The Role of CRFR1 in Addiction and Anxiety Disorders

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Abstract

Addiction and anxiety disorders are highly co-morbid, and represent a huge burden on society. The central role of stress-reactivity in the pathogenesis and maintenance of both of these diseases has led to the identification of corticotropin-releasing factor (CRF) signalling as a key factor in these effects. The focus of this thesis was the ventral tegmental area (VTA), as it is a site where reward- and fear-related circuitry converge and can be modulated by CRF. The broad aims of this thesis were to examine the role of VTA CRF receptor 1 (CRFR1) in animal models of reward-seeking and conditioned fear to understand how these systems can become dysregulated in addiction and anxiety disorders. To this end, chapter 3 of this thesis validated a technique for the viral-mediated downregulation of CRFR1 within the VTA, and chapter 4 established a novel model of stress-induced reinstatement of cocaine-seeking in mice. These techniques were then implemented to examine the effects of VTA CRFR1 knockdown on the acquisition, extinction, and reinstatement behaviours.

Chapters 5 and 6 are two separate publications demonstrating that VTA CRFR1 signalling is differentially involved in various components of cocaine-seeking and conditioned fear. In chapter 5, knockdown of CRFR1 in the VTA blocked stress-induced reinstatement of cocaine-seeking and attenuated cued cocaine-seeking, without any effects on self-administration or extinction responding. This was a specific effect on drug-related behaviours, as there were no changes to operant responding for sucrose rewards. In chapter 6, VTA CRFR1 knockdown enhanced the expression of conditioned fear, but had no effects on fear extinction or reinstatement. This evidence suggests that CRFR1 participates in distinct subcircuits within the VTA which mediate fear and reward-seeking.
Declaration

This is to certify that:

(1) The thesis comprises only my original work towards the degree of Doctor of Philosophy, except where indicated in the Preface.

(2) Due acknowledgement has been made in the text to all other materials used.

(3) The thesis is fewer than 100,000 words in length, exclusive of tables, figures, references, and appendices.

Signed,

Nicola Alexandra Chen
Preface

The author would like to acknowledge the following people for their contributions of data collection and technical assistance. Data that were collected by the author which contributed to the award of a previous qualification [Bachelor of Science (Honours)] are also acknowledged.

Mice used for all intravenous self-administration studies were cannulated by Professor Andrew J Lawrence. These surgeries were performed with the assistance from the following laboratory members at various times: Dr Jee Hyun Kim, Dr Rose Chesworth, Sarah Ch’ng, and Dr Bianca Jupp. Stereotaxic microinjection surgeries were performed with the assistance of Dr Jee Hyun Kim and Shawn Tan. Behavioural results presented in chapter 4 were obtained with the assistance of Dr Jee Hyun Kim and Dr Rose Chesworth. qPCR data presented in chapter 3 were obtained under the guidance of Dr Despina Ganella and Dr Chris Bye. Fear conditioning data were collected with the assistance of Johnny Park and Dr Jee Hyun Kim.

Chapter 5 includes a manuscript that was accepted for publication in The Journal of Neuroscience. I, Nicola Chen, am the primary author of this paper and contributed more than 50% of the experimental planning, execution, and preparation of the research data for publication. This has been acknowledged by completion of ‘co-author authorisation’ and ‘declaration of thesis with publication’ forms that were submitted with this thesis, in line with The University of Melbourne requirements. Approximately 30% of the data presented in this paper had been collected by the author, prior to the commencement of her PhD candidature, and contributed to the award of a previous qualification [Bachelor of Science (Honours)]. In the studies described, Dr Bianca Jupp performed some of the stereotaxic surgeries and assisted with behavioural data collection for the cued cocaine-seeking sections of the paper, along with Dr Yehezkel Sztainberg and Dr Robyn M Brown and myself. Viral vectors and qPCR validation data in this paper were produced by the co-authors Dr Maya Lebow and Professor Alon Chen. All other viral vectors used in this study were produced by Sharon Layfield and Professor Ross Bathgate.

Chapter 6 includes a manuscript that was accepted for publication in the scientific journal European Neuropsychopharmacology. I, Nicola Chen, am the primary author of this paper and contributed more than 50% of the experimental planning, execution, and preparation of the research data for publication. The viral constructs described in this paper were provided by Professor Alon Chen. All viral vectors used in this study were produced by Sharon Layfield and Professor Ross Bathgate. qPCR data was obtained with the assistance of Dr Despina Ganella.
Acknowledgements

Firstly, I would like to thank my supervisors Professor Andrew Lawrence and Dr Jee Hyun Kim, who have shaped me as a scientist as well as a person. Thank you Andy for taking me into your lab as a fresh-faced 21-year-old honours student. I didn’t realise at the time how lucky I was to have this as my introduction to lab life, but now it is obvious. I really appreciated your open door policy and ability to decipher my half-formed thoughts and know exactly what I was talking about, even though you had a dozen other projects going at the same time. Thank you for your invaluable guidance over the years. Thank you Jee for your excitement and enthusiasm for science. One of my earliest lab memories is of you showing me how to do immuno, side-by-side at the lab bench. You always took this hands-on approach to teaching me and showed that you really cared about my learning. Whenever I came into your office freaking out, you could show me the best way forward, no matter how lost I was. Thank you for imparting so much of your wisdom on all aspects of lab and life, and for being such a boss. I can only hope that one day, like you two, I will also have the seemingly magical ability to solve whatever conundrums I run into, but guess that comes with experience; slowly gained, bit-by-bit, through years of hard work and dedication.

I would like to thank the members of my PhD advisory committee, Dr Peter Kitchener and Professor Andrew Gundlach, for all their advice and support—they always had my best interests at heart. I would also like to thank my collaborators Sharon Layfield and Professor Ross Bathgate for assenting to my unending requests for more virus—none of the studies in this thesis would have been possible without you. Thank you to Dr Chris Bye for your expert advice on qPCR work. I would also like to thank the staff of the core animal facility. Thank you to Daniel Drieberg, for your constant assistance in caring for my mice, and to Brett Purcell for making sure all the behavioural equipement ran smoothly.

Many thanks to past and present members of the Lawrence lab and the Kim lab. I am indebted to the post docs, Dr Robyn Brown, Dr Heather Madsen, Dr Christina Perry, Dr Jhodie Duncan, Dr Craig Smith, and Dr Bianca Jupp, who generously gave their time to help me when I ambushed them with random problems and questions. You have all rescued me from disaster on countless occasions. Thank you to Dr Despina Ganella for teaching me everything I know about qPCR, and for all your patience in guiding this dirty mouse trainer through the world of molecular biology. Thank you to Luba Lee-Kardashyan for keeping the lab running smoothly—we all know the place would burst into flames if you left. To my labmates who shared the struggle, Dr Rose Chesworth, Dr Alec Dick, Dr Hanna Kastman and Dr Andrew Walker, thank you for all your help and for encouraging me when things seemed impossible. And to my labmates Sophia Luikinga, Felicia Reed, Leigh Walker, Annabeth Simpson, Jan Koelmann, Shaun Khoo, Sarah Gibbs, Russell Coulthard, and Marionne Tolentino, for the much-needed laughs along the way. Thank you to my stereotaxic surgery buddy
Shawn Tan, for all the hours spent in that small windowless room together, unwittingly gassing ourselves with iso and dealing with cabin fever. A special thank you to my partner in crime, Isabel Zbukvic—you kept my head up for this marathon and dragged my limp body behind you when necessary. It would only be fitting if we could throw our hats up together.

I would like to thank my mom and sister and the rest of the family for all their love and support over the years. I’m looking forward to getting to spend more time in the same country. Many thanks to Bridie and Eve who have taken such good care of me when I needed it the most. Thank you to all of my friends who were there for a drink or seven when things all seemed too hard, gave me perspective when it seemed like the end of the world, and forgave my absences when I really should have been there. I will now crawl out of my thesis hole and come party.

Lastly, to my partner Jorja, in this thesis I demonstrate the extent of my vocabulary and scientific rigor when interrogating the neurobiological mechanisms of addition and anxiety disorders, but I simply do not have the words to express my gratitude for the unwavering care and support you show me on a daily basis. You motivate me to do the best that I can and make you proud. I love you, and I would not have been able to do this without you.
This thesis is dedicated to all the people around the world who suffer from addiction and anxiety disorders, and their loved ones.
Publications

Original peer-reviewed articles arising from this research:


Original peer-reviewed articles arising from other works:


Conference poster presentations arising from this research:


Awards

Australian Postgraduate Award—3 year stipend (2012-2015)

1st prize poster presentation in ‘Behavioural Neuroscience and Neurophysiology’ category. SOBR symposium.
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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>avidin-biotin complex</td>
</tr>
<tr>
<td>AON</td>
<td>anterior olfactory nucleus</td>
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<tr>
<td>AMB</td>
<td>nucleus ambiguus</td>
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<td>AMG</td>
<td>amygdala</td>
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<td>β2-AR</td>
<td>β2 adrenergic receptor</td>
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<td>basolateral amygdala</td>
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<td>BNST</td>
<td>bed nucleus of the stria terminalis</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>cc</td>
<td>corpus callosum</td>
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<td>central amygdala</td>
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<td>cerebellum</td>
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<td>conditioned place aversion</td>
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<td>conditioned place preference</td>
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<td>caudoputamen</td>
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<td>CRF</td>
<td>corticotropin-releasing factor</td>
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<td>CRFBP</td>
<td>corticotropin-releasing factor binding protein</td>
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<td>corticotropin-releasing factor type 1 receptor</td>
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<td>CS, CS+</td>
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<td>C&lt;sub&gt;r&lt;/sub&gt;</td>
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<td>double stranded DNA</td>
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<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>Acronym</td>
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<td>EW</td>
<td>Erdinger-Westphal nucleus</td>
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<td>hypoxanthine phosphoribosyltransferase 1</td>
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<td>ICSS</td>
<td>intracranial self-stimulation</td>
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<td>ID</td>
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<td>optimal cutting temperature</td>
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<td>PAG</td>
<td>periaqueductal grey</td>
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<tr>
<td>PB</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SEPT</td>
<td>septal region</td>
</tr>
<tr>
<td>S. COL.</td>
<td>superior colliculus</td>
</tr>
<tr>
<td>shCRFR1</td>
<td>short hairpin corticotropin-releasing factor receptor subtype 1</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SO</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>SUM</td>
<td>supramammillary nucleus</td>
</tr>
<tr>
<td>TX100</td>
<td>triton X-100</td>
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<tr>
<td>UR</td>
<td>unconditioned response</td>
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<tr>
<td>US</td>
<td>unconditioned stimulus</td>
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<tr>
<td>VENT THAL</td>
<td>ventral thalamus</td>
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<tr>
<td>VMH</td>
<td>ventromedial nucleus of the hypothalamus</td>
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<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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CHAPTER 1:
GENERAL
INTRODUCTION
1.1 Preface

Fear and reward-seeking are essential emotions that drive behaviours necessary for survival. However, when expressed inappropriately, both can lead to maladaptive behaviour, manifesting as anxiety disorders and drug addiction. These disorders are highly prevalent and constitute a leading worldwide healthcare problem and cause of death (figure 1-1) (Kalivas & Volkow, 2005; Whiteford et al., 2013); indeed, 1 in 4 Australian adults are diagnosed with an anxiety disorder and/or substance abuse at least once in their life (Australia Bureau of Statistics, 2007). According to the Global Burden of Disease Study 2013, there were an estimated 266 million prevalent cases of anxiety disorders, and 127 million cases of substance use disorders (Global Burden of Disease Study Collaborators, 2015).

![Figure 1-1](image)

Figure 1-1 Global impacts of addiction and anxiety disorders. Proportion of years of life lost and disability-adjusted life years attributable to addiction and anxiety disorders compared to all other mental illness, based on findings from the World Health Organisation 2010 Global Burden of Disease report. Adapted from Whiteford et al. (2013).

These disorders significantly increase risk of injury, illness and death, and are associated with depression, cardiovascular disease, HIV, and suicide (Albert et al., 2005; Degenhardt & Hall, 2012; Grant et al., 2009; Kawachi et al., 1994; Nepon et al., 2010; Pompili et al., 2010; Smoller et al., 2007). They also have a large impact on the community through urban crime, road accidents, domestic violence, loss of productivity, and other social and emotional burdens (Collins & Lapsley, 2008). These factors contribute to the cost incurred, which is estimated at over $700 billion per year for substance abuse (Pouletty, 2002; Volkow et al., 2016), and $150 billion per year for anxiety disorders, in the USA alone (DuPont et al., 1996; Greenberg et al., 1999; Kessler & Greenberg, 2002). Recent Australian estimates suggest the national cost of all licit and illicit drug use was $56.1 billion in 2004/05 (Collins & Lapsley, 2008).
Drug addiction is a chronic, relapsing neurological disease characterized by enduring compulsive drug use and the compromised ability to suppress drug seeking, even when confronted with serious adverse consequences. As indicated in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), the specific cognitive, behavioural, and physiological symptoms of drug addiction (classified as substance use disorders) indicate significant impairment and distress (table 1-1) (American Psychiatric Association, 2013). An important characteristic of substance use disorders is an underlying change in brain circuits that persist beyond detoxification. These brain changes may manifest behaviourally in the repeated relapses and intense drug craving when the individuals are exposed to drug-related stimuli.

Table 1-1 DSM-5 diagnostic criteria for substance use disorders. Diagnosis of substance use disorders requires at least two of these symptoms within 12 months.

<table>
<thead>
<tr>
<th>Diagnostic Criteria for Substance Use Disorders</th>
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<tr>
<td>• Drugs are often taken in larger amounts or over a longer period than was intended.</td>
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<td>• There is a persistent desire or unsuccessful efforts to cut down or control drug use.</td>
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<td>• A great deal of time is spent in activities necessary to obtain drugs, use drugs, or recover from its effects.</td>
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<td>• Craving, or a strong desire or urge to use drugs.</td>
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<td>• Recurrent drug use resulting in a failure to fulfill major obligations at work, school, or home.</td>
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<tr>
<td>• Continued drug use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of drug use.</td>
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<tr>
<td>• Important social, occupational, or recreational activities are given up or reduced because of drug use.</td>
</tr>
<tr>
<td>• Recurrent drug use in situations in which it is physically hazardous.</td>
</tr>
<tr>
<td>• Drug use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by drugs.</td>
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<tr>
<td>• Tolerance, as defined by either of the following:</td>
</tr>
<tr>
<td>o A need for markedly increased amounts of drug to achieve intoxication or desired effect.</td>
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<tr>
<td>o Markedly diminished effect with continued use of the same amount of drug.</td>
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<tr>
<td>• Withdrawal, as manifested by either of the following:</td>
</tr>
<tr>
<td>o Characteristic withdrawal syndrome of the drug.</td>
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<tr>
<td>o Taking the drug or another closely related substance to relieve or avoid withdrawal symptoms.</td>
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Anxiety disorders are a collection of related mental disorders all involving pervasive, uncontrollable fear and worrying and related behavioural disturbances (table 1-2) (American Psychiatric Association, 2013). They differ from normative fear or anxiety by being excessive or
persisting beyond appropriate periods, typically lasting 6 months or more—long after the initial fear-evoking or stressful event has passed.

Table 1-2 List of DSM-5 anxiety disorders

<table>
<thead>
<tr>
<th>Anxiety disorders</th>
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<tbody>
<tr>
<td>• Separation Anxiety Disorder</td>
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<td>• Selective Mutism</td>
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<tr>
<td>• Specific Phobia</td>
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<tr>
<td>• Social Anxiety Disorder (Social Phobia)</td>
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<tr>
<td>• Panic Disorder</td>
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<tr>
<td>• Panic Attack (specifier)</td>
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<tr>
<td>• Agoraphobia</td>
</tr>
<tr>
<td>• Generalised Anxiety Disorder</td>
</tr>
<tr>
<td>• Other Specified Anxiety Disorder</td>
</tr>
<tr>
<td>• Unspecified Anxiety Disorder</td>
</tr>
</tbody>
</table>

1.2 Comorbidity of addiction and anxiety disorders

Importantly, addiction and anxiety disorders have a bidirectional relationship, and are highly correlated (Burns et al., 2001; Grant et al., 2014). Epidemiological studies provide evidence for a pattern of comorbidity between these disorders (Burns et al., 2001; Compton et al, 2007; Grant et al, 2004, 2009; Hasin et al., 2007; Merikangas et al., 1998; Regier et al., 1990), with anxiety being highly correlated with drug (r=0.412) and alcohol dependence (r=0.281) (Hodgson et al., 2016). Increased rates of anxiety disorders have been found across all classes of drug use disorders including cocaine, alcohol, tobacco, cannabis, sedatives, opiates, and other stimulants (Vorspan et al., 2015). Cocaine use was found to be associated with lifetime anxiety disorders with an odds ratio between 1.5 and 3 (Sareen et al., 2006). A considerable amount of evidence suggests anxiety disorders are more prevalent in cocaine-dependent individuals, including a three- to four-fold increase in panic attacks (Katerndahl & Realini, 1999; O’Brien et al., 2005). Furthermore, in clinical samples of patients in care for cocaine addiction, the proportion of those diagnosed with current anxiety disorders has been found to be around 10%-15%, and up to 40% if lifetime diagnosis is taken into account (Vorspan et al., 2015).

Not only are they comorbid, but there is also evidence for mutual maintenance of one another—both anxiety disorders and substance use disorders impact the course and treatment outcome for the counterpart condition. Indeed, as the severity of substance use disorders increases, so does the observed rate of anxiety comorbidity (Compton et al., 2007; Merikangas et al., 1998). Among individuals with addictive disorders, comorbid anxiety disorders are associated with more severe
symptomatology, greater social and functional impairments, and higher relapse rates (Driessen et al., 2001; Johnston et al., 1991; Perkonigg et al., 2006; Sannibale & Hall, 2001; Thomas et al., 1999). Alternatively, the presence of a substance use disorder is associated with decreased recovery rates and increased recurrence of anxiety disorders (Bruce et al., 2005), as well as elevated risk of suicide in patients with panic disorder (Hornig & McNally, 1995).

Shared familial risk factors for alcoholism and anxiety disorders suggest that common genetic factors play an important role in these high rates of comorbidity (Goodwin et al., 2011; Merikangas et al., 1994, 1998; Tambs et al., 1997). This was demonstrated in a recent genome-wide linkage study which observed that anxiety had genetic correlations with alcohol (0.655) and drug dependence (0.619), and identified a number of significant quantitative trait loci that underlie this genetic overlap (Hodgson et al., 2016).

Despite this evidence for a shared aetiology, these disorders are rarely examined together in preclinical research, and still lack effective treatments. Therefore, considering their high comorbidity and overlapping neural circuitry (Avery et al., 2016; Conway et al., 2006; Daniel & Rainnie, 2016; Jennings et al., 2013; Lammel et al., 2012; Luthi & Luscher, 2014; Peters et al., 2009), it is important to examine reward- and aversive-learning in parallel. Taken together, these collective findings highlight the need to investigate these shared neurobiological mechanisms in order to develop new and innovative approaches to treating co-occurring anxiety and substance use disorders.

1.3 Animal models

Rodent models of addiction and anxiety can be used to investigate the fundamental mechanisms of these disorders (Sanchis-Segura & Spanagel, 2006; Uys & Stein, 2003).

1.3.1 Intravenous self-administration model

Intravenous self-administration is one of the most widely utilized models assessing rodent analogues of the major elements of addiction: acquisition, compulsive drug-seeking and reinstatement (or relapse). This experimental design minimises human intervention, and consequently has a robust reproducibility index and high face value for the clinical situation. Indeed, the abuse potential of a substance in humans can be predicted from self-administration in rodents (Collins et al., 1984). In this paradigm, rodents are typically presented with two levers (or nose pokes). Depression of the ‘active’ lever produces the delivery of drug through a venous (typically jugular) catheter, and is coupled to a cue such as a tone and/or light, whereas the ‘inactive’ lever either delivers saline or has no programmed consequence. In this way, the tone/light cue becomes a conditioned stimulus (CS) that signals drug delivery. These drug-associated stimuli can be used to represent cues/contexts that induce craving and trigger relapse (Jupp & Lawrence, 2010).
**1.3.2 Fear conditioning**

The most widely-employed laboratory paradigm to study anxiety disorders is Pavlovian fear conditioning which models the learning that can occur in a fearful situation that may lead to anxious behaviours. Typically, it involves a neutral stimulus (e.g., tone or light) that is presented with an aversive unconditioned stimulus (US), such as a foot-shock. After repeated pairings, the tone/light becomes a CS, and its presentation alone elicits fear responses, which is referred to as a conditioned response (CR). Pavlovian fear conditioning is a robust paradigm to examine anxiety disorders because it is readily acquired over a range of different species, including humans. This paradigm is adaptable in that different stimuli can be used as US/CS. Moreover, there are different measures to report fear. In humans, heart rate and galvanic skin responses can be used. In rodents, a protective response known as freezing, where the animal becomes entirely still except for breathing, is widely used as a reliable index of fear (Blanchard & Blanchard, 1969). Although Pavlovian conditioned fear does not comprehensively model all the elements that can be involved in anxiety disorders, it is important to understand how learned fear is normally regulated to understand how fear can go astray. Also, learned fear is directly involved in common forms of anxiety disorders such as post-traumatic stress disorder and phobias.

**1.3.3 Extinction as a common mechanism underlying the treatment of substance abuse and anxiety disorders**

Behavioural treatments for anxiety disorders and substance abuse both rely on learning to inhibit fear or drug-seeking through a process called extinction (Peters et al., 2009). Extinction is the decrease in conditioned fear/drug-seeking responses to a stimulus that is brought about by repeated presentation of the stimulus without reinforcement by the US. Specifically, if the tone CS is no longer paired with a shock, or the lever press no longer delivers cocaine, the conditioned responding (e.g., freezing or lever pressing) will diminish over time. This reduction in CRs by extinction does not reflect erasure of the initial CS-US association. Rather a new association is formed (CS-No US) that suppresses the conditioned memory (Milad & Quirk, 2012). However, this extinction memory is not as robust as the original conditioned memory. Since the original association is still available for retrieval, the CRs can be reinstated without any retraining. Therefore, it has been proposed that strengthening the formation of extinction memory may be a promising strategy to for the treatment of these disorders (Kaplan et al., 2011; Maren & Holmes, 2015; Myers & Carlezon, 2010; Perry et al., 2014; Peters et al., 2009).

**1.3.4 Reinstatement as a model of relapse**

Addiction and anxiety disorders are chronic, long-lasting disorders, due in part to the high risk of relapse even after extended periods of time (Keller et al., 1994; O’Brien, 2008; Penninx et al., 2011;
Pollack et al., 1990; Scholten et al., 2013; Yonkers et al., 2000, 2003). Prospective studies of patients with anxiety disorders have reported relapse rates of 38-58% over 12 years (Bruce et al., 2005), and up to 65% recurrence during a 3-year follow-up, in women (Calkins et al., 2009). Additionally, it has been estimated that up to 90% of addicted individuals relapse to drug-taking within 12 months of abstinence, making relapse prevention a central problem for successful treatment (DeJong, 1994). There are three main factors that are known to induce relapse in both anxious and drug dependent individuals: (1) exposure to the drug/fear-inducing stimuli (Dirikx et al., 2004, 2007; Hermans et al., 2005; Jaffe et al., 1989; Ludwig et al., 1974; Norrholm et al., 2006), (2) exposure to associated cues (Childress et al., 1999; O’Brien et al., 1992; Sell et al., 2000), and (3) exposure to stressful stimuli (Brown et al., 1995; Sinha et al., 2000, 2003, 2006; Wade et al., 1993).

In animal models, these same factors can recover extinguished responding in both fear and drug self-administration paradigms. These interventions include (1) ‘primes’ of sub-threshold shocks/drug (de Wit & Stewart, 1981, 1983, 1996; Rescorla & Heth, 1975), (2) exposure to conditioned stimuli (Bouton & Bolles, 1979; Crombag & Shaham, 2002; Meil & See, 1996), and (3) a variety of stressors (Deschaux et al., 2013; Mantsch et al., 2016; Morris et al., 2005). These animal models have significantly advanced our understanding of the various components of addiction and anxiety disorders, as well as alluding to potential interactions between these paradigms. For example, chronic cocaine exposure has been shown to impair the extinction of conditioned fear in rodents (Burke et al., 2006). Nevertheless, there remains an urgent need to further investigate the neurobiological mechanisms that mediate these behaviours.

1.4 Neural substrates of fear and reward-seeking

In the past, independent lines of research have largely ascribed fear- and reward-learning to distinct brain circuits. However, it has become evident that these circuits overlap in several structures, including the ventral tegmental area (VTA), which can control both aversively and appetitively motivated behaviours (Bromberg-Martin et al., 2010; Lüthi & Lüscher, 2014; Wang & Tsien, 2011). The VTA is widely known to be a major substrate for the rewarding effects of drugs, primarily through its dopaminergic (DA) projections to the nucleus accumbens (Nestler, 2005). The VTA is also necessary for stress-, cue-, and drug-primed reinstatement of cocaine- (Mahler et al., 2013; McFarland et al., 2004; McFarland & Kalivas, 2001) and heroin-seeking (Bossert et al., 2004; Stewart, 1984). In addition to its established role in reward-learning, the VTA is an integral component of fear neurocircuitry, and has been shown to encode aversive experiences (Brischoux et al., 2009; Cohen et al., 2012; Jhoul et al., 2009), as well their conditioned stimuli (Guarraci & Kapp, 1999), and is necessary for the expression of conditioned fear (Borowski & Kokkinidis, 1996; de Oliveira et al., 2009, 2011; Greba et al., 2000; Munro & Kokkinidis, 1997).
Figure 1-2 The mesocorticolimbic dopamine system in rodents. Simplified diagram of structures and circuitry of the mesocorticolimbic dopamine system which involves the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala (AMG) and bed nucleus of the stria terminalis (BNST). Dopaminergic activity (red) is influenced by excitatory glutamatergic projections (blue) as well as inhibitory GABAergic projections (orange). Adapted from Kauer & Malenka, 2007.

The diverse functions of the VTA are reflected in the heterogeneity of the neuronal phenotypes and connectivity found within this region. A comprehensive summary of the numerous studies describing the behavioural phenotypes mediated by VTA circuitry is beyond the scope of this general introduction, however, recent optogenetic studies have shown that stimulation of certain VTA pathways can be aversive or rewarding depending on the neurotransmitter population and projection targets involved, and figure 1-3 summarises the current status.
Figure 1-3 Diagram of VTA neural circuitry showing the behavioural effects of optogenetic stimulation of VTA-containing pathways (red: aversive, green: rewarding; LDT: laterodorsal tegmentum, LH: lateral hypothalamus, RMTg: rostromedial tegmental nucleus).
Table 1-3 Description of VTA circuitry involved in aversive and reward-related behaviours in figure 1-3.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phasic activation of VTA DA neurons elicits conditioned place preference (CPP) in mice (Ilango et al., 2014; Tsai et al., 2009) and supports operant responding to receive photostimulation (intracranial self-stimulation; ICSS) in mice and rats (Ilango et al., 2014; Witten et al., 2011)</td>
</tr>
<tr>
<td>2</td>
<td>Photostimulation of terminals of DAergic VTA projections in the NAc drives ICSS in rats, which is blocked by infusion of either DA D1 or D2 receptor antagonists in the NAc (Steinberg et al., 2014).</td>
</tr>
<tr>
<td>3</td>
<td>Activation of glutamatergic VTA neurons increase firing of mesoaccumbal DA neurons, and produces real-time place preference (RTPP) in mice which persists when tested the following day in the absence of photostimulation (CPP). This intervention also supports ICSS by wheel turning and reversal learning when the active and inactive wheels are switched. These behavioural effects are blocked by intra-VTA injection of AMPA or NMDA receptor antagonists (Wang et al., 2015)</td>
</tr>
<tr>
<td>4</td>
<td>Activation of GABAergic VTA neurons inhibits local DA neurons via GABA-A receptor transmission (Tan et al., 2012; van Zessen et al., 2012). This photostimulation disrupts reward consumption (van Zessen et al., 2012) and produces real-time place aversion (RTPA) and conditioned place aversion (CPA) (Tan et al., 2012). Direct optogenetic inhibition of VTA DA neurons also elicits RTPA and CPA, but to a lesser degree (Tan et al., 2012)</td>
</tr>
<tr>
<td>5</td>
<td>Photostimulation of glutamatergic projections from the VTA to NAc shell elicits RTPA and CPA in mice, and also drives operant avoidance; mice would perform an operant task (wheel turn) to pause the photostimulation. Glutamatergic projections from the VTA synapse on parvalbumin (PV) GABAergic interneurons in the NAc, which in turn inhibit accumbal medium spiny output neurons. Microinjection of a combination of either AMPA/NMDA glutamate receptor antagonists or GABA-A/GABA-B receptor antagonists in the NAc also blocked the aversive effects of activation of the VTA—NAc pathway. Direct photostimulation of PV GABAergic interneurons is also aversive (Qi et al., 2016).</td>
</tr>
<tr>
<td>6</td>
<td>Photostimulation of GABAergic VTA—LHB projections elicits RTPP and supports ICSS and reversal learning in mice, which is blocked by GABA-A receptor antagonists in the LHB (Lammel et al., 2015; Stamatakis et al., 2013).</td>
</tr>
<tr>
<td>7</td>
<td>Photostimulation of glutamatergic VTA—LHb projections produces RTPA and CPA in mice, which is blocked by AMPA/NMDA receptor antagonists in the LHb (Lammel et al., 2015; Root et al., 2014).</td>
</tr>
</tbody>
</table>
Glutamatergic projections from the LDT synapse onto DAergic neurons in the lateral VTA which project to the NAc lateral shell. Photostimulation of this LDT—VTA pathway elicits CPP in mice, and is blocked by DA receptor antagonists in the NAc lateral shell (Lammel et al., 2012).

Glutamatergic projections of the LHb synapse on to GABAergic neurons of the RMTg as well as medial VTA DA neurons which project to the mPFC in mice. Photostimulation of these glutamatergic LHb neurons elicits CPA (Stamataki & Stuber, 2012), which can be blocked by intra-mPFC injection of a D1 receptor antagonist (Lammel et al., 2012).

Photostimulation of GABAergic projections from the LH to VTA produces RTPP and CPP in mice, and supports ICSS as well as reversal learning. These effects can be blocked by intra-VTA GABA-A/GABA-B receptor antagonists (Barbano, 2016).

Photostimulation of glutamatergic BNST—VTA projections elicits RTPA in mice, which can be blocked by ionotropic glutamate receptor antagonists in the VTA (Jennings et al., 2013).

Photostimulation of GABAergic BNST—VTA projections supports ICSS and RTPA in mice, which is blocked by GABA-A receptor antagonists in the VTA. Both glutamatergic and GABAergic BNST projections synapse primarily on non-dopaminergic VTA neurons. Direct photoinhibition of VTA GABA neurons is rewarding, as it produces CPP for the inhibition-paired chamber and supports robust operant responding; mice will nose-poke to obtain photoinhibition of VTA GABA neurons (Jennings et al., 2013).

Importantly, the VTA receives projections from major fear processing structures such as the central nucleus of the amygdala (CeA) and the BNST (figure 1-2) (Kauer & Malenka, 2007). These inputs can also influence bivalent functioning of the VTA (figure 1-3); glutamatergic stimulation induces anxiety and avoidance behaviour, whereas GABAergic projections affect responding to rewards (Jennings et al., 2013). Reciprocal connections also exist between the VTA and medial prefrontal cortex (mPFC), an area implicated in the expression as well as inhibition of conditioned fear and drug-seeking (Peters et al., 2009). Therefore, the VTA is well positioned to integrate diverse emotional states to control an organism’s affective drives, such as freezing when fearful or seeking rewards, as observed in anxiety and substance abuse disorders respectively (de Oliveira et al., 2011).

Current theories of addiction and anxiety hypothesise that aberrant function and remodelling of these neural circuits gives rise to the pathological behaviours seen in these disorders, beginning with unusually strong activation by external stimuli (Everitt & Robbins, 2005; Hyman et al., 2006; Lüthi & Lüscher, 2014). For example post-traumatic stress disorder (PTSD), by definition, is precipitated by a traumatic event (American Psychiatric Association, 2013). All drugs of abuse,
regardless of different pharmacological mechanisms of action, can acutely affect extrasynaptic DA levels within this system (Lüscher & Ungless, 2006; Nestler 2005). For example, cocaine potentiates dopaminergic neurotransmission and consequent acute positive effects by blocking presynaptic dopamine transporters (DAT) to inhibit dopamine reuptake (Kuhar et al., 1991) (figure 1-4). Generally, habituation to natural rewards occur with repeated exposure such that they no longer cause dramatic changes to DA levels upon re-exposure. However, drugs of abuse do not produce this habituation and the continuing induction of DA release and resulting neuroplasticity drives excessive reinforcement of drug-seeking behaviour (Kalivas, 2005).

Likewise, this persistent over-expression of originally adaptive emotions is thought to underlie the excessive and pervasive fear seen in anxiety disorders, and may be due to similar changes in these circuits—for example, both addictive drugs and acute stressors induce similar synaptic adaptations to enhance excitatory neurotransmission in VTA DA neurons (Saal et al., 2003; Ungless et al., 2001). In this way, addiction and anxiety disorders represent a pathological expropriation of the neural mechanisms of learning and memory which, evolutionarily, served to develop adaptive behaviours related to punishment and rewards, as well as the cues that predict them (Hyman, 2005; Kauer & Malenka, 2007).

A number of neuromodulatory peptides are also found in the VTA. These neuropeptides are commonly produced within the same neuron as classical neurotransmitters (Hokfelt et al., 1987; Merighi, 2002; Salio et al., 2006; Zupanc, 1996), and can be co-packaged and co-released (Reyes et al., 2011; Waselus & Van Bockstaele, 2007). However, neuropeptides are typically released in a less precise manner (Nusbaum, 2002), and often require higher stimulus frequencies (Arrigoni & Saper, 1987).
2014; Nassel, 2009). They also can act at longer distances from their release site via volume neurotransmission, where the peptide can diffuse outside the synaptic cleft at biologically relevant concentrations (Barbour & Hausser, 1997; Zoli & Agnati, 1996; Zoli et al., 1999), and can even act on different target neurons than their classical neurotransmitter partners (Nassel, 2009).

Of particular interest is corticotropin-releasing factor (CRF), a stress-related neuropeptide. A number of studies have shown that CRF within the VTA is able to regulate dopaminergic activity as well as associative learning. However the mechanism, including identification of receptor subtype(s) subserving CRF’s role in different behaviours remain to be characterized (Borgland et al., 2010; Wise & Morales, 2010).

1.5 The role of CRF in anxiety and addiction

Psychological stress is a prominent contributor to the development of anxiety and addictive disorders, and a significant precipitant of relapse in both situations (Goeders, 2003; Koob, 2009; Maren & Holmes, 2015; McGowan, 2012; Preston & Epstein, 2011; Sinha, 2001; Torres & O’Dell, 2016). Altered stress reactivity increases the risk for both disorders (Lovatto, 2006; Villada et al., 2016), and may impact disease severity. For example, a clinical study of 49 treatment-seeking cocaine addicts found that higher levels of drug craving in response to stress imagery was associated with a shorter latency to relapse (Sinha et al., 2006). This has led to the identification of CRF as a mediator of the effects of stress on anxiety and addictive disorders, and a crucial factor in the pathogenesis of these psychopathologies (Hauger et al., 2006, 2009). This is supported by reports of hypersecretion of CRF (Sautter et al., 2003), as well as CRF receptor single nucleotide polymorphisms (SNPs) in patients with anxiety disorders (Amstadter et al., 2011; Ishitobi et al., 2012; Keck et al., 2008; Weber et al., 2015). Furthermore, individuals with certain CRF receptor 1 (CRFR1) SNPs show decreased fear responses to conditioned threat cues, which is associated with heightened generalised anxiety (Grillon, 2002; Heitland et al., 2013, 2016; Weber et al., 2015). Consistent with those findings, in studies of healthy humans it has been shown that CRFR1 antagonists increased fear responses to a predictable threat (Grillon et al., 2015), and had anxiolytic effects on CO2-induced general anxiety (Bailey et al., 2011).

1.6 The CRF system

1.6.1 CRF-family peptides

CRF is a 41-amino acid neuropeptide hormone which regulates the autonomic, immune, and endocrine responses to stress via activation of the hypothalamic-pituitary-adrenal (HPA) axis (Rivier et al., 1981; Rivier et al, 1982; Rivier & Vale, 1983). CRF also acts as a neurotransmitter in extrahypothalamic regions of the brain to modulate cognitive function, including anxiety- and addiction-related
behaviours (Koob & Heinrichs, 1999). The CRF system is highly conserved across species. Mouse, rat, and human CRF is identical at the amino acid level (Rivier et al., 1983; Seasholtz et al., 1991), and act via two receptor subtypes: CRF receptor 1 (CRFR1) and CRF receptor 2 (CRFR2) (Dautzenberg & Hauger, 2002). Both belong to the family of seven-transmembrane-domain G protein-coupled receptors (GPCRs) and predominantly link to adenylate cyclase activation through Gαs. However, CRF receptors can activate multiple different Gα subunits resulting in diverse downstream signalling cascades (Hillhouse & Grammatopoulos, 2006). The different CRF receptors are produced from distinct genes, but there is 70% sequence homology between them at the amino acid level, and greater than 80% homology at the transmembrane and intracellular domains (Lovenberg et al., 1995). The main differences (~47%) are found at the N-terminal extracellular domain which determine ligand binding and receptor specificity (Dautzenberg & Hauger, 2002; Dautzenberg et al., 2002; Grammatopoulos, 2012). CRF has at least 10-fold higher affinity for CRFR1 over CRFR2 (Behan et al., 1996; Chen et al., 1993; Dautzenberg et al., 2002; Lovenberg et al., 1995). In fact, other CRF-like peptides (urocortins 2 and 3) have higher affinity to CRFR2 compared with CRF, suggesting that these urocortins and not CRF may be the endogenous ligand of CRFR2 (De Souza, 1997).

CRF also has high affinity for CRF binding protein (CRFBP)—several-fold higher than that of either CRF receptor (Huising et al., 2008). Binding by CRFBP prevents CRF from activating its receptors (Herringa et al., 2004; Suda et al., 1988) and promotes uptake and degradation of the CRF-CRFBP complex (Burrows et al., 1998; Karolyi et al., 1999). CRFBP levels are approximately 10-fold higher than CRF levels in most brain regions and bind between 40-90% of extracellular CRF (Behan et al., 1996; Suda et al., 1998). In this way, CRFBP sequesters free CRF and buffers CRF signalling (Chan et al., 2000).

1.6.2 Distribution of CRF-family peptides in brain

CRF-containing neurons and CRF receptors are expressed throughout the brain with partially overlapping expression patterns, and display strong homology in rat and mouse (figure 1-5) (Van Pett et al., 2000). The highest concentrations of CRF containing cell bodies are found in the paraventricular nucleus of the hypothalamus (PVN), parabrachial nucleus (PBN), BNST, and CeA. CRF containing neurons are also found in the nucleus accumbens, hippocampus, cortex, periaqueductal gray columns (PAG), septum, and olfactory bulb (Merchenthaler et al., 1982; Olschowka et al., 1982; Potter et al., 1994; Swanson et al., 1983). These brain regions regulate a broad spectrum of behaviours from affective and motivational states to gustatory and pain processing. Interestingly, CRF receptor mRNA is low or undetectable in several cell groups implicated as central sites of CRF action, suggesting that major effects are largely dependent on the source structure and downstream circuitry (Refojo et al., 2011).
Figure 1-5 Distribution of CRF receptor mRNA in the rodent brain detected by *in situ* hybridization (ISH) (Van Pett et al., 2000).

CRFR1 is widely distributed with high expression in the neocortex, cerebellum, hippocampus, amygdala, BNST, olfactory bulb, lateral septum, thalamus, basal ganglia, the raphé nuclei, and pituitary (Justice et al., 2008; Korosi et al., 2006; Van Pett et al., 2000). CRFR2 is confined to subcortical structures, showing high expression levels in the pituitary, lateral septum, hypothalamus, dorsal and median raphé, nucleus of the solitary tract (NTS), and extended amygdala (Bittencourt & Sawchenko, 2000; Korosi et al., 2006, 2007; Lawrence et al., 2002; Lukkes et al., 2011; Van Pett et al., 2000). Importantly, both of these receptors as well as CRFBP (Wang & Morales, 2008) are found in the VTA. CRFR1 expression has been observed using genetically engineered CRFR1-reporter mice (Justice et al., 2008), and CRFR1 mRNA is consistently detected by ISH (Lein et al., 2007; Van Pett et al., 2000) (figure 1-6). These same ISH studies did not find CRFR2 mRNA within the VTA, however it has been detected by single-cell qPCR (Korotkova et al., 2006), and in the synaptosomes of inputs to the VTA, indicating presynaptic CRFR2 (Slater et al., 2016).
Figure 1-6 CRF receptor mRNA within the mouse VTA. Coronal sections showing CRF receptor mRNA detected by *in situ* hybridization within the mouse VTA, and corresponding computerized expression level images (red; strong signal, blue; weak signal). Image adapted from the Allen Mouse Brain Atlas (Lein et al., 2007)

1.7 The role of CRF in rodent conditioned fear

The role of CRF in aversive learning has been extensively characterized using rodent models, being shown to increase fear responses to unconditioned stimuli, as well as learned fear responses. In these studies, intra-cerebroventricular (ICV) administration of CRF increased shock-elicited freezing in rats (Kalin et al., 1988; Sherman & Kalin, 1988) and potentiated acoustic startle response in rats and mice (Risbrough et al., 2003; Swerdlow et al., 1989), whereas CRF receptor antagonists had the opposite effect, though neither altered pain sensitivity (Sherman & Kalin, 1988). The two treatments had similar effects on the expression of conditioned fear in rats when administered just before the CS test session (Kalin & Takahashi, 1990; Skórzewska et al., 2008; Swerdlow et al., 1989). More specifically, CRFR1 signalling has been implicated in the induction and expression of conditioned fear, as central CRFR1 inhibition by pharmacological antagonists or antisense oligonucleotides can reduce conditioned fear in rats when administered prior to conditioning, or prior to the CS test session (Deak et al., 1999; Hikichi et al., 2000; Ho et al., 2001). Interestingly, mice with disrupted GABAergic signalling to CRF neurons in the extended amygdala showed deficits in fear extinction, suggesting that excessive CRF impairs extinction (Gafford et al., 2012). This region is a major source of CRF input to
the VTA (Rodaros et al., 2007; Silberman et al., 2013), however it is not known whether these projections are critical for extinction learning. Furthermore, the BNST (Asok et al., 2016; Lee & Davis, 1997; Sink et al., 2013a, b), amygdala (Bakshi et al., 2002; Hubbard et al., 2007; Isogawa et al., 2013; Pitts & Takahashi, 2011), hippocampus (Blank et al., 2003a; Radulovic et al., 1999), and PAG (Borelli et al., 2013) are known sites of action of CRF in some of these behaviours. However, the role of VTA CRF has not yet been thoroughly investigated.

1.8 The role of CRF in rodent models of addiction

CRF has also been shown to have an important modulatory role on drug-taking behaviour across many classes of abused substances including psychostimulants, heroin, alcohol, nicotine, and cannabinoids (see Sarnyai et al., 2001 for review), and a great amount of research has focused on cocaine, which will be the focus herein. One study (Goeders & Guerin, 2000) reported that systemic administration of a centrally active CRFR1 selective antagonist significantly decreased drug self-administration across a range of doses of cocaine, without effecting food-maintained responding. This finding was corroborated by experiments by Specio et al., (2008), in which two different CRFR1 selective antagonists attenuated escalation of self-administration in rats that were allowed self-administration sessions of 6 hours, daily, as opposed to the standard 1-2 hour sessions. Specifically, cocaine self-administration was attenuated in both session lengths; however, the increased rate of cocaine intake of the rats given extended access was impacted upon to a greater extent. Przegaliński et al. (2005), provided more support for the role of CRFR1 in a series of experiments investigating the effects of a different CRFR1 selective antagonist on a range of cocaine-related behaviours. The antagonist did not reduce self-administration, suggesting that the rewarding effects of cocaine were not decreased; however, it was able to significantly, and dose-dependently decrease cocaine-primed reinstatement of cocaine-seeking, following a 10-day extinction period.

CRF has also been implicated in stress-induced reinstatement of cocaine-seeking. Indeed, central administration of CRF itself can reinstate cocaine-seeking (Buffalari et al., 2012; Erb et al., 1998; Erb et al., 2014; Graf et al., 2011; Mantsch et al., 2008a, b), even after post-injection delays of up to 3 hours (Erb et al., 2006). Conversely, CRF receptor antagonists can attenuate footshock reinstatement of cocaine-seeking (Erb et al., 1998), through a CRFR1-dependent mechanism (Shaham et al., 1998), and also block reinstatement induced by central noradrenaline administration (Brown et al., 2009). D1/5-like receptor signalling is a crucial factor for CRF-induced cocaine-seeking (Brown et al., 2012), which suggests a role for VTA in this effect, as there is an abundance of CRF receptors found on DAergic neurons in this area, and CRF application excites these neurons directly (Korotkova et al., 2006; Wanat et al., 2008). Indeed, a number of studies indicate that the VTA is a key site for the actions of CRF on cocaine-related behaviours. For example, administration of a CRFR1 antagonist
into this region can inhibit the development of social defeat stress-induced escalation of cocaine self-administration (Boyson et al., 2011; Burke et al., 2016; Holly et al., 2016).

Within the VTA, CRF causes synaptic neuroadaptations in dopaminergic neurons, which occur more readily after cocaine experience (Corominas et al., 2010; Kauer & Malenka, 2007; Saal et al., 2003). A study by Wang et al. (2005) demonstrated that footshock stress increases both CRF and DA levels within the VTA. This increased dopamine release was mimicked by direct infusion of CRF into the VTA. In both instances, the DA increase was attenuated by local administration of a CRF receptor antagonist, suggesting that DA release is downstream of CRF activation. Intra-VTA CRF infusion also led to the reinstatement of cocaine-seeking behaviour in rats extinguished for cocaine self-administration. Interestingly, the CRF-induced increase of DA was not observed in cocaine-naïve rats, suggesting that CRF potentiation of DA release develops after cocaine exposure. This cocaine-induced sensitization of the VTA to stress via CRF provides a putative physiological basis for the correlation between stress and drug abuse (Wang, 2005).

1.8.1 VTA CRF receptor specificity in stress-induced reinstatement

CRF in the VTA is seemingly critical for footshock-induced reinstatement of cocaine seeking, although there are conflicting reports regarding which receptor subtypes are involved in this effect. In initial studies using intra-VTA microdialysis, the CRFR2 antagonist antisauvagine-30 perfused at a concentration of 1μM blocked footshock-induced reinstatement, whereas the CRFR1 antagonist NBI had no effect at either 1μM or 10μM (Wang et al., 2007). Although the antagonist results would predict that the CRFR2 agonists alone would be effective to induce reinstatement, this was not the case when 6 different agonists were tested. The differences between the agonists in their effectiveness in initiating reinstatement did not relate to their receptor subtype selectivity; two were non-selective and one was CRFR2 selective. Rather, the three agonists that reinstated the response all bound CRFBP, while the agonists that were ineffective, failed to bind CRFBP (Wang et al., 2007). The involvement of CRFBP was further investigated by co-administration of the effective agonists and CRF_{6,33}, a high-affinity CRFBP ligand inhibitor, capable of displacing CRF (Ungless et al., 2003). All three agonists were ineffective in the presence of CRF_{6,33}, while the administration of CRF_{6,33} alone had no effect. These results tentatively pose two criteria for CRF receptor ligands to influence drug-seeking behaviour in the VTA: affinity for CRFR2 and affinity for CRFBP. Although the established function of CRFBP is to buffer CRF levels, ICV infusion of CRFBP inhibitors have behavioural effects that seem dissociated from the according rise in free CRF (Heinrichs & Joppa, 2001). The role of CRFBP in neurotransmission is still unclear, but it has been hypothesized by that binding by CRFBP could induce functionally relevant conformational changes in the substrate that could change its affinities for CRFR1 and/or CRFR2 (Ungless et al., 2003).
Although Wang et al. (2007) proposes a role of CRFR2 in the VTA, via a CRFBP interaction, as crucial for stress induced reinstatement, a contradictory study has highlighted the role of CRFR1 and not CRFR2 in the VTA (Blacktop et al., 2011). In this series of experiments, it was demonstrated that both intra-VTA CRF injection and footshock stress-induced reinstatement of drug-seeking could be attenuated by either of two CRFR1 selective antagonists microinjected into the VTA, whereas CRFR2 selective antagonists were not able to, even at much higher doses. This effect was recapitulated in experiments where CRFR1 selective agonists were capable of reinstating cocaine seeking behaviour, whereas CRFR2 selective agonists were not. These inconsistencies in the current literature could possibly be caused by different doses used of agonists and antagonists, experimental design (including rodents strains used) and/or the method of delivery. Regardless, there is seemingly a role for CRF within the VTA in effecting stress-induced reinstatement, though it is still unclear which CRF receptor subtype is essential.

1.9 Limitations of pharmacological manipulations

The majority of studies to date have utilized pharmacological approaches to ascertain the contribution of different receptor subtypes to the role of CRF in cocaine addiction. While this is a valid approach, there are significant limitations associated with the interpretation of such experiments due to off-target effects of antagonists. Firstly, the difficulty in directly gauging the spread of the microinjected drug makes it unclear which neurons are actually being affected (Fong, 2006). As the volume of distribution can only be estimated, the actual brain concentration of the injected drug is unknown. Additionally, the binding affinities of the antagonists determined by in vitro binding assays under controlled pH, temperature, and ionic composition may not be accurate under in vivo conditions (Baghdoyan & Lydic, 1999). Secondly, because nanolitre volumes of the drug are required in order to target discrete brain regions in rodents, extremely high concentrations of antagonist are used in microinjection studies (Boyson et al., 2011). This poses complications for experiments using selective antagonists as the selectivity can change as a function of concentration, which may lead to increased non-specific binding, and a compromised ability to parse the actions mediated by CRFR1 and CRFR2. Furthermore, these interventions do not differentiate between presynaptic and postsynaptic CRF receptors.

1.10 Viral-mediated gene silencing

These potential confounds can be minimized by implementation of sequence-specific post-transcriptional gene silencing via RNA interference. This process employs viral vectors containing expression cassettes to reliably deliver short-hairpin RNA (shRNA) into transfected cells. The shRNA is cleaved by the Dicer endonuclease into short segments called small interfering RNA (siRNA), the effector of gene inactivation (Paddison et al., 2002). Once the siRNA is introduced into cells it is able
to suppress complementary messenger RNA (mRNA) via two pathways simultaneously. In one pathway, the siRNA binds homologous mRNA, which blocks its translation into protein. Alternately, the siRNA is incorporated into an RNA-induced silencing complex (RISC), which contains the endonuclease Slicer (Sharp, 2001). The siRNA is used to direct nuclease activity towards the corresponding mRNA, which is cleaved and then further degraded by other nucleases in the cell. Both of these mechanisms lead to decreased expression of the gene from which the mRNA was transcribed and consequent protein translation (figure 1-7).

This less invasive form of gene regulation is used ubiquitously in the normal functioning of cells to regulate developmental timing in many organisms (Hannon, 2002). It does not require global knockout of the gene of interest, or the creation of mutant animals, and thus limits possible confounding developmental effects. The utility of this technique is contingent on the stable expression of the shRNA. Although this approach has previously had problems with the efficient delivery of these shRNAs, the use of lentiviral vector-mediated delivery was reported to stably suppress genes over several months (Hommel et al., 2003; Zufferey et al., 1998), even in non-dividing cells. Furthermore, Van den Haute et al., (2003) described persistent local knockdown of GFP expression in an adult mouse brain for up to six months after stereotaxic microinjection. Decreased mRNA levels can be measured by quantitative real-time PCR (qRT-PCR) and western blot analysis, or immunohistochemistry can be used to determine whether this is reflected in decreased expression of the actual protein. In the majority of cases, this method has been shown to be able to reduce expression of the cognate target gene to less than 10% of control levels (McCaffrey et al., 2002). Additionally, shRNA vectors can be concurrently expressed with a GFP reporter fluorophore to indicate which cells have been transfected (Hasuwa et al., 2002), to facilitate discrete anatomical targeting of the VTA. Viral knockdown can be precisely targeted to CRFR1 because expression cassettes can be designed to specify any sequence of mRNA.
Figure 1-7 shRNA mediated gene silencing. shRNA is processed by Dicer into siRNA which activates RISC and guides degradation of complementary mRNA leading to highly specific decreased gene expression. Adapted from Genetic Engineering and Biotechnology News, 2006. Volume 26, No. 4.


**1.11 Summary**

It is clear from the literature that through the mesocorticolimbic system, CRF is implicated in stress-induced relapse following drug self-administration. Additionally, nothing is known about the role of CRF in the VTA in conditioned fear, despite the importance of the stress system in anxiety behaviours. It is not definitively known through which receptor subtype(s) and in which brain region(s) CRF is acting to exert these effects. Additionally, involvement of CRF in extinction learning for both appetitive and aversive behaviours is not yet clear. By using a viral-mediated genetic knockdown approach it is possible to specifically attenuate the expression of CRFR1 within VTA neurons. This allows the direct investigation of these postsynaptic receptors and avoids the complications of CRFBP interactions with pharmacological antagonists.

**1.12 Hypothesis and aim**

Given that VTA neurons produce CRF, as well as receiving dense CRF-containing inputs from key fear structures, it is hypothesised that CRFR1 expressed on neurons intrinsic to the VTA are involved addiction and anxiety-related behaviours. Therefore, the aim of this thesis is to examine the effects of viral-mediated knockdown of VTA CRFR1 on cocaine-seeking and condition fear in mice. With this strategy, it is possible to specifically attenuate expression of CRFR1, and the addition of a green fluorescent protein (GFP) marker will facilitate validation of the microinjection sites.
CHAPTER 2:
GENERAL METHODS
2.1 Animals

All experiments were performed in adherence to the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia and approved by the Animal Ethics Committee at the Florey Institute of Neuroscience and Mental Health (approval number 09-037, plus amendments). Adult male C57BL/6J mice (Animal Resource Center, Perth, Australia) were housed in temperature- and humidity-controlled rooms maintained on a reversed 12 h light/dark cycle (dark period 0700-1900) with ad libitum access to food and water in their home cage unless otherwise stated. Mice were allowed a period of 10 days to acclimate to the facilities before experimentation. All experiments were conducted during the dark phase of the light/dark cycle.

2.2 Lentiviral vectors

Lentiviral constructs were designed by collaborators Dr Yehezkel Sztainberg and Professor Alon Chen of the Weizmann Institute of Science, Israel, as described previously (Sztainberg et al., 2010). Lentiviral vectors used in my studies were produced in-house by Sharon Layfield and Professor Ross Bathgate of The Florey Institute of Neuroscience and Mental Health, Australia. shRNA was cloned into expression cassettes driven by the H1 RNA polymerase III (Pol III) promoter in the p156RRLsinPPTCMV-GFP-PREU3Nhe lentiviral construct. The shRNA constructs consisted of a 83-mer oligonucleotide with a 5’ end containing a unique XbaI restriction site, a stretch of 5 adenosines as a template for the Pol III promoter termination signal, 19-nucleotide sense and antisense strands separated by a 9 nucleotide loop, and 20 nucleotides complementary to the 3’ end of the Pol III H1 promoter (figure 2-1).

![Diagram of plasmid used for shCRFR1 and shControl viruses.](image-url)
The oligonucleotide sequence for shRNA targeted against CRFR1 mRNA (shCRFR1) was 5’-CTGTTCTAGACAAAAATCTTTTCTTCAACATTTGTTCTCTTGA-3’ (in italics the sense and antisense strands; in bold the nine nucleotide loop; underlined is the XbaI restriction site). The control virus (shControl) consisted of the same lentiviral construct containing a scramble non-relevant shRNA sequence, which ensures that the cellular microRNA machinery will be activated to serve as an appropriate control. Both lentiviruses expressed enhanced GFP to mark transduced cells.

### 2.3 Injectors for stereotaxic surgery

Injectors for stereotaxic were constructed in-house (figure 2-2). Injector needles were made from Clark Eletromedical Instruments borosilicate glass pipettes (model number GC120-7.5, ID: 0.69 mm, OD: 1.2 mm; SDR Clinical Technology, NSW, Australia) pulled into needles using a Narishige PE-22 micropipette puller (Leica, NSW, Australia) set at 95°C. The tips of the glass capillaries were broken off to create an approximately 8 µm opening, under a microscope; smaller diameter openings were found to become clogged often, whereas larger diameters did not allow sufficient pressure for viral injections. The pulled sections of the glass capillaries were kept at least 5 mm long to reduce damage to brain tissue during injections (figure 2-2B). These were fitted over a 23G needle using a sleeve of heatshrink tubing. The sleeve was heated to form a water-tight seal between the glass and metal needle, and flushed with sterile saline to ensure no leakage from either end of the heatshrink seal. The 23G needle was then attached to a 10 µL Gastight Hamilton syringe (model number 1701, Grace Discovery Science, VIC, Australia), which was placed in a syringe driver (model number 53311; Stoelting, IL, USA) mounted on the stereotaxic frame.

**Figure 2-2** Injector for stereotaxic microinjections. (A) Injector attached to 10 µL Hamilton syringe. (B) Close up of injector showing 5 mm long tip of glass capillary with a drop of saline at the end.
2.4 Stereotaxic injection of lentiviral vectors

Mice were initially anaesthetized using 5% v/v isoflurane (in air) in an induction chamber until the loss of righting reflex, anaesthesia was then maintained through an isoflurane nosecone (1-1.5% in air 0.5L/min). Depth of anaesthesia was tested by foot-pinocch response and mice were given an intraperitoneal (i.p.) injection of meloxicam (3mg/kg; Boeringher Ingleheim, Inglehiem, Germany) for perioperative analgesia before being mounted into a stereotaxic frame (Stoelting, Dublin, Ireland). Once the skull was stably positioned, the head was shaved and disinfected with 10% povidine-iodine solution and an incision was made in the scalp. Bregma and lambda were used as landmarks to ensure the skull was level across the sagittal, coronal, and horizontal planes, before drilling holes for corresponding injection sites. VTA injection co-ordinates relative to bregma were posterior -3.0 mm; lateral ±0.45 mm, and ventral -4.4 mm from the skull surface (Paxinos and Franklin, 2004), based on a calibration study using injections of methylene blue (1µl/side) (figure 2-3A). Mice received intra-VTA injections of either shCRFR1 or shControl viruses injected bilaterally (1µl/side) using automated syringe driver at a rate of 0.25µl/min. This volume was chosen based on pilot studies using 0.25, 0.5, and 1µl infusions which found that 1µl infusions led to optimal spread of transduced neurons within the VTA (data not shown). This is concordant with a previous study by Sztainberg et al. (2010), which used this same injection volume to target the BLA, a structure of similar size to the VTA. The injector was left in place for 5 min following virus infusion for pressure equilibration and then raised 0.1mm for 2 min before being fully withdrawn from the brain in order to allow diffusion of the virus from the micropipette and minimize virus being drawn up the injection tract. The scalp incision was sutured closed and treated with Tricin antibiotic and mice were allowed to recover for at least 1hr in an incubator (27°C). They were then returned to their home cage and monitored closely after surgery to look for signs of distress such as weight loss or hunched postures/piloerection, as well as any infection at the incision site. The mice then remained in their home cages for three weeks while the virus was transduced before any behavioural testing to allow maximal virus expression and complete turnover of CRF-1 receptor protein (Sztainberg et al., 2010).
Figure 2-3 Stereotaxic targeting of VTA. (A) Sagittal brain diagram showing intended placement of injector tip with measurements relative to bregma in mm. (B) Coronal section with methylene blue staining showing successful targeting of the VTA. Sections were counterstained with neutral red to visualise anatomy (EW: Edinger Westphal nucleus; ML: white matter tracts of medial lemniscus; SN: substantia nigra).
2.5 Histology

After behavioural studies were complete, the level and distribution of viral transduction in each mouse was confirmed by observing the associated GFP expression.

2.5.1 Tissue preparation

Mice were anaesthetised by i.p. injection of sodium pentobarbitone (80 mg/kg, 0.1 ml/10 g body weight) and transcardially perfused with 40ml phosphate buffered saline (PBS; 0.1M, pH 7.4) followed by 40ml 4% paraformaldehyde (PFA, Sigma, Australia) in PBS. The mice were then decapitated; their brains removed and post-fixed overnight in 4% PFA in PBS. The following day brains were transferred to a solution of 20% sucrose in PBS where they would remain overnight at 4°C before being frozen over liquid nitrogen and stored at -80°C. They were then sliced on a Leica cryostat CM1950 (Leica Biosystems, North Ryde, Australia). Coronal sections (40μm) through the VTA, from bregma -1.82 to bregma -4.04mm, according to The Mouse Brain Atlas (Paxinos & Franklin, 2004), were collected in 1 in 4 series into cryoprotectant (table 2-1) and stored at -20°C. These co-ordinates were chosen in order to capture the entire region of transduced cells, based on the observed spread of viral transduction in optimization studies, with a wide margin for error to account for individual variations in injection sites.

To prepare sections for direct fluorescent imaging of GFP expression sections were washed (3 x 5 min, 0.1M PBS), then sorted in 0.9% saline and mounted onto gelatin subbed slides. Slides were allowed to dry before being coverslipped using fluorescent mounting media (Dako North America Inc., USA). Sections were covered by foil during washing and drying steps to prevent bleaching.

2.5.2 Immunohistochemistry

Chromogenic immunohistochemistry for the GFP protein was performed in order to visualize GFP under a light microscope and allow counterstaining of neuroanatomical landmarks to more accurately assess injection placement and viral spread. The sections were first washed of cryoprotectant (3 x 5 min, 0.1M PBS). They were then pretreated in a solution of 10% methanol, 0.03% hydrogen peroxide in PBS for 15 minutes to quench endogenous peroxidases. After another PBS wash (3 x 5 min), sections were incubated overnight in a solution of anti-GFP primary antibody (1:1000 dilution; Invitrogen, Australia) with 1% normal goat serum (NGS; Millipore, USA) and 0.05% Triton X-100 (TX100, BDH Chemicals, Australia) in PBS. The following day the sections were washed (3 x 5 min; PBS), pre-blocked with 10% NGS in PBS for 45 minutes, and incubated for two hours in biotinylated goat anti-rabbit secondary antibody (1:500 dilution; Vector laboratories Inc, USA) with 1% NGS and 0.05% TX100 in PBS. After another PBS wash (3 x 5 min), the sections were incubated with avidin-biotinylated horseradish peroxidase complex (ABC; Vector, USA) in 1% NGS and 0.05% TX100. The
sections were then washed (3 x 5 min; PBS) and placed in a nickel-enhanced 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma, Australia) solution for 20 mins. The chromogenic reaction was initiated by addition of 10% v/v hydrogen peroxide and allowed to develop before being terminated by a final 3 x 5 minute PBS wash. The sections were then slide-mounted using 0.5% gelatin in PBS and air dried overnight. They were lightly counterstained in 1% neutral red (Sigma-Aldrich, Australia) for 1 min, differentiated, and serially dehydrated in ethanol (50%, 70%, 90% and 2 x 100%; 30 s each) and cleared in X-3B (2 x 3 min washes; BDH Chemicals, Australia), then cover-slipped with Depex Mounting Medium (Gurr, Germany).

2.6 Microscopy

Endogenous GFP fluorescence was visualised using a Leica DM600B microscope (excitation filter: BP 485; emission filter: BP 537). Images were captured using a Hamamatsu C10600-10B digital camera (RS Components, Australia). DAB staining was observed under an Olympus BH-2 light microscope. For each section, the areas of staining were transposed onto a corresponding page of the Mouse Brain Atlas (Paxinos & Franklin, 2004) to generate a representation of injection site location, as well as the brain regions which contained transfected cells.
Table 2-4 Composition of reagents used.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Details</th>
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<tbody>
<tr>
<td>cryoprotectant</td>
<td>30% ethylene glycol&lt;br&gt;15% sucrose&lt;br&gt;1% polyvinylpyrrolidone&lt;br&gt;0.9% NaCl&lt;br&gt;0.68995% Na$_2$HPO$_4$$\cdot$H$_2$O&lt;br&gt;0.541% Na$_2$HPO$_4$&lt;br&gt;in distilled water</td>
</tr>
<tr>
<td>methylene blue</td>
<td>4% methylene blue trihydrate&lt;br&gt;in distilled water</td>
</tr>
<tr>
<td>neutral red</td>
<td>1% neutral red powder&lt;br&gt;1.53% sodium acetate (anhydrous)&lt;br&gt;1.2% 17.4 M acetic acid&lt;br&gt;in distilled water</td>
</tr>
<tr>
<td>Nickel-enhanced DAB solution</td>
<td>1% DAB&lt;br&gt;0.004% NH$_4$Cl&lt;br&gt;0.004% ammonium nickel (II) sulfate hexahydrate&lt;br&gt;in PBS</td>
</tr>
<tr>
<td>PBS</td>
<td>8% NaCl&lt;br&gt;1.44% Na$_3$HPO$_4$&lt;br&gt;0.24% KH$_2$PO$_4$&lt;br&gt;0.2% KCl&lt;br&gt;in distilled water</td>
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CHAPTER 3:
VALIDATION OF
VIRAL-MEDIATED
GENE KNOCKDOWN
3.1 Introduction

Before conducting behavioural experiments with lenti-shCRFR1 virus, I first had to confirm that my knockdown strategy resulted in decreased CRFR1 levels in the VTA. The lenti-shCRFR1 viral construct was developed and validated by our collaborators, as published in Sztainberg et al. (2010). In that study, knockdown of CRFR1 protein expression in vitro was assessed using HEK293T cells expressing myc-tagged CRFR1 that were treated with either lenti-shCRFR1, or a non-related virus as a negative control. Western blot analysis showed an approximately 80% decrease of myc in the lenti-shCRFR1 treated group with no effect from the control. To determine whether the reduction of CRFR1 protein resulted in decreased receptor signalling, these same cell lines were assessed for CRF-induced cyclic adenosine monophosphate (cAMP) production using a luciferase assay. shCRFR1 virus treated cells showed a 2.5-fold decrease in CRF-stimulated activation of cAMP signalling compared to controls. They then tested the effects of shCRFR1 in vivo. Four mice were given bilateral injections of either lenti-shCRFR1 or control virus in the basolateral amygdala (BLA), and the knockdown of CRFR1 mRNA expression was confirmed by semi-quantitative reverse transcription PCR (RT-PCR). These experiments demonstrated the efficacy of the shCRFR1 viral knockdown on multiple levels – protein expression, receptor signalling, and mRNA expression – and this same construct was used in my studies. However, the viral vectors used in my studies were produced by a different group of collaborators and I was targeting a different brain region. Therefore, my aim was to quantify the decrease of CRFR1 achieved in the VTA using virus produced in-house in the present chapter.

Ideally, validation of the knockdown of CRFR1 would be done using immunohistochemistry to visualise receptor expression and quantify the number of CRFR1 positive cells. However, current commercially available antibodies for CRFR1 show cross-reactivity with other species and so this approach was not chosen. To circumvent this problem, I used CRFR1 mRNA expression as a surrogate measure of CRFR1 levels. CRFR1 mRNA can be measured using a quantitative reverse transcription PCR (RT-qPCR). In this method, total RNA is extracted from the target structure and reverse transcribed into cDNA, which serves as a template for qPCR. Specifically designed primers are then used to amplify the DNA sequence of interest. During each cycle of the qPCR process the amount of target DNA is doubled, which increases the fluorescence of a DNA-binding dye. The intensity of this fluorescence is measured every cycle, and the cycle number at which the fluorescence exceeds the threshold for detection is called the threshold cycle (C_T) (Vet & Marras, 2005). This C_T value is inversely related to the amount of initial target DNA template (ie. the higher the C_T value, the lower the amount of DNA template), and relative expression of the gene of interest (GOI) can be determined by comparison with an internal control gene or housekeeping gene (HKG) (Ullmannová & Haskovec, 2003). For example, if the C_T value of the GOI is 3 cycles higher than that of the internal control, there is $2^3 = 8$ times less template of the GOI than the HKG. By choosing an internal control
gene that is stably expressed across samples, the difference in gene expression levels across different treatment groups can be quantified.

qRT-PCR is an established methodology for measuring the effects of RNAi-mediated knockdown (Van Maerken et al., 2008; Zhao et al., 2008), and allows for the simultaneous quantification of multiple genes from a single sample. Obtaining high quality RNA, primer design and evaluation/validation, selection of an appropriate housekeeping gene, normalization strategies and accurate data analysis are all essential factors in the success of these experiments. Therefore, the first step was to carefully optimise this process to generate robust and reproducible results. This protocol was then implemented to quantify the relative expression of different CRF binding partners in the VTA of naïve mice, as well as the effect of intra-VTA injections of the lenti-shCRFR1.

3.2 Methods

3.2.1 Tissue collection

Working with RNA is a precarious task because of the chemical instability of RNA and the ubiquitous presence of RNases. Unlike DNases, RNases do not need metal ion co-factors and can maintain activity even after autoclaving (Tuzmen et al., 2007). The VTA is a small, but diffuse structure that lacks clear boundaries and is intermingled with other structures such as the substantia nigra (Paxinos & Franklin, 2004). These small tissue samples provide a limited amount of starting material from which to extract RNA, and are more prone to degradation during processing. Dissecting a larger tissue sample would be easier in terms of capturing the VTA, and would be a viable option if one was measuring the presence of a transgene, but as I am trying to quantify the knockdown of an endogenous gene, capturing too much of the surrounding tissue would dilute the amount of virus affected tissue and misrepresent the amount of CRFR1 knockdown. Initial dissection of the VTA from fresh tissue using razor blades led to large (>50%) variations in tissue sample sizes. Therefore, I opted to collect tissue using 1.2 mm micro-punches, which allowed uniform sample weights of approximately 0.8 mg.

For virus injected mice (see chapter 2 for methods), tissue was collected three weeks after virus incubation to match the onset of behavioural testing in my other studies. All surgical equipment was cleaned with RNAse free 100% ethanol, RNAse zap (Qiagen), and diethylpyrocarbonate (DEPC)-treated water prior to use and between each mouse, and kept on wet ice. Mice were terminally anaesthetised (80 mg/kg, i.p.; 0.1 ml/10 g pentobarbitone) and decapitated, the brain quickly removed and rinsed in cold sterile saline before being placed ventral side up in a metal mouse brain matrix (model number 51386, Stoelting, USA) pre-cooled on wet ice (figure 3-1A). The arterial circle of Willis was used as a landmark to guide dissection of a 2 mm thick coronal slice containing the VTA, spanning approximately -2.8 to 4.8mm from bregma (Paxinos & Franklin, 2004) (figure 3-1B). The section was removed and immediately placed rostral side up on dry ice and the left and right VTA
were separately punched out using a 1.2mm punch (Harris Uni Core™ micro-punches; Harris, USA) and snap frozen on liquid nitrogen to be stored at -80°C (figure 3-1C). Each punch was stored individually (ie. 2 samples/mouse). Punching the slice before it was adequately frozen fails to capture the sample, and attempting to take the punch after the slice was completely frozen can shatter the slice. Therefore, both punches had to be taken within a few seconds of the slice beginning to freeze.

**Figure 3-1** Tissue collection. ~2 mm thick coronal brain slice was dissected and the left- and right-VTA were separately collected using a micro-punch. (A) Photo of the ventral surface of a mouse brain in a steel brain matrix after dissection. (B) Schematic diagrams showing the location of the targeted coronal section (approximately -2.8 mm to -4.8 mm from bregma), and the punched regions (C). Images adapted from Paxinos & Franklin (2004).
3.2.2 RNA extraction

Total RNA was extracted using an RNeasy Plus Universal Mini kit (Qiagen, NL). All work areas and equipment for RNA extraction were decontaminated with RNase free 100% ethanol, RNase zap (Qiagen), and DEPC-treated water. In order to reduce the processing time and the risk of RNA degradation, no more than six samples were extracted in each run. Efficient disruption and homogenisation of the tissue sample is an absolute requirement for RNA purification procedures. Micro-dissected tissue was added to 900µl of QIAzol lysis reagent (Qiagen, NL), and immediately placed in a Bioruptor sonicator (UCD-300, Diagenode, USA) set on high intensity (300W) for 5 cycles (10 s on/ 10 s off). This ensures sufficient disruption of plasma membranes of cells to allow RNA to be solvated, as well as homogenisation of the resulting lysates. Incomplete disruption or homogenisation of the sample results in significantly reduced RNA yields, and over homogenisation can lead to degradation of RNA (Burden, 2012). Sonication was chosen over vortexing, syringe and needle aspiration, and fixed blade variable speed Tissue Tearor (Biospec Products Inc) methods because it led to higher yields of intact RNA in preliminary testing with this type of tissue sample (data not shown). 100µl of gDNA eliminator was then added to the homogenate followed by 180µl of chloroform and the sample was then centrifuged at 12,000 g for 15 min (4°C) to separate RNA, DNA and protein into fractions; all further steps were performed at room temperature. The upper aqueous phase containing RNA was collected and mixed with an equal volume of 70% Ethanol (v/v in nuclease free water). The mixture was then passed through an RNeasy spin column. 700µl Buffer RWT was passed through the spin column once by centrifugation at 8,000 g for 15 s and then 500µl Buffer RPE was passed through the spin column twice (8,000 g, 15 s; 8,000 g, 2 min) to wash the column membrane prior to elution of the RNA with 35 µl RNase-free water. 3 µl of each sample was aliquoted to assess the concentration, purity, and integrity using an Agilent 2100 Bioanalyzer (Agilent, USA) that produces RNA integrity number (RIN). RINs for all samples used in qPCR studies were above 7 (figure 3-2). RNA samples were stored at -80°C until required.
Figure 3-2 Electropherogram RNA analysis. Example of bioanalyzer report showing the RNA concentration of 43 ng/µl in this sample. Integrity of the RNA is shown by a high RNA Integrity Number (RIN), in this case 8.2, and is also shown by strong 18S and 28S bands of eukaryotic RNA. A RIN higher than 5 is considered good RNA quality, and above 8 as perfect total RNA quality for RT-qPCR application (Fleige & Pfaffl, 2006).
3.2.3 Reverse transcription PCR

RNA (500 ng) was reverse transcribed into cDNA using a SuperScript® VILO™ cDNA synthesis Kit (Invitrogen, Mulgrave, Victoria). Reactions were run in a GeneAmp PCR System (Model 2400; Perkin-Elmer Life Sciences, Emeryville, CA, USA). Two samples were run either without RNA or without RT enzyme to serve as controls for genomic DNA contamination. Thermocycler conditions are described in Table 3-1. cDNA products were stored at -20°C until further use.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer annealing</td>
<td>25°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Extension</td>
<td>42°C</td>
<td>60 minutes</td>
</tr>
<tr>
<td>Reaction termination</td>
<td>85°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>indefinite</td>
</tr>
</tbody>
</table>

Table 3-1 Thermocycler conditions for reverse transcription.

3.2.4 Real-time quantitative PCR

Gene expression was measured using real-time quantitative PCR (qPCR) performed on an Applied Biosystems ViiA™7 real-time PCR system (Applied Biosystems, USA). All qPCR experiments were conducted in 10 µL reactions in MicroAmp® Optical 386-well plates (Applied Biosystems, USA) using SYBR® Green Master Mix (Applied Biosystems, USA). Reaction mixture per sample are specified in Table 3-2.

<table>
<thead>
<tr>
<th>Item</th>
<th>Volume per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR® Green Master Mix</td>
<td>5 µL</td>
</tr>
<tr>
<td>20 µM forward primer</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>20 µM reverse primer</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>cDNA</td>
<td>4 µL</td>
</tr>
<tr>
<td>Total</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Table 3-2 Reagent mixture for each qPCR reaction.
Such small total reaction volumes are vulnerable to pipetting errors, most importantly in the amount of cDNA. Two steps were taken to guard against this. Firstly, the proportions of the reaction mixture were refined to allow a larger volume of more dilute cDNA to be added. Secondly, three technical replicates (triplicates) of each reaction were run to assess within-sample variability and generate an average value.

**Table 3-3** Thermocycler conditions for qPCR experiments

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Ramp rate</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA polymerase activation</td>
<td>Step 1</td>
<td>1.6°C/s</td>
<td>50°C</td>
<td>2 min</td>
</tr>
<tr>
<td></td>
<td>Step 2</td>
<td>1.6°C/s</td>
<td>95°C</td>
<td>10 min</td>
</tr>
<tr>
<td>PCR stage (40 cycles)</td>
<td>Step 1 dissociation of dsDNA</td>
<td>1.6°C/s</td>
<td>95°C</td>
<td>15 s</td>
</tr>
<tr>
<td></td>
<td>Step 2 primer annealing and</td>
<td>1.6°C/s</td>
<td>60°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>extension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melt curve</td>
<td>Step 1</td>
<td>1.6°C/s</td>
<td>95°C</td>
<td>15 s</td>
</tr>
<tr>
<td></td>
<td>Step 2</td>
<td>1.6°C/s</td>
<td>60°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>Step 3</td>
<td>0.05°C/s</td>
<td>95°C</td>
<td>15 s</td>
</tr>
</tbody>
</table>

This process quantitated the amount of PCR product for every cycle of amplification by measuring the fluorescence of DNA-intercalating fluorophore SYBR-Green ($\lambda_{\text{max}} = 522$ nm), which increases in the presence of double stranded DNA (dsDNA). As the fluorescent signal can be altered by primer dimerisation or generation of non-specific PCR products (Vet & Marras, 2005), a melt curve analysis is performed after every qPCR experiment to ensure there is only one type of PCR product. This analysis takes advantage of the fact that the energy required to break base-base hydrogen bonding between two strands of DNA is dependent on their length, GC content and complementarity (Nelson & Cox, 2008). As the temperature is increased, dsDNA dissociates and SYBR green is unbound from minor groove. The decrease in fluorescence at a certain temperature ($T_m$) can be used to identify different PCR products. Thermocycler conditions are described in Table 3-3. Data were acquired by the ViiA™ 7 software (Applied Biosystems, USA) at the end of each primer extension stage, and during the melt curve stage. Hypoxanthine phosphoribosyltransferase (HPRT) was chosen as an internal control gene as it has been previously shown by our collaborators to be unaffected by viral treatment (Sztainberg et al., 2010).
**3.2.5 Primer sequence design**

CRFR1, CRFR2, CRFBP, and HPRT mRNA levels were measured in all samples. For samples from virus injected tissue, GFP levels were also measured to identify missed/failed virus injections. qPCR primer sets for HPRT, CRFR1, and GFP were designed by our collaborators, as described in previous publications (Sztainberg et al., 2010). I designed primers for CRFR2 and CRFBP using Primer-BLAST software (Ye et al., 2012). Forward and reverse primers were selected to be located at exon-exon junctions, or span an exon-intron boundary to avoid false-positive results caused by amplification of contaminating genomic DNA. Primer sequences for each gene are provided in table 3-4.

**Table 3-4** Sequences of primers used for real-time quantitative PCR. Shown in 5’-3’ notation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>5’GCAGTACAGCCCCAAAATGGG3’</td>
<td>5’GGTCCCTTTTCACCAGCAAGCT3’</td>
</tr>
<tr>
<td>CRFR1</td>
<td>5’TGCCAGGAGATTCTCAACGAA3’</td>
<td>5’AAAGCCGAGATGAGGTCCAG3’</td>
</tr>
<tr>
<td>CRFR2</td>
<td>5’TACCGAATCGCCCTCATTGT3’</td>
<td>5’CCACGCGATGTTTCTCAGAAT3’</td>
</tr>
<tr>
<td>CRFR2</td>
<td>5’GGTCCACGAAACCAGAAATG3’</td>
<td>5’CTCTGTAGGCTTCCGGGTC3’</td>
</tr>
<tr>
<td>CRFBP</td>
<td>5’GGTCCACGAAACCAGAAATG3’</td>
<td>5’ATGCAAGTGTCCGAGGTAA3’</td>
</tr>
<tr>
<td>GFP</td>
<td>5’CATGCCCGAAGGCTACGT3’</td>
<td>5’CGATGCCCTTCAGCTCGAT3’</td>
</tr>
</tbody>
</table>

**3.2.6 Primer efficiency assessment**

Before performing relative gene expression experiments, I needed to ensure that primer sets for the HPRT and GOI amplified the target cDNA template with similar efficiencies across a range of initial cDNA concentrations. If the efficiencies of the primers differ depending on the starting level of cDNA, then the relative quantification will be incorrect. Ideally, the amplification efficiency of each primer set should be 100%, meaning that during the exponential phase of the reaction, the amount of amplicon doubles with each cycle. This allows for fold difference values to be calculated from C_T values. A standard curve, generated by performing qPCR with a serial dilution of template was used to test assay efficiency, precision, sensitivity, and working range. Neat cDNA reverse transcribed from an experimentally naïve mouse was diluted to 1:10 which from then on was considered a 1:1 dilution. From this stock, serial dilutions of 1:2, 1:5, 1:10, 1:20, 1:50 and 1:100 were made. qPCR was performed for each dilution using each primer set. C_T values were plotted against the log of the cDNA
39 dilutions and then a linear regression analysis was performed on these data using GraphPad Prism 6.0 software to generate lines of best fit. The $r^2$ and gradient values of each line were used to calculate the amplification efficiency for individual primer sets according to the equation below. Primer sets with amplification efficiency values that were not 100% ± 10% were rejected and re-designed.

\[
E = \text{amplification efficiency} \\
E = 10^{(-\frac{1}{m})} - 1\times100 \% \\
m = \text{gradient of line of best fit}
\]

3.2.7 Relative gene expression analysis

The expression of each gene of GOI was determined relative to the housekeeping gene HPRT. cDNA samples generated from reverse transcription were diluted 1:10 to obtain a working concentration for qPCR and run according to section 1.2.4. On each 384-well plate, qPCR reactions were run in triplicate for HPRT as well as GOIs of each sample. In order to normalize for the amount of cDNA in each sample, the average $C_T$ value of each GOI was compared against HPRT; $\Delta C_T (\text{GOI}) = C_T (\text{HPRT}) - C_T (\text{GOI})$. The difference in $\Delta C_T$ values is used to infer the fold difference between genes or treatment groups; fold difference = $2^{\Delta C_T}$.

3.3 Results

3.3.1 Primer efficiency assessment

Primer efficiencies, $r^2$ values and gradient of lines of best fit are described in table 3-5. 100% PCR efficiency will demonstrate a change of 3.3 cycles between 10 fold dilutions of template, reflected in the line of best fit gradient of -3.3. An $r^2$ value of 1 indicates a linear correlation between cDNA dilution and $C_T$ value. CRFR2 primer set B was rejected, and set A was used for all further experiments. All primer sets used in relative gene expression experiments had an efficiency within 100 ± 5%. Primer efficiency curves (figure 3-3) showed that the concentration of cDNA template at each dilution was within the range for reliable quantification using qPCR; i.e. $C_T < 35$ (Goni et al., 2009).
Figure 3-3 Primer efficiency curves. Standard curves generated for each primer set by performing qPCR on a serial dilution of cDNA template. Dots represent average C_T values of triplicate reactions for each cDNA dilution. Lines of best fit were determined using linear regression analysis.

Table 3-5 Amplification efficiency of qPCR primer sets. r^2 values and gradients are shown for each line of best fit for standard curves.

<table>
<thead>
<tr>
<th>Gene</th>
<th>r^2</th>
<th>Gradient</th>
<th>Amplification efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>0.9975</td>
<td>-3.242</td>
<td>103%</td>
</tr>
<tr>
<td>CRFR1</td>
<td>0.9980</td>
<td>-3.419</td>
<td>96%</td>
</tr>
<tr>
<td>CRFR2 (set A)</td>
<td>0.9782</td>
<td>-3.364</td>
<td>98%</td>
</tr>
<tr>
<td>CRFR2 (set B)</td>
<td>0.5716</td>
<td>0.6622</td>
<td>-96.9%</td>
</tr>
<tr>
<td>CRFBP</td>
<td>0.9969</td>
<td>-3.234</td>
<td>103%</td>
</tr>
<tr>
<td>GFP</td>
<td>0.9995</td>
<td>-3.318</td>
<td>100%</td>
</tr>
</tbody>
</table>
3.3.2 Melt curve analysis

After the final amplification stage of qPCR experiments, a dissociation analysis was run on all qPCR products to generate melt curves. qPCR data was transformed so that decreases in fluorescence represented as peaks at certain temperatures. The number of peaks shows how many amplicons with unique melting temperatures are in a given sample. Melt curve analysis of each primer set yielded one clean peak indicating a single template product with a unique melting temperature. This shows that the primers were specifically amplifying their intended cDNA target, and not affected by potential genomic DNA contamination.

![Melt curve graphs](image)

**Figure 3-4** Representative examples of melt curves generated for each primer set. Graphs show melt curves generated after a primer efficiency qPCR experiment. Each line represents a separate qPCR reaction. Negative control graph shows no signals from samples without cDNA.
3.3.3 Stability of endogenous control gene

To confirm that HPRT is not altered by the viral treatment, I compared the average $C_T$ values of HPRT for naïve mice and those receiving shControl or shCRFR1 viral injections. One-way ANOVA showed no differences in the average $C_T$ values of HPRT across treatment groups ($F<1, p=0.42$) (figure 3-5). This shows that HPRT is suitable for use as an internal control gene.

![Figure 3-5 Average $C_T$ values for HPRT from VTA samples. Box plots show median and interquartile range, with whiskers showing 10 and 90 percentiles. Naïve, n=8; shControl, n=8; shCRFR1, n=7. Mean shown as “+” symbol.](image)
3.3.4 Relative gene expression analysis

The basal levels of CRF receptor mRNA were examined using qPCR on mRNA extracted from the VTA of naïve mice. Analysis of qPCR data using one-way ANOVA showed an overall difference in delta C_T values between groups ($F_{(2,21)}=63.87, p<0.0001$) (figure 3-6). Tukey’s multiple comparisons tests revealed a significantly higher C_T value for CRFR2 compared to CRFR1, with a difference of $4.62 \pm 0.44$ indicating 24 times less CRFR2 mRNA compared to CRFR1, and a $1.20 \pm 0.29$ fold difference in CRFBP mRNA compared to CRFR1, equivalent to approximately 2.3 times less CRFBP mRNA than CRFR1.

Figure 3-6 Relative expression of CRF receptor transcripts in the VTA of 4 naïve mice (n=8 VTA samples) normalized to HPRT. Box plots show median and interquartile range, with whiskers showing 10 and 90 percentiles. Mean shown as “+” symbol. ****p<0.0001 *p<0.05.
I then assessed viral knockdown of CRFR1. Analysis of virus injected VTA tissue showed significantly less CRFR1 mRNA in mice receiving shCRFR1 virus compared to shControl treated mice (t(11)=2.419, p<0.05) (figure 3-7) with a mean difference in delta Ct value of 0.85 ± 0.35, equivalent to an approximately ~50% reduction. There were no differences between virus treatment groups for either CRFR2 or CRFBP (ts<1).

Figure 3-7 Relative expression of CRFR1 (A) CRFR2 (B) and CRFBP transcripts (C) in shControl and shCRFR1 virus treated groups (n=8). Box plots show median and interquartile range, with whiskers showing 10 and 90 percentiles. Mean shown as “+” symbol. *p<0.05.
3.4 Discussion

Using qPCR analysis, the present chapter shows for the first time the relative expression of CRF receptor and CRFBP mRNA in the VTA of naïve mice, which is concordant with in situ hybridization (ISH) and GFP reporter mouse studies of CRFR1 (Refojo et al., 2011; Van Pett et al., 2000) and CRFBP mRNA (Wang & Morales, 2008). CRFR2 has not yet been identified in the VTA by ISH, but it has been consistently detected in VTA neurons by single cell reverse transcription polymerase chain reaction (RT-PCR) and functional assays (Ungless et al., 2003).

I then demonstrated that shCRFR1 injected in the mouse VTA decreases CRFR1 mRNA. This is concordant with the previously mentioned in vitro and qualitative in vivo BLA validation by Sztainberg et al. (2010), as well as in situ hybridization (ISH) studies showing decreased CRFR1 positive cells in the external globus pallidus (GPe) of shCRFR1 virus injected mice (Sztainberg et al., 2011). Due to conflicting reports of the effect of CRF1 receptor antagonism on addiction-related behaviours in rats (Blacktop et al., 2011; Wang et al, 2005), possibly arising from problems with the selectivity of different antagonists, it was important to show that shCRFR1 does not alter the levels of CRFR2 or CRFBP. Here I showed that shCRFR1 virus specifically decreased CRFR1 mRNA levels without altering the expression of CRFR2 or CRFBP, implying no compensatory up- or down-regulation of these other two species.

mRNA is frequently used to measure changes in gene expression and as a proxy of apparent changes in protein levels; for example, qPCR has been used to validate the viral-mediated downregulation of CRF within the VTA (Greider et al., 2014). However, using qPCR to infer knockdown of CRF1 receptors is problematic because GPCR mRNA levels do not necessarily correlate with protein levels (Vogel & Marcotte, 2012). This is a major caveat when examining the upregulation of GPCR mRNA, as the functional consequences rely on efficient translation into protein and trafficking to the membrane surface. Conversely, when down-regulating mRNA, one can measure a limiting factor for protein expression. Considering the relatively modest reduction in CRFR1 mRNA seen in this study compared to in vitro assessment of CRFR1 protein expression and receptor signalling (Sztainberg et al., 2010), it may be that measuring CRFR1 mRNA underestimates the impact of the shCRFR1 treatment. This may be further complicated by the qPCR amplification of fragments of CRFR1 mRNA that are not completely degraded, but nonetheless fail to form functional CRF1 receptors. Indeed, it has been shown that detection of RNAi-mediated gene silencing is dependent on the location of the primers relative to the mRNA cleavage site (Van Maerken et al., 2008).

A major obstacle in investigating CRF receptors is the lack of specific antibodies for immunohistochemical analysis. Antibodies against GPCRs are notoriously unreliable. Often GPCRs within the same family have high degrees of homology, and hence antibodies may cross-react between
different receptor subtypes. For example, CRFR1 and CRFR2 share approximately 70% of amino acid sequence homology. Most of the variation is in the N-terminal extracellular domain with 40% homology between the two receptors, the rest of the sequence is highly conserved (Zmijewski & Slominski, 2010). However, even antibodies directed against the N-terminus of GPCRs can lack specificity just as those targeting more conserved domains (Michel et al., 2009). Systematic testing of seven different CRFR1 antibodies did not produce a single one suitable for identifying CRFR1 (Refojo et al., 2011). Notably, a recently published study that employed an anti-CRFR1 antibody for immunohistochemistry (Lemos et al., 2012), failed to replicate and be validated in my hands (data not shown), and this antibody has since been recalled by the manufacturer.

At this time, these qPCR studies, along with in vitro western blot, luciferase assay, ISH, and semi-quantitative PCR experiments (Sztainberg et al., 2010, 2011), provide the best practical evidence for the effective knockdown of CRFR1. Furthermore, our collaborators have successfully implemented this technique in behavioural assays, confirming that viral-mediated downregulation of GPe CRFR1 recapitulates the anxiogenic effect of pharmacological blockade by local infusion of a selective CRFR1 antagonist (Sztainberg et al., 2011). Now that the VTA-targeted knockdown using virus produced in-house has been validated, this method can be used to characterise the behavioural effects of this decreased VTA CRFR1 in relation to cocaine-seeking and conditioned fear.
CHAPTER 4:
DEVELOPMENT OF A
PARADIGM FOR
STRESS-INDUCED
REINSTATEMENT OF
COCAINE-SEEKING
IN MICE
4.1 Introduction

Stress is a potent trigger of relapse in abstinent drug addicts. Stress-induced relapse has been regularly modelled as ‘stress-induced reinstatement’ in animals using an intravenous self-administration (IVSA) model. In this paradigm, drug-seeking following IVSA is first extinguished by allowing the animals to lever-press in the absence of the drug. Over time the animals will decrease lever-pressing. Once drug-seeking has been extinguished, animals are exposed to a stressor which causes them to resume the previously drug-enforced behaviour, in the absence of drug reward. This model has a high degree of construct and face validity for the study of addiction (O’Brien & Gardner, 2005), and researchers have shown that a variety of stressors will reinstate drug-seeking behaviour across many drug classes (see Mantsch et al., 2016 for review).

Nevertheless, stress-induced reinstatement of drug-seeking has proved troublesome to implement in mice. Most studies investigating relapse-like behaviour in mice have used a CPP approach to examine the ability of stressful stimuli to re-establish an extinguished place preference for a previously cocaine-paired environment (Carey et al., 2007; Kreibich & Blendy, 2004; Redila & Chavkin, 2008; Mantsch et al., 2010). However, this paradigm does not involve volitional drug consumption and cannot be used to measure active drug-seeking or motivation to obtain drug reward which are crucial components of addiction. There are currently only two studies describing a protocol for stress-induced reinstatement of cocaine-seeking in mice (Highfield et al., 2002; Soria et al, 2008), neither of which have been utilized in other subsequent publications. Given the variety of transgenic mouse lines currently available, there is a need for a robust and reliable stress-induced reinstatement paradigm in conjunction with IVSA.

To date, my lab’s protocol for examining drug seeking in mice models drug-seeking after extended enforced abstinence (eg Brown et al., 2009), or cue-induced reinstatement following extinction of methamphetamine-seeking behaviour (Chesworth et al., 2013) and as such I did not have access to an established protocol for extinction and stress-induced reinstatement of cocaine-seeking in mice. Therefore, in order to investigate the effect of CRFR1 knockdown in the VTA on stress-induced reinstatement of cocaine self-administration in mice, I first needed to develop a methodology to effectively extinguish lever responding for cocaine so that it could then be reinstated with a stressor.

In the laboratory, stress can be operationally defined as ‘forced exposure to events or conditions that are normally avoided by the non-human lab subject’ (Lu et al., 2003). One of the most commonly used stressors for the reinstatement of drug-seeking in rats is inescapable footshock (Buczek et al., 1999; Erb et al., 1996; Le et al., 1998; Shaham and Stewart., 1995; Shepard et al., 2004); however, this was not considered a viable option as it did not reinstate lever discrimination in CD1 mice (Soria et al., 2008). In developing translational approaches to the study of drug relapse, it would be advantageous to use stressors that have a high degree of homology across species. An
experimental approach with clear translational potential is the administration of pharmacological agents known to induce stress states, by acting via similar neurobiological mechanisms, in both humans and animal models. A commonly used pharmacological stressor is the \( \alpha_2 \) adrenoceptor antagonist yohimbine, which increases synaptic NE, elicits an increase in subjective anxiety, and activates autonomic stress measures. This has been extensively used to elicit stress and anxiety responses in clinical studies (Bremner et al., 1996; Southwick et al., 1999) and to produce stress states in rodent models (Pellow et al., 1985). Further, yohimbine administration induced anxiety and drug craving in abstinent opiate addicts and alcoholics (McDougle et al., 1995; Stine et al., 2002; Umhau et al., 2011), and increased opioid-seeking in heroin-dependent individuals (Greenwald et al., 2013), thus lending support for the use of yohimbine as a pharmacological probe to activate stress responses and subsequent craving and drug-seeking.

In operant self-administration studies using rats, yohimbine has been shown to reinstate drug-seeking for cocaine (Feltenstein & See, 2006), methamphetamine (Shepard et al., 2004), alcohol (Le et al., 2005; Ryan et al., 2013; Simms et al., 2011), nicotine (Feltenstein et al., 2012), and heroin (Banna et al., 2010). This effect is also seen in other species, for example cocaine-seeking in monkeys (Lee et al., 2004). Although yohimbine has not been tested in the reinstatement of drug-seeking following IVSA in mice, it has been shown to reinstate a cocaine CPP (Mantsch et al., 2010).

Food-deprivation is another widely-used stressor, and has been shown to reinstate heroin-seeking (Maric et al., 2011; Sedki et al., 2015; Tobin et al., 2009, 2013), as well as cocaine-seeking in rats (Shalev et al., 2003) and in 129X1/SvJ mice (Highfield et al., 2002). Importantly, both yohimbine and acute food-deprivation are dependent on CRF signalling. Food deprivation-induced heroin seeking is dependent on extrahypothalamic CRF (Shalev et al., 2006). Yohimbine-induced reinstatement of alcohol-seeking (Marinelli et al., 2007) and palatable food-seeking (Ghitza et al., 2006) is prevented by systemic CRFR1 antagonism (Marinelli et al., 2007). Therefore, both of these stressors were tested in this chapter.

### 4.2 Methods

#### 4.2.1 Animals

Refer to general methods. Mouse weights were recorded daily.

#### 4.2.2 Drugs

Cocaine hydrochloride (Johnson Matthey Macfarlan Smith, Edinburgh, UK) was dissolved in 0.9% sterile saline at a concentration of 0.5mg/kg/infusion. Heparin anticoagulant (CSL Limited, Melbourne, Australia) was made up in 0.9% sterile saline to 10U and 90U concentrations. Neomycin sulphate (Delta Veterinary Laboratories, Hornsby, Australia) was diluted 1:50 with heparinised (90U) 0.9% saline. Yohimbine (Tocris Bioscience, Bristol, UK) was dissolved in distilled water.
4.2.3 Apparatus

All self-administration tasks took place in operant chambers (model ENV-307W, Med Associates Inc®, USA), singly-housed in sound-attenuating, fan ventilated boxes. Each chamber was equipped with two levers; an active lever which led to delivery of a sucrose/cocaine reward and an inactive lever that had no programmed consequences. The conditioned cue light (CS+) was located above the active lever and was coupled to reward delivery and lasted 10s. A 1cm square of paper towel was infused with a drop of vanilla essence to serve as a non-conditioned olfactory cue (S+) and placed under the active lever. Operant sessions took place at approximately the same time each day during the dark phase. MED-PC IV software (Med Associates®, USA) was used to record active lever presses as well as inactive lever presses and contacts with the sucrose receptacle.

4.2.4 Sucrose self-administration/instrumental training

One-session daily instrumental learning program was used across 8 days to train mice to press the active lever in order to obtain a reward - in this case 5μL of 10% (w/v) sucrose delivered in a receptacle located between the two levers. A fixed-ratio-of-one (FR1) reinforcement schedule was used, so that each active lever press led to sucrose delivery. Mice were started on a single lever program where only the active lever was present. After 3 days, when mice had made more than 100 lever presses in a session, the inactive lever was introduced for a further 5 days of training.

4.2.5 Implantation of jugular catheters

Indwelling jugular vein catheters were implanted to facilitate intravenous delivery of cocaine.

4.2.5.1 Construction of catheters

All catheters were constructed in-house essentially as previously described (Brown et al., 2009). The tips of 22G x 1¼” needles were removed and then threaded through 2 cm lengths of BCOEX-T22 tubing (0.024” inner diameter (ID) x 0.064” outer diameter (OD), Instech Solomon, PA, USA). The needles were then bent at a right angle to the luer and then bent into a U shape. 3.8 cm lengths of silastic tubing (0.3 mm ID x 0.64 mm OD, Dow Corning, USA) were attached to the end and sealed with silicone. The overhang of silastic tubing was trimmed to 3.3 cm and a dab of silicone was placed 1 cm from the end of the tubing to mark the length to be inserted into the jugular vein.
Figure 4-1 Mouse jugular catheter.

### 4.2.5.2 Jugular cannulation surgery

Surgery was performed according to Brown et al. (2009). Mice were initially anaesthetized using 5% v/v isoflurane in an induction chamber until the loss of righting reflex, anaesthesia was then maintained through an isoflurane nosecone (1-1.5% in air 0.5L/min). Depth of anaesthesia was tested by foot-pinach response and mice were given an i.p. injection of meloxicam (3mg/kg; Boeringher Ingleheim, Ingleheim, Germany) for perioperative analgesia. The left jugular was isolated and the silastic tubing was inserted 1 cm into the vein and secured with sutures. The rest of the tubing ran subcutaneously behind the left ear and exited on top of the head, where the catheter port was glued to the skull using Loctite 454 instant adhesive (Loctite Australia Pty Ltd, Caringbah, Australia) and dental cement (Vertex-Dental, Zeist, Netherlands). The catheter was then flushed with an antibiotic in heparinised saline (0.02 ml neomycin sulfate 4 mg/ml in 10U heparin) and drawn back so a small amount of blood was visible moving through the tubing to check catheter patency. Mice were allowed to recover in an incubator before being placed in a clean home cage. The day after surgery, catheters were flushed with 0.02 ml of a heparinised neomycin solution (10U heparin in the morning, 90U heparin in the afternoon). To minimize risk of infection, heparinised antibiotic solution was used to flush catheters for two days following surgery before switching to heparinised saline without antibiotic. They were allowed to recover for at least 2-3 days before any operant task. Mice were flushed with 0.02ml of heparin before (10U) and after (90U) each behavioural session to maintain catheter patency.
4.2.6 Intravenous self-administration of cocaine

The catheters were connected via flexible tubing (0.5mm ID, 1.5mm OD, Tygon, USA) to a 22-gauge swivel (Instech Solomon, PA, USA) which then connected to a syringe pump (model PHM-100SVA; Med Associates) by BCOEX-T22 tubing (6 mm ID, 0.64 mm OD, Intech Solomon). The cocaine self-administration program followed an FR1 schedule of reinforcement. Active lever presses lead to a single intravenous infusion (0.5mg/kg body weight) of cocaine (20 µL delivered over 1.7 seconds). The sessions were usually 2 hours long, however, in order to prevent overdose mice were allowed a maximum of 80 infusions before the session would be terminated. Each infusion also resulted in a 10s timeout, during which, lever presses would no longer lead to drug infusion. During the timeout period, active lever presses were still recorded resulting in a total number of active lever presses made in the session being a combination of active lever presses that lead to drug infusions and those that were made during the timeout periods. Mice were randomly checked for catheter patency by administering 0.02ml ketamine (15 mg/ml) via the catheter and observing immediate prominent signs of hypnosis.

![Image of a mouse in an operant chamber. The olfactory cue is positioned under the grid floor in front of the active lever. Pressing the active lever activated the cue light for 10 seconds and cocaine is delivered via flexible tubing connected to their indwelling jugular catheters secured to the head. The same chambers were used for sucrose training. Sucrose could be delivered via a receptacle that is not shown (behind the mouse).](image)

**Figure 4-2** Example of a mouse in an operant chamber. The olfactory cue is positioned under the grid floor in front of the active lever. Pressing the active lever activated the cue light for 10 seconds and cocaine is delivered via flexible tubing connected to their indwelling jugular catheters secured to the head. The same chambers were used for sucrose training. Sucrose could be delivered via a receptacle that is not shown (behind the mouse).

4.2.7 Extinction

Extinction training was performed in the same operant chambers as self-administration and no cocaine was available during this time. Mice were deemed to be extinguished once active lever presses were < 50% of initial extinction responding for two consecutive days. Different forms of extinction training previously described from our laboratory (Zbukvic et al., 2016) were tested.
CS + lever extinction training – one hour long sessions where both levers were available and active lever presses led to the illumination of the cue light for 10 seconds.

CS-only extinction – one hour long sessions where mice were placed in operant chambers with both levers retracted. The cue light above the previously reward-paired lever was illuminated 120 times for 2.7 seconds.

Lever-only extinction - both levers were available, but active lever presses no longer lead to illumination of the cue light. In experiment 1, lever-only extinction sessions were one hour long, in experiment 2 they were 30 minutes long.

4.2.8 Stress-induced reinstatement

Following extinction of cocaine-seeking, mice were exposed to different stressors and the reinstatement of cocaine-seeking was measured during 30-minute test sessions without any cocaine. Reinstatement was calculated for each individual mouse and defined as a significant increase in active lever responses compared to the final day of extinction training.

4.2.8.1 Yohimbine

Mice were given intra-peritoneal (i.p) saline injections 20 minutes before their extinction sessions for at least three consecutive days in order to habituate them to receiving injections and avoid confounding effects of the stress on the injection. Once a mouse had reached extinction criteria, it was tested for yohimbine-induced reinstatement the following day. On yohimbine test day mice were given an i.p injection of 2mg/kg yohimbine (Tocris Bioscience, Briston, UK) dissolved in distilled water 20 minutes before reinstatement testing. This dose of yohimbine was chosen as it has been shown to reinstate cocaine CPP in mice (Mantsch et al., 2010).

4.2.8.2 Acute food-deprivation

Upon completing their final extinction session all food was removed before mice were returned to their home cage. Reinstatement testing was conducted either during the following dark phase (24-hours later) or after a complete dark phase of food deprivation (48-hours later). Mice remained in their home cage during the food deprivation period. Food was returned to their home cage immediately after reinstatement testing.

4.2.9 Analysis

Self-administration data was analysed using a mixed-design repeated measures ANOVA with Bonferroni and Tukey’s post hoc multiple comparisons where appropriate, and the more conservative statistics is reported in this chapter. The level of significance in all tests was $p < 0.05$. All data
analyses were conducted using SPSS (IBM SPSS Statistics Version 20.0, IBM Corp., New York, USA).

4.3 Results

4.3.1 Experiment 1: Extinction of cocaine-seeking in mice

In experiment 1, mice first underwent sucrose training. Mice readily acquired an instrumental learning task for the operant self-administration of sucrose. Two-way RM ANOVA of days 4-8 showed a main effect of day \( F_{(4,56)} = 7.529, p < 0.0001 \), a main effect of lever type \( F_{(1,14)} = 125.365, p < 0.0001 \) and a significant lever \( \times \) day interaction \( F_{(4,56)} = 10.998, p < 0.0001 \) (figure 4-4A).

Figure 4-3 Experiment 1 timeline.

I analysed each lever type separately due to the significant interaction. One-way RM ANOVA of active lever pressing days 4-8 showed a main effect of day \( F_{(4,56)} = 12.63, p < 0.0001 \). The same analyses of inactive lever pressing days 4-8 showed no effect of day \( F < 1 \).

Mice were then allowed to self-administer cocaine for 12 days. Two-way RM ANOVA of lever pressing data showed preference for the active lever over the inactive lever: main effect of lever.
type \(F_{(1,6)} = 27.613, p < 0.05\), but no effect of day \(F_{(11,66)} = 1.230, p = 0.286\), and no day × lever interaction \(F_{(11,66)} = 1.236, p = 0.282\) (figure 4-4B). Mice earned an average of 47.41 ± 3.7 infusions per day equating to 23.7 ± 1.85 mg/kg of cocaine/day.

Mice then underwent CS + lever extinction training. After 10 days of extinction training in the presence of the CS, mice had not reached extinction criteria. Two-way RM ANOVA showed a main effect of lever type \(F_{(1,3)} = 14.259, p < 0.05\), indicating a preference for the active over inactive lever (fig. 4-5A). There was also a main effect of day \(F_{(9,27)} = 2.648, p < 0.05\), and a significant lever × day interaction \(F_{(9,27)} = 2.747, p < 0.05\). Separate one-way RM ANOVA of active and inactive lever pressing showed a significant effect of day on both active lever presses \(F_{(9,27)} = 2.709, p < 0.05\), and inactive lever presses \(F_{(9,27)} = 2.355, p < 0.05\). However, analysis of lever responding on first vs final CS + lever extinction day showed a main effect of lever type \(F_{(1,3)} = 16.370, p < 0.05\), but no effect of day \(F_{(1,3)} = 9.118, p = 0.057\) nor any interaction \(F < 1\) (figure 4-5D). Taken together, these analyses show that while lever responding has fluctuated day-to-day, it never significantly decreased over the 10 days.

**Figure 4-5** Extinction of cocaine-seeking in sucrose-trained mice. Mean ± SEM daily active and inactive lever presses during (A) CS + lever extinction \((n = 15)\), (B) with a subset receiving additional cue extinction sessions (no CS extinction, \(n = 8\); CS extinction, \(n = 7\)), and (C) lever only extinction \((n = 15)\). (D-F) show first and final lever presses for each phase of extinction training.
I then examined the effect of CS-only extinction on CS + lever operant responding. Mice were first randomly assigned to two groups just for 8 days – one group received CS-only extinction sessions while the other group remained in their home cage. During those days both groups still received daily CS + lever extinction sessions prior to CS-only extinction sessions. Three-way RM ANOVA (between-subjects factor: CS extinction, within-subjects factors: day & lever type) showed a main effect of lever type \[F_{1,13} = 26.996, p < 0.0001\], but no effect of CS extinction \[F < 1\], no effect of day \[F_{7.91} = 1.485, p = 0.182\], nor any significant interactions \[Fs < 1\] (fig. 4-5B). Similarly, analysis of day 11 compared to day 18 of lever responding showed a main effect of lever type \[F_{1,13} = 30.771, p < 0.0001\], but no effect of day, CS extinction, or any significant interactions \[Fs < 1\] (figure 4-5E).

To examine lever pressing behaviour in the absence of the CS, all mice received 8 days of lever-only extinction. Two-way RM ANOVA revealed a main effect of lever \[F_{1,6} = 7.106, p < 0.05\], day \[F_{7,42} = 2.361, p < 0.05\], and a significant lever × day interaction \[F_{3.364} = 3.664, p < 0.01\] (figure 4-5C). Separate one-way RM ANOVA of active and inactive lever pressing showed a significant effect of day on active lever presses \[F_{7,42} = 3.068, p < 0.05\], but not inactive lever presses \[F < 1\]. Again, analysis of first vs final days of this phase of extinction training showed a main effect of lever type \[F_{1,6} = 6.296, p < 0.05\], but no effect of day \[F_{1,6} = 3.844, p = 0.084\] nor a lever × day interaction \[F_{1,6} = 2.214, p = 0.183\] (fig. 4-5F).

The fact that these mice received 26 consecutive days of extinction training with barely any decrease in active lever responding shows that mice are extremely resistant to extinction of drug-seeking behaviour, at least under the conditions of this experiment.

### 4.3.2 Experiment 2: Stress-induced reinstatement of cocaine-seeking

![Figure 4-6](image-url) Experiment 2 timeline.
In experiment 2, mice underwent cocaine IVSA without prior sucrose training. This is because I hypothesised that less reinforcement of operant responding may reduce the significance of the active lever and facilitate extinction of drug-seeking. Since they were not familiar with the operant self-administration paradigm, mice were given three sessions of active-lever only cocaine IVSA to facilitate acquisition of lever pressing for cocaine. By day three of single-lever cocaine IVSA, all mice acquired high levels of cocaine self-administration, and the inactive lever was introduced for further IVSA sessions for 10 days. Two-way RM ANOVA of double-lever cocaine IVSA showed preference for the active over the inactive lever [main effect of lever type: $F_{(1,15)} = 35.309, p < 0.0001$], but there was no effect of day, nor day × lever interaction [$F$s < 1] (figure 4-7). Interestingly, compared to experiment 1, a lower number of active lever presses was observed. Consistent with that finding, mice consumed slightly less cocaine in experiment 2 compared to experiment 1, earning an average of 39.8 ± 0.5 infusions per day equating to 19.9 ± 0.25 mg/kg of cocaine/day [significant difference in cocaine intake: $t(31) = 2.23, p<0.05$; unpaired t-test].

![Figure 4-7](image)

**Figure 4-7** Acquisition of cocaine self-administration in mice that did not receive prior sucrose training. Mean ± SEM daily active and inactive lever presses (n = 18).

Examination of within-session lever pressing during extinction in experiment 1 showed that the majority of the lever pressing activity occurred in this initial 30 minutes of the extinction session, with very few lever presses occurring in the last 30 minutes of the session. Therefore, to shorten experimental procedures, daily 30-minute extinction and reinstatement sessions were used in experiment 2. After 4-7 days of extinction training, mice that had reached extinction criteria were tested for 24-hour food-deprivation stress-induced reinstatement, and then returned to their home cage with *ad libitum* access to food. This was followed by yohimbine-induced reinstatement testing the next day. Two-way RM ANOVA showed a main effect of lever type [$F_{(1,6)} = 29.88, p < 0.01$], day [$F_{(3,18)} = 55.19, p < 0.0001$] and a significant lever × day interaction [$F_{(3,18)} = 31.14, p < 0.0001$] (figure 4-8A &
B). As there was an interaction, Bonferroni post hoc analysis of active vs inactive lever pressing showed that mice made significantly more presses on the active compared to inactive lever on the first extinction day \( p < 0.0001 \) and 24-hour food-deprivation test day \( p<0.001 \) only. To further investigate significant main effects Tukey’s post hoc comparisons was carried out within each lever type, with all results displayed in table 4-1A & B. Note that mice made significantly fewer active lever presses on final extinction day compared to first extinction day, indicating that active lever pressing had been extinguished, but there were no differences between final extinction day and 24-hour food-deprivation reinstatement test, showing a lack of reinstatement. Additionally, rather than inducing reinstatement, active lever presses were significantly reduced on the yohimbine test day. There was also a reduction of inactive lever presses on yohimbine test day compared to final extinction day.

![Graph](image_url)

**Figure 4-8** Mean + SEM of (A) active and (B) inactive lever pressing under extinction conditions \((n = 7)\).
Table 4-1 Results of Tukey’s post hoc multiple comparisons of (A) active and (B) inactive lever pressing under different extinction conditions.

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<th>Active lever</th>
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<td><strong>Active lever</strong></td>
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<td>first extinction day</td>
<td>final extinction day</td>
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<td>24-hour food-deprivation</td>
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<td>yohimbine</td>
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<td>final extinction day</td>
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<td>24-hour food deprivation</td>
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<th>Inactive lever</th>
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<td><strong>Inactive lever</strong></td>
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<td>first extinction day</td>
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These mice were given 4-5 days of further lever-only extinction sessions, and during this time three mice from the previous ‘non extinguished’ group reached extinction criteria. All extinguished mice were then tested for 48-hour food-deprivation stress-induced reinstatement. Two-way RM ANOVA revealed a main effect of lever type \( [F_{(1,9)} = 15.53, p < 0.01] \), a main effect of day \( [F_{(2,18)} = 18.83, p < 0.0001] \), and significant lever × day interaction \( [F_{(2,18)} = 59.63, p < 0.0001] \) (figure 4-9). Bonferroni post hoc analysis showed that mice made significantly more presses on the active compared to inactive lever on first extinction day \( [p < 0.0001] \) and 48-hour food-deprivation test day \( [p < 0.0001] \) only. Tukey’s post hoc analysis showed significant differences in active lever presses between each of the test days (figure 4-9A), but no differences in inactive lever presses (figure 4-9B).
Figure 4-9 Effect of 48 hour food deprivation on (A) active and (B) inactive lever pressing in extinguished mice (n=10). Black dots represent mice who were previously tested for effects of 24-hour food deprivation and yohimbine. Blue dots represent mice who did not receive prior 24-hour food deprivation or yohimbine.

The effect of 48-hour food-deprivation stress on operant responding was also tested in mice that had not reached extinction criteria after 17-21 days of lever-only extinction training. Two-way RM ANOVA showed a main effect of lever type \([F_{(1,7)} = 88.80, p < 0.0001]\), but no effect of treatment \([F < 1]\), and no lever \(\times\) treatment interaction \([F_{(2,14)} = 2.897, p = 0.0886]\) (figure 4-10).
4.4 Discussion

4.4.1 Extinction of drug-seeking in mice

In experiment 1, mice received 8 days of sucrose training followed by 12 days of cocaine IVSA before commencing extinction training. After 10 days of lever + CS extinction, mice still showed high levels of cocaine-seeking behaviour. Considering that our lab has shown lever-only extinction of methamphetamine-seeking behaviour in this timeframe (Chesworth et al., 2013), and others have demonstrated that extinction is impaired by CS presentation (Highfield et al., 2002), I reasoned that the CS may be promoting lever pressing behaviour even in the absence of reward delivery. In an attempt to decrease the salience of the CS, half of the mice were given CS-only extinction sessions, which is effective in reducing extinction responding in rats previously trained to self-administer cocaine (Zbukvic et al., 2016). However, this had no effect on operant responding during lever + CS extinctions in mice.

The CS was then removed from subsequent extinction sessions. For a further 8 days, mice received lever-only extinction sessions but still did not extinguish active lever pressing. In experiment 2, mice without prior sucrose training took 4-12 days of lever-only extinction training to reach criteria.

Figure 4-10 Effect of 48 hour food deprivation on operant responding in non-extinguished mice. Data presented as Mean + SEM of total active and inactive lever presses (n=8).
4.4.1.1 Sucrose training

Sucrose training is routinely used in rodent self-administration studies to facilitate the acquisition of drug consumption (Kmiotek et al., 2012; Rocha et al., 1997, 1998), however, it is possible that the large number of stimulus-reward pairings during sucrose self-administration strengthened the conditioned reinforcing effects of the reward-paired stimuli and thus contributed to impaired extinction seen in experiment 1. Indeed, extended food training has been shown to maintain lever responding in during CS+ extinction for several weeks even in cocaine naïve mice (Thomsen & Caine, 2011).

Fuchs et al. (2003) reported an average of 18 days of lever-only extinction was required to extinguish cocaine-seeking in previously sucrose trained mice. But in their study sucrose training was brief, consisting of one overnight (16-hour) session. Also, the difference in latency to extinguish can largely be explained by the different extinction criteria used in this study; ie. < 25 active lever presses (< 20% of group average initial extinction responding). At a group level, by day 7 active lever presses were reduced to 55% of initial extinction levels – following a similar trajectory to the C57BL/6J mice in our study. A similar extinction latency is seen in non-sucrose trained CD1 mice which, at a group level, decreased cocaine-seeking to ~50% of initial extinction levels by day 8 of lever-only extinction training (Soria et al., 2008).

The experiments in this chapter show that mice can acquire high levels cocaine self-administration without prior sucrose training and that these mice extinguish cocaine-seeking more readily. Importantly, in order to keep food-deprivation as a viable stressor for reinstatement, the active lever must never be associated with food. Therefore, the two requirements for extinction of cocaine-seeking in this study were: 1) no sucrose training 2) lever-only extinction.

4.4.1.2 Mouse strain differences

In experiment 1, C57BL/6J mice received 26 consecutive days of extinction training with barely any decrease in active lever responding. In contrast, 129X1/SvJ mice reduced cocaine-seeking to ~30% of initial extinction responding after 21 days of CS+ extinction training (Highfield et al., 2002). These differences in extinction learning are also reported by Thomsen & Caine (2006), which examined both strains under identical conditions.

Increased latency to extinction in C57BL/6J mice is in line with evidence that cocaine-trained C57BL/6J mice show strong cue-reactivity during cue-reinstatement tests, i.e. 17 times extinction levels (Thomsen & Caine, 2011), 3- or 4-fold above extinction levels in CD1 mice (Soria et al., 2008), double extinction levels in 129X1/SvJ mice (Highfield et al., 2002). While direct comparisons between these studies cannot be made due to differences in extinction criteria, these results are consistent with previous findings that 129X1/SvJ mice do not acquire cocaine conditioned place preference in doses that were effective in C57BL/6J mice (Miner, 1997), which suggest that
129X1/SvJ mice not be as sensitive to surrounding cues in an environment compared to C57BL/6J mice. Thus, cue-reactivity may be one of the reasons for the difference in CS+ extinction performance between these two strains of mice.

It should also be noted that 129X1/SvJ mice as well as other 129 substrains exhibit cognitive and cocaine-related deficits which may make them unsuitable for examining the complex behaviours involved in IVSA studies (Crawley et al., 1997). For example, 129X1/SvJ mice require longer training (Thomsen & Caine, 2006), whereas the 129/OlaHsd substrain fails to acquire cocaine self-administration behaviour compared to C57BL/6J mice (Kuzmin & Johansson, 2000).

4.4.1.3 Methodological considerations

Aside from strain differences, there are also other methodological factors that could explain the lack of CS+ extinction in the present study compared to Highfield et al. (2002), namely the decreased active lever pressing and cocaine intake in their 129X1/SvJ mice and twice-daily extinction sessions. Additionally, the authors point out that the lever position had to be elevated to 5.5cm above the grid floor, as the mice would not extinguish with the levers at the standard height.

A major limitation in making meaningful comparisons between extinction behaviour in different mouse strains is the inconsistency in the definition and application of extinction criteria in these studies. In the study by Highfield et al. (2002) extinction criterion was applied as a group mean instead of monitoring extinction behaviour for each individual mouse. This may be problematic as there is variation between mice in the latency to reach extinction criteria, some may not have reached extinction criteria and cannot truly be tested for reinstatement of cocaine-seeking. Some studies defined extinction responding relative to self-administration, varying from < 80% (Thomsen et al., 2005; Thomsen & Caine, 2006) to < 30% of final self-administration levels (Soria et al., 2008; Thomsen & Caine, 2011), while others chose a threshold of active lever presses set at < 15 (Highfield et al., 2002; Kruzich et al., 2007), or < 25 (Fuchs et al., 2003). Examining final extinction levels compared to baseline self-administration levels may provide useful information about the responding maintained by intravenous drug infusions and thus the reinforcing effect of the drug. However, because we are interested in the extinction and reinstatement of conditioned drug memory rather than the response to the unconditioned stimuli, we need to compare initial and final drug-seeking behaviour entirely under drug-free conditions and without an enforced maximum number of active lever presses. Therefore, in this study extinction criteria were < 50% of initial extinction levels for two consecutive days. In practice, the final extinction levels reached ~25% of average initial responding (figure 4-9).
4.4.2 Stress-induced reinstatement of drug-seeking in mice

4.4.2.1 Food deprivation

After the final extinction session, food was removed from the home cage and mice were tested for reinstatement during the following dark-phase (24 hours later) or after a complete dark-phase of food deprivation (48 hours later). Interestingly, 24-hour food deprivation had no effect on operant responding. While this is contrary to other food deprivation studies (Highfield et al., 2002; Shalev et al., 2003, 2006), at least one reported weight loss after this period of time (Shalev et al., 2003), whereas in my study mice only lost weight after 48 hours of food deprivation. It is also possible that the effective food deprivation period needed to be longer for two reasons. Firstly, chow was removed from the home cage by hand to avoid the additional stress of cage changes, and small crumbs may have remained in my study. Secondly, the timing of food deprivation may not properly coincide with their feeding habits; rodents usually resume feeding at the onset of the dark phase (Harkness & Wagner, 1977), but food was removed immediately after the conclusion of the final extinction session— which was well into the dark phase. That is, in the 24-hr deprivation condition only a brief period of food unavailability occurred during their usual feeding time, with the majority of the food deprivation occurring during sleep time.

After 48-hour food-deprivation stress, mice increased their responding specifically on the active lever. The intact preference for the previously cocaine-paired lever is an important feature of reinstatement testing as it indicates that mice are displaying cocaine-seeking behaviour rather than a non-specific increase in lever pressing. This is a distinct advantage of this stressor over footshock stress in mice, where lever discrimination is not evident during reinstatement (Soria et al., 2008). In mice that did not reach extinction criteria food deprivation had no effect on operant responding, suggesting a specific reinstatement effect as opposed to a general increase in cocaine-seeking.

In previous food deprivation studies, the CSs were present during all phases of the experiments (self-administration, extinction, and reinstatement). Here I show that acute food deprivation reinstates cocaine-seeking in the absence of the motivational effects of the CS, and can be effective in conditions which are different to self-administration. Together these results show that acute food deprivation is a viable and robust stressor for the reinstatement of drug-seeking across a range of different experimental methodologies, and may be highly useful in rodent models of drug addiction. From a translational perspective, food deprivation appears to be a relevant environmental stressor for humans, as circulating leptin levels are associated with increased self-reported craving in abstinent alcoholics and smokers (al’Absi et al., 2011; Kiefer & Wiedemann, 2004), and there is evidence to suggest that food restriction increases relapse rates in smokers (Hall et al., 2011).
4.4.2.2 Yohimbine

Rather than reinstating operant responding, systemic administration of yohimbine (2mg/kg) significantly decreased active lever pressing compared to the previous extinction session. This result is reminiscent of anecdotal reports of inhibition of footshock-induced reinstatement in rats, due to freezing behaviour when baseline stress levels are high (Mantsch et al., 2016). A decrease in locomotion cannot definitively be concluded here, as inactive lever pressing is only decreased in comparison to final extinction day and not when compared to 24-hour food deprivation test day, possibly due to a floor effect. Nonetheless, this dose of yohimbine has been shown to decrease locomotor activity in mice elsewhere (Blanchard et al., 1993; Shimada et al., 1995). The lack of reinstatement of cocaine-seeking is unexpected given that the same dose of yohimbine apparently reinstates cocaine CPP (Mantsch et al., 2010), but this discrepancy must be interpreted with caution; unlike CPP which measures the relative amount of time spent in each compartment, reinstatement of operant responding requires performing an active task of lever pressing and this may be more sensitive to decreases in locomotor activity. Additionally, in the CPP paradigm, mice never self-administer cocaine. Reinstatement in CPP represents restoration of the significance of drug-associated cues rather than drug-seeking behaviour itself.

In fact, yohimbine may not induce drug-seeking in the absence of the CS in mice. Previous IVSA studies with rats have shown that yohimbine can reliably reinstate drug-seeking when the CS is present during all phases of the experiments (Shepard et al., 2004; Simms et al., 2011; Ryan et al., 2013). While yohimbine greatly potentiates cue-induced cocaine- (Feltenstein & See, 2006), heroin- (Banna et al., 2010), and nicotine-seeking (Feltenstein et al., 2012), there is only a weak effect of yohimbine alone. Indeed, there is emerging evidence which challenges the notion that yohimbine induces a stress-like state that motivates drug-seeking. Rather than being aversive, a dose of yohimbine commonly used to reinstate drug-seeking produces a modest CPP in rats (Chen et al., 2015). These authors also demonstrated that yohimbine potently and dose-dependently increased operant responding for a compound tone + light CS that had never been reinforced by food- or drug-reward delivery. From this evidence it is possible that the primary action of yohimbine in reinstatement studies is to invigorate responding for visual or auditory stimuli/cues that under normal circumstances have weak or moderate rewarding effects in rodents. That is, the effect of yohimbine on operant responding in rat reinstatement studies may be independent of the drug-seeking, and is not related to the commonly assumed stress-like effects of yohimbine. However, it should be noted that yohimbine-induced reinstatement of ethanol seeking can be prevented by treatments that do not impact upon yohimbine-induced reinstatement of sucrose seeking (Ryan et al., 2013), suggesting that cue reactivity is not the only factor behind a yohimbine-induced reinstatement of reward seeking. The exact mechanisms driving the effect of yohimbine on operant responding still require elucidation, and may involve more multiple actions operating in concert to precipitate reinstatement.
4.5 Conclusion

From these pilot studies I have developed a reliable protocol for the extinction and stress-induced reinstatement of cocaine-seeking in C57BL/6J mice. In summary, the procedure involves 10 days of double-lever cocaine self-administration without prior sucrose training, followed by daily 30-minute extinction sessions without the cue light. Once an individual mouse had reached extinction criteria of <50% of active lever presses compared to the first extinction day for two consecutive sessions, they were tested for 48-hour food-deprivation stress-induced reinstatement. Reinstatement tests were conducted under the same operant parameters as extinction sessions.

These results extend previous studies of food deprivation-induced reinstatement in 129X1/SvJ mice, which along with C57BL/6J is the most common strain of mice used in drug-seeking studies. There are some advantages found in my protocol, namely, the much shorter period of extinction training as well as absence of the CS during extinction and reinstatement. There are a wide range of established genetically modified C57BL/6J mouse lines which can now be implemented in this stress-induced drug-seeking model to examine the neurobiological basis of these behaviours. Reporter mice will be particularly important for studying CRF receptors, and many other GPCRs which lack specific antibodies to label them (Refojo et al., 2011). Viral tracers and optogenetics can provide the temporal and circuit specificity necessary to address the complex challenges posed by disorders of learning and memory systems. The results of this chapter should be useful to researchers interested in using transgenic mice to investigate candidate genes and dissect the neural circuitry that may be involved in drug abuse in humans.
CHAPTER 5:
THE ROLE OF
VTA CRFR1 IN
COCAINE-SEEKING
Behavioral/Cognitive

Knockdown of CRF1 Receptors in the Ventral Tegmental Area Attenuates Cue- and Acute Food Deprivation Stress-Induced Cocaine Seeking in Mice

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Corticosteroid-releasing factor (CRF) modulates the influence of stress on cocaine reward and reward seeking acting at multiple sites, including the ventral tegmental area (VTA). There is controversy, however, concerning the contribution of CRF receptor type 1 (CRFR1) to this effect and whether CRF within the VTA is involved in other aspects of reward seeking independent of acute stress. Here we examine the role of CRFR1 within the VTA in relation to cocaine and natural reward using viral delivery of short hairpin RNAs (lenti-shCRFR1) and investigate the effect on operant self-administration and motivation to self-administer, as well as stress- and cue-induced reward seeking in mice. While knockdown of CRFR1 in the VTA had no effect on self-administration behavior for either cocaine or sucrose, it effectively blocked acute food deprivation stress-induced reinstatement of cocaine seeking. We also observed reduced cue-induced cocaine seeking assessed in a single extinction session after extended abstinence, but cue-induced sucrose seeking was unaffected, suggesting dissociation between the contribution of CRFR1 in the VTA in cocaine reward and sucrose and cocaine seeking. Further, our data indicate a role for VTA CRFR1 signaling in cocaine seeking associated with, and independent of, stress potentially involving conditioning and/or salience attribution of cocaine reward-related cues. CRFR1 signaling in the VTA therefore presents a target for convergent effects of both cue- and stress-induced cocaine-seeking pathways.

Key words: cocaine; cue; mouse; relapse; stress; VTA

Introduction

A major problem in the treatment of substance use disorders is the enduring propensity to relapse, even after extended periods of abstinence (O’Brien, 1997). Indeed, 40–60% of patients treated for drug or alcohol dependence will return to active substance use within 1 year (McLellan et al., 2000). Craving and relapse to drug use can be triggered in individuals by environmental stimuli that have become conditioned to the action of drugs (O’Brien et al., 2012). Indeed, 40–60% of patients treated for drug or alcohol dependence will return to active substance use within 1 year (McLellan et al., 2000). Craving and relapse to drug use can be triggered in individuals by environmental stimuli that have become conditioned to the action of drugs (O’Brien et al., 2012). Here we examine the role of CRFR1 within the VTA in relation to cocaine and natural reward using viral delivery of short hairpin RNAs (lenti-shCRFR1) and investigate the effect on operant self-administration and motivation to self-administer, as well as stress- and cue-induced reward seeking in mice. While knockdown of CRFR1 in the VTA had no effect on self-administration behavior for either cocaine or sucrose, it effectively blocked acute food deprivation stress-induced reinstatement of cocaine seeking. We also observed reduced cue-induced cocaine seeking assessed in a single extinction session after extended abstinence, but cue-induced sucrose seeking was unaffected, suggesting dissociation between the contribution of CRFR1 in the VTA in cocaine reward and sucrose and cocaine seeking. Further, our data indicate a role for VTA CRFR1 signaling in cocaine seeking associated with, and independent of, stress potentially involving conditioning and/or salience attribution of cocaine reward-related cues. CRFR1 signaling in the VTA therefore presents a target for convergent effects of both cue- and stress-induced cocaine-seeking pathways.

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also observed in rodent models of drug use, with both stress and cue exposure capable of inducing drug seeking in abstinence animals (Bossert et al., 2013). Corticosteroid-releasing factor (CRF) is a neuropeptide mediating hormonal, autonomic, and behavioral responses to stress, and a considerable literature implicates CRF in mediating the link between stress and addiction (Zorrilla et al., 2014). As such, rodent models of relapse suggest CRF as a key mediator of stress-induced reinstatement of drug seeking (Shalev et al., 2010). There is, however, also evidence for a role of CRF in the learned association between drug experience and environmental cues (DeVries et al., 1998), and CRF has been implicated in cue-induced drug seeking (Goeders and Clampitt, 2002; Moffett and Goeders, 2007), potentially as a result of the stress associated with exposure to such cues (DeVries and Pert, 1998; Sinha et al., 2003). Systemic receptor antagonist studies implicate CRF receptor type 1 (CRFR1) in both stress- and cue-induced drug seeking (Shaham et al., 1998; Goeders and Clampitt, 2002; Moffett and Goeders, 2007); however, the loci of this action are still equivocal.

The ventral tegmental area (VTA) is a major neural substrate underlying reward-seeking behavior, and is implicated in cue conditioning, as well as cue- and stress-induced cocaine seeking in rats (McFarland et al., 2004; Feltenstein and See, 2008). Prior cocaine exposure allows CRF control over firing of VTA neurons (Wang et al., 2005), and infusion of CRF into the VTA can rein-
state cocaine-seeking rats, though controversy surrounds which CRF receptor subtype within the VTA mediates stress-induced reinstatement (Wang et al., 2005, 2007; Blacktop et al., 2011). In this study we took a novel approach using viral-mediated knockdown to investigate the contribution of CRFRI within the VTA in aspects of cocaine and natural reward self-administration, and cue-induced as well as stress-induced reward seeking.

Materials and Methods

Animals. All experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia and approved by the Animal Ethics Committee at the Florey Institute of Neuroscience and Mental Health. Adult male C57BL/6j mice (Animal Resource Center), were used for all experiments and were maintained on a reversed 12 h light/dark cycle with ad libitum access to water and were maintained at ~95% of free-feeding weight.

Validation of VTA-CRFR1 and CRFR2 transcript levels, plus validation of viral knockdown. To determine the endogenous levels of CRFRI and CRFR2 in the VTA, adult C57BL/6j mice were used (n = 8). Immediately after decapitation, the brain was removed and placed in a steel brain matrix, 1.0 mm, coronal (model 51386: Stoelting). The brains were sliced into 2 mm slices using standard razor blades and were quickly frozen on dry ice. The right and left VTA were punched out using a 16G microdissecting needle and immediately stored at ~80°C.

RNA extraction was performed using 5 PRIME PerfectPure RNA Cell & Tissue kit (5 Prime). RNA preparations were reverse transcribed to generate cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA products were used as templates for Real-Time PCR analysis. Primer sequences as follows: CRFR2:TACCGAATGGCCCTCATGTT, CCAGGGATGTTCTCCAGAT; CRFR1: TGCCAGAGATTCTCCAAGAA, AAAGCCGAGATGAGGTTCCAG; GFP: CATGCCCGAAGGCTACGT, CGATGCCCTTCAGCTCGAT; DMRTA2: CGGAATCGCCCTCATTGT, CCACGCGATGTTTCTCAGAAT; CRFRI: TGCCAGGAGATTCTCAACGAA, AAAGCCGAGATGAGGTTCCAG;

Quantitative RT-PCR was performed using 5 PRIME PerfectPure RNA Cell & Tissue kit (5 Prime). RNA preparations were reverse transcribed to generate cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA products were used as templates for Real-Time PCR analysis. Primer sequences as follows: CRFR2:TACCGAATGGCCCTCATGTT, CCAGGGATGTTCTCCAGAT; CRFR1: TGCCAGAGATTCTCCAAGAA, AAAGCCGAGATGAGGTTCCAG; GFP: CATGCCCGAAGGCTACGT, CGATGCCCTTCAGCTCGAT; DMRTA2: CGGAATCGCCCTCATTGT, CCACGCGATGTTTCTCAGAAT; CRFRI: TGCCAGGAGATTCTCAACGAA, AAAGCCGAGATGAGGTTCCAG;

Quantification of VTA-CRFR1 and CRFR2 transcript levels, plus validation of viral knockdown. To determine the endogenous levels of CRFRI and CRFR2 in the VTA, adult C57BL/6j mice were used (n = 8). Immediately after decapitation, the brain was removed and placed in a steel brain matrix, 1.0 mm, coronal (model 51386: Stoelting). The brains were sliced into 2 mm slices using standard razor blades and were quickly frozen on dry ice. The right and left VTA were punched out using a 16G microdissecting needle and immediately stored at ~80°C.

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Validation of VTA CRFR1 knockdown in vivo was performed in a similar manner to our previous studies. Adult C57BL/6j mice (n = 6) were injected directly into the VTA with shCRFR1 or shControl lentiviruses, as detailed below and previously described (Sztainberg et al., 2010, 2007). One week post injection, the VTA was microdissected as above, and right and left punches stored separately to accurately measure GFP n with minimal GFP mRNA were also measured as a marker for VTA-specific microdissection to control for substantia nigra contamination of the sample. Samples with minimal GFP mRNA were excluded (n = 2). Dmrt2a mRNA was also measured as a marker for VTA-specific microdissection to control for substantia nigra contamination of the sample. Samples with minimal Dmrt2a mRNA were thus also excluded (n = 1).

Intracerebral injections of lentiviral vectors. Mice received bilateral VTA injections (1 µl per side, 0.25 µl/min) of lentivirus with a GFP reporter containing either shRNA targeted against CRFRI mRNA (shCRFR1) or a control construct containing a scrambled shRNA sequence (shControl virus; Sztainberg et al., 2010, 2011). Stereotaxic coordinates for injections relative to bregma were posterior ~3.0 mm; lateral 0.45 mm, and ventral ~4.4 mm ( Paxinos and Franklin, 2001). An additional group of mice received shCRFR1 virus in the lateral thalamus (posterior ~3.0 mm; lateral 1.45 mm, and ventral ~5.0 mm). Mice were allowed to recover for 3 weeks to allow for optimal viral expression before commencing behavioral studies (Szainberg et al., 2010).

Operant self-administration and cue-induced reward seeking. Operant self-administration of oral sucrose (10% w/v; shCRFR1 n = 20; shControl n = 9) or intravenous cocaine (0.5 mg/kg/infusion; shCRFR1 n = 27; shControl n = 10) was conducted as previously described (Brown et al., 2009; McPherson et al., 2010).

Briefly, mice undergoing cocaine self-administration were surgically implanted under isoflurane anesthesia (1.5-1.4% in 1 L/min air) and perioperative meloxicam analgesia (3 mg/kg, i.p.) with a jugular catheter to enable intravenous infusion of cocaine and allowed to recover for 2–3 d before commencement of self-administration sessions. Catheters were flushed daily with 0.02 ml of a solution of 10 U heparinized saline before self-administration and 90 U following completion of each session. The latency of the catheters was evaluated periodically using 0.02 ml of ketamine (15 mg/ml). If prominent signs of hypnosis were not apparent within 3 s of infusion the mouse was removed from the experiment (Brown et al., 2009).

Mice were conditioned using a two-lever procedure on a fixed ratio of one (FR1) schedule of reinforcement. Active lever presses were paired with a contingent light cue and all sessions were performed in the presence of a discriminative olfactory cue (vanilla essence) located behind the active lever. Inactive lever responses had no effect.

On acquisition of stable responding (≥15 drug rewards; cocaine 0.5 mg/kg/infusion), ≥100 rewards (sucrose 5 ml 10% w/v; ≥75% active lever discrimination over 3 consecutive days), motivation for self-administration was assessed using a progressive ratio (PR) schedule of reinforcement as previously described (Brown et al., 2009; Cahill et al., 2011). Break point was defined as the last completed ratio within the session. Following evaluation of self-administration, mice were kept in their home cage for 3 weeks of abstinence before assessment of reward seeking during a 1 h extinction test in which drug cues were present, but no cocaine or sucrose was available.

Acute food deprivation stress-induced reinstatement of cocaine seeking. To examine the role of VTA CRFRI in acute food deprivation stress-induced reinstatement another cohort of mice received either shCRFR1 (n = 11) or shControl virus (n = 11) in the VTA. Additionally, a third group of mice was injected with shCRFR1 virus in the lateral thalamus and served as a control to examine the anatomic specificity of the effects of CRFRI knockdown (n = 7). Following 10 d of cocaine self-administration, the mice underwent daily 30 min extinction sessions in which there was no cocaine delivery or light cue. Once mice met extinction criteria of <50% of day 1 extinction lever pressing for 2 consecutive days, they were returned to the home cage and subject to a full 12 h dark-phase food-deprivation stress (Highfield, 2002), a CRF-dependent stressor (Shalev et al., 2006). Mice were then tested for reinstatement in the following dark phase, under extinction conditions.

Histological validation of injection sites. We confirmed the level and distribution of shRNA expression in each mouse by immunofluorescence for GFP. Mice were terminally anesthetized (80 mg/kg, 0.1 ml/10 g pentobarbitone) and transcardially perfused. Brains were postfixed over-night in 4% paraformaldehyde and cryoprotected in 20% sucrose in PBS between steps. The reaction product was visualized with fluorescent SYBR Green technology (Applied Biosystems). Reaction protocols had the following format: 10 min at 95°C for enzyme activation followed by 40 cycles of 15 s at 94°C and 60 s at 60°C. Melting curve analysis checked the specificity of the amplification products. All reactions contained the same amount of DNA, 10 µl Master Mix, and 250 nm primers to a final volume of 20 µl. Real-time data were normalised to the housekeeping gene HPRT1.

Validation of VTA CRFR1 knockdown in vivo was performed in a similar manner to our previous studies. Adult C57BL/6j mice (n = 6) were injected directly into the VTA with shCRFR1 or shControl lentiviruses, as detailed below and previously described (Sztainberg et al., 2010, 2007). One week post injection, the VTA was microdissected as above, and right and left punches stored separately to accurately measure GFP n with minimal GFP mRNA were excluded (n = 2). Dmrt2a mRNA was also measured as a marker for VTA-specific microdissection to control for substantia nigra contamination of the sample. Samples with minimal Dmrt2a mRNA were thus also excluded (n = 1).
sites fell outside the target area were excluded from the study (cocaine cue: shCRFR1/H11005, sucrose cue: shCRFR1/H11005, shControl/H11005, stress: shCRFR1 VTA/H11005, shControl VTA/H11005, shCRFR1 LT/H11005).

Statistical analysis. qPCR data were analyzed using Student’s t test. Behavioral data were analyzed retrospectively following assessment of GFP-IR at injection sites by a reviewer blind to treatment group. All data were tested for normality (Kolmogorov–Smirnov) before assigning appropriate statistical tests. Self-administration and reward-seeking data were analyzed by two-way ANOVA with Student–Newman–Keuls post hoc comparisons to examine any significant main effects or interactions.
interactions. Differences in break point on PR responding were assessed using a Mann–Whitney Rank Sum test. Significance was set at $p \leq 0.05$.

**Results**

Quantification of CRF receptor transcript levels in the VTA qPCR analysis of VTA tissue from naive mice showed significantly greater levels of CRFR1 mRNA compared with CRFR2, equivalent to a 30-fold difference (Fig. 1A). This finding is consistent with in situ hybridization studies showing abundant expression of CRFR1 in the VTA, but little CRFR2 (Van Pett et al., 2000), although CRFR2 is measurable in the VTA by single-cell RT-PCR (Korotkova et al., 2006). Moreover, low transcript expression in and of itself should not be directly interpreted as a lack of functional CRFR2 receptors, since presynaptic receptors may still regulate VTA synaptic activity. Additionally, CRF has 10-fold higher affinity for CRFR1 than for CRFR2 (Dautzenberg et al., 2001). Given the prominence of CRFR1, we assessed the ability of a lenti-shCRFR1 virus to knockdown expression of CRFR1 mRNA in the VTA, and observed a nine-fold decrease of CRFR1 mRNA compared with the control virus (Fig. 1B).

VTA CRFR1 knockdown has no effect on self-administration or motivation to self-administer sucrose or cocaine

No effect of VTA CRFR1 knockdown (Fig. 2) was observed on lever responding for sucrose ($F_{(1,23)} = 0.027, p = 0.87$; Fig. 3A, B) or cocaine ($F_{(1,31)} = 0.025, p = 0.88$; Fig. 4A, B) on an FR1 schedule of reinforcement. Similarly, no effect of intra-VTA lenti-shCRFR1 was observed on lever responding or break point on a PR schedule of reinforcement for sucrose (responding, $U = 67, p = 0.98$; Fig. 3C, D) or cocaine (responding, $F_{(1,31)} = 0.025, p = 0.88$; break point $U = 58, p = 0.66$; Fig. 4C, D).

For all experiments mice demonstrated a significant preference for active over inactive lever responses (main effect of lever: sucrose: $F_{(1,23)} = 654.08, p < 0.0001$, PR $F_{(1,23)} = 62.08, p < 0.001$; cocaine: $F_{(1,31)} = 102.35, p < 0.001$, PR $F_{(1,31)} = 11.28, p < 0.01$).

VTA CRFR1 knockdown reduces cue-induced cocaine seeking, but not sucrose seeking

Following 3 weeks of abstinence, mice were assessed for the effect of VTA CRFR1 knockdown on cue-induced cocaine or sucrose...
seeking. While no effect was observed for sucrose seeking (treatment: $F(1,23)=0.25$, $p=0.62$; lever: $F(1,23)=88.24$, $p<0.001$; Fig. 5A), lenti-shCRFR1 treatment significantly reduced responding during cocaine seeking (effect of treatment: $F(1,32)=9.8$, $p=0.01$; lever: $F(1,32)=51.41$, $p<0.001$; interaction: $F(1,32)=7.16$, $p=0.05$; Fig. 5B). Subsequent post hoc analysis of these effects revealed that those treated with shCRFR1 virus displayed significantly reduced responding for the active lever when compared with shControl-treated mice ($p<0.001$), while both groups demonstrated a significant preference for the active lever ($p<0.001$).

CRFR1 knockdown in the VTA effectively blocks acute food deprivation stress-induced reinstatement of cocaine seeking

Again, there was no effect of viral treatment (Fig. 6) on self-administration of cocaine ($F(1,26)=0.0334$, $p=0.96$; Fig. 7A,B). There was also no observed effect on responding during extinction ($F(1,26)=0.49$, $p=0.61$; Fig. 7C); all mice had similar extinction bursts and extinguished to a similar level. Treatment groups also showed similar rates of extinction, as there was no effect of treatment on the number of days to reach extinction criteria (average of 6 d, $F(2,25)=0.078$, $p=0.85$; one-way ANOVA; Fig. 7D).

Acute food-deprivation stress reinstated lever pressing in both the control group receiving shControl virus in the VTA and the anatomic control group injected with lenti-shCRFR1 virus in the lateral thalamus. However, in VTA CRFR1 knockdown mice, there were no significant differences in lever pressing during the reinstatement session compared with extinction levels (Fig. 8). All mice showed high discrimination for the active over inactive lever ($p<0.05$ active versus inactive lever responses. shCRFR1, $n=27$; shControl, $n=10$).

**Discussion**

CRFR1 receptors in the VTA do not critically contribute to self-administration of cocaine

Our data suggest that, at least within the VTA, CRFR1 receptors are not critically involved in mediating cocaine self-administration un-
under a short access operant paradigm, as no effect of CRFR1 knockdown was observed on FR1 or PR schedules of reinforcement. This concurs with studies that found no effect of systemic CRFR1 antagonism on self-administration and/or motivation for cocaine under restricted access conditions (Przegaliński et al., 2005; Mello et al., 2006; Boyson et al., 2011); but note in contrast, Goeders and Guerin (2000). While it is possible that CRFR1 may exert an influence on cocaine self-administration through other

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**Figure 5.** Effect of VTA-directed shCRFR1 viral transfection on cue-induced sucrose and cocaine seeking. A, While both shCRFR1 and control viral-treated mice responded significantly more for the active than inactive lever during cue-induced sucrose seeking, no differences were observed between the two treatments. B, VTA directed shCRF-R1 transfection, however, significantly reduced responding for the active lever during cue-induced cocaine seeking, while maintaining the preference for active over inactive lever responses in both treatment types. All data presented as mean ± SEM, #p < 0.05 active versus inactive lever responses; *p < 0.05 control versus shCRFR1. Sucrose: shCRFR1, n = 20; shControl, n = 9; cocaine: shCRFR1, n = 27; shControl, n = 18.
brain nuclei, this may be dependent upon the level of cocaine exposure (Specio et al., 2008) and stress (Boyson et al., 2011). A similar effect has also been observed for other drugs of abuse. CRFR1 antagonists typically only reduce ethanol self-administration in rats made dependent (Funk et al., 2007) and/or with elevated stress levels (Lodge and Lawrence, 2003), and in certain alcohol-prefering strains (Gehlert et al., 2007), a phenomenon suggested to involve dysregulation of the CRF system. Indeed, extended access to cocaine can cross-sensitize locomotor responses to CRF (Erb et al., 2003) and alter levels of CRF differentially within the brain when compared with animals administering cocaine on a short access paradigm (Zorrilla et al., 2012). Chronic stress may also alter brain CRF and CRFR1 levels (Bonaz and Rivest, 1998; Sutherland et al., 2010). It is therefore possible that the schedule of cocaine access/level of stress experienced by the mice in our study did not alter CRF levels sufficiently to recruit an action of VTA CRFR1 in cocaine intake.

CRF1 receptors within the VTA are necessary for stress-induced reinstatement of cocaine seeking

The role of VTA CRF in mediating stress-induced reinstatement of cocaine seeking is well established. Wang et al. (2007) reported that intra-VTA perfusion of CRFR2 but not CRFR1 antagonists prevented stress-induced reinstatement of cocaine seeking in rats. Conversely, using direct intra-VTA microinjections Blacktop et al. (2011) found that only CRFR1, and not CRFR2, antagonists prevented stress-induced cocaine seeking. The reason for the discrepancy is unclear, but may relate to differences in the method of delivery (reverse dialysis vs microinjection), potential off-target effects of some of the drugs (Zorrilla et al., 2013), and differences in cocaine intake.

Here we show that CRFR1 knockdown in the VTA prevented stress-induced reinstatement of cocaine seeking independent of differences in self-administration or extinction behavior. Moreover, control virus within the VTA and targeted lenti-shCRFR1

Figure 6. Illustration of injection sites confirming VTA-specific lentiviral transfection of mice included in stress-induced reinstatement experiment. A, Representative expression of GFP in the VTA and lateral thalamus (B). C, Neuroanatomical representation of viral injection sites. Open and closed circles represent intra-VTA injection sites of control and shCRFR1 virus, respectively. Triangles represent injection sites of shCRFR1 virus targeting the lateral thalamus. Adapted from Paxinos and Franklin, 2001.
infusion outside the VTA both had no such effect, suggesting anatomic specificity of this effect. This supports a critical role of CRFR1 signaling in the VTA in stress-induced reinstatement of cocaine seeking.

CRFR1 receptors within the VTA contribute to cue-induced cocaine seeking

To address the role of VTA CRFR1 in stress-induced reinstatement we used an extinction-reinstatement model of relapse. This model has been used extensively, however, is associated with certain limitations. Extinction is an active process where rodents learn to inhibit active lever responding in response to devaluation of this behavior in the absence of drug reward. Although extinction procedures are sometimes used clinically, the majority of individuals with substance use disorders do not enter formal rehabilitation (Substance Abuse and Mental Health Services Administration 2010). Whether by incarceration, hospitalization, or self-imposed, dependent individuals can remain abstinent for extended periods of time before relapsing. An alternative model measuring drug seeking after an extended period of enforced abstinence, away from the drug-taking environment, may better reflect the human experience of enduring propensity to relapse.

Using this paradigm, we observed that knockdown of CRFR1 in the VTA significantly attenuated cue-induced cocaine seeking. These findings are consistent with previous data demonstrating a reduction in cue-induced reinstatement of cocaine seeking following systemic administration of the CRFR1 antagonist CP-154,526 (Goeders and Clampitt, 2002), and provide evidence for a role for CRFR1 in the VTA in cue-induced cocaine seeking. Further, these data also suggest a dissociation between the contribution of CRFR1 in the VTA to primary versus secondary reinforcement, consonant with a previous study demonstrating dose-dependent reductions of drug-primed reinstatement by systemic CRFR1 antagonism, independent of any effects on cocaine self-administration (Przegalinski et al., 2005).

There is increasing evidence for a role of CRF in cue conditioning and salience attribution. Salient visual, olfactory, and auditory cue exposure increases levels of CRF in the prefrontal cortex (Merali et al., 2004) and central amygdala (Merali et al., 2003), while CRF antagonists reduce conditioned anxiety elicited from cocaine-related cue exposure (DeVries and Pert, 1998) and hypothalamic pituitary adrenal axis activation (DeVries et al., 1998). It is possible these effects are driven by actions at CRFR1, as expression of fear-potentiated startle is disrupted in mice lacking CRFR1 (Risbrough et al., 2009). Beyond a role in condition-
ing, CRF has also been implicated in the attribution of incentive salience to reward-related cues in a pavlovian-instrumental transfer paradigm (Peciná et al., 2006). The VTA has also been implicated in mediating both cue conditioning (Lee et al., 2011) and incentive salience (Corbit et al., 2007), particularly via connections with the central amygdala. Together, these findings and those of the current study suggest that targeting CRFR1 within the VTA may reduce cue-induced cocaine seeking by disrupting the secondary reinforcement of and/or attribution of incentive salience to cocaine-related cues, beyond generalized effects of cue exposure on stress. A possible source of CRF to drive both stress- and cue-induced cocaine seeking is the ventral bed nucleus of the stria terminals (BNST). The BNST sends dense CRF-containing projections to the VTA, and is selectively activated during cue- and stress-induced reinstatement in rats (Erb et al., 1999; Rodros et al., 2007; Mahler and Aston-Jones, 2012). Undoubtedly, future studies will identify the source(s) of CRF input to the VTA implicated in relapse-like drug seeking.

CRFR1 receptors within the VTA are not critical for self-administration of sucrose or cue-induced sucrose seeking

The lack of impact on sucrose-related behaviors confirms the effects of VTA CRFR1 knockdown are not attributable to motor impairment, suggests that CRF action in the VTA during cue-induced “relapse” is specific to drug seeking, and does not generalize to natural rewards. While CRFR1 knock-out and antagonist studies have previously shown no effect on binge consumption of sucrose (Sparta et al., 2008; Kaur et al., 2012), there is evidence for a role of CRFR1 in stress-induced reinstatement of food seeking (Ghitza et al., 2006) and the integration of natural reward-related cues (Peciná et al., 2006). While this finding suggests CRF may act via different receptors or anatomical loci for natural rewards than for cocaine, the discrepancy may be due to the differential effect of sucrose and cocaine on VTA signaling. Though both sucrose and cocaine self-administration result in the potentiation of glutamatergic synapses onto VTA DA neurons, sucrose self-administration produces only transient potentiation, which dissipates within a 3 week period of abstinence, while cocaine-induced LTP is persistent (Chen et al., 2008). Furthermore, the ability of VTA CRF to control local glutamate release and consequent dopaminergic activation is dependent on CRFR1; however, it is only found in animals with a previous history of cocaine exposure (Hahn et al., 2009). In this way neuroadaptations within the CRF system induced by cocaine exposure, particularly within the VTA, may explain how cocaine-related cues attain greater motivational salience when compared with food-related cues. In this regard, our collective data also suggest that VTA CRFR1 appears more implicated in cue-driven, as opposed to context-driven, cocaine seeking. Thus, in our “stress experiment” there was no effect of VTA CRFR1 knockdown on active extinction performed in the absence of cues paired with drug availability, yet in our “cue experiment” we saw marked reduction of cue-induced cocaine seeking after abstinence in the same drug context. More studies are clearly required to disentangle this question.

Conclusion

Our study indicates a role for CRF within the VTA, acting via CRFR1, in stress-induced reinstatement, as well as cue-induced cocaine seeking, independent of effects on cocaine or sucrose self-administration. It also suggests that regardless of the form of abstinence (≥ extinction), both cue- and stress-induced drug seeking involves CRFR1 in the VTA. While presynaptic CRFRs can modulate excitatory input onto dopaminergic VTA cells (Wang et al., 2007), CRFR1 on the terminals of afferent glutamatergic projections are unlikely to be responsible for the effects we observed. Given the knockdown approach used, it is more likely that cocaine seeking was modulated by CRFR1 expressed on cell bodies and dendritic processes of neurons intrinsic to the VTA, including dopaminergic VTA neurons (Refojo et al., 2011). Neve...
etherless, we cannot rule out potential involvement of CRFR1 expressed on distal projections of VTA neurons; for example, CRFR1 is present on dopaminergic terminals in the nucleus accumbens (Lemos et al., 2012).

Psychological stress and exposure to drug-associated cues are major factors that promote craving and relapse in humans and animals (O’Brien et al., 1992; Brown et al., 1995; Sinha, 2001). Given that the underlying circuitry of stress- and cue-driven drug seeking is overlapping to some degree, targeting these concurrences is an important goal for effective treatment (Shaham et al., 2003).

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CHAPTER 6: 
THE ROLE OF VTA 
CRFR1 IN 
CONDITIONED FEAR
SHORT COMMUNICATION

Knockdown of corticotropin-releasing factor 1 receptors in the ventral tegmental area enhances conditioned fear

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Abstract

The neuropeptide corticotropin-releasing factor (CRF) coordinates the physiological and behavioural responses to stress. CRF receptors are highly expressed in the ventral tegmental area (VTA), an important region for motivated behaviour. Therefore, we examined the role of CRF receptor type 1 (CRFR1) in the VTA in conditioned fear, using a viral-mediated RNA interference approach. Following stereotaxic injection of a lentivirus that contained either shCRF-R1 or a control sequence, mice received tone-footshock pairings. Intra-VTA shCRF-R1 did not affect tone-elicited freezing during conditioning. Once conditioned fear was acquired, however, shCRF-R1 mice consistently showed stronger freezing to the tone even after extinction and reinstatement. These results implicate a novel role of VTA CRF-R1 in conditioned fear, and suggest how stress may modulate aversive learning and memory.

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1. Introduction

Corticotropin-releasing factor (CRF) is localised in numerous extrahypothalamic brain regions including the ventral tegmental area (VTA), a major structure responsible for motivated behaviour and associative learning (Koob and Heinrichs, 1999; Wise and Morales, 2010). Specifically, CRF signalling within the VTA can influence behaviours related to reward learning, including stress-induced reinstatement of cocaine-seeking (Wang et al., 2005; Blacktop et al., 2011; Chen et al., 2014). The VTA also appears important for aversive learning. For example, VTA dopamine signalling is necessary for the expression of conditioned fear (de Oliveira et al., 2009). However, little is known of the role of CRF in the VTA for conditioned fear. Therefore, we used a lentiviral vector to deliver shRNA targeting CRF receptor 1 (CRFR1) mRNA and examined the effect of CRFR1 knockdown on conditioned fear in mice. This method enables visualisation of the viral transduction in the targeted area, as well as a high level of specificity for CRFR1 over other CRF binding partners. We chose CRFR1 because it is more highly expressed in the VTA and binds CRF with a 10-fold higher affinity than CRF receptor 2 (CRFR2) (Chen et al., 2014; Van Pett et al., 2000).

Stress has diverse effects on learning and memory, and VTA activity can be up- or down-regulated depending on the nature of the stressor (Valenti et al., 2012). If VTA CRFR1 promotes an anxiogenic state that increases the strength of the unconditioned stimulus (US) to promote fear learning, then knockdown of CRFR1 signalling in the VTA may dampen conditioned fear. However, CRFR1 signalling is necessary for stress-induced impairment in memory, and antagonism of CRFR1 reverses memory deficits caused by stress (Urani et al., 2011). Therefore, it is also possible that knockdown of CRFR1 in the VTA may enhance fear learning by alleviating stress. With these two opposing hypotheses in mind, we examined the effects of a viral knockdown of CRFR1 in the VTA on conditioned fear.

2. Experimental procedures

2.1. Animals

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia and approved by the Animal Ethics Committee at the Florey Institute of Neuroscience and Mental Health. In each experiment, naïve adult male C57BL/6J mice (Animal Resource Center, Perth, Australia) were used, and maintained on a reversed 12 h light/dark cycle (lights off: 08:00 with ad libitum access to food and water throughout the entire study).

2.2. Surgery

Following 10 days of acclimatisation to the facility, mice were placed under isoflurane anaesthesia (1.5-1.8% in 1 L/min air) with perioperative meloxicam analgesia (3 mg/kg, i.p.), and received bilateral VTA injections (1 μl per side, 0.25 μl/min) of lentivirus with a GFP reporter containing either shRNA targeted against CRFR1 mRNA (shCRFR1) or a control construct containing a scrambled shRNA sequence (shControl virus; Sztainberg et al., 2010; Chen et al., 2014). Stereotaxic coordinates for injections relative to bregma were posterior −3.0 mm; lateral 0.45 mm, and ventral −4.4 mm (Paxinos and Franklin, 2004). Mice were allowed to recover for 3 weeks for optimal viral expression before commencing behavioural studies, and were weighed daily during this period (Sztainberg et al., 2010; Chen et al., 2014).

2.3. qPCR validation of viral knockdown

Adult C57BL/6J mice (n=8) were injected with virus and allowed 3 weeks of recovery. Mice were then deeply anaesthetised (80 mg/kg, 0.1 ml/10 g pentobarbitone) before being decapitated. Each brain was immediately placed in a steel brain matrix (model 51386: Stoelting). The brains were sliced 2 mm thick using standard razor blades and frozen over dry ice. The right and left VTA were separately punched out using a 1.2 mm micro disecting needle, then snap frozen over liquid nitrogen and stored at −80 °C (n=16; two separate samples per mouse). RNA was extracted using RNeasy Plus Universal Minikit (Qiagen) and reverse transcribed to generate cDNA using a SuperScript VILO cDNA synthesis kit in concert with the manufacturers protocol (Invitrogen). Primer sequences used in qPCR reactions were: HPRT1: 5′-GAGGTTCCAG-3′ (sense) and 5′-GTCCTTTTCACCAGCAAGCT-3′ (antisense); CRFR1: 5′-TGCCAGAAGCTCTTCAACGA-3′ (sense) and 5′-AAAGCCGAGATGGTTTCCAG-3′ (antisense); CRFR2: 5′-TACCAATGCGCTCATTGTG-3′ (sense) and 5′-CAACCGCATGTTCTTCTAGA-3′ (antisense); CRFPB: 5′-GTCCTCGAAGACGAAATG-3′ (sense) and 5′-ATGCAATGTTCCCAGGTTA-3′ (antisense); GFP: 5′-CATGCCGAGGCTACGT-3′ (sense) and 5′-CTGATGCCTCTAGCTG-3′ (antisense). qPCR was performed on a Viia7 real-time PCR system using fluorescent SYBR Green Master Mix (Applied Biosystems). Thermocycler conditions were: 10 min at 95 °C for enzyme activation followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C. Melt curve analysis verified the specificity of amplification products for each primer set. All reactions contained the 4 μl cDNA, 5 μl SYBR Green Master Mix, and 1 μl total primers. Real-time data were normalised to the housekeeping gene HPRT1. GFP mRNA was measured as an indicator of site-specific viral injection.

2.4. Behaviour

2.4.1. Apparatus

Each chamber was equipped with a programmable tone generator and speaker used to deliver auditory cues and a constant-current shock generator to deliver electric shock through the stainless steel grid floor (Med Associates, USA). Chambers were set up in one of two different configurations to act as distinct contexts, as previously described (Handford et al., 2014). All groups were counterbalanced across both contexts. Each session was video recorded and freezing behaviour was measured using a Med Associates Video Freeze system.

2.4.2. Conditioning

In all experiments baseline freezing activity was measured for the initial 2 min of the conditioning session. Mice then received 6 tone-footshock pairings. Each pairing consisted of a 10 s tone (conditioned stimulus; CS, vol: 80 dB; frequency: 5000 Hz) that co-terminated with a 1 s footshock (unconditioned stimulus; US, 0.6 mA). Inter-trial intervals (ITI) ranged from 85 to 135 s with an average of 110 s. Freezing was calculated from the first 9 s of each CS presentation to avoid confounding effects of the shock on movement.

2.4.3. Retrieval/extinction

Retrieval/extinction was carried out the following day in the alternate context to the conditioning session. All mice were first allowed a 2 min period during which baseline freezing was
measured. In experiment 1, mice then received 15 presentations of a 10 s tone in the absence of footshock with an ITI of 10 s. In experiment 2, mice were given 45 CS presentations to extinguish tone-elicited freezing. Percentage freezing reported for all experiments is based on 10 s of tone with each CS block representing the average freezing of five tone presentations.

2.4.4. Reinstatement
In experiment 2, mice received an additional session the next day where they were placed in the extinction context for 2 min and half of each virus group received a single unsignaled 0.6 mA shock in order to reinstate extinguished CS-elicited freezing. The remaining half were also placed in the extinction context for 2 min but did not receive a shock.

2.4.5. Test
Baseline freezing was measured for 2 min followed by 15 presentations of 10 s tone in the absence of footshock with an ITI of 10 s in the same context as the extinction session. Percentage freezing was reported as average tone-elicited freezing across all the trials.

2.5. Scoring, data analysis, and baseline levels of freezing
Freezing was assessed using automated near-infrared video tracking equipment and computer software (Video Freeze, Med Associates). The motion index threshold of the Video Freeze program was set at 50, and minimum freeze duration was 30 frames (1 s). These parameters were chosen based on calibration experiments showing high concordance with manual scoring (Handford et al., 2014). Analyses of variance (ANOVA) were used for all behavioural experiments. qPCR data were analysed using Student’s t-test. The level of significance for all analyses was p<0.05.

Table 1 reports the group mean (±SEM) levels of freezing during all baseline periods during all behavioural sessions. There were no significant differences in baseline levels of freezing in any experiment (p>0.05).

3. Results

3.1. Quantification of CRF receptor transcript levels in the VTA
qPCR analysis of naive mouse VTA tissue showed significantly higher levels of CRFR1 mRNA compared to CRFR2 indicated by an average ΔΔCt value of 4.68 relative to CRFR1 (t(14) =10.21, p<0.0001). There were also greater levels of CRFR1 than CRFBP mRNA, which showed an average ΔΔCt of 1.25 (t(14)=3.94, p<0.01) (Figure 1A). Further, shCRFR1 virus treated mice showed decreased expression of CRFR1 mRNA compared to the shControl treated mice (t(11)=2.42, p<0.05). No differences were seen in CRFR2 or CRFBP (ts<1) levels between shCRFR1 or shControl virus treated mice (Figure 1B).

3.2. Validation of injection sites
For all mice that received behavioural testing, we confirmed the level and distribution of shRNA expression by chromogenic GFP immunohistochemistry (Figure 2) as previously described (Chen et al., 2014). All animals whose injection sites fell outside the target area were excluded from all analyses (Figure 2C).

3.2.1. Experiment 1: knockdown of CRFR1 in the VTA enhances fear memory
To examine the role of VTA CRFR1 in auditory conditioned fear, mice were injected with either shControl virus (n=10) or shCRFR1 (n=9) and trained on a Pavlovian fear conditioning procedure (Figure 3A). No effects of VTA CRFR1 knockdown on acquisition of tone-elicited freezing were observed. Repeated measures (RM) ANOVA of the data during conditioning (Figure 3B) revealed a main effect of conditioning trial (F(5,85)=25.17, p<0.01), but no significant differences between virus groups, and no significant interaction (F<1).

The next day mice were tested for retrieval of conditioned fear. All mice received 15 CS-alone presentations averaged into blocks of 5 CS presentations (Figure 3C). Mice that received shCRFR1 showed substantially higher levels of CS-elicited freezing compared to shControl treated mice (main effect of virus group: F(1,17)=8.422, p<0.01). There was also a main effect of block (F(2,34)=6.52, p<0.01), but no significant interaction (F(2,34)=1.163, p=0.3246). This difference was also present when tested again the following day, with shCRFR1 mice showing higher levels of CS-elicited freezing (Figure 3D) (t(17)=2.16, p<0.05; unpaired t-test), which suggests that VTA CRFR1 knockdown produces a persistent enhanced fear memory.

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<th>Table 1 Baseline levels of freezing.</th>
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<td><strong>Experiment</strong></td>
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Mean (±SEM) % freezing at baseline for all phases of behavioural experiments.
There was also a main effect of viral treatment, with freezing (block (shock group). There was also a main effect of extinction (to their non-shocked counterparts across the virus condition

significantly decreases CRFR1 signalling and functionality (Sztainberg et al., 2010). Importantly, we observed no difference across virus groups in the levels of CRFR2 or CRFBP mRNA, indicating virus specificity for CRFR1 and no evidence of compensatory up-regulation of CRFR2 or CRFBP caused by decreased CRFR1 levels (Figure 1B).

Our data show no difference across virus condition in the acquisition of tone-shock association as measured during conditioning (Figure 3B, E). The lack of main effect or an interaction indicates that knockdown of CRFR1 in VTA does not affect locomotor activity, overall freezing expression, or rate of conditioning. These results strongly suggest that intra-VTA CRFR1 knockdown does not affect the experience of the US and/or formation of the CS-US association, which is consistent with the finding that stress does not cause changes to footshock sensitivity during conditioning (Todorovic et al., 2007), and argues against the hypothesis that CRFR1 within the VTA increases the strength of the US to promote fear learning.

In contrast, enhanced CS-elicited freezing the day after conditioning and following extinction was consistently observed in the lenti-shCRFR1 group (Figure 3C, F), which suggests that CRFR1 signalling in the VTA may normally serve to dampen fear memories. This idea is supported by previous findings that stress can impair fear conditioning, and this deficit can be reversed by systemic administration of a CRFR1 antagonist (Blank et al., 2003; Todorovic et al., 2007). As footshocks are highly stressful and lead to CRF release in the VTA (Wang et al., 2005), this lends evidence to the hypothesis that CRFR1 knockdown alleviates stress-induced impairment of conditioned fear memory by interfering with action of VTA CRF in the consolidation of the CS-US association, leading to enhanced fear learning.

Increased CRF impairs extinction memory in areas critical for fear extinction such as the amygdala and BNST (Gafford et al., 2012; Abiri et al., 2014). Although VTA neurons encode a level of fear elicited by CS presentation (Alileykovskiy and Morales, 2011), their role in extinction learning is poorly understood. Our data show that whilst there was a consistent main effect of virus in overall levels of freezing, once conditioned fear is acquired the virus condition did not interact with the rate of extinction. This implies that acquisition of extinction is not mediated by CRFR1 in VTA. Furthermore, the knockdown group showed persistently higher levels of freezing during and after extinction, and following reinstatement. These results

3.2.2. Experiment 2: knockdown of CRFR1 in the VTA leads to a stronger fear memory in extinction and reinstatement

The effects of VTA CRFR1 knockdown on extinction and footshock-induced reinstatement were examined in a separate group of virus injected mice (shControl, n=22; shCRFR1, n=18). All mice similarly acquired CS-induced freezing (Figure 3E) (main effect of conditioning trial: F < 1). During extinction, shCRFR1 mice again showed higher CS-elicited freezing compared to control mice (Figure 3F) (main effect of virus: F = 4.57, p < 0.05; no effect of shock group). There was also a main effect of extinction block (F = 41.51, p < 0.0001), but no interaction between factors (F < 1).

The following day, mice either received reinstatement treatment or were merely placed in the chamber (shControl – no shock, n=11; shControl – shock, n=11; shCRFR1 – no shock, n=9; shCRFR1 – shock, n=9). When tested the next day, those that received a reinstatement shock showed significantly higher levels of CS-elicited freezing compared to their non-shocked counterparts across the virus condition (Figure 3G) (main effect of shock: F = 4.632, p < 0.05). There was also a main effect of virus treatment, with significantly higher levels of freezing in shCRFR1 mice (F = 6.031, p < 0.05), and no interaction (F < 1).

4. Discussion

We investigated the role of VTA CRFR1 in conditioned fear. qPCR analysis of naïve mouse VTA showed that there is approximately 23 times more CRFR1 mRNA compared to CRFR2 (Figure 1A). This finding is consistent with in situ hybridisation studies showing abundant expression of CRFR1 in the VTA, but little CRFR2 (Van Pett et al., 2000). We also detected CRF-BP mRNA in the VTA at much higher levels than CRFR2, and approximately 50% of CRFR1 levels. We then demonstrated the ability of a lenti-shCRFR1 virus to significantly decrease CRFR1 mRNA in the VTA compared to the control virus (Figure 1B). We have previously shown that this virus also decreases CRFR1 signalling and functionality (Sztainberg et al., 2010). Importantly, we observed no difference across virus groups in the levels of CRFR2 or CRFBP mRNA, indicating virus specificity for CRFR1 and no evidence of compensatory up-regulation of CRFR2 or CRFBP caused by decreased CRFR1 levels (Figure 1B).

Our data show no difference across virus condition in the acquisition of tone-shock association as measured during conditioning (Figure 3B, E). The lack of main effect or an interaction indicates that knockdown of CRFR1 in VTA does not affect locomotor activity, overall freezing expression, or rate of conditioning. These results strongly suggest that intra-VTA CRFR1 knockdown does not affect the experience of the US and/or formation of the CS-US association, which is consistent with the finding that stress does not cause changes to footshock sensitivity during conditioning (Todorovic et al., 2007), and argues against the hypothesis that CRFR1 within the VTA increases the strength of the US to promote fear learning.

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indicate that extinction consolidation and retrieval processes are unaffected by chronic knockdown of CRFR1 in VTA, inline with our previous study showing no effects on the extinction of appetitively motivated operant responding (Chen et al., 2014). Unlike Chen et al. (2014), however, our present results show that the magnitude of fear reinstatement is not impaired by CRFR1 knockdown in the VTA. This may be due to the difference in the reinstatement stimulus used (shock vs food deprivation). Future studies can examine this dissociation.

Figure 2  Validation of virus injection sites. Neuroanatomical representation of viral injection sites included in experiment 1 (A) and experiment 2 (B). Circles and squares represent location of shCRFR1 and control constructs, respectively. Open shapes represent those in the no shock condition, hatched shapes received reminder shock. (C) Injections that fell outside the target area and were excluded from behavioural analyses. 3 mice were not depicted due to lack of GFP expression. (D) Representative immunohistochemical expression of GFP within the VTA. Dark grey area shows GFP positive cells visualised by DAB staining. Scale bar=0.5 mm.
Our findings propose a novel role for CRFR1 within the VTA in conditioned fear to a tone. Considering the knockdown approach used, it is likely that conditioned fear was modulated by CRFR1 expressed on cell bodies and dendritic processes of neurons intrinsic to the VTA (Justice et al., 2008; Refojo et al., 2011). Although dopaminergic VTA signalling has a key role in conditioned fear (de Oliveira et al., 2009; Fadok et al., 2009), CRFR1 deletion in dopaminergic neurons does not affect auditory conditioned fear (Refojo et al., 2011) suggesting that the effects seen in this study may be mediated by CRFR1 expressed on GABAergic neurons (Korotkova et al., 2006), rather than directly affecting dopaminergic transmission. In the VTA, footshock stimuli transiently and potently increases firing of GABAergic neurons, which can then modulate the transmission of information between neurons. This interaction between CRFR1 and GABAergic signalling may provide a mechanism by which CRFR1 expression influences conditioned fear in the VTA.
interneurons and inhibits DA neurons, an effect which is blocked by GABA- but not DA receptor antagonism indicating that the effects on DA activity are downstream of increased activity of GABA neurons (Tan et al., 2012). Optogenetic stimulation of this pathway can produce aversion and freezing behaviour within a single real-time conditioned place aversion session which persists when tested the next day, demonstrating a strong learning effect resulting from GABAergic activity (Tan et al., 2012). CRFR1 modulation of VTA GABA signalling has been implicated in a range of aversively motivated behaviours (Blacktop et al., 2016; Griender et al., 2014). However, the subcellular localisation as well as the phenotype(s) are yet unclear. One must also consider the potential involvement of VTA GABAergic projection neurons, which enhance auditory conditioned fear learning (Brown et al., 2012). Given the heterogeneity in the neurochemical profiles of VTA neurons, and the diversity of behavioural effects elicited by specific VTA inputs (Lammel et al., 2012; Beier et al., 2015; Holly and Miczek, 2016) future studies targeting specific CRF inputs to VTA sub-regions are necessary to determine the underlying circuits controlled by CRFR1 that modulate conditioned fear. Nevertheless, our data increase understanding of how CRF receptors contribute to emotional learning, and may aid in the identification of therapeutic targets for anxiety-related disorders.

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Contribution

NAC, AJL, and JHK designed the study. NAC and DEG conducted the study. All authors contributed to writing of the manuscript.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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CHAPTER 7:
GENERAL DISCUSSION
7.1 Summary

Addiction and anxiety disorders are a tremendous burden on our society. Not only are these diseases highly comorbid, but they also display mutual maintenance of one another, and this interaction likely contributes to their persistence and poor outcomes of current treatments. The overlap of neural circuitry underlying these diseases, as well as common genetic and environmental risk factors, suggests a shared aetiology of addiction and anxiety disorders. The central role of stress-reactivity in pathogenesis and maintenance of both of these diseases has led to the identification of CRF signalling as a key factor in these effects. This highlights the importance of the investigation of brain areas where stress- and motivational circuitry intersect. The focus of this thesis was the VTA, as it is a site where fear- and reward-related circuitry converge and can be modulated by stress through actions of CRF. In these studies, animal models of reward-seeking and conditioned fear were used to examine the neurobiological mechanisms of these forms of learning in order to understand how these systems can become dysregulated in addiction and anxiety disorders.

Chapters 5 and 6 of this thesis demonstrated that VTA CRFR1 signalling is differentially involved in various components of cocaine IVSA and conditioned fear (table 7-1). Viral-mediated knockdown of CRFR1 expression in the VTA had no effect on the acquisition of cocaine IVSA or conditioned fear, however there were differing effects on the expression of these learned behaviours. Specifically, decreased VTA CRFR1 signalling enhanced conditioned freezing behaviour, but did not affect the self-administration of cocaine or natural rewards. This intervention had no effect on the extinction of either cocaine self-administration or conditioned fear, but again there were contrasting effects during reinstatement testing. VTA CRFR1 knockdown blocked stress-induced reinstatement as well as cued cocaine-seeking, but no effect was seen on the reinstatement of conditioned fear, nor were there any effects on cued sucrose-seeking.

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Table 7-1 Comparison of the behavioural effects of VTA CRFR1 knockdown in cocaine IVSA and conditioned fear.
7.2 Methodological differences in the assessment of reward- and fear-related behaviours

When interpreting these results, one must consider the methodological differences in the behavioural assessment of acquisition, extinction, and reinstatement in these two paradigms that may contribute to the differential effects of VTA CRFR1 knockdown on cocaine IVSA and conditioned fear. The majority of these differences were due to the unique requirements of each paradigm. Firstly, in cocaine IVSA, the active behaviour of lever pressing is used as an indicator of drug-seeking, whereas conditioned freezing involves a lack of movement. Secondly, different forms of extinction training were used; in conditioned fear experiments mice underwent extinction by cue-exposure, while cocaine IVSA experiments examined instrumental (operant) extinction without contingent CS presentations. This was necessitated by lack of extinction of cocaine-seeking that was observed when lever-pressing was reinforced by presentation of the CS (described in chapter 4). Consequently, the CS was also absent during stress-induced reinstatement of cocaine-seeking, but not conditioned fear experiments. Thus, stress-potentiation of the CS could not be examined in relation to cocaine-seeking, but note that cued cocaine-seeking was examined after a period of enforced abstinence. Lastly, different stressors were used for the reinstatement of cocaine-seeking and conditioned fear that may have different motivational mechanisms in these two contexts. In the case of stress-induced cocaine-seeking, acute food deprivation was used to create a stressful emotional state which precipitates reinstatement, whereas the footshock given to fear-conditioned mice functions not only as a non-specific stressor which changes arousal, but also has a cognitive function which influences the retrieval of the fear conditioning episode; in other words, it acts as a reminder to bring back the memory of previous shocks to reinstate CS-elicited freezing. Therefore, it may not be possible to draw direct comparisons between these two paradigms in terms of stress-induced reinstatement.

Nevertheless, from these experiments it is clear that VTA CRFR1 signalling is differentially involved in these behaviours, suggesting that CRF1 participates in distinct subcircuits underlying fear and reward within the VTA. Further research should aim to thoroughly characterize these subcircuits and disentangle the CRF1 mediated VTA circuits underlying fear- and reward-related behaviours.

7.3 VTA CRF-CRFR1 system

CRF is released in the VTA in response to aversive stimuli (Blacktop et al., 2011, 2016; Hauger et al., 2006; Twining et al., 2015; Wang et al., 2005, 2007, 2012), and may arise from various sources. CRF immunoreactivity has been observed in both axon terminals and dendrites in the VTA (Tagliaferro & Morales, 2008). CRF mRNA is found in cell bodies within the VTA (Grieder et al., 2014). In mice and humans, 95-98% of CRF expressing neurons in the VTA are dopaminergic (Grieder et al., 2014). In
addition to CRF produced by cells within the VTA, there are also GABAergic and glutamatergic CRF-containing inputs to this region (Tagliaferro & Morales, 2008). Early studies using retrograde tracers combined with immunohistochemical analysis showed that the main CRF inputs to the VTA are from the CeA, BNST, and PVN (Rodaros et al., 2007). However, studies using transgenic CRF reporter rats showed the CeA only has very minor CRF-containing projections to the VTA (Pomrenze et al., 2015). CRF-containing projections from the BNST to VTA have been confirmed in rats and mice (Rinker et al., 2016; Vranjkovic et al., 2014), and appear to be exclusively GABAergic (Dabrowska et al., 2013).

CRF actions in the VTA are complex and involve both excitatory and inhibitory effects that vary depending on the receptor and site. *In vitro* electrophysiological studies showed that bath application of CRF excites DAergic and non-DAergic neurons directly (Korotkova et al., 2006). CRFR1 is expressed on dopaminergic neurons (Grieder et al., 2014; Korotkova et al., 2006; Refojo et al., 2011; Wanat et al., 2008) as well as GABAergic neurons (Korotkova et al., 2006) within the VTA. Activation of CRFR1 on VTA DA neurons increases DA neuron firing through a PKC-dependent mechanism (Wanat et al., 2008). It also indirectly facilitates excitatory signalling by promoting glutamate release in the VTA (Hahn et al., 2009; Wang et al., 2005, 2012), and potentiates NMDA receptor-mediated synaptic transmission in VTA DA neurons (Ungless et al., 2003). Interestingly, CRF has been shown to promote GABAergic input onto VTA DA neurons (Beckstead et al., 2009), including via presynaptic regulation of GABA release (Williams et al., 2014), but can also block GABAergic transmission in DA neurons under certain circumstances, via CRFR1 activation (Grieder et al., 2014).

Although the distribution of cells containing CRFR1 and CRFR2 mRNA has been extensively studied (Justice et al., 2008; Van Pett et al., 2000), the neurochemical identity of neurons expressing CRFR1 protein, as well as their subcellular localisation, remains largely unknown and awaits the development of specific antibodies that can reliably target CRFR1 for immunohistochemical labelling (see chapter 3).

### 7.4 VTA CRFR1 in reinstatement of drug-seeking

Several studies have shown that CRF signalling in the VTA is necessary for stress-induced reinstatement of cocaine (Blacktop et al., 2011, 2016; Vranjkovic et al., 2014; Wang et al., 2005, 2007), heroin (Wang et al., 2006), and nicotine-seeking (Grieder et al., 2014), and that exogenously applied CRF within the VTA is sufficient to reinstate cocaine-seeking in rats (Blacktop et al., 2011, 2016; Wang et al., 2005, 2009). However, pharmacological manipulations using CRF receptor agonists/antagonists have produced conflicting reports of whether this effect on cocaine-seeking is mediated by CRFR1 (Blacktop et al., 2011, 2016), or CRFR2 (Wang et al., 2007). Furthermore, the subcellular localisation of these CRF receptors was unknown. The experiments in chapter 5 of this thesis demonstrate that CRFR1 expressed on neurons intrinsic to the VTA are not only crucial for
stress-induced reinstatement of cocaine-seeking, but are also necessary for cued cocaine-seeking after a period of enforced abstinence. These results build on previous studies which found that stress-induced reinstatement of cocaine-seeking is inhibited by ventricular administration of CRFR1 antagonists (Shaham et al., 1998), and are concordant with reports that intra-VTA administration of CRFR1, but not CRFR2, agonists are sufficient to reinstate drug-seeking in a similar way to intra-VTA CRF infusion (Blacktop et al., 2011). Together this provides strong evidence that VTA CRFR1 activation is both necessary and sufficient for stress-induced reinstatement of cocaine-seeking.

However, these findings are inconsistent with studies by Wang et al. (2007) which implicate VTA CRFR2 signalling through interactions with CRFBP, instead of CRFR1 signalling. In these experiments, VTA CRF-induced reinstatement of cocaine seeking was prevented by administration of a CRFR2 antagonist, but not a CRFR1 antagonist in the VTA. Furthermore, only CRF receptor agonists that bound to both CRFR1 and CRFR2 as well as CRFBP induced reinstatement. In contrast, Blacktop et al. (2011) showed that intra-VTA delivery of the CRFR1 selective agonist cortagine, which does not bind to CRFBP (Tezval et al., 2004), reinstated cocaine seeking, whereas the CRFR2 agonist, rat Urocortin 2 (rUcn2) did not—despite its binding to CRFBP (Jahn et al., 2004). The apparent involvement of CRFR2, and not CRFR1, is also inconsistent with early studies which found that stress-induced reinstatement is inhibited by systemic or ventricular administration of CRFR1 (Lu et al., 2001; Shaham et al., 1998), whereas CRFR2 antagonists have no effect on stress-induced reinstatement of cocaine CPP in rats (Lu et al., 2001).

The reasons behind these inconsistencies are unclear but may be due to the different experimental methodologies, including the mode of intra-VTA delivery (microinjection vs. reverse dialysis) and dose of pharmacological agents (90 µM to 5.5 mM vs 1 to 10µM), as well as different rat strains used (Sprague Dawley vs Long-Evans). Importantly, rats in Blacktop et al. (2011) had a significantly higher total cocaine intake (~70mg/kg/day) compared to 33 mg/kg/day in Wang et al. (2007). Thus, it is possible that regulation of cocaine-seeking by VTA CRFR1 is intake-dependent and requires a prior history of very high levels of cocaine-intake. This is supported by previous reports that excessive cocaine intake is required for stress-induced reinstatement (Mantsch et al., 2008), and that CRFR1 antagonists only reduce cocaine self-administration following escalation in rats given extended access to cocaine (Specio et al., 2008).

**7.4.1 Downstream effects of VTA CRFR1 activation in cocaine-seeking**

VTA CRF can modulate inhibitory and excitatory effects upon dopamine signalling to regulate reinstatement. Various studies have shown that ionotropic glutamate receptor signalling (Wang et al., 2005, 2007), as well as GABA-B receptor signalling (Blacktop et al., 2016) is necessary for stress- and intra-VTA CRF induced reinstatement of cocaine-seeking. Studies by Wang et al., (2005, 2007) demonstrated that stress-induced reinstatement of cocaine-seeking is associated with increased VTA
glutamate levels, and that intra-VTA administration of kynurenic acid, an ionotropic glutamate receptor antagonist, can prevent reinstatement in response to footshock or intra-VTA CRF. This implies that VTA CRF drives reinstatement through local release of glutamate, and therefore antagonism of AMPA or NMDA ionotropic glutamate receptors should prevent reinstatement. However, intra-VTA infusion of AMPA or NMDA receptor antagonists alone were found not to block intra-VTA CRF-induced reinstatement, and this effect may require simultaneous inactivation of both receptors, as would be achieved with kynurenic acid (Blacktop et al., unpublished results). Instead, Blacktop et al. (2016) found that GABA-B, but not GABA-A receptor antagonists in the VTA prevent reinstatement. Given that I showed knockdown of CRFR1 in VTA neurons also blocks reinstatement, it is unlikely that this effect is due to presynaptic regulation of CRFR1 on the terminals of VTA inputs. Nevertheless, we cannot rule out potential involvement of CRFR1 expressed on distal projections of VTA neurons; for example, CRFR1 is present on dopaminergic terminals in the NAc (Lemos et al., 2012).

The neurochemical identity of CRFR1 expressing VTA neurons mediating reinstatement of cocaine-seeking has not yet been identified, but likely include mesocortical DA cells, which are activated by stress (Deutch et al., 1991), as elevated mPFC DA is necessary for stress-induced reinstatement (Capriles et al., 2003; McFarland et al., 2004). Indeed, selective deletion of CRFR1 in midbrain DAergic neurons blunts DA release in the PFC in response to footshock stress (Refojo et al., 2011). This suggests that VTA CRFR1 activation can regulate stress-induced cocaine-seeking by increasing mPFC dopamine release. The finding that selective deletion of CRFR1 in midbrain DA neurons is anxiogenic (Refojo et al., 2011) is not entirely consistent with our findings that VTA CRFR1 KD blocks stress-induced reinstatement of cocaine seeking. However, this may be explained by synaptic adaptations within the VTA CRF system induced by cocaine self-administration. For example, CRF only causes glutamate release in the VTA of cocaine experienced rats, and not cocaine naïve rats (Wang et al., 2005). Furthermore, cocaine self-administration, but not passive cocaine infusions, increases the ratio of AMPA/NMDA receptor-mediated currents in VTA DA neurons, which persists after 3-weeks of daily extinction training, and even after 3 months of abstinence. In contrast, this potentiation of excitatory signalling is only transiently expressed after sucrose self-administration (Chen et al., 2008). Importantly, the role of VTA CRFR1 in reward-seeking after a 3-week period of enforced abstinence is only evident in cocaine-experienced mice; no effect was observed in sucrose-experienced mice (chapter 5).

7.4.2 Sources of VTA CRF involved in cocaine-seeking

The source(s) of VTA CRF involved in stress-induced drug-seeking have been investigated by others. One region that has received particular attention is the BNST, as CRF-containing BNST neurons are activated by stressors (Partridge et al., 2016). Transient inhibition of this region blocks stress-
reinstatement of cocaine-seeking in rats (McFarland et al., 2004), and reinstatement of cocaine CPP is associated with activation of a BNST to VTA pathway in mice (Briand et al., 2010). Importantly, Vranjkovic et al. (2014) has demonstrated that CRF input from the vBNST to the VTA is necessary for stress-induced reinstatement of cocaine-seeking in rats. In this study, footshock reinstatement was blocked by β2 adrenergic receptor (β2-AR) antagonists within the vBNST, and local activation of these receptors using a β2-AR agonist was sufficient to reinitiate cocaine-seeking. By pharmacological disconnection of this pathway it was shown that reinstatement induced by vBNST β2-AR activation was blocked by VTA administration of the CRFR1 antagonist antalarmin (Vranjkovic et al., 2014). As the dose of antalarmin used may also block CRF-R2, the findings are not definitive regarding which CRF receptor subtype is involved. The results of chapter 5 suggests that this effect is mediated, at least in part, by CRFR1 expressed on VTA neurons, as knockdown of these receptors is sufficient for inhibition of stress-induced cocaine-seeking (figure 7-1).

![Proposed mechanism of CRF action within the VTA during stress-induced reinstatement of cocaine-seeking. Stress activates VTA-projecting CRF/GABAergic neurons in the BNST. CRF released from these neurons activates CRFR1 expressed on VTA neurons to drive cocaine-seeking.](image)

**Figure 7-1** Proposed mechanism of CRF action within the VTA during stress-induced reinstatement of cocaine-seeking. Stress activates VTA-projecting CRF/GABAergic neurons in the BNST. CRF released from these neurons activates CRFR1 expressed on VTA neurons to drive cocaine-seeking.
Interestingly, the magnitude of cocaine seeking induced by β2-AR activation was lower than the effect of footshock, while cocaine seeking was completely blocked by intra-VTA antalarmin, suggesting that other sources of VTA CRF may be involved. The neurochemical identity of CRF-containing BNST neurons has been thoroughly investigated using immunohistochemical, electrophysiological, and single-cell qPCR evidence showing that these neurons are exclusively GABAergic (Dabrowska et al., 2013). Whether this BNST-VTA CRF-containing pathway is the sole source of VTA CRF involved in drug-seeking is unknown.

Recent studies provide evidence that locally produced VTA CRF may be differentially involved in drug-associated behaviours compared to extrinsic sources of CRF input. While binge-like ethanol consumption increases CRF mRNA within the VTA (but not the BNST), chemogenetic inhibition of CRF projections from the BNST to the VTA reduced binge-like ethanol drinking in mice, whereas inhibition of CRF neurons in the VTA did not (Rinker et al., 2016). This suggests that although VTA CRF neurons are engaged by acute ethanol consumption, these neurons do not modulate this behaviour. Conversely, Grieder et al. (2014) demonstrated that abstinence-induced escalation of nicotine intake is driven by CRF released from VTA DA neurons which function in an autocrine fashion to activate CRFR1. This intrinsic source of VTA CRF also mediates the aversive effects of nicotine withdrawal (Grieder et al., 2014). Therefore, the possibility that VTA CRF produced by cells resident to this area drives stress-induced reinstatement and/or cocaine-seeking after enforced abstinence cannot yet be ruled out. To address this question, future studies could examine whether viral-mediated downregulation of CRF mRNA within the VTA has a similar effect on cocaine-seeking.

7.5 VTA CRFR1 in conditioned fear

The findings of chapter 6 indicate a role for CRF within the VTA, acting through CRFR1, in conditioned fear to a tone. It was observed that knockdown of VTA CRFR1 enhanced the expression of conditioned freezing to a tone CS, suggesting that the endogenous function of these receptors impairs aversive learning. This is consistent with evidence that acute exposure to uncontrollable stress, including footshock, has deleterious effects on learning and memory (Blank et al., 2003b; El Hage et al., 2004; Park et al., 2008; Philbert et al., 2012, 2013; Sandi et al., 2005; Todorovic et al., 2007; Urani et al., 2011), which can be reversed by CRFR1 antagonists (Blank et al., 2003b; Philbert et al., 2012, 2013; Todorovic et al., 2007; Udani et al., 2011). Such memory enhancement is not observed in non-stressed mice, indicating that CRFR1 antagonism specifically blocks stress-induced cognitive impairment, rather than enhancing memory per se (Urani et al., 2011). Furthermore, the mechanism by which a strong footshock stress (2 x 2 s 1.5 mA) impairs memory appears to be independent of alterations on HPA axis functioning, and may be mediated by central CRFR1 (Philbert et al., 2012).
CRFR1 signalling can also act to impair fear conditioning (Blank et al., 2003b; Isogawa et al., 2013; Todorovic et al., 2007). Acute restraint stress immediately prior to conditioning (1 x 2 s 0.7 mA footshock) decreases the expression of conditioned freezing when tested the next day, and this can also be reversed by systemic CRFR1 antagonist administration (Blank et al., 2003b). Despite reports that hippocampal CRF can modulate conditioned fear via CRFR1 (Blank et al., 2003a; Radulovic et al., 1999), intra-hippocampal injection of a CRF receptor antagonist failed to replicate such rescue (Blank et al., 2003b). It appears that the action of CRF was through a different CRFR1 expressing site within the fear circuitry. One such region may be the lateral amygdala (LA), where direct infusion of CRF before or after conditioning (1 x 1 s 0.7 mA footshock) can disrupt the consolidation of fear memories to impair the expression of conditioned freezing to auditory cues (Isogawa et al., 2013). In contrast, this effect is not observed by CRF infusions in CeA (Isogawa et al., 2013). Considering that the fear conditioning paradigm used in chapter 6 may be a highly stressful situation involving footshock presentations (6 x 0.6 mA footshock), and can increase CRF in the VTA (Wang et al., 2005), VTA CRFR1 knockdown may modulate CRF release following tone-shock pairings to increase conditioned fear.

### 7.5.1 VTA CRFR1 in stress-impairment of fear learning

Given the chronic suppression of VTA CRFR1 in this study, the exact nature of the mnemonic deficit is unclear, and may involve effects on the acquisition, consolidation, or retrieval (figure 7-2).

**Figure 7-2** Mnemonic processes involved in conditioned fear learning. During fear conditioning on day 1, mice are exposed to repeated tone-footshock pairings. The increase in CS-elicited freezing represents acquisition of conditioned fear. Freezing during early extinction training on day 2 reflects the consolidation of fear learning as well as the retrieval of conditioned fear memories. The decrease of freezing during extinction training represents the acquisition of extinction learning. Test sessions on day 3 or day 4 test the expression of fear memories vs. extinction memories.
However, my data show no differences in the acquisition of tone-shock association as measured during conditioning, nor were there any differences in baseline freezing across all tests. This argues against the hypothesis that CRFR1 within the VTA affects arousal or anxiety during fear conditioning to alter the experience of the US and/or formation of the CS-US association. Furthermore, it makes it unlikely that knockdown of VTA CRFR1 altered locomotor or attention to affect learning or freezing behavior. This is consistent with reports that acute stress does not cause changes to footshock sensitivity (Todorovic et al., 2007), and it appears that VTA CRFR1 is not involved in unconditioned fear responses. This suggests that enhanced expression of conditioned freezing is due to effects on the consolidation and/or retrieval of the conditioned fear memory. Stress can impair both these processes independently (Park et al., 2008). As re-exposure to fear conditioned cues is also stressful (Hagewoud et al., 2011; Philbert et al., 2012), it is unclear whether CRF release in response to tone presentations impairs retrieval of conditioned fear.

However, VTA CRFR1 knockdown mice showed no deficits in extinction learning—although they expressed higher levels of auditory conditioned fear, once conditioned fear was acquired, the rate of extinction was unaffected. This is noteworthy because extinction is not a process of ‘unlearning’ but rather a process by which new learning of fear inhibition occurs and is superimposed over the initially acquired learned response. The fact that both short- and long-term extinction are unaffected suggests that extinction training that does not involve footshock is not sufficiently stressful to recruit VTA CRFR1 signalling. Therefore, it is unlikely that the effects seen in the study are due to effects on memory retrieval processes, and are probably due to effects on the consolidation of fear memories. This idea is supported by the finding that systemic administration of CRFR1 antagonists prior to conditioning reverses stress-impairment of conditioned fear memories (Blank et al., 2003b). It appears that knockdown of VTA CRFR1 disrupts the action of CRF in the consolidation of the CS-US association to enhance conditioned fear, and that in this situation, the endogenous function of these receptors decreases conditioned fear.

Clearly, my results warrant further investigation into the role of VTA CRFR1 signalling in stress-induced impairments of learning and memory. Firstly, to test the hypothesis that VTA CRFR1 knockdown increases the consolidation of fear conditioning, future studies could examine the effects of acute administration of a CRFR1 antagonist in the VTA either immediately after conditioning or before retrieval testing the following day. Secondly, to test the hypothesis that these effects were due to the excessively stressful nature of the conditioning paradigm, a single tone-shock pairing could be used for conditioning (Blank et al., 2003a,b; Refojo et al., 2011; Todorovic et al., 2008). It may be the case that when fear conditioning is extremely stressful VTA CRFR1 reduces fear learning as an adaptive mechanism to prevent traumatic memories, and in mildly stressful situations VTA CRFR1 has no effects or may even enhance fear learning. Lastly, it is unknown whether the VTA participates
in other forms of stress-induced cognitive impairments known to be mediated by CRFR1, such as contextual conditioned fear (Blank et al., 2003b).

### 7.5.2 Downstream effects of VTA CRFR1

As is the case with stress-induced cocaine-seeking, the identity of the CRFR1 expressing neurons involved in this effect is unknown. Although DAergic VTA signalling has a key role in conditioned fear (de Oliveira et al., 2009; Fadok et al., 2009), Refojo and colleagues reported that CRFR1 KO in midbrain DA neurons had no effect on auditory conditioned fear. It should be noted that, contrary to many studies showing that CRFR1 is involved in auditory conditioned fear, Refojo et al. (2011) found KO of CRFR1 in any neurotransmitter population had no effects. This may be due to methodological differences in their fear conditioning paradigm. Specifically, mice were conditioned using a single-tone shock pairing (20 s tone; 0.7 mA footshock). Fear expression was assessed by total freezing over a 5-minute tone presentation on the following day. In this situation, control mice only showed weak expression of auditory conditioned fear (~10-30 % across control groups), and any differential effects of CRFR1 KO may have been masked by within-session extinction.

Regardless, the possibility remains that CRFR1 expressed on VTA DA neurons is specifically involved in stress-impairment of conditioned fear, which may not occur when mice are given a single tone-shock pairing. Indeed, using the same conditioning paradigm described in Refojo et al. (2011), Blank et al. (2003b) found no effects of CRFR1 antagonism on conditioned freezing in non-stressed mice. Notably, distinct populations of VTA DA neurons exhibit differential responses to aversive stimuli and have divergent projection targets (Matsumoto & Hikosaka, 2009). Identification of the subset of these cells which express CRFR1 would facilitate understanding of the role of CRF signalling in these behaviours.

The effects seen in chapter 6 may also be mediated by CRFR1 expressed on non-DAergic neurons. In the VTA, footshock stimuli transiently and potently increase firing of GABAergic interneurons and inhibit DA neurons, an effect which is blocked by GABA-A but not DA receptor antagonism indicating that the effects on DA activity are downstream of increased activity of GABA neurons (Tan et al., 2012). Optogenetic stimulation of this pathway can produce aversion and freezing behaviour within a single real-time conditioned place aversion session which persists when tested the next day, demonstrating a strong learning effect resulting from GABAergic VTA activity (Tan et al., 2012). CRFR1 modulation of VTA GABA signalling has been implicated in a range of averingly motivated behaviours (Blacktop et al., 2016), including nicotine withdrawal-induced anxiety (Grieder et al., 2014). One must also consider the potential involvement of VTA GABAergic projection neurons, which can enhance auditory conditioned fear learning through connections with cholinergic interneurons in the NAc (Brown et al., 2012).
7.6 Limitations

As discussed in chapter 3, a major limitation of these studies is the inability to assess knockdown of the CRFR1 protein \textit{in vivo}. At this time, these qPCR studies, along with semi-quantitative PCR and ISH experiments (Sztainberg et al., 2010; 2011), provide the best practical evidence for the effective knockdown of CRFR1 mRNA, and the functional effects of CRFR1 signalling has been assessed \textit{in vitro} using a luciferase assay (Sztainberg et al., 2010). Furthermore, downregulation of CRFR1 using the shCRFR1 virus recapitulates the behavioural effects of CRFR1 antagonists (Blacktop et al., 2016; Sztainberg et al., 2011). Nevertheless, in the future, confirmation of reduced CRFR1 protein levels will be mandatory.

7.7 Clinical applications of CRFR1 antagonists

In line with CRF-mediated cognitive deficits in animal models, patients with anxiety disorders also suffer from impairments in cognitive processes including impaired executive function, episodic- and declarative memory (Aupperle et al., 2012; Brewin et al., 2007; Samuelson et al., 2011). Individuals with certain CRFR1 SNPs also show altered fear responses to conditioned threat cues, which is associated with heightened generalised anxiety (Grillon, 2002; Heitland et al., 2013, 2016; Weber et al., 2015). Changes in the mnemonic aspects of fear processing may have significant impact on the development of anxiety disorders. PTSD in particular is thought to be caused, at least in part, by dysregulated fear learning, including enhanced memories of trauma-associated cues (Blechert et al., 2007; Mahan & Ressler, 2012; Pitman, 1989; Wessa & Flor, 2007). This can occur at the time of the traumatic event when associations between the trauma and various environmental cues become so strong that they later trigger excessive and persistent fear (Orr et al., 2000; Pitman, 1989). Preclinical studies suggest that hypersecretion of CRF could contribute to these mnemonic changes, depending on the timing and brain region involved (Isogawa et al., 2013). Therefore, it is possible that in situations of traumatic stress exposure, VTA CRFR1 signalling has a protective function to dampen the formation of fear memories, and dysregulation of this system may have deleterious consequences on anxiety symptoms.

Despite a large amount of evidence from preclinical studies in addiction and anxiety models, including non-human primate models of anxiety (Kalin et al., 2016), no CRFR1 antagonists have to date shown efficacy in clinical trials for treatment of anxiety disorders or drug addiction. Early studies showed that the CRFR1 antagonists pexacerfont had no effect on suicidal ideation in patients with anxiety disorders (Coric et al., 2009), nor did it have any positive results reported in a multi-centre, randomized, double-blind active comparator and placebo-controlled trial for GAD (Coric et al., 2010). Trials of two other CRFR1 antagonists, verucerfont and emicerfont, for social anxiety disorder were completed in 2008 with undisclosed results (clinicaltrials.gov ID: NCT00555139), while trials for
verucerfont in women with PTSD are ongoing (Dunlop et al., 2014). A clinical trial of pexacerfont was initiated for the treatment of stress-induced tobacco craving in smokers attempting to quit, but has since been withdrawn (clinicaltrials.gov ID: NCT01557556). Pexacerfont was also ineffective for the treatment of stress-induced alcohol craving in anxious alcoholics (Kwako et al., 2015). Most recently, clinical trials of verucerfont in anxious alcohol-dependent women showed no effects on alcohol cravings (Schwandt et al., 2016).

The reasons behind the overwhelmingly negative clinical results of CRFR1 antagonists in addiction and anxiety disorders are unknown, but may be due to the complex and, at times, potentially opposing behavioural effects of CRF signalling. Indeed, from a translational perspective, even targeting VTA CRFR1 may be a double-edged sword as it would block stress-induced reinstatement, but enhance conditioned fear.

7.8 Future Directions

The involvement of VTA CRFR1 in fear- and cocaine-related behaviours has now been investigated separately. Given the clinical evidence that addiction and anxiety disorders bi-directionally influence one another, the underlying behavioural processes are likely intertwined. The effects of acute cocaine administration on fear conditioning are well established; low doses of cocaine before conditioning enhances CS-elicited freezing in mice when tested the next day (Wood et al., 2007). Cocaine administration prior to retrieval testing also increases acoustic startle responses to fear conditioned cues in rats (Borowski & Kokkinidis, 1994; Borowski & Kokkinidis, 1998). This suggests that acute cocaine intensifies the fear-eliciting properties of the CS. The effects of chronic cocaine exposure are less clear. While studies have shown that chronic cocaine treatment prior to fear conditioning attenuates fear-potentiated startle (Borowski & Kokkinidis, 1994), no effects were seen on the acquisition or expression of conditioned freezing or conditioned suppression of operant responding for sucrose rewards in rats, but extinction learning was impaired (Burke et al., 2006). The anxiogenic effects of cocaine in rats is mediated by CRF receptors in the VTA (Ettenberg et al., 2015). VTA CRFR1 signalling is also involved in anxiety induced by drug withdrawal, including through actions in the VTA (Breese et al., 2004; Grieder et al., 2014). Whether VTA CRFR1 contributes to alterations in processing following chronic cocaine is unknown. Furthermore, voluntary cocaine consumption as well as different dosing schedules may have distinct effects on fear conditioning compared to experimenter administered bolus i.p cocaine injections (Chen et al., 2008). In future studies, conditioned fear and cocaine IVSA should be performed concurrently, to examine the role of VTA CRFR1 in potential interactions between these behaviours.

Future studies should also aim to identify the neurochemical identity and projection targets of the CRFR1-expressing cells involved in fear and reward-seeking. For example, viruses with cell-type specific promotors could be used to selectively transduce dopaminergic or GABAergic neurons to
differentiate between the effects of knocking down CRFR1 in these neuronal populations. A better understanding of the neurobiological mechanisms of these circuits is necessary to enable the development of effective pharmacotherapies for these disorders, including adjunctive treatments to enhance existing cognitive behavioural modification therapies (Manning et al., 2016; Opriş et al., 2011; Rabinak & Phan, 2014; Rodrigues et al., 2014). Future treatments may also involve selective targeting of certain disease-related circuits within the brain, which awaits the development of less invasive methods of site-specific brain manipulation (Lüscher et al., 2015; Marin et al., 2014).
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