session in adolescent rodents[2]. However, I expect that extinction-induced increases in anandamide would be observed following the second extinction training session, or after one extinction session if fear was acquired as a juvenile (i.e., in two situations where the adolescent rat exhibits good extinction retention).

While currently there are no pharmacological agents which block anandamide, anandamide signalling can be augmented by preventing the breakdown/reuptake of the ligand. This can be achieved by administration of a FAAH inhibitor, as FAAH is the enzyme responsible for anandamide breakdown/reuptake (Otrubova, Ezzili, & Boger,

[2] In 2018, I attempted to examine extinction-induced changes in anandamide in adolescent rats. Specifically, I collected tissue samples and then sent them to the Mass Spectrometry Facility at the University of Sydney for liquid chromatography/mass spectrometry analysis. Due to technical difficulties in identifying the anandamide signal by researchers at that facility, the experiment had to be terminated.

2011). The administration of FAAH inhibitors prior to extinction training leads to improved extinction learning and retention in adult rodents (Bitencourt, Pamplona, & Takahashi, 2008; Gunduz-Cinar et al., 2013) and humans (Mayo et al., 2019). In particular, administration of FAAH inhibitors promote extinction in the inbred S1 mouse strain (Gunduz-Cinar et al., 2013), a species of mice that show impaired extinction retention in adulthood that is accompanied by a lack of synaptic plasticity in the mPFC and amygdala following extinction training (Hefner et al., 2008). As this pattern of impaired extinction and reduced synaptic plasticity is also seen in adolescent rats following a single extinction training session (Baker & Richardson, 2015), increasing anandamide signalling may also improve fear extinction in adolescent rodents[3]. However, as anandamide has been shown to work through CB1R activation (Bitencourt et al., 2008; Gunduz-Cinar et al., 2013), and there are adolescent-specific changes in anandamide signalling (for review, see Meyer, Lee, & Gee, 2018b), it is also possible that administration of FAAH inhibitors may not facilitate extinction during this developmental period.

In humans, variation in the FAAH genotype is associated with differences in anandamide levels, and these naturally occurring individual differences have been implicated in extinction. In particular, the FAAH polymorphism C385A is present in approximately 40% of people and reduces the level of FAAH protein, leading to increased concentrations of anandamide throughout the brain (Boileau et al., 2015; Chiang, Gerber, Sipe, & Cravatt, 2004). In other words, individuals who carry the A variant (AA/AC) have higher concentrations of anandamide compared to those who

[3] In 2016, I examined the effects of administering the FAAH inhibitor AM404 prior to extinction training in adolescent rats. Mid-way through the experiment, Tocris Biosciences (the supplier) discontinued production of AM404 (and this agent was not manufactured elsewhere). Due to the small sample size (n = 6-9), the experiment was not included in this thesis. See Appendix 2 for results.

only carry the C variant (CC). This increase in anandamide is associated with enhanced connectivity between the prefrontal cortex and amygdala (assessed using the neuronal activity marker c-Fos in mice and fMRI in humans), as well as a faster reduction in fear expression across extinction training (Dincheva et al., 2015). As central concentrations of anandamide (e.g., brain, cerebrospinal fluid) do not correlate well with peripheral concentrations (e.g., plasma; Jumpertz, Guijarro, Pratley, Piomelli, & Krakoff, 2011), the use of the FAAH polymorphism C385A would likely be more beneficial as a biomarker in predicting extinction performance compared to measuring an individual's baseline peripheral anandamide levels. Somewhat surprisingly, there has yet to be a comparison between allele variants for extinction retention performance as one would expect the enhanced acquisition of extinction observed in individuals who carry the A allele to persist to test. Interestingly, the differences in functional connectivity between the allele variants emerges during adolescence, implicating adolescence as a critical time in endocannabinoid (and particularly anandamide) signalling (Gee et al., 2016). This suggests that there may be individual differences in endocannabinoid signalling that are associated with greater risk to impaired extinction during adolescence.

Summary

The involvement of each of the three neurotransmitter systems investigated in this thesis in adolescent extinction varies across different extinction conditions. In adolescent rats that receive one session of extinction training, impaired opioid signalling likely contributes to deficits in extinction learning that either completely prevent extinction consolidation, or which are then further compounded by aberrant extinction consolidation due to impaired NMDAR signalling. Unlike in adult rats, administration of the CB1R agonist WIN55212-2 prior to the first session of extinction training did not improve extinction retention in adolescent rats. In contrast, when a second extinction

training session is given both opioid receptor-dependent extinction learning and NMDAR-dependent extinction consolidation mechanisms seem to be engaged in the adolescent rat. In other words, extinction learning and consolidation appears to be quantitatively, rather than qualitatively, different in rats conditioned and extinguished as adolescents compared to adult rats.

A very different picture of neurotransmitter systems engaged in extinction emerges when the performance of rats conditioned as juveniles and extinguished as adolescents is examined. Not only does this group of adolescent rats show good extinction retention after only one session of extinction training, but they do not engage NMDARs (in contrast to juvenile rats, adolescent rats given two sessions of extinction training, and adult rats). Although this group of adolescent rats engage opioid receptor signalling, presumably to acquire extinction learning, the consolidation of such learning occurs in the absence of NMDARs. Overall, my findings highlight the inherent complexity of the mechanisms of extinction during adolescence, and across development more broadly.

Sex differences

In each of the experiments reported in this thesis, only male rats were used. This is quite typical of preclinical research (Lebron-Milad & Milad, 2012), and is the case for the majority of studies which have investigated extinction retention during adolescence. While the use of male rats is methodologically simpler due to bypassing the need to assess for estrous cycling in female rats, this siloed approach restricts research applicability to only half of the general population, and less than half of the individuals who will be diagnosed with an anxiety disorder. Specifically, over half of those who are diagnosed with an anxiety disorder are women, and adolescent girls are more likely to

be diagnosed with an anxiety disorder than adolescent boys (Australian Institute of Health and Welfare, 2014; Dalsgaard et al., 2019; McLean, Asnaani, Litz, & Hofmann, 2011). Therefore, it is important for the preclinical research which has been conducted in male rodents to be expanded to female rodents prior to translation of this work to human research and eventually, dissemination in clinical settings.

One important reason for examining the mechanisms of extinction in adolescence in both males and females is that there are numerous examples of sex differences in the neurobiology of fear extinction. For instance, there are sex-dependent patterns of neuronal activation during extinction learning and retention, as well as differential neurotransmitter involvement (for review, see Velasco, Florido, Milad, & Andero, 2019). In adult females, extinction retention (as well as the effects of pharmacological adjuncts on extinction) is dependent on endogenous levels of the sex hormones estrogen and progesterone. Across both rodent and human studies, females show impaired extinction retention when estrogen and progesterone levels are low (e.g., in the metestrus phase in rodents), but not when estrogen and progesterone levels are high (e.g., in the proestrus phase in rodents; Graham & Milad, 2013; Milad, Igoe, Lebron-Milad, & Novales, 2009).

A similar pattern of extinction retention performance is observed in adolescent female rats that have begun puberty. At the onset of puberty, estrogen and progesterone increase and begin to fluctuate in line with the estrous cycle (Döhler & Wuttke, 1974; Mohr, Wong, Tomm, Soma, & Micevych, 2019; Swerdloff & Odell, 1975). In earlier studies which did not assess for estrous phase or include a male control group, adolescent females were reported as demonstrating either intact (e.g., McCormick, Mongillo, & Simone, 2013) or impaired extinction retention (e.g., Riddle et al., 2013). However, in more recent studies, which did assess for estrous phase and included a

male control group, it has become clear that extinction retention fluctuates in adolescent female rats depending on the stage of estrous cycle at extinction training. Specifically, adolescent females that were extinguished in the metestrus phase exhibited impaired extinction retention which was equivalent to age-matched male rodents, whereas extinction retention was improved in adolescent females in the proestrus phase (Baker, Gable, & Richardson, 2019). Therefore, as has been observed in adult females, extinction performance fluctuates across the estrous cycle in adolescent females once they have entered puberty. While it would be interesting to investigate how the opioid, glutamatergic, and endocannabinoid systems are involved in extinction in adolescent females, such investigations were outside the scope of my thesis and should be followed up in future research.

Improving fear extinction in adolescence

Here, I consider two potential pharmacological adjuncts, neither of which were discussed above, as well as two potential behavioural approaches to enhancing extinction in adolescent rodents and humans. In both cases, I will discuss why these treatments may or may not be effective during this developmental period. As the ultimate aim of my research is to further our understanding of how clinical treatments can be advanced, in this section I will focus on strategies which are most likely to have clinical viability.

Pharmacological adjuncts

Fluoxetine. In preclinical research, chronic administration of the selective serotonin reuptake inhibitor fluoxetine for 2 weeks improves fear extinction and decreases anxiety- and depressive-like behaviours in adult rodents (Deschaux, Spennato, Moreau, & Garcia, 2011; Karpova et al., 2012). The benefits of fluoxetine for

fear extinction have even been extended to the S1 mouse strain which, as noted earlier, show impaired extinction retention (Gunduz-Cinar et al., 2016). While it is well-known that fluoxetine works by facilitating serotonergic transmission (Stahl, 1998), the drug's actions on extinction are also mediated through endocannabinoid signalling. Specifically, systemic or intra-amygdala administration of the CB1R antagonist SR141716A prior to extinction training prevents the extinction-enhancing effects of fluoxetine in adult S1 mice (Gunduz-Cinar et al., 2016). If the effects of fluoxetine are indeed dependent on CB1R activation during extinction training, the failure of the CB1/2R agonist WIN55212-2 to augment extinction in adolescent rats suggests that fluoxetine may offer limited benefit during adolescence.

Although fluoxetine is prescribed to young people with anxiety and/or depression in general practice settings (Bachmann et al., 2016), the evidence supporting its use in youth is limited. For instance, in a recent multi-centre clinical trial, Davey et al. (2019) found that the addition of fluoxetine to cognitive behaviour therapy had no impact on treatment outcomes relative to placebo in a sample of young people aged 15-25 years diagnosed with moderate to severe depression. To date, an investigation into the effects of fluoxetine in young people with a primary diagnosis of anxiety has yet to be published. However, given that 63% of the young people in the sample reported by Davey et al. (2019) were also diagnosed with an anxiety disorder, similar null effects may be expected. In support of this suggestion, null effects of fluoxetine have been observed for extinction performance in adolescent rats. Indeed, unpublished data from our laboratory indicates that fluoxetine fails to enhance fear extinction in adolescent rats unlike its effects in adults (Chan, 2018). Specifically, chronic administration of fluoxetine (10mg/kg) for 2 weeks did not ameliorate impaired extinction retention in adolescent rats (the same dose improved extinction in adult rats). As the actions of

fluoxetine on extinction are dependent on endocannabinoid signalling, and specifically CB1Rs in the amygdala (Gunduz-Cinar et al., 2016), fluoxetine may fail to improve extinction during adolescence due to the immaturity of the endocannabinoid system.

Cannabidiol. There are two major constituents to cannabis, one of which is cannabidiol (Marco, 2011; Mechoulam, Parker, & Gallily, 2002). As cannabidiol is not responsible for the psychoactive and anxiogenic properties of cannabis, it is well-placed to be a therapeutic adjunct (Amminger et al., 2017; Patel, Hill, Cheer, Wotjak, & Holmes, 2017). Furthermore, cannabidiol has been shown to improve extinction in both adult rodents (Bitencourt et al., 2008; Do Monte, Souza, Bitencourt, Kroon, & Takahashi, 2013) and healthy adult humans (Das et al., 2013). When compared to other endocannabinoid-derived agents such as WIN55212-2, cannabidiol may have different effects on extinction due to divergent actions at receptor sites. While both agents target CB1Rs, the two agents have contrasting effects on transient receptor potential vanilloid-type 1 receptors, which are involved in synaptic plasticity (Cui, Perez, & Venance, 2018; Gibson, Edwards, Page, Van Hook, & Kauer, 2008). Whereas cannabidiol stimulates the activity of this receptor, WIN55212-2 inhibits it (Bisogno et al., 2001; De Petrocellis et al., 2011; Patwardhan et al., 2006). Therefore, administration of cannabidiol may not result in increased fear expression during extinction training in adolescent rats, as was observed following WIN55212-2 administration. Instead, cannabidiol could lead to improved extinction learning and retention during adolescence, as has been observed in adult rodents and humans.

Despite the fact that the effects of cannabidiol on extinction during adolescence remain to be tested in preclinical models (rodent or human), clinical research has begun to examine cannabidiol's therapeutic potential. In a case study of a 10 year old girl with post-traumatic stress disorder, cannabidiol was reported to lead to a reduction in anxiety

symptoms (Shannon & Opila-Lehman, 2016). In addition, an open-label pilot trial of cannabidiol in young people (12-25 years old) with treatment-resistant anxiety is currently underway in Australia (Trial Registration #: ACTRN12617000825358; Principal Investigator: P.A. Amminger). Alongside this pilot trial, future research is required to investigate how cannabidiol acts in the adolescent brain to influence treatment outcomes. Such research is particularly important in the case of cannabidiol, as the findings of my thesis indicate that developing animals may respond differently to some cannabinoid agents (e.g., WIN55212-2).

Behavioural strategies

Exercise. The benefits of increased exercise are numerous for not only an individual's physical health, but also their mental health. To illustrate, evidence supports the benefits of prescribing exercise for a range of psychiatric, neurological, cardiovascular, and musculoskeletal disorders (for review, see Pedersen & Saltin, 2015). Of interest to the present thesis, a short bout of exercise immediately following extinction training has been shown to improve extinction retention the following day in both rodent and human adults (Bouchet et al., 2017; Keyan & Bryant, 2019; but see Jacquart et al., 2017). There are several likely pathways by which exercise could maximise extinction learning. First, exercise impacts the endocannabinoid system, such that 2 weeks of daily exercise enhances the sensitivity of CB1Rs in adult rodents at least in the striatum, the only region assessed in this study (De Chiara et al., 2010), and 1-h of moderate intensity exercise increases peripheral anandamide levels in individuals who regularly engage in exercise (Sparling, Giu, Piomelli, Rosskopf, & Dietrich, 2003). Given demonstrations of how increased activity of the endocannabinoid system leads to enhanced extinction, adding exercise following extinction training may benefit extinction retention in adolescent rats. In support of this suggestion, exercise

downregulates CB1R expression in adolescent rats (note that there were no other age groups included in this study; Gomes da Silva et al., 2010). As CB1R density in the mPFC and amygdala is greater during adolescence compared to adulthood (Experiment 2.6), exercise may be beneficial for enhancing extinction during this developmental period. In saying this, it must be acknowledged that this suggestion is tempered by the findings reported in Chapter 2, in which increased CB1R activation failed to lead to improved extinction in adolescent rats. Indeed, it may be the case that any interventions – whether pharmacological or behavioural – that target CB1Rs to augment extinction have limited efficacy during development.

The extinction-enhancing effects of exercise during adulthood are also mediated by brain-derived neurotrophic factor (BDNF). The neurotrophin BDNF is involved in activity-dependent synaptic plasticity, and directly augmenting BDNF levels facilitates fear extinction in adult rodents (Leßmann & Brigadski, 2009; Peters, Dieppa-Perea, Melendez, & Quirk, 2010). BDNF levels have been found to increase following exercise dose-dependently, such that more exercise leads to a greater increase in BDNF (Ferris, Williams, & Shen, 2007; Rojas Vega et al., 2006; Sleiman et al., 2016). There are, however, individual differences in the extinction-enhancing effects of exercise that are a function of the BDNF polymorphism Val66Met. When compared to individuals who carry the Val allele, individuals who carry the Met allele show impaired fear extinction and compromised functional connectivity between the mPFC and amygdala (Soliman et al., 2010). Furthermore, engaging in exercise following extinction training is only beneficial for individuals who carry the Val allele; engaging in exercise was actually associated with a greater return of fear in individuals who carry the Met allele (Keyan & Bryant, 2019). In any case, there has yet to be an investigation into whether exercise following extinction training improves extinction retention in adolescent rats,

or impacts exposure therapy outcomes in clinical populations. I expect that exercise would facilitate extinction retention during adolescence, as moderate to high intensity exercise increases BDNF serum levels in adolescent humans (Jeon & Ha, 2017) and increasing BDNF levels via the agonist 7, 8-DHF facilitates extinction retention in adolescent rats (Stylianakis, Baker, & Richardson, 2019). If engaging in exercise was able to result in improved extinction performance via BDNF pathways independently of the endocannabinoid system, this behavioural intervention holds substantial potential as a candidate adjunct to exposure-based therapies.

It should be noted that the benefits of engaging in exercise following extinction training may differ between adolescent males and females. In adult rodents, 2-h of acute exercise immediately following extinction training improved extinction retention in adult male rodents, but had no benefit for adult female rodents (Bouchet et al., 2017; Tanner, Hake, Bouchet, & Greenwood, 2018). This discrepancy may be, at least in part, a result of sex differences in one of the putative mechanisms of exercise on extinction: BDNF. Whereas administration of the BDNF mimetic 7, 8-DHF enhanced extinction in adult male rodents, the same drug impaired extinction in adult female rodents (Tohyama, Matsuda, & Mizutani, 2020). If the same pattern observed in adults across male versus female rodents occurs during adolescence, post-extinction exercise may only be beneficial for adolescent males.

Novelty-facilitated extinction. As adolescent rats use opioid-receptor related prediction error mechanisms when given additional extinction training (Experiment 4.3), this suggests that extinction/exposure in human adolescents could be facilitated by maximising negative prediction error. Theoretically, this could be achieved pharmacologically by temporarily increasing opioid neurotransmission at the time of extinction training but the use of opiates (e.g., morphine) raises important issues of

efficacy and safety, especially in paediatric populations. An alternative, perhaps more practical and safe, approach would be to maximise prediction error by modifying extinction/exposure procedures. This could be achieved through a procedure termed novelty-facilitated extinction.

As novel stimuli lead to faster learning than familiar stimuli, it is not surprising that the pairing of a novel cue with the conditioned stimulus (CS) during extinction training facilitates extinction retention in adult rodents (Dunsmoor et al., 2015). During standard extinction training, there is a difference between the expected outcome (CS = US) and the actual outcome (CS = no US; i.e., the *absence* of the shock). During novelty-facilitated extinction training, there is an even larger difference between the expected outcome (CS = US) and the actual outcome (CS = novel stimulus [NS], i.e., the *absence* of the shock in addition to the *presence* of a novel stimulus). As put forward by Dunsmoor et al. (2015), the inclusion of the NS alongside the CS during extinction training is surprising, and therefore leads to increased prediction error and enhanced learning. As the findings of Chapter 4 clearly demonstrated that adolescent rats acquired extinction when given additional time to recruit prediction error mechanisms, modifying extinction training as a 'short-cut' to enhanced opioid receptor activity is a particularly encouraging possibility for augmenting exposure-based therapies.

The benefits of novelty-facilitated extinction training have also been extended from preclinical studies to adult humans. During novelty-facilitated extinction training, the CS (e.g., an angry face) is followed by the NS (e.g., a low-volume tone), and extinction retention involves presentations of the CS without the NS. Compared to standard extinction, novelty-facilitated extinction leads to faster extinction learning, reduced spontaneous recovery, and reduced reinstatement in adult humans (as assessed

using skin conductance responding; Dunsmoor et al., 2015, 2019; Lucas, Luck, & Lipp, 2018). One theorised neurobiological mechanism underpinning the success of novelty-facilitated extinction is greater recruitment of the PFC. Using functional neuroimaging techniques, Dunsmoor et al. (2019) demonstrated that adult participants who underwent novelty-facilitated extinction training showed increased activation of the PFC relative to those participants who underwent standard extinction training. As the adolescent extinction deficit is characterised by an under-recruitment of the PFC, adapting standard extinction/exposure procedures to include a novel stimulus may ameliorate this impairment by enabling increased engagement of the PFC. In clinical settings, novelty-facilitated exposure therapy could involve following exposure trials (e.g., of a picture of a needle in blood-injury-injection phobia) with a short, low-volume tone. By adapting traditional extinction/exposure methods to specifically target key deficits in the neurobiology in safety learning during adolescence (e.g., under-recruitment of the PFC, slowed engagement of opioid receptors), treatment outcomes are likely to improve.

Limitations

Overall, it is important to acknowledge the primary limitation of my work in understanding the mechanisms of anxiety treatment in adolescent humans – namely, that the work was conducted using an animal model. While the use of animal models allows for unparalleled experimental manipulation, as well as the opportunity to examine the neural consequences of experimental procedures, it does limit the extent to which conclusions can be drawn regarding psychological treatment in humans. However, in saying this, extinction and exposure therapy are based on the same underlying principle – that repeated exposures to a feared stimulus will eventually lead to the acquisition of a safety memory (Craske et al., 2018; Scheveneels, Boddez, Vervliet, & Hermans, 2016). Furthermore, the neural mechanisms which underlie the

two procedures are evolutionarily conserved across species, even when considering differences between the rodent and human brains (VanElzakker, Dahlgren, Davis, Dubois, & Shin, 2014). In my view, the use of preclinical models is critical in developing a preliminary understanding of the brain regions and neurotransmitters which are involved in extinction, as this knowledge then informs the development of pharmacological adjuncts, as well as modifications to exposure therapy.

Secondly, the experiments presented in this thesis all employed systemic administration of pharmacological agents. That is, the agents were injected into either the nape of the neck (Chapters 3-4) or the intraperitoneal cavity of the abdomen (Chapter 2). While these routes of administration lead to reliable behavioural effects, as observed in the current thesis, they do not allow for conclusions to be drawn regarding the locus of their action within the brain (i.e., the medial prefrontal cortex versus the amygdala). Instead, direct intracranial injections would be required. Therefore, the conclusions drawn about *where* in the brain the neurotransmitter systems act (or fail to act) during extinction in adolescent rats are speculative, and future research should follow up these results using targeted, regionally specific administration of the pharmacological agents within the brain.

Concluding remarks

Adolescence is a time of not only profound vulnerability to developing anxiety, but also one of opportunity for early intervention. In order to understand how to optimise treatments for anxiety within this window of opportunity, my thesis examined how the adolescent brain processes learned threat and acquires safety learning. To begin, I investigated the potential of the CB1/2R agonist WIN55212-2 to improve extinction retention when administered prior to the first session of extinction training in

adolescent rats. Unexpectedly, administration of WIN55212-2 increased fear expression during extinction training and had no impact on extinction retention performance. In subsequent chapters, I found that the first session of extinction training in rats conditioned and extinguished as adolescents is associated with impaired extinction retention alongside a failure to recruit NMDARs and opioid receptors.

However, an additional extinction training session facilitated the engagement of NMDARs and opioid receptors in rats conditioned and extinguished as adolescents, leading to good extinction retention. This set of findings indicates that the adolescent brain can successfully adapt and acquire safety learning when given additional training – likely a reflection of a quantitatively slower, but qualitatively similar, neural circuitry which underpins extinction in rats conditioned and extinguished as adolescents compared to adulthood.

In contrast, opioid receptors were involved during the first session of extinction training in rats conditioned as juveniles and extinguished as adolescents and led to good extinction retention. Somewhat surprisingly, the consolidation of extinction learning occurred without NMDARs in this group of adolescent rats; the neurobiological mechanisms which enable extinction consolidation are, as yet, undetermined. In contrast to rats conditioned and extinguished as adolescents, this latter population of adolescent rats recruit a qualitatively different neural circuitry to consolidate extinction.

While further research is needed to understand the neurotransmitters involved in extinction learning and consolidation in adolescent females, my findings highlight the incredibly complex and dynamic nature of the neural circuitry underpinning extinction during adolescence. The differential involvement of the opioid, glutamatergic, and endocannabinoid systems during extinction training (in addition to developmentally

appropriate structural alterations in the neurotransmitter systems) will impact the effectiveness of novel behavioural/pharmacological interventions designed to augment extinction/exposure during adolescence. Taken together, the experiments presented in this thesis highlight the importance of understanding an individual's age at fear acquisition during assessment, and extending the duration of exposure activities during treatment, to optimise treatment outcomes for adolescents with anxiety.

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