THE ROLE OF COCAINE- AND AMPHETAMINE-
REGULATED TRANSCRIPT (CART) AND OREXIN IN DRUG-
SEEKING AND ADDICTION-RELATED BEHAVIOURS

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A thesis submitted in fulfillment
of the requirements for the degree of
Doctor of Philosophy

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September, 2013
DECLARATION

STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository*, subject to the provisions of the Copyright Act 1968.

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________________________
MORGAN H JAMES
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A final thank you must be extended to my family – particularly my parents – without whom, this PhD would not have been possible. Mum and Dad, you have always encouraged and supported my academic pursuits, and for this I am eternally grateful.
MANUSCRIPTS

The work described in this thesis is divided into five manuscripts:

Chapter 1:


Chapter 2:


Chapter 3:


Chapter 4:


Chapter 5:


Two additional publications are referred to in this thesis and are included in the appendices:

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ABSTRACT

Over the past decade, two hypothalamic neuropeptides, cocaine- and amphetamine-regulated transcript (CART) and orexin (hypocretin), have been shown to play important roles in regulating addiction-relevant behaviours, including ‘reinstatement’ of drug-seeking, a model of relapse-like behaviour. Interestingly, CART generally opposes the rewarding effects of psychostimulant drugs such as cocaine and negatively regulates reinstatement of drug-seeking. In contrast, orexin generally promotes reinstatement of cocaine seeking. Despite intense interest in CART and orexin as potential novel targets for pharmacotherapies designed to treat cocaine addiction, very little is known as to where in the brain these peptides act to regulate addiction-relevant behaviour.

The work presented in this thesis investigated the paraventricular thalamus (PVT) as a potential important site of convergence for CART and orexin signaling in the regulation of reinstatement behaviour. As a first step, I provide evidence that PVT signaling is important for reinstatement behaviour. In Chapter 1, I present data demonstrating that activation of PVT, as gauged by levels of Fos-protein, a marker of neuronal activity, is strongly correlated with cocaine reinstatement elicited by drug-associated cues. In Chapter 2, I show that intra-PVT injections of tetrodotoxin (TTX), a sodium-channel blocker, attenuated drug-primed reinstatement of cocaine seeking. Taken together with evidence implicating the PVT in stress responsivity, these findings point to the PVT as an important substrate that may be common to all forms of reinstatement (cue-, drug- and stress-induced).

In Chapter 2 I also show that microinfusions of the CART peptide directly into the PVT attenuated drug-primed reinstatement, suggesting that CART acts in this region to negatively regulate cocaine-seeking behaviour. In contrast, data presented in Chapter 3 suggests that orexin signaling in the PVT is not important for reinstatement behaviour, as intra-PVT injections of the orexin receptor-1 antagonist SB-334867 had no effect on cocaine seeking elicited by drug cues. This Chapter raises the possibility that orexin receptor-2 signaling in the PVT may be more important in reinstatement behaviour.
Subsequent studies in *Chapter 3* point to the importance of orexin signaling in the ventral tegmental area (VTA) in regulating reinstatement behaviour. Intra-VTA infusions of SB-334867 significantly attenuated cue-induced reinstatement of cocaine seeking without having any effect on spontaneous locomotor activity. These findings are further explored in *Chapter 4*, where I show that intra-VTA SB-334867 administration altered the activity of key drug-seeking substrates, including the PVT. I also present evidence that intra-VTA SB-334867 treatment does not affect reinstatement responding for a natural reward, suggesting that at low doses, SB-334867 can reduce drug seeking but not affect normal motivated behaviour. This *Chapter* also summarises the findings from each of the former chapters and discusses the implications of these findings in the context of the existing literature relating to the role of hypothalamic peptides in regulating drug-seeking behaviour.

In the final chapter of this thesis, I focus on the orexin system as a possible neurobiological link between addiction and stress-related disorders such as depression and anxiety. In this chapter (*Chapter 5*), I show that animals exposed to early life stress exhibit hypoactivity of the orexin system following exposure to an additional stressor in adulthood. These findings are consistent with recent clinical evidence of reduced orexin activity in depressed patients. Interestingly, findings in this chapter also demonstrate that access to voluntary wheel running, an intervention known to have beneficial effects for depressive-like symptomology, protected against changes in orexin function and stress-related behaviour in male rats, but not female rats. Whilst these findings represent only the first step in our understanding, they point to the possibility that dysregulated orexin function may represent a neurobiological factor contributing to the high comorbidity of addiction and stress-related disorders.
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<table>
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<tr>
<td>1-AG</td>
<td>1-allylglycine</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
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<tr>
<td>BLA</td>
<td>Basolateral amygdala</td>
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<td>CART</td>
<td>Cocaine- and amphetamine- regulated transcript</td>
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<td>CBT</td>
<td>Cognitive behavioural therapy</td>
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<td>CPP</td>
<td>Conditioned place preference</td>
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<td>CPA</td>
<td>Conditioned place aversion</td>
</tr>
<tr>
<td>CPRS</td>
<td>Comprehensive pathological rating scale</td>
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<tr>
<td>CRF</td>
<td>Corticotropin-releasing factor</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DMH</td>
<td>Dorsal medial hypothalamus</td>
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<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
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<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
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<tr>
<td>FR</td>
<td>Fixed ratio</td>
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<td>FST</td>
<td>Forced swim test</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HPA axis</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
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<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
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<tr>
<td>ICSS</td>
<td>Intracranial self-stimulation</td>
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<td>KOR</td>
<td>Kappa opioid receptor</td>
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<tr>
<td>LH</td>
<td>Lateral hypothalamus</td>
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<tr>
<td>MCH</td>
<td>Melanin concentrating hormone</td>
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<tr>
<td>mEPSCs</td>
<td>Miniature excitatory post-synaptic currents</td>
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<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
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<tr>
<td>NAC</td>
<td>Nucleus accumbens</td>
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<tr>
<td>NACe</td>
<td>Nucleus accumbens core</td>
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<tr>
<td>NACsh</td>
<td>Nucleus accumbens shell</td>
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<tr>
<td>NMDAR</td>
<td>N-Methyl-D-aspartic acid receptor</td>
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<tr>
<td>OF</td>
<td>Open field</td>
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<tr>
<td>OX₁</td>
<td>Orexin receptor 1</td>
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<td>OX₂</td>
<td>Orexin receptor 2</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PF</td>
<td>Perifornial area</td>
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<td>PFC</td>
<td>Prefrontal cortex</td>
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<td>POMC</td>
<td>Pro-opiomelanocortin</td>
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<td>PR</td>
<td>Progressive ratio</td>
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<tr>
<td>PTSD</td>
<td>Post-traumatic stress disorder</td>
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<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
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<td>PVT</td>
<td>Paraventricular thalamus</td>
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<tr>
<td>rPP</td>
<td>Rat pancreatic polypeptide</td>
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<tr>
<td>shRNAs</td>
<td>Short hairpin RNAs</td>
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<tr>
<td>TRH</td>
<td>Thyrotropin-releasing hormone</td>
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<tr>
<td>UCMS</td>
<td>Unpredictable chronic mild stress</td>
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<tr>
<td>VGAT</td>
<td>GABA transporter</td>
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<tr>
<td>VGLUT2</td>
<td>Vesicular glutamate transporter 2</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventral medial hypothalamus</td>
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<tr>
<td>VP</td>
<td>Ventral pallidum</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<td>WKY</td>
<td>Wistar Kyoto rat</td>
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GLOSSARY

**Behavioural sensitisation:** A progressive increase in the locomotor-activating effects of drug following repeated exposure to a set drug dose (typically 5-10 days). The expression of behavioural sensitisation is typically assessed during withdrawal following an acute drug challenge at a dose equivalent to that administered during the sensitisation exposure.

**Conditioned place preference (CPP):** A classical (Pavlovian) conditioning paradigm whereby animals are administered either drug or saline (delivered intraperitoneally). After injection animals are immediately placed into one of two distinct contexts that differ in terms of their contextual cues (e.g. wall colour/pattern, floor texture). Following conditioning, the animals are tested for their preference of the drug- vs. saline-paired chamber, as measured by time spent in each chamber. An increase in preference for the drug-paired context is thought to indicate a positive reinforcing effect of the drug.

**Conditioned place aversion (CPA):** Similar to CPP, however, one context is associated with an aversive stimulus, whilst the other is associated with a neutral stimulus. Following conditioning, animals are tested for their preference for the contexts. An increase in preference for the neutral context indicates an aversive effect of the test stimulus.

**Intracranial self-stimulation (ICSS):** A procedure whereby animals learn to respond for direct electrical stimulation via an electrode implanted into a specific region of the brain. Higher rates of responding for ICSS are thought to be the result of decreased ‘reward’ pathway sensitivity whereas lower rates of responding for ICSS are thought to result from increased ‘reward’ pathway sensitivity.

**Progressive ratio schedule:** A reinforcement schedule applied to the drug self-administration procedure to assess an animals’ motivation to obtain a natural or drug reward. Under this schedule, the number of responses required to obtain a drug reward is increased following every reward. The point at which the animal ceases to press is referred to as the animals’ ‘break point’.

**Reward:** Outcomes such as food, sex and drugs of abuse that are positive reinforcers and animals will work to obtain.

**Drug-seeking behaviour:** The active seeking of either illicit and licit drug rewards.

**Reward-seeking behaviour:** A broader term that describes the seeking of drug and/or non-drug rewards (such as food).
GENERAL INTRODUCTION
GENERAL INTRODUCTION

0.1 Addiction, relapse and animal models of addiction and relapse

0.1.1 Cocaine addiction, relapse and current treatments

Drug use represents a significant health and social burden for Australian society. In 2004-2005, the direct costs associated with compulsive drug use (both legal and illicit) were estimated to exceed $56 billion Australian dollars per year (Australian Institute of Health and Welfare, 2008). Whilst alcohol and tobacco represent the primary drugs of abuse, a significant proportion of the economic burden associated with drug use (~15%) is attributable to the use of illicit drugs. In particular, the use of psychostimulants, including cocaine, has been increasing steadily in recent years. In 2010, an estimated 2.3% of the population used cocaine, the highest rate on record for this drug (AIHW, 2011). Of those who experiment with cocaine, it is estimated that 16-17% develop a dependence disorder, which compares to 9% of persons who use marijuana and 15% of those who consume alcohol (Wagner and Anthony, 2002). People who report using cocaine (both recreationally and compulsively) are more likely than non-users to have been diagnosed with a mental illness and to suffer from very high levels of psychological distress. Further, cocaine use is associated with increased risk of HIV/AIDS, hepatitis C, malnutrition and death by overdose (AIHW, 2011).

Like other substance-use disorders, cocaine addiction is characterised by a compulsive need to seek and take the drug, a loss of control over the amount of drug consumed, and by periods of abstinence that are closely followed by relapse to drug-taking behaviour (DSM-IV-TR, 2004). Indeed, relapse is often regarded as the most significant hurdle to the successful treatment of drug addiction. It is estimated that approximately 69% of cocaine addicts completing outpatient treatment programs relapse within one year, and 80% of addicts completing long-term residential treatment relapse within one year (Institute for Health Policy, 2001). Factors that may increase the risk for relapse include re-exposure to drug-associated environments, discrete conditioned stimuli (eg. the materials used to self-administer the drug, such as needles), stress, or taking the drug.
itself (Jaffe et al., 1989; Childress et al., 1993; O'Brien et al., 1998; Sinha et al., 1999; Sinha et al., 2000).

At present, treatment options for cocaine addiction are limited to psychological interventions, such as cognitive-behavioural therapy (CBT) and motivational interviewing (Sanchez et al., 2011; Schierenberg et al., 2012). However, as evidenced by the high rates of relapse in treatment populations, the long-term effectiveness of these interventions is generally limited. As with other psychiatric disorders (such as depression), it is likely that the clinical utility of these psychological therapies would be significantly strengthened if used in combination with an effective medication (Penberthy et al., 2010). At present however, there are no FDA-approved medications for the treatment of cocaine addiction (Kalivas and Volkow, 2011; Jonkman and Kenny, 2013), nor are any chemical entities specifically designed for the treatment of cocaine addiction currently in clinical testing (see www.clinicaltrials.gov). This is likely due, at least in part, to an inadequate understanding of the complex neurobiological mechanisms of the disorder. To this end, significant effort has been focused on understanding how the brain is altered by chronic drug taking and the neural mechanisms responsible for protracted relapse risk.

0.1.2 Animal models of addiction

Recent advances in functional imaging techniques, including fMRI and PET, have provided insight into the brain mechanisms involved in relapse processes in humans. However, such approaches are limited in terms of their spatial and temporal resolution, and are often largely correlational in nature. Accordingly, much of the progress in our understanding of the neurobiology of addiction is the result of experimental research using laboratory animals, which allows for invasive neurobiological investigations not feasible in humans (Ahmed, 2012). Indeed, significant efforts over the past 50 years have resulted in increasingly sophisticated models of drug-seeking in experimental animals (particularly rats) with strong phenomenological similarity (face validity) to the human drug-taking experience (Marchant et al., 2013).
Training animals to self-administer drug is widely considered the most valid way to mimic human drug taking. Indeed, laboratory animals reliably self-administer most drugs of abuse. To achieve this, animals (typically rodents) are trained to perform an instrumental behavioural task, such as a lever press or nose poke, to receive a drug reinforcer. Whereas route of delivery can differ across drugs, cocaine, other psychostimulants and opiates are typically administered via a chronically indwelling catheter that is surgically inserted into the jugular vein. Alcohol, on the other hand, is usually delivered into a receptacle, allowing animals oral access to the drug. The schedule of reinforcement is experimenter-determined, such that the number or pattern of responses necessary to earn a drug reward/infusion can be altered. In a simple fixed ratio (FR) schedule of reinforcement, the number of responses necessary to earn a reward is set to a constant number. For example, an animal on an FR1 schedule would need to lever press once in order to earn drug reinforcement. Whilst FR1 schedules are often used during the acquisition stages of self-administration training, it is common for the fixed ratio schedule to then be increased such that a higher number of responses are necessary for a single drug reward (eg. FR3 or FR5 schedules).

0.1.3 Using the self-administration model to study reinstatement or ‘relapse’

The most widely employed animal model of drug relapse is the extinction-reinstatement model (Weiss, 2010; Marchant et al., 2013). In this model, animals are trained to self-administer drugs before being subjected to a period of extinction training whereby lever responses that normally produce a drug infusion are no longer rewarded. Extinction training is typically continued until an experimenter-determined ‘criterion’ is met (eg. <6 lever responses per session over three consecutive days). Relapse-like behaviour can be assessed by measuring the reinstatement of lever pressing following exposure to either stress (e.g. intermittent foot shock), non-contingent priming injections of the drug, or exposure to cues associated with drug intake. Extinction conditions remain in effect during reinstatement testing, meaning that drug rewards are not available.

With respect to ‘cue-induced’ drug-seeking, three types of conditioned cues are
typically used: discrete, discriminative or contextual cues. Discrete cues are thought to mimic drug-associated stimuli that are linked with the acute rewarding effects of the drug, such as drug paraphernalia (described above). To model discrete cue-induced reinstatement, animals are typically trained to self-administer drug and each drug-reinforced response is paired with a brief presentation of one or more environmental stimuli (conditioned stimuli (CS) such as a light or tone). Thus, both drug delivery and presentation of the discrete cue are contingent on lever pressing. Once stable drug self-administration is achieved, responding is extinguished by withholding both the drug and the discrete cue. During the reinstatement test, responding on the previously drug-paired operandum, which now results in a response-contingent presentation of the discrete cue only, serves as a measure of relapse (Weiss, 2010).

Discriminative and context-induced cues, on the other hand, are thought to mimic environmental cues that predict drug availability, such as a specific place where drugs are often taken. In the discriminative cue model, animals are trained to self-administer either cocaine or saline in the presence of distinct discriminative stimuli that are not response-contingent (Weiss, 2010). For example, cocaine availability is signalled by an environmental stimulus (S+), such as a background white noise, that is present for the entire duration of the drug-taking session. On the other hand, non-drug availability (saline) is signalled by a distinct stimulus (S-), such as a house light. Owing to their predictive nature of drug availability, discriminative cues are often said to ‘set the occasion’ for engaging in drug-seeking behaviour (Weiss, 2010). During extinction training, discriminative cues are withheld, along with drug infusions. Reinstatement of drug-seeking can then be assessed by presenting the discriminative cue previously associated with drug-availability (S+).

Finally, the context-induced reinstatement procedure is based on the ‘renewal’ procedure first described by Bouton and Bolles (1979) as a means of studying context-specificity of fear extinction. This procedure was later adapted by Crombag & Shaham (2002) to show renewal of extinguished drug seeking. In this procedure, animals are trained to self-administer drug in a distinct environment (A) that provides compound contextual cues (eg. light, sound, olfactory or tactile cues). Responding is then
extinguished in a second distinct environment (B) before animals are tested for renewal of drug seeking in the original (drug-paired) context (A). Context-induced reinstatement, or ABA renewal, is a robust phenomenon and has been observed across many separate investigations and drug types (Crombag et al., 2002; Crombag et al., 2008; Hamlin et al., 2008; Janak and Chaudhri, 2010; Bossert et al., 2011; Millan and McNally, 2012; Perry and McNally, 2013).

Each of the addiction-related experiments described in this thesis utilise the extinction-reinstatement model, whereby drug-seeking is reinstated by either a drug prime or discriminative cues previously paired with drug availability. Whilst it is beyond the scope of this thesis, it is important to acknowledge that this model has attracted some criticism, mostly relating to the extent to which the operant extinction phase accurately reflects the abstinence phase of the human condition. A number of alternative models, including the incubation of craving model, seek to address these criticisms. These models were recently reviewed by Marchant et al. (2013). Regardless, the extinction-reinstatement model offers strong face validity in terms of recapitulating the processes by which relapse is initiated in the human condition and thereby provides an approach through which the neural substrates of relapse-like behaviour can be studied.
0.2 The hypothalamus and reinstatement behaviour

0.2.1 Hypothalamus and motivated behaviour

The hypothalamus has long been implicated in motivated behaviour. Early studies demonstrated that rats readily learn to lever press to receive brief electrical stimulation of the hypothalamus in an intracranial self-stimulation model (ICSS) (Olds and Milner, 1954; Gallistel et al., 1981). Similarly, lesions of the hypothalamus were shown to disrupt appetitive motivational states, including feeding, drinking and sex behaviours (Stellar, 1954). These seminal studies identified the lateral portion of the hypothalamus (lateral hypothalamus; LH) as being important for promoting appetitive behaviour and the medial portion (ventromedial hypothalamus; VMH) as being involved in inhibiting such behaviour. Although this ‘dual-centre’ hypothesis is oversimplified, this body of work was highly influential in establishing the hypothalamus, and the LH in particular, as a key component of a brain reward-seeking network (Panksepp, 1998; Elmquist et al., 1999; Kishi and Elmquist, 2005).

Indirect evidence for hypothalamic involvement in drug-seeking was first demonstrated in a report by Carroll (1985), who showed that 24 h of food restriction (previously shown to increase the rewarding effects of LH self-stimulation) reinstated extinguished cocaine seeking. More than a decade later, food deprivation was shown to increase self-administration of psychostimulant and opioid drugs (Carroll, 1999), as well as reinstate heroin seeking (Shalev et al., 2000). The late 1990s also saw a number of elegant molecular studies that led to the identification of several novel neuropeptide families expressed in the hypothalamus that were subsequently implicated in motivated behaviour, including drug-seeking. Two such peptides include *cocaine and amphetamine regulated transcript* (CART), which is expressed in various hypothalamic regions including the LH and arcuate nucleus (ARC) (Douglass and Daoud, 1996), and *orexin* (hypocretin), which is restricted to the perifornical (PF) and LH divisions (de Lecea et al., 1998; Sakurai et al., 1998b) see Figure 1. Soon after their identification, both peptides were initially implicated in feeding behaviour, with CART generally found to inhibit feeding and orexin generally found to have pro-feeding effects. Subsequent studies established a role for both CART and orexin in drug-seeking
behaviours, including reinstatement, leading to considerable effort dedicated to determining how and where these peptides act to promote this behaviour. Accordingly, a comprehensive overview of studies implicating CART and orexin in drug seeking behaviour is provided below, followed by evidence that these peptides may influence the drug-seeking circuitry via projections to the paraventricular thalamus (PVT) and ventral tegmental area (VTA).

0.2.2 Cocaine- and amphetamine-regulated transcript (CART)

In 1996, Douglass and colleagues identified an mRNA that was upregulated in the striatum of rats following acute cocaine and amphetamine administration. Interestingly, this mRNA was found to encode a gene product that had previously been identified by Spiess et al. (1981) in the ovine hypothalamus. This peptide was subsequently named cocaine- and amphetamine-regulated transcript (CART). Human and rat CART mRNA share 91% sequence homology, but despite this similarity, the rat has both long and short splice variants of the CART peptide whereas only the short form is present in humans (Larsen et al., 2000). Two active fragments of the rat long form (CART 55-102 and CART 61-102) have been identified, however, the CART receptor(s) has yet to be cloned, meaning that selective CART receptor antagonists are yet to be developed. However, recent in-vitro experiments indicate that the CART receptor is a G-protein coupled receptor that signals via G_i/o, suggesting an inhibitory post-synaptic action for CART (Rogge et al., 2008).

CART-positive cell bodies are found in a number of hypothalamic regions, including the LH, the paraventricular (PVN), supraoptic and the arcuate nuclei (ARC) (Douglass and Daoud, 1996; Koylu et al., 1998; Elias et al., 2001). In addition to this distribution, CART is co-expressed with a number of other neuropeptides including thyrotropin-releasing hormone (TRH) in the PVN, melanin concentrating hormone (MCH) in the DMH/LH, and pro-opiomelanocortin (POMC) in the ARC nucleus and retrochiasmatic area (RCA). It is interesting and somewhat surprising that within the ARC nucleus, CART is co-expressed with the POMC-derived anti-feeding peptide α-melanocyte stimulating hormone (α-MSH), whereas in the DMH/LH CART is co-expressed with
the pro-feeding peptide MCH (Broberger, 1999; Elias et al., 2001; Kishi and Elmquist, 2005). Although not a focus of this thesis, it is interesting that MCH appears to have pro drug-seeking effects within the NAC (Chung et al., 2009). These findings raise the possibility that different populations of CART neurons may subserve different functions. Beyond the hypothalamus, CART peptide-expressing neurons are also found in the NAC, VTA and amygdala (Koylu et al., 1998; Dallvechia-Adams et al., 2002). Interestingly, approximately 15% of CART terminals in the VTA contain MCH, whilst CART peptides also co-localise, albeit to a lesser extent, with GABA and dynorphin in both the VTA and substantia nigra (Dallvechia-Adams et al., 2002). CART-cell bodies are also found in the peripheral nervous system, including myenteric neurons in the gastrointestinal tract, sympathetic preganglionic neurons and in the adrenal glands (Dun et al., 2000; Ekblad et al., 2003). In keeping with this broad distribution and diverse neurochemical phenotypes, CART has been shown to be involved in a number of functions, including regulation of food intake, maintenance of body weight, reward and endocrine functions (for review, see Rogge et al. (2008)).

Figure 1. Distribution of CART- and orexin- expressing neurons. CART-expressing neurons are located in a number of hypothalamic subregions, including the arcuate nucleus and lateral hypothalamus, whereas orexin-expressing neurons are restricted to the perifornical/lateral hypothalamus. Importantly, CART-expressing neurons are found in other brain regions, including the nucleus accumbens, ventral tegmental area and amygdala. 3V: third ventricle; ARC: arcuate nucleus; DMH: dorsomedial hypothalamus; F: fornix; LH: lateral hypothalamus; MT: mammillary tract; PFA: perifornical area; VMH: ventromedial hypothalamus.
0.2.2.1 CART and feeding Behaviour

Early studies firmly established a role for CART in the regulation of feeding behaviour. Acute intracerebroventricular (i.c.v.) CART administration dose-dependently suppressed feeding behaviour in rats (Kristensen et al., 1998; Lambert et al., 1998), whilst chronic CART treatment prevents body weight gain in both lean and obese animals (Larsen et al., 2000). Because the identity of the CART receptor is unknown, pharmacological manipulations to antagonize the CART system have been limited to the use of antibodies raised against the active peptide sequence. Central administration of CART antiserum increases feeding, corroborating findings that CART peptide injections exert an inhibitory influence on feeding behaviour (Kristensen et al., 1998; Lambert et al., 1998). Consistent with these functional effects, administration of rimonabant, a CB(1) receptor antagonist with anorectic effects, increases Fos-protein expression in hypothalamic CART-positive cells (Verty et al., 2009). Further, food deprived animals show a marked decrease in CART mRNA expression in the ARC nucleus, and CART mRNA is almost completely absent from obese animals with disrupted leptin signaling (Kristensen et al., 1998). Somewhat surprisingly though, intra-DMH infusions of CART have been shown to increase feeding (Abbott et al., 2001), which may be caused by a disinhibitory effect of CART on neurons in the DMH that normally oppose food-seeking (Marchant et al., 2011).

0.2.2.2 CART and addiction-relevant behaviours

Evidence that CART is involved in feeding behaviour led to studies investigating a role for this peptide in other reward-related behaviours, including drug seeking. Indeed, the original report by Douglass and colleagues (1996) alluded to a role for CART in psychostimulant reward, whereby acute cocaine and amphetamine administration increased CART mRNA in the striatum of the rat brain. This finding has been somewhat difficult to replicate (Marie-Claire et al., 2003), and some studies have suggested that binge/repeated, rather than acute, cocaine administration is required to reliably increase CART transcript expression in the brain (Fagergren and Hurd, 1999; Hunter et al., 2005). Despite these technical caveats, acute cocaine has been shown to
increase Fos-like immunoreactivity in CART neurons in the NAC, even under conditions that do not produce changes in CART mRNA levels (Hubert and Kuhar, 2008). Finally, strong evidence for a role of CART in psychostimulant use comes from reports that CART mRNA levels are increased in the VTA and NAC of human cocaine overdose victims (Tang et al., 2003; Albertson et al., 2004). In addition, alcoholism is associated with a mutation of the CART gene in a Korean population (Jung et al., 2004), and acute administration of ethanol increases CART mRNA and peptide expression in the rat NAC (Salinas et al., 2006).

Pre-clinical studies have demonstrated an important, but complicated, role for CART in mediating the effects of psychostimulants. For example, intraperotential (i.p.) administration of CART (Job & Kuhar, 2012), and administration of CART into the VTA attenuates psychostimulant induced-locomotor activation (Jaworski et al., 2007). Intra-NAC infusions of CART reduce cocaine self-administration (Jaworski et al., 2008), attenuate the locomotor-activating effects of both cocaine and amphetamine (Jaworski et al., 2003), and prevent the expression of conditioned hyper-locomotion (Yoon et al., 2010). Similarly, intra-NAC injections of small hairpin RNAs (shRNAs), which reduce the expression of CART peptide in this region, produces an exaggerated locomotor response to cocaine (Job et al., 2012). Consistent with these findings, injections of CART into the ventral pallidum (VP), one of the main nuclei that receive accumbal efferents, attenuates cocaine-induced locomotion (Hubert et al., 2010).

Whilst these data suggest that CART tends to negatively regulate the rewarding effects of psychostimulants, other studies suggest that the role of CART in modulating reward-seeking may be more complicated. For example, infusions of CART 55-102 peptide fragment into the VTA produces an efflux of dopamine release in the NAC resulting in a cocaine-like increase in locomotor activity. Further, when administered on its own into the NAC, CART produces a conditioned place preference (CPP; see Glossary) (Kimmel et al., 2000). Finally, Rademacher et al (2010) showed that at low doses (2µg/side), intra-basolateral amygdala injections of CART produce CPP, whilst higher doses (4µg/side) produce conditioned place aversion (CPA; see Glossary). Taken together with the data described previously, it would appear that the effects of CART on
addition-related behaviours is both dose- and region-specific and further work is required to understand these effects completely.

**0.2.2.3 CART and reinstatement behaviour**

With respect to reinstatement of drug-seeking behaviour, a potential role for CART was first alluded to in the study of Mattson and Morrell (2005). These authors showed that conditioned cues associated with passive cocaine administration increased the number of CART-positive neurons in the NAC. Since this report, a number of subsequent studies have provided evidence for a role of hypothalamic CART cells in regulating alcohol-seeking behaviours. With respect to context-induced reinstatement, Dayas et al. (2008) showed that exposure to drug-associated discriminative cues was associated with an increase in Fos-like immunoreactivity in CART neurons of the ARC nucleus, but not the DMH/LH. Interestingly, Millan et al. (2010) showed that reinstatement of alcohol-seeking elicited by inactivation of the NACsh is associated with an increase in PF hypothalamic CART cells expressing c-Fos. These same authors later showed that i.c.v. (King et al., 2010) or intra-NACsh (Millan and McNally, 2012) infusion of CART 55-102 attenuates contextual renewal of alcohol seeking, whilst infusions of the same peptide into the DMH prevents extinction (promotes reinstatement) of alcohol-seeking (Marchant et al., 2010).

Taken together, there is clear evidence supporting a role for CART signalling in reward-related behaviours. Generally, CART tends to suppress feeding behaviour and oppose the rewarding effects of psychostimulant drugs, including cocaine. With respect to reinstatement behaviour, CART cells are responsive to alcohol-associated discriminative cues, and exogenous CART administration negatively regulates context-induced renewal of alcohol seeking. **Despite these findings, it is currently unclear whether a role for CART in alcohol reinstatement behaviour extends to other drugs, including cocaine.** Further, with the exception of the recent study by Millan & McNally (2012), very little is known about the specific brain site(s) at which CART acts to modulate reinstatement behaviour.
0.2.3 The orexin neuropeptide system

Orexin-expressing neurons are located in the DMH, PF and LH areas and secrete two peptides (orexin A & orexin B), which are derived from the same precursor gene (prepro-orexin) (Sakurai et al., 1998a). These peptides were identified almost concurrently by two independent research groups, with one group naming these peptides orexins (Sakurai et al., 1998a) and the other naming them hypocretins (de Lecea et al., 1998). The orexin peptides are highly conserved between humans and rodents, with identical orexin-A sequences and just two amino acid substitutions in orexin-B. Unlike the CART system, two G-protein-coupled receptors have been identified: Orexin receptor 1 (OX_R1) and orexin-receptor 2 (OX_R2). Orexin A binds to both OX_R1 and OX_R2 with equal affinity, whereas orexin B binds to OX_R2 with a higher affinity than OX_R1 (Mieda and Yanagisawa, 2002). OX_R1s are found in the PFC IL, hippocampus, PVT, VTA, VMH, dorsal raphe nucleus, and locus coeruleus. OX_R2s are expressed in regions including the prefrontal and insular cortices, septal nuclei, hippocampus, medial thalamic groups, VTA, raphe nuclei, and hypothalamic nuclei including the tuberomammillary nucleus, DMH, PVN, and ventral premammillary nucleus (see Figure 2) (Trivedi et al., 1998; Lu et al., 2000; Marcus et al., 2001). Consistent with this distribution, the function of orexin peptides is diverse. Thus, in addition to reward seeking, orexins have been implicated in the regulation of sleep (Chemelli et al., 1999), energy metabolism, and the maintenance of arousal (Sutcliffe and de Lecea, 2002; Taheri et al., 2002).

0.2.3.1 Orexin and feeding behaviour

Consistent with the anatomical location of orexin-expressing cells in feeding behaviour-related nuclei of the hypothalamus, central administration of both orexin A and B has been found to dose-dependently initiate food-seeking (Sakurai et al., 1998a; Yamanaka et al., 1999; Rodgers et al., 2000), whilst systemic administration of the OX_R1 antagonist SB-334867 has the opposite effect (Haynes et al., 1999; Rodgers et al., 2001; Ishii et al., 2004; Ishii et al., 2005). Furthermore, prepro-orexin mRNA is upregulated following fasting (Sakurai et al., 1998a), whereas obese mice (ob/ob and db/db) show
decreased prepro-orexin gene expression (Yamamoto et al., 1999). Interestingly, SB-334867 reduces responding for sucrose under an FR3 schedule, but has no effect under higher effort schedules such as progressive ratio (Jupp et al., 2011b). Consistent with this finding, systemic SB-334867 administration has no effect on reinstatement of high-fat food (Nair et al., 2008) or sucrose (Cason and Aston-Jones, 2013) under normal conditions, but prevents reinstatement of sucrose seeking under food restricted conditions. These findings clearly suggest a pro-feeding role for orexin, but suggest that endogenous orexin signalling may only regulate food seeking under circumstances where reward salience is increased.

Figure 2. Summary of orexinergic projections in the rat brain. Orexin-expressing neurons are located in the perifornical/lateral hypothalamus and project widely throughout the brain. Importantly, orexin neurons innervate a number of key regions in the brain reward network. Hipp: hippocampus; LC: locus coeruleus; LH: lateral hypothalamus; NAC: nucleus accumbens; NTS: nucleus tractus solitarii; PFC: prefrontal cortex; PVT: paraventricular thalamus; VTA: ventral tegmental area.
0.2.3.2 Orexin and addiction-relevant behaviours

An interesting observation highlighting the potential role for orexin in addiction is the lack of drug dependence in narcolepsy patients despite receiving amphetamine as a treatment for this condition (Sakurai, 2007). These patients also have relatively low CSF orexin-A levels (Nishino et al., 2000; Nishino et al., 2001; Mignot et al., 2002). Additional evidence implicating orexin in addiction is the demonstration that orexin knock-out mice display an attenuated morphine withdrawal and dependence (Georgescu et al., 2003). More recently, increased Fos-protein immunoreactivity has been reported in PF/LH orexin cells following acute and repeated administration of psychostimulants (Cornish et al., 2012) and alcohol (Macedo et al., 2013). Further, blockade of the OX₁ receptors reduces self-administration of alcohol (Lawrence et al., 2006; Jupp et al., 2011a; Jupp et al., 2011c), nicotine (Hollander et al., 2008; LeSage et al., 2010) and high-fat food (Nair et al., 2008). With respect to psychostimulants, OX₁ blockade does not appear to reduce cocaine self-administration under low effort (FR1) conditions (Smith et al., 2009b), but does affect self-administration under higher effort FR5 (Hollander et al., 2012) or progressive ratio (PR) schedules (Borgland et al., 2009).

These findings from self-administration studies suggest that orexin may not play a significant role in the primary rewarding effects of psychostimulants, but may modulate circuitry that drives the motivated behaviour (Borgland et al., 2009; Boutrel et al., 2010) (but see Ho and Berridge (2013) for evidence that orexin mediates hedonic ‘liking’ of natural reinforcers). Interestingly however, orexin signaling does appear to modulate the rewarding properties of psychostimulants under some experimental conditions, as treatment with SB-334867 reduces the acquisition and expression of cocaine-conditioned reinforcement and the expression of amphetamine-induced CPP (Hutcheson et al., 2011). A clear role for OX₁ signaling has also been shown in the development of behavioural sensitisation (see Glossary) to psychostimulants. For example, SB-334867 treatment prevents sensitization following repeated cocaine and amphetamine treatment (Borgland et al., 2006; Quarta et al., 2010). In comparison, whereas OX₂ signaling appears to primarily mediate wakefulness and arousal and play less of a role in
mediating reward seeking, infusions of orexin B (presumably acting on OX<sub>R2</sub>) into the VTA increases preference for morphine (Narita et al., 2006b), and repeated cocaine exposure produces an up-regulation of OX<sub>R2</sub> levels in the NAC (Zhang et al., 2007). It is also worth highlighting recent evidence that blockade of the OX<sub>R2</sub> can prevent ethanol self-administration, CPP and reinstatement of extinguished CPP (Shoblock et al., 2011), but not cue-induced reinstatement of ethanol seeking in a self-administration paradigm (Brown et al., 2013).

With respect to drug-seeking behaviour, activation of orexin cells, as assessed by Fos-protein, has been associated with reinstatement of drug seeking using different procedures. Importantly, Harris et al (2005) showed that re-exposure to cocaine- and morphine- associated contexts in a CPP paradigm increased the percentage of LH orexin neurons that express Fos. Further, the proportion of Fos-positive LH orexin neurons was strongly correlated with the propensity for reinstatement of a CPP. These authors also provided evidence that Fos-protein expression increased specifically within LH orexin neurons, as correlations between Fos expression and reinstatement of CPP was not observed with PF or DMH orexin neuron populations, nor for non-orexin neurons within the LH (Harris et al., 2005). In contrast, Dayas et al. (2008) showed in an operant self-administration reinstatement procedure, that re-exposure to alcohol-associated cues increases the numbers of DMH and PF/LH Fos-positive orexin neurons. Further, Hamlin et al. (2007) showed that renewal of cocaine-seeking behaviour is associated with increased Fos-protein induction in DMH and PF regions, but that the majority of renewal-associated Fos expression in the LH is restricted to orexin-negative cells. These findings are interesting given the proposal that different roles may exist for DMH/PF (arousal) vs LH (reward) orexin cell populations (Harris and Aston-Jones, 2006), and point to the need for further understanding of how these different populations may differentially influence drug-seeking behaviour.

0.2.3.3 Orexin and reinstatement behaviour

In order to confirm a functional role for orexin cell activation in reinstatement of CPP, Harris and colleagues (2005) microinfused the excitatory Y4 receptor agonist rat
pancreatic polypeptide (rPP) directly into the LH. As predicted by the expression of Y4 receptors on orexin cells, this manipulation induced Fos expression in LH orexin neurons and robustly reinstated extinguished morphine CPP. A functional role for orexin signaling in psychostimulant reinstatement has also been well established by experiments using operant-based procedures. In a key study, Boutrel and colleagues (2005) demonstrated that i.c.v. infusion of orexin-A reinstated extinguished cocaine-seeking and that the OX_R1 antagonist SB-334867 reduced reinstatement elicited by exposure to acute footshock stress. Similarly, Lawrence et al. (2006) found that pretreatment with SB-334867 (i.p.) abolished olfactory cue-induced reinstatement of alcohol-seeking behaviour. Since these studies, the orexin system has been shown to mediate various forms of reinstatement. For example, systemic administration of the OX_R1 antagonist SB-334867 (10-30mg/kg) blocks reinstatement of extinguished cocaine-seeking elicited by and discrete cues (Smith et al., 2009b) and discriminative contexts previously associated with cocaine availability (Smith et al., 2010). These findings extend to non-extinguished cocaine seeking, as SB-334867 pretreatment (10-30mg/kg) attenuates drug seeking in cocaine-associated contexts following two weeks of abstinence in the home cage (Smith et al., 2010).

The orexin system also appears to interact with stress pathways to regulate footshock-induced reinstatement (Boutrel et al., 2005). Specifically, in this paradigm, administration of the corticotropin-releasing factor (CRF) anatagonist D-Phe-CRF (12-41) and the α2-noradrenergic agonist clonidine prevents reinstatement induced by i.c.v. orexin, suggesting that orexins may mediate footshock-induced reinstatement by recruiting these stress systems. Importantly, antagonism of OX_R1 does not appear to block the priming effects of psychostimulants on reinstatement behaviour, as pretreatment with SB-334867 (10-30mg/kg, i.p.) had no effect on reinstatement of cocaine-seeking induced by an acute cocaine injection (Smith et al., 2009a). These findings would appear consistent with the previously described evidence that orexin signalling does not play a role in mediating the primary rewarding effects of cocaine.

Taken together, there is strong evidence to suggest that orexin signalling is important in mediating reinstatement of cocaine-seeking behaviour elicited by drug-related cues and
stress, but not cocaine prime. *Importantly however, these conclusions have been drawn from studies that have made use of systemic (peripheral) administration of the orexin receptor antagonist SB-334867. Indeed, no attempt has been made to identify where in the brain orexin acts to mediate reinstatement behaviour, despite the fact that a great deal is known about the projection patterns of orexin neurons and the distribution of orexin receptors.*

0.2.4 Hypothalamic neuropeptides and drug-seeking behaviour: Summary

Since their discovery almost fifteen years ago, significant evidence has accumulated implicating CART and orexin in regulating motivated behaviour. Early studies generally showed opposing roles for these peptides in feeding behaviour, and subsequent studies suggest that a similar relationship may exist for drug-seeking behaviour. That is, the majority of studies suggest that CART negatively regulates the effects of drugs, whereas orexin generally has pro-drug seeking effects. With respect to reinstatement behaviour, recent evidence has shown that CART-expressing cells are activated by alcohol-associated cues, and that central CART administration can inhibit context-induced reinstatement of alcohol seeking. *At present however, it is unclear whether findings implicating CART in cue/context-induced reinstatement of alcohol seeking extend to cocaine or other priming modalities (such as drug-primed reinstatement). It is also unclear whether/how CART acts in the brain to negatively regulate reinstatement behaviour.* With respect to orexin, studies have demonstrated a role for this peptide in both cue- and stress- (but not drug-prime) induced reinstatement of various drugs of abuse, including cocaine. *These studies however, have primarily utilised systemic injections of orexin receptor antagonists, meaning that the site of orexin signalling in mediating reinstatement behaviour is unknown.*

Central to this thesis therefore, is the identification of potential candidate regions through which CART and orexin may influence reinstatement behaviour. Particular attention was given to the paraventricular thalamus, as this structure has previously been highlighted as a key target of hypothalamic neuropeptide activity with strong projections to brain regions involved in drug seeking (Kelley et al., 2005). The ventral tegmental area was also investigated as a potential target of orexin signalling, in light of
recent evidence that orexin signalling potentiates dopamine release (Korotkova et al., 2003). This evidence is discussed below.
0.3. Potential central sites of action of CART and orexin

0.3.1 The Paraventricular Thalamus (PVT)

The paraventricular thalamus (PVT), a component of the dorsal midline thalamic group (Groenenwegen & Berendse, 1994), has received significant interest in recent years in relation to its role in drug-seeking behaviour. This has been prompted, at least in part, by an influential proposal by Kelley and colleagues (2005) that argued that the PVT is a key relay in a hypothalamic-thalamic-striatal axis that regulates motivation and reward-seeking behaviour. This proposal was based on anatomical evidence that the PVT is densely innervated by peptide-expressing neurons originating from the hypothalamus (Kirouac et al., 2005; Kirouac et al., 2006; Parsons et al., 2006), as well as midbrain dopamine and brainstem catecholamine neurons (Van der Werf et al., 2002). In fact, the PVT receives more CART and orexin peptide inputs than any other forebrain structure (Kirouac et al., 2005; Kirouac et al., 2006; Parsons et al., 2006), and it appears that this information is relayed to important reward-related regions. For example, Parsons et al. (2007) showed that NACsh-projecting neurons in the PVT are innervated by both CART and orexin terminals (see Figure 3). The PVT also projects to other important substrates of the reinstatement circuitry, including the prefrontal cortex (PFC) and basolateral amygdala (BLA) (Berendse and Groenewegen, 1991; Moga et al., 1995; Bubser and Deutch, 1998; Otake and Nakamura, 1998; Parsons et al., 2007; Li and Kirouac, 2008; Vertes and Hoover, 2008; Hamlin et al., 2009). Interestingly, single PVT neurons have been shown to send branched projections to both PFC and NACsh, suggesting that the PVT can stimulate both of these regions simultaneously (Bubser and Deutch, 1998; Otake and Nakamura, 1998). Together, these anatomical findings point to the PVT as a strong candidate as a potential site of action for both CART and orexin in the regulation of drug-seeking behaviour, including reinstatement.

It is noteworthy that early studies suggested an important role for the PVT in drug-seeking behaviour. For example, Young & Deutch (1998) showed that lesions of the PVT blocked behavioural sensitisation to cocaine, whilst other studies showed that Fos protein is increased in the PVT following re-exposure to cocaine-paired
environments after experimenter administered drug injections (Brown et al., 1992; Franklin and Druhan, 2000). More recently, work by McNally and colleagues has implicated the PVT in alcohol-seeking behaviour. These authors showed that that Fos protein in the PVT is increased following reinstatement of alcoholic beer seeking (Millan et al., 2010) and that excitotoxic lesions of the PVT prevented renewal of beer seeking (Hamlin et al., 2009). Further, intra-PVT infusions of a kappa opioid receptor (KOR) agonist prevented reinstatement of beer seeking, suggesting that PVT KOR activation in this region may be important in the expression of alcohol-seeking extinction behaviour (Marchant et al., 2010). Whilst these studies provide strong evidence for a role for the PVT in alcohol-seeking behaviour, it is currently unclear whether these findings extend to the reinstatement of cocaine seeking.

Figure 3. The paraventricular thalamus (PVT) as a key relay in the drug-seeking circuitry. The PVT is densely innervated by CART- and orexin- expressing neurons as well as midbrain dopamine neurons. The PVT relays this input to key regions in the drug reward-seeking circuitry, including the prefrontal cortex and nucleus accumbens. In fact, single PVT neurons send branched projections to the prefrontal cortex and nucleus accumbens shell, thereby stimulating both regions simultaneously. Importantly, whilst not included in this Figure, strong evidence suggests nucleus accumbens shell-lateral hypothalamus projections are important for inhibiting drug seeking after extinction training (Millan et al., 2010). ARC: arcuate nucleus; LH: lateral hypothalamus; NAC: nucleus accumbens, PFC: prefrontal cortex; PVT: paraventricular thalamus; VP: ventral pallidum; VTA: ventral tegmental area.
In Chapters 1 and 2, I present evidence supporting the hypothesis that the PVT is an important component of the neural circuitry mediating cocaine reinstatement behaviour. In Chapter 1, I present evidence that Fos-protein expression, a marker of neuronal activation (Bullitt, 1990), is increased within the PVT in animals that exhibit robust reinstatement behaviour following the presentation of a cocaine-associated discriminative (S+) cue. I also show that a strong, positive correlation exists between PVT Fos expression and reinstatement behaviour. In Chapter 2, I show that inactivation of the PVT, using site-specific injections of the sodium channel blocker tetrodotoxin, attenuates drug-primed reinstatement of cocaine seeking. Together, these findings indicate that the PVT is involved in mediating both cue- and drug-induced reinstatement of cocaine seeking. Whilst not directly investigated in this thesis, the possibility that the PVT is also involved in stress-induced reinstatement is discussed in a published ‘Commentary’ article included in the appendix of this thesis (James et al., 2013; Appendix 1).

Despite strong anatomical evidence that the PVT is a possible site of action for both CART and orexin in their regulation of reinstatement behaviour, very few studies have explored this hypothesis directly. Dayas et al. (2008) showed that alcohol-associated cues activate PVT neurons that are innervated by CART and orexin terminals. More recently, unpublished findings from Martin-Fardon and colleagues suggest that at small doses, cocaine seeking can be reinstated by intra-PVT infusions of orexin-A (Martin-Fardon et al., 2010). Beyond these findings, no study has provided direct functional evidence that CART and orexin act in the PVT to regulate reinstatement of drug-seeking behaviour. Based on evidence outlined above that suggests CART and orexin play generally opposing roles in the regulation of drug-seeking behaviours, this thesis aims to address two important unanswered questions: 1) Does CART act in the PVT to negatively regulate the reinstatement of cocaine-seeking behaviour? If so, do infusions of CART into the PVT attenuate reinstatement behaviour? And conversely: 2) Does orexin act in the PVT to promote reinstatement of cocaine seeking? If so, do infusions of an orexin receptor-1 antagonist into the PVT attenuate reinstatement?
In Chapter 2, I show that infusions of CART peptide directly into the PVT attenuate drug-primed reinstatement of cocaine-seeking behaviour. Importantly, this effect was specific to the PVT, as misplaced infusions of CART into surrounding structures had no effect on reinstatement behaviour. In contrast, Chapter 3 shows that intra-PVT infusions of the orexin antagonist SB-334867 have no effect on cocaine reinstatement behaviour elicited by drug-associated discriminative ($S^*$) stimuli.

0.3.2 The Ventral Tegmental Area

Recent findings have highlighted the significance of orexinergic projections onto midbrain dopamine neurons. Although there are reportedly few ‘classic’ synaptic contacts between orexin axons and VTA dopamine neurons (Balcita-Pedicino and Sesack, 2007), both OX$_R$1 and OX$_R$2 have been identified in VTA dopamine cells (Narita et al., 2006a). Importantly, functional electrophysiological studies have convincingly shown that orexins modulate dopaminergic neuronal activity within the VTA (Korotkova et al., 2003; Moorman and Aston-Jones, 2010). For example, Korotkova et al. (2003) showed that orexins increase the firing rate of VTA dopamine neurons and can also elicit burst firing of these cells. These authors also showed that subpopulations of dopamine neurons appear to respond differentially to orexin A and B and express OX$_R$1, OX$_R$2, or both receptor subtypes. In work that described a potential pathway for orexins to enhance VTA dopamine neuron activity, Borgland et al., (2006) reported that orexin-A potentiates NMDAR currents in this population through protein-kinase C-dependent trafficking of NMDARs. Systemic SB-334867 has also been shown to block cocaine sensitisation and to elevate the AMPA/NMDA ratio in VTA DA neurons – a surrogate measure of synaptic plasticity (Kauer and Malenka, 2007).

Supporting these anatomical and physiological data, there is now a significant body of behavioural evidence to suggest that orexin signaling in the VTA plays a key role in mediating drug seeking. For example, intra-VTA injections of orexin-A reinstate morphine preference in a CPP model (Harris et al., 2005) as well as reinstate extinguished cocaine-seeking behaviour in a self-administration paradigm (Wang et al.,
2009). Moreover, intra-VTA infusions of SB-334867 suppress morphine CPP (Narita et al., 2006a). To date however, no study has investigated the role of endogenous orexin signalling in the VTA in a cocaine reinstatement model.

Chapter 3 provides evidence that supports a role for orexin receptor 1 signalling in the VTA in cocaine reinstatement behaviour. Using doses that had no effect when injected into the PVT, I show that intra-VTA infusions of SB-334867 block cue-induced reinstatement of cocaine seeking. Importantly, this treatment was not associated with a change in general locomotor activity, suggesting that the effect of SB-334867 on reinstatement was specific to drug-seeking behaviour. These findings are further explored in Chapter 4, where I show that intra-VTA SB-334867 administration altered the activity of key drug-seeking substrates, including the PVT. I also show that intra-VTA infusions of SB-334867 have no effect on reinstatement of responding for a natural reward, sweetened-condensed milk, suggesting that at low doses, SB-334867 can reduce drug seeking but not affect normal motivated behaviour. Finally, Chapter 4 summarises the findings from Chapters 1, 2 and 3 and discusses the implications of these findings in the context of the existing literature relating to the role of hypothalamic peptides in regulating drug-seeking behaviour.
0.4 Plasticity of hypothalamic neurons in response to drug exposure and other stressors

0.4.1 Plasticity of hypothalamic neurons

Despite evidence that CART- and orexin-expressing cells are involved in drug-seeking behaviours, very few studies have addressed how the functional properties of these neurons may be altered by exposure to drugs. Evidence from other fields suggests that orexin cells are ‘re-wired’ in response to other environmental stressors. For example, Horvarth & Gao (2005) showed that overnight food restriction increased the frequency of miniature post-synaptic currents in orexin neurons and promoted the formation of new excitatory, vesicular glutamate transporter 2 (VGLUT2)-positive inputs onto orexin neurons. Importantly, these changes were reversed entirely by re-feeding. Similarly, Rao et al. (2007) showed that sleep deprivation promotes plasticity at hypothalamic glutamatergic synapses, increasing both the firing frequency and amplitude of miniature excitatory post-synaptic currents (mEPSCs) in orexin neurons. Together, these studies indicate that orexin cells are ‘soft wired’ and undergo significant functional changes in response to stress, therefore highlighting the possibility that these cells may also be susceptible to drug-induced plasticity.

Our group has recently addressed the possibility that cocaine can modulate hypothalamic orexin circuitry by using anatomical and electrophysiological techniques (Yeoh et al., 2012; see Appendix 2). Rats given seven days of passive cocaine exposure exhibited increased excitatory, vesicular glutamate transported 2 (VGLUT2)-positive puncta closely apposed to orexin neurons, whereas vesicular γ-aminobutyric acid (GABA) transporter (VGAT)-positive inputs were unchanged. This finding was reinforced by electrophysiological studies that identified an increased frequency, but not amplitude, of miniature excitatory post-synaptic currents (mEPSCs) in PF/LHA of cocaine-exposed rats. Similar electrophysiological differences were observed in animals that were trained to self-administer cocaine for two weeks, when compared to rats that were trained to respond for food. Self-administered cocaine resulted in a reduced paired-pulse ratio of evoked excitatory inputs, but had no effect on AMPA/NMDA
ratio, indicative of a presynaptic locus for synaptic plasticity. Importantly, these electrophysiological effects were observed in a sub-population of cells that were confirmed as orexin-positive, supporting the hypothesis that orexin cells are susceptible to drug-induced plasticity. Whilst not addressed directly in this study, it is possible that changes in excitatory input onto orexin cells in long-term cocaine users may increase the responsivity of these cells to drug, drug cues or stress, contributing to an increased risk of relapse in these individuals.

The vulnerability of LH circuits to environmental and pharmacological insults is interesting in light of recent evidence that dysregulated orexin signaling is associated with psychiatric conditions such as depression and anxiety. As discussed below, animal studies generally show that acute stress upregulates the activity of the orexin system and produces increased anxiety-like behaviour, whereas chronic stress is associated with a hypoactive orexin system and depression-like behaviours. It is noteworthy that recent clinical data tends to support these observations in animals, with heightened orexin activity observed in patients with anxiety disorders whereas low orexin activity reported in depressed patients.

These findings are also interesting given that the rate of depression is significantly increased amongst addicted individuals (Grant et al., 2004). A factor that may link vulnerability to depression and addiction is exposure to early life emotional stress (ELS), as this form of environmental insult is a strong predictor of the development of both pathologies (Agid et al., 1999; Kendler et al., 2000). To date however, little is know about the long-term effects of ELS on orexin function and the reactivity of this system to psychological stress in adulthood. As a first step towards understanding the possible link between altered orexin function produced by ELS and the expression of addiction or depression in later life, the final Chapter (Chapter 5) mapped the expression of stress-induced Fos-protein in orexin neurons in animals exposed to ELS versus controls. Because exercise is known to increase stress coping, we also assessed the effects of exercise on orexin system function in ELS animals. Below is a summary of the evidence implicating dysregulated orexin function in depression and anxiety pathology, as well as an overview of the experimental model of early life stress (maternal separation) that was utilised in this Chapter.
0.4.2 Orexins and depression

Clinical studies have highlighted a potential role for dysregulated orexin signalling in the aetiology of depression. For example, Salamon and colleagues (Salomon et al., 2003) reported that the peak amplitude of orexin-A CSF levels across the diurnal cycle is significantly reduced in depressed patients compared to healthy controls, but when averaged across a 24hr period, orexin CSF levels were increased in depressed patients. Interestingly, this increase in average CSF orexin levels was reduced by five weeks of treatment with the antidepressant sertraline. Similarly, Palhagen et al. (2010) showed that CSF orexin-A levels were higher in depressed patients when compared to patients with Parkinson’s disease without depression. Treatment with the antidepressant citalopram tended to decrease the CSF level of orexin-A in depressed patients.

In contrast, recent studies by Brundin and colleagues report reduced orexin levels in depression. In their first study, these authors examined orexin-A CSF levels from patients admitted to a psychiatric hospital after a suicide attempt. They showed that the level of orexin-A was negatively correlated with two psychiatric symptoms of the Comprehensive Psychopathological Rating Scale (CPRS; lassitude and slowness of movement), as well as the ratings of global illness (Brundin et al., 2007b). In a separate study a few months later, these authors reported that orexin-A CSF levels were significantly lower in suicide-attempters diagnosed with depression as compared to suicide-attempters with adjustment disorders or dysthymia (Brundin et al., 2007a). Interestingly, amongst all patients, there was a positive correlation between orexin-A and CRF levels. In a follow-up study, Brundin and colleagues (2009) reported that at 6 and 12 months following the suicide attempt orexin-A levels had increased and that these increases were associated with significantly lower scores on the CPRS rating of global illness. It is noteworthy however, that some patients received pharmacological treatment for their condition whereas others did not, and the number of patients that participated in the follow-up study was low (n=4 at 6 months, n=5 at 12 months). Consistent with these findings, Rotter et al. (2011) reported a trend towards decreased orexin A mRNA expression in the blood of depressed patients upon admission to
hospital, as well as a negative correlation between orexin-A levels and the severity of symptoms observed.

Importantly, there are also two published reports that failed to observe evidence of dysregulated orexin levels in depression. Schmidt and colleagues (2010) found that orexin-A CSF levels in patients with major depression did not differ significantly to manic patients or healthy controls. These same authors showed in a separate study that orexin-A CSF levels were similar in patients with unipolar disorder (without psychiatric comorbidities) and healthy controls (Schmidt et al., 2011). Importantly, in both studies, patients were medicated with a range of antidepressants and sample sizes were relatively small.

Dysregulated orexinergic signalling has also been observed in studies utilising experimental models of depression. For example, Lutter and colleagues (2008) utilised the chronic (21d) social defeat model to induce depressive-like behaviour, as indicated by reduced sucrose preference and increased immobility in the forced swim test. Social defeat stress was associated with reduced expression of prepro-orexin mRNA and orexin cell numbers in the lateral hypothalamus. Consistent with this finding, Nocjar and colleagues (2012) also reported reduced orexin mRNA in the hypothalamus of animals exhibiting depressive-like behaviour following chronic social defeat. These authors also showed that social defeat was associated with reduced orexin peptide levels in a number of stress-related regions, including the VTA and PFC.

In contrast, Nollet and colleagues showed that unpredictable chronic mild stress-(UCMS) induced depression-like behaviour, as assessed by the resident-intruder and tail suspension tests, is associated with increased activity of orexin cells in the PF (Nollet et al., 2011). Interestingly, chronic treatment with almorexant, a dual orexin-receptor antagonist, was shown to have an anti-depressant effect on the tail suspension test. In a subsequent study, these authors reported that chronic almorexant treatment was also able to reverse UCMS-induced dysregulation of the HPA axis, as measured by corticosterone levels and Fos protein expression in the PVN following dexamethasone exposure (Nollet et al., 2012). It is difficult to speculate why the relationship between
orexin activity and depression-like behaviour in these studies is opposite to those of Lutter et al. (2008), however it is possible that this may be related to the type (UCMS vs social defeat) and duration (8 vs 3 weeks) of stress exposure.

Allard et al. (2004) examined orexin cell expression in Wistar Kyoto rats (WKY), a selectively bred rat strain often used as a model of depression-like behaviour, as they display increased immobility on the forced swim test (often interpreted as learned helplessness) and dysregulated neuroendocrine functioning (Will et al., 2003). These authors reported that WKY rats had 18% fewer orexin immunoreactive neurons than control Wistar animals, and that these cells were, on average, 15% smaller than in controls. This finding was consistent with an earlier study by Taheri et al. (2001), who reported lower prepro-orexin mRNA expression and orexin-A levels in various brain areas of WKY rats. Interestingly, the Flinders Sensitive Line of rats, which also exhibit increased immobility in the forced swim test (Mikrouli et al., 2011), appear to have larger numbers of orexin immunoreactive neurons. Further study is required to compare the depressive-like behavioural phenotype of WKY versus FSL rats in order to understand the significance of these findings.

Taking both clinical and preclinical studies together, it is clear that findings from studies investigating the involvement of orexin signalling in depressive symptomology are somewhat conflicting. Whilst early clinical studies suggested that orexin levels are elevated in depressive patients, more recent reports indicate that orexin activity may in fact be reduced in these patients. Similarly, genetic and experimental animal models of depression have shown that depressive-like behaviour is associated with either hyper- or hypoactivity of the orexinergic system. This discrepancy in the literature perhaps reflects the heterogeneity of depression, in that not all depressed individuals share the exact same set of symptoms. Indeed, depending on the depression subtype (melancholic, atypical etc), it is possible for patients to report insomnia or hypersomnia, weight loss or weight gain, and increased or decreased HPA axis activity (Nollet and Leman, 2013). These findings may also reflect the fact that depression is commonly associated with comorbid anxiety disorders, which are typically associated with increased orexinergic signalling (discussed below). Indeed, it seems plausible that
unipolar depression may be associated with hypoactive orexin signalling, whereas the orexin profile in depressed patients with comorbid anxiety may be more variable depending on the relative severity of each disorder. It is therefore important that both clinical and preclinical studies seek to further explore the relationship between orexin system activity and specific depressive-like and comorbid symptomologies.

0.4.3 Orexins and anxiety

Johnson and colleagues (2010) reported that orexin-A CSF levels are elevated in patients with increased anxiety and panic associated symptoms compared to healthy controls. Interestingly however, amongst those patients that reported that their panic state was accompanied by depressive-like symptomology, orexin levels tended to be lower than healthy controls. This finding is consistent with the findings outlined above pointing to the possibility that depressive symptomology, depending on its severity, may be associated with reduced orexin function in patients with comorbid psychiatric disorders. A separate study by Strawn and colleagues (2010) examined levels of orexin-A in both CSF and plasma in patients with combat-related posttraumatic stress disorder (PTSD). CSF and plasma orexin-A concentrations were significantly lower in PTSD patients, as compared to healthy controls, and CSF orexin-A levels strongly and negatively correlated with PTSD severity as measured by the Clinician-Administered PTSD scale. It is interesting that the orexin profile in PTSD patients is opposite to those with panic disorder, particularly given that patients in the PTSD population had no signs of clinical depression at the time of the study. These findings are likely to reflect the heterogeneity of anxiety disorders. For example, PTSD is generally characterized by hypocortisolism (Rohleder et al., 2004) whereas some studies suggest that cortisol levels are elevated in panic patients (Abelson et al., 2007), and orexin is known to have an excitatory effect on HPA axis activity (Ida et al., 2000; Winsky-Sommerer et al., 2004). It will be important for future studies to examine orexin levels in other anxiety disorders, including generalized anxiety disorder and obsessive-compulsive disorder, so that the role of orexin in neuroendocrine control and anxiety behaviour can be better understood.
Animal models have provided significant insight into the role that orexin plays in regulating anxiety-like behaviour. In a recent series of studies, Shekhar and colleagues have firmly implicated PF/DMH orexin cells in the expression of panic-like behaviour. These authors showed that administration of the inverse benzodiazepine agonist FG-7142, which induces panic-like behaviour, increases orexin neuronal activity in the PF/DMH (Johnson et al., 2012a). In a separate study, these authors increased the activity of orexinergic cells through chronic reduction of GABA synthesis in the PF/DMH of rats using site-specific injections of 1-allylglycine (1-AG). This produced anxiety-like behaviours, as assessed by social interaction and elevated plus maze tests, and a vulnerability to panic-like states following i.v. infusions of sodium lactate (Johnson et al., 2010). Importantly, these panic-like states were prevented by systemic pretreatment with either SB-334867 or another OX<sub>R1</sub> antagonist SB408124 (Johnson et al., 2010).

The findings of Shekhar and colleagues are consistent with earlier work showing that exposure to anxiogenic/stressful stimuli including footshock (Harris and Aston-Jones, 2006) or exposure to a novel environment (Furlong et al., 2009) increase Fos-protein immunoreactivity in orexin cells. Similarly, exogenous administration of orexin-A can induce anxiety-like behaviour. For example, Suzuki et al. (2005) showed that i.c.v. injections of orexin-A increase anxiety-like behaviour in the light/dark exploration and the elevated plus maze (EPM) apparatuses. Similarly, Longwitz (2012) reported that infusions of orexin-A directly into the BNST increases anxiety-like behaviour on the EPM and in social interaction tasks, whilst Li et al. (2010) showed that intra-PVT orexin elicits anxiety-like behaviour on the EPM. Interestingly however, orexin receptor antagonists do not affect anxiety-like behaviours under basal conditions. For example, systemic injections of either SB-334867 (Rodgers et al., 2013) or almorexant (Steiner et al., 2013) have no effect on the expression of anxiety-like behaviour on the EPM in animals with no history of stress exposure. In contrast however, increasing evidence suggests that orexin antagonists have anxiolytic-like effects when anxiety levels are exacerbated. Using their model of panic-prone rats, Johnson et al. (2012a) showed that pretreatment with SB-334867 attenuates the anxiogenic effects of FG-7142, including anxiety-like behaviour, elevated heart rate and neuronal activation in panic-related...
centres. These authors also reported that systemic SB-334867 attenuates sodium-lactate induced anxiety-like behaviour and elevated heart rate and blood pressure in panic prone rats (Johnson et al., 2010), as well as anxiety responses in response to hypercapnic gas exposure (Johnson et al., 2012b). Finally, Li and colleagues (2010) showed that intra-PVT administration of the OX_{R2} antagonist TCSOX229 attenuates the anxiogenic effects produced by previous exposure to footshock stress.

In summary, both clinical and animal studies point to a role for hyperactive orexin signaling in panic disorder. Orexin CSF levels are elevated in panic patients, and manipulations that increase the activity of PF/DMH orexin cells produces a vulnerability to a panic-like phenotype. In addition to these studies, other investigations have demonstrated that exogenous application of orexin results in increased anxiety-like behaviour on a variety of behavioural assays, including the open field (OF), EPM and social interaction tests. Interestingly, orexin antagonists only prevent anxiety-like behaviour in panic-prone animals or in animals with a history of stress exposure. These findings suggest that endogenous orexin signaling plays a minor role in stress behaviour under normal conditions, but may become more involved when baseline anxiety levels are elevated, such is the case in anxiety disorders. A better understanding of this area will likely be achieved through studies that build upon studies focused on panic disorder by utilizing alternative animal models of anxiety-like behaviour. It is also important that further investigation of orexin function is carried out in cases where anxiety is comorbid with other psychiatric disorders, including depression.

0.4.4 Maternal separation as an experimental model of stress-related psychopathology

Evidence from a variety of studies suggests that exposure to adverse events in early life is a major risk factor for the development of mental disorders in adulthood, particularly depression and anxiety (Famularo et al., 1992; Pelcovitz et al., 1994). Perhaps representative of many other studies, a community-based study of almost 2000 adult women revealed that those with a history of childhood sexual or physical abuse exhibited more symptoms of depression and anxiety, as well as more frequent suicide attempts, as compared to non-abused women (McCauley et al., 1997). Similarly, early parental loss is associated with unipolar and bipolar depression, as well as anxiety.
disorders (Kendler et al., 1992; Kendler et al., 1993; Agid et al., 2000), and prenatal stress has been related to an increased risk for major depression in adulthood (Hulshoff Pol et al., 2000). Moreover, early life stress, including childhood sexual abuse, has been strongly associated with increased risk of substance abuse disorders (Agid et al., 1999; Kendler et al., 2000).

Preclinical studies using rodents have contributed significantly to our understanding of the behavioural and neural consequences of early life stress. One of the most widely used models of early life stress is the maternal separation model, whereby rat pups are repeatedly separated from their mothers during the neonatal period. A large number of studies have shown that maternal separation results in an increase in depressive-like behaviours, as determined in the forced swim (El Khoury et al., 2006), tail suspension and sucrose preference tests, and also increased anxiety-like behaviours on the EPM and OF apparatuses (Ladd et al., 2000; Daniels et al., 2004). Maternal separation is also associated with dysregulated basal levels of ACTH, corticosterone, and hypothalamic CRF mRNA (Plotsky and Meaney, 1993; Slotten et al., 2006), as well as altered expression of hippocampal mineralocorticoid (MR) and glucocorticoid (GR) receptors (Meaney et al., 1996; Ladd et al., 2004; Maniam and Morris, 2010). Moreover, animals exposed to maternal separation stress exhibit increased cell death in the hippocampus and cerebral cortex (Zhang et al., 2002; Mirescu et al., 2004) which reflect structural changes seen in depressed patients (Bremmer et al., 2000; Sheline et al., 1999; Coffey et al., 1992; Drevets et al., 1997; Krishnan et al., 1992; Husain et al., 1991). Consistent with these findings, maternally separated animals exhibit greater behavioural and neuroendocrine abnormalities when challenged with an additional stressor, such as acute restraint stress, in adulthood (Uchida et al., 2010; Marias et al., 2008; Veenema et al., 2006; Daniels et al., 2004).

In light of evidence that stress results in persistent alterations in orexin function, it seems plausible that maternal separation stress may result in a dysregulation of the orexinergic system, and that these changes are associated with the depressive- and or an anxiety-like phenotype often observed in separated animals. Indeed, a study by Feng et al. (2007) showed that maternal separation stress is associated with changes in
orexinergic signalling, as evidenced by increased basal hypothalamic orexin-A peptide expression. To date however, no study has directly explored the link between increased stress-related behaviour in animals exposed to maternal separation stress and aberrant orexinergic signalling.

In Chapter 5, I present evidence that orexin cell functioning is significantly impaired in animals exposed to maternal separation stress and that these changes are associated with dysregulated stress-related behaviour. Specifically, I show that separated animals exhibit hypoactivity of the orexinergic system when challenged with an additional stressor (restraint stress) in adulthood. These animals also exhibit reduced activation of stress-related regions in the brain, including the PVT, as well as reduced activation of putative dopamine cells within the VTA. These neural deficits were accompanied by changes in stress-related behaviour, as evidenced by reduced exploratory behaviour in the open field apparatus, possibly reflective of depressive-like behaviour. Interestingly, access to voluntary wheel running protected against the neural and behavioural effects of maternal separation in male rats, whereas wheel running tended to exacerbate these effects in female rats. Together, these findings suggest that early life stress results in persistent alterations on orexin cell function, resulting in dysregulated neural and behavioural responses to a subsequent stressor in adulthood. This Chapter discusses the implications of these findings in the context of the development of psychopathology and highlights the possibility that altered functioning of the orexin system may underlie the increased rates of anxiety- and depression-like pathologies in individuals with substance abuse disorders.
0.5 References


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CHAPTER ONE

Propensity to ‘relapse’ following exposure to cocaine cues is associated the recruitment of thalamic and epithalamic nuclei
**CHAPTER ONE**

**PROPENSITY TO ‘RELAPSE’ FOLLOWING EXPOSURE TO COCAINE CUES IS ASSOCIATED WITH THE RECRUITMENT OF SPECIFIC THALAMIC AND EPITHALAMIC NUCLEI**

*Morgan H. James*, Janine L. Charnley, Jamie R. Flynn, Doug W. Smith & Christopher V. Dayas

*Neuroscience (2011) pp 235-242*

Statement I: Author contribution to Chapter 1 manuscript

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CHAPTER ONE

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PROPENSITY TO ‘RELAPSE’ FOLLOWING EXPOSURE TO COCAINE CUES IS ASSOCIATED WITH THE RECRUITMENT OF SPECIFIC THALAMIC AND EPITHALAMIC NUCLEI

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Abstract—The thalamus is considered an important interface between the ventral striatopallidum and the dorsal striatum, and may therefore contribute to compulsive drug-seeking behaviour. Recent evidence suggests that the paraventricular thalamus (PVT), a dorsal midline thalamic nucleus, and the mediodorsal thalamus (MD) are involved in drug self-administration and respond to drug-associated cues. At present, however, the role of these thalamic regions in mediating cue-induced reinstatement of cocaine-seeking is unclear. Similarly, the habenula complex, part of the epithalamus, has been implicated in nicotine self-administration and cue-induced reinstatement of heroin seeking, but the role of this region in cocaine reinstatement behaviour has received little attention. Rats (n=20) were trained to self-administer cocaine in the presence of discriminative stimuli associated with drug availability (S⁺) or drug non-availability (S⁻). Once a stable level of responding was reached, lever pressing was extinguished. Animals were then tested for reinstatement and sacrificed immediately following the presentation of either the S⁺ or S⁻ discriminative stimuli, and Fos-protein expression was assessed in the thalamic and epithalamic regions. Interestingly, significant variation was observed in reinstatement behaviour, allowing a comparison between high-reinstating (HR), low-reinstating (LR) and control animals. Compared with LR animals, HR animals exhibited increased Fos-protein expression in the PVT, mediodorsal thalamus and the medial and lateral divisions of the habenula. Our data provide evidence that activation of thalamic and epithalamic nuclei is associated with propensity to reinitiate to cocaine-seeking elicited by drug-related cues. We also build upon existing data highlighting the importance of the PVT in reinstatement behaviour.

Key words: addiction, cocaine, paraventricular thalamus, rat, relapse.

The prevention of relapse is considered critical to the successful treatment of addiction. A known risk factor for triggering drug-seeking and elevating relapse risk is re-exposure to environmental stimuli linked with prior drug taking. In this regard, significant advances in our knowledge of the brain pathways that control relapse-like behaviour have been made, particularly through the use of preclinical, that is, animal studies. Brain regions that are thought to be involved in relapse-like behaviours and have been revealed through these experimental approaches include the medial prefrontal cortex, nucleus accumbens, ventral pallidum and midbrain A10 (ventral tegmental area; VTA) dopamine cells (for review, see McFarland and Kalivas, 2001; McFarland et al., 2004; Feltenstein and See, 2008; Kalivas and O’Brien, 2008). However, a more comprehensive understanding of the brain regions involved in mediating relapse-like behaviour is presumably required before efficacious pharmacotherapies can be successfully developed for the prevention of psychostimulant relapse.

The thalamus is considered an important interface between the ventral striatopallidum and the dorsal striatum, and may therefore contribute to the development of compulsive drug-seeking behaviour (Pierce and Vanderschuren, 2010). One thalamic nucleus that is of particular interest is the paraventricular thalamus (PVT), a component of the dorsal midline thalamic group (Groenewegen and Berendse, 1994). The PVT has been implicated in stress reactivity (Spencer et al., 2004), reward (Kelley et al., 2005) and general arousal (Hernandez et al., 2005). Interestingly, several recent studies have shown that the PVT might play a role in drug-seeking and relapse-like behaviour. For example, re-exposure to an alcohol self-administration environment, or presentation of discriminative stimuli previously linked with alcohol reward, increases the activation of neurons within the PVT (Wedzony et al., 2003; Dayas et al., 2008). Furthermore, inactivation of the PVT prevents context-induced reinstatement of alcohol-seeking (Hamlin et al., 2009) and cocaine-primed reinstatement (James et al., 2010). These findings are consistent with those of earlier reports using experimenter-administered drug injections demonstrating that re-exposure to a cocaine-paired environment activates PVT neurons (Brown et al., 1992; Franklin and Druhan, 2000) and that PVT lesions block cocaine sensitization (Young and Deutch, 1998). However, it is not known whether the PVT responds to cues linked with cocaine availability in a reinstatement model of drug-seeking. Such a response would support a hypothesis that the PVT is a common site of activation in response to multiple drug relapse cues and drug classes (Dayas et al., 2008; Hamlin et al., 2009).

Apart from the literature pertaining to the PVT, there is only limited evidence that other thalamic and epithalamic structures are involved in mediating cocaine relapse. For...
example, the mediiodorsal thalamus (MD) has been shown to be important for reward-associated learning assessed using conditioned place preference (CPP; McAlonan et al., 1993). Further, the habenula has been implicated in reward-based decision making (Hikosaka, 2010), and a recent study showed that the medial habenula plays an important role in the regulation of nicotine intake (Fowler et al., 2011). Activation of the lateral habenula is associated with cue-evoked heroin-seeking (Zhang et al., 2005) as well as reinstatement of cocaine-seeking in a conditioned place preference paradigm (Brown et al., 2010). The role of habenula nuclei in cocaine cue-induced relapse-like behaviour has, to our knowledge, not been reported.

The aim of this study was therefore threefold. Firstly, given the lack of studies assessing the role of thalamic and epithalamic structures in reinstatement behaviour, we sought to map Fos-protein expression within these regions following presentation of drug-associated cues. Thalamic structures included the dorsal midline thalamic group (PVT and ventromediodorsal thalamus, VMd) and the mediiodorsal thalamus, whereas epithalamic structures included the medial and lateral divisions of the habenula complex. Secondly, to understand the role that these structures play in relapse propensity, Fos expression was compared across animals that showed high- versus low-reinstatement behaviour. Finally, we sought to build upon existing literature implicating the PVT in cue-induced reinstatement of cocaine-seeking.

EXPERIMENTAL PROCEDURES

Subjects
Male Sprague-Dawley rats (Central Animal House, University of Newcastle, NSW, Australia; weighing 200–250 g upon arrival) were housed two per cage on a reverse 12-h light/dark cycle (lights off at 7:00 AM) with ad libitum access to food and water. All procedures were performed in accordance with protocols approved by the University of Newcastle Animal Care and Ethics Committee, New South Wales Animal Research Act.

Drugs
For cocaine self-administration, cocaine hydrochloride (Johnson Matthey, Edinburgh, UK) was dissolved in sterile physiological saline (2.5 mg/ml) as per previous studies (Brown et al., 2011; James et al., 2010, 2011). During self-administration, cocaine or saline was infused intravenously at a volume of 0.1 ml over 4 s.

Surgery
Rats (n=20, 250–300 g) were anaesthetized with isoflurane (1–3% with a flow rate of 2 L/min) and, using aseptic procedures, a Silastic catheter was surgically implanted into the right jugular vein as described in detail previously (Brown et al., 2011; James et al., 2010, 2011). Before surgery, rats were injected intramuscularly with 0.5 ml of a broad-spectrum antibiotic (150 mg/ml procaine penicillin, 112.5 mg/ml benzathine penicillin; Norbrook Laboratories, UK) and subcutaneously with 0.2 ml of a 50 mg/ml solution of carprofen (Norbrook Laboratories, UK). Post-surgery, catheter lines were flushed with 0.3 ml of 50 mg/ml cephalixin (Mayne Pharma, Australia). Rats were allowed 7 days’ recovery before starting cocaine self-administration training. To ensure catheter patency throughout the course of the experiment, catheters were flushed daily with 0.2 ml of 50-unit heparinized saline.

Behavioural testing equipment
Behavioural procedures were conducted in standard operant conditioning chambers located inside sound-attenuating, ventilated cubicules (Med Associates, VT, USA). Chambers were equipped with two retractable levers (6 cm above the floor), white cue lights (one above each lever), two speakers to deliver auditory stimuli and a house light located at the top of the chamber wall opposing the levers. The auditory stimuli were produced by (1) a white noise generator/speaker adjacent to the house light (0.5 Hz at 70 dB), a syringe pump (5-rpm motor, Med Associates) located outside the sound-attenuating cubicule delivering the cocaine and saline vehicle solutions. Data acquisition and behavioural testing equipment were controlled by a Windows-based PC, running MED-PC IV software (Med Associates).

Self-administration training, conditioning and extinction
Seven days after surgery, rats were trained to self-administer cocaine in 3-h sessions conducted daily for 5 days per week. Responding on the active (right) lever resulted in a 4-s infusion of cocaine (0.25 mg cocaine/infusion) via the i.v. catheter and activation of a white cue light above the active lever that remained illuminated for 20 s, signalling a time out period. The inactive (left) lever was not extended during training. Training was continued until stable responding for cocaine was achieved (>70% over three sessions) at which time animals were subjected to daily 1-h randomised conditioning sessions for cocaine or saline infusions (FR1), in the presence of distinct discriminative stimuli. This involved, for cocaine (S+), a constant 70-dB white noise and illumination of the white cue light (20 s) above the active lever and, for saline (S−), constant illumination of the white house light and a 20-s intermittent beeping tone coupled with responding on the active lever (Martin-Fardon et al., 2009). Conditioning sessions were initiated by the extension of both levers and the introduction of the appropriate discriminative stimuli. Responding on the inactive lever was recorded but did not result in cocaine or saline infusions. After 16 days of conditioning (8 days of cocaine, 8 days of saline), lever responding was extinguished in daily 1-h extinction sessions until a criterion of ≤6 responses per session on the active lever over three consecutive days was achieved. During extinction, discriminative stimuli were withheld alongside with i.v. infusions.

Conditioned reinstatement testing
One day after animals met the extinction criteria, rats were presented with the S− discriminative stimuli (i.e. house light) under extinction conditions for 1 h. A subgroup of animals that were destined for Fos-expression analysis (n=6) remained in the operant chamber for an additional hour after the S− test and were then perfused as described later. All remaining animals (n=14) were tested for a recovery of responding with the S+ discriminative stimuli on the following day (i.e. 1 day following S− testing). This involved placing the animals in the operant chamber and presenting them with the S+ discriminative stimuli (i.e. white noise) for 1 h. As with the S− subgroup used for Fos-expression analysis, S− animals remained in the operant chamber for 1 h after the S+ test until they were sacrificed and perfused as described later. Responses on both the active and inactive levers were recorded during S− and S+ reinstatement tests.
Immunohistochemistry

Two hours after initiation of either the S^- or S^+ reinstatement test, animals were deeply anaesthetised with sodium pentobarbital (200 mg/kg i.p.) and transcardially perfused with 150 ml of normal saline followed by 500 ml of 4% paraformaldehyde (pH 7.4). Animals were perfused 2 h after commencing the reinstatement test consistent with previous reports (Zhao et al., 2006; Kovács, 1996). Brains were removed, post-fixed and cryoprotected (24 h, at 4 °C) in the same fixative solution with the addition of 10% sucrose. Brains were then stored in 15% sucrose in 0.1 M phosphate buffer (pH 7.4 at 4 °C). Serial 40-μm coronal sections of the forebrain were cut on a freezing microtome (Leica SM 2000R, Leica Biosystems, Germany) and a 1-in-4 series of sections were processed for immunohistochemical detection of Fos protein (72 h, 1:5000, rabbit polyclonal, Santa Cruz Biotechnology, CA, USA) as described previously and in detail by Dayas et al. (2008), in the PVT and IMD, the habenula (medial and lateral divisions) and the MD (medial and central/lateral divisions; see Fig. 1).

Analysis

Behavioural analysis. Reinstatement scores following presentation of both S^- and S^+ cues were calculated as the percentage change from extinction responding, and all subsequent analyses were carried out on these transformed data. Significant variability was observed with respect to reinstatement behaviour in S^-exposed rats. To examine potential differences in Fos-protein expression between animals that displayed heightened propensity to reinstatet compared with animals with a lower propensity to reinstatement, a median split was performed on the S^- responding under S^- extinction criterion being met after 22.3 ± 1.4 (mean ± SEM) days of training. During conditioning, the number of self-administered infusions was significantly higher (719.5 ± 86.69, P < 0.001) for cocaine compared with saline (Fig. 2a). After conditioning, animals underwent extinction training with the extinction criterion being met after 22.3 ± 3.7 (mean ± SEM) days. Total group data indicated that re-exposure to the cocaine-linked S^- produced a significant recovery of responding on the previously active lever, as compared with responding under S^- conditions (F(1,28) = 14.122, P < 0.01; Fig. 2a). Inactive lever responses during reinstatement tests did not differ from extinction levels (data not shown).

Patterns of Fos-protein expression in S^- versus S^+ animals

We first compared Fos-protein ‘group’ data, that is, counts across animals exposed to the S^- versus those exposed to the S^+ discriminative stimuli, without consideration of individual variability in reinstatement. Across the midline thalamic nuclei, a significant main effect of ‘group’ was observed (F(2,36) = 5.06, P < 0.05), and significantly greater Fos counts were observed across the PVT and IMD regions of S^- as compared with S^+ animals (P < 0.05, Tukey post hoc analyses). Similar results were observed across the mediodorsal thalamic nuclei (F(2,36) = 8.60, P < 0.01), where greater Fos counts were observed in the medial and central/lateral divisions (P < 0.01). The same trend was observed in the habenula (F(2,36) = 5.47, P < 0.05), where greater Fos counts were observed across the medial and lateral divisions of the S^- animals, as compared with the S^+ animals (P < 0.05).

Propensity to reinstate—high vs. low reinstaters

An analysis of S^- reinstatement data revealed substantial variability in propensity to relapse. As outlined in the Experimental procedures, a median split was made on the S^- reinstatement data to examine potential differences in Fos-protein expression between animals that displayed heightened propensity to relapse, compared with those with a lower propensity to reinstate. Thus, the ‘high-reinstating’ (HR) group (n = 6) and the ‘low-reinstating’ (LR) group (n = 8) differed significantly in their overall reinstatement

Fig. 1. Thalamic and epithalamic regions assessed for Fos-protein expression. Fos-protein expression was assessed in the dorsal midline thalamic group (paraventricular thalamus, PVT, and intermediodorsal thalamus, IMD), mediodorsal thalamic complex (medial portion, MDM, and lateral portion, LMD), and the habenula complex (medial portion, mHb, and lateral portion, LHb). Figure (bregma level = −2.80) adapted from (Paxinos and Watson, 1997).
in active lever responding that did not differ to baseline (extinction) responding. Presentation of the S' cue resulted in active lever responding during S' cocaine sessions as compared with saline sessions (†P < .01). Following extinction, presentation of the S' cue resulted in active lever responding that did not differ to baseline (extinction) responding. Presentation of the S' cue in the absence of drug reward produced a significant recovery of responding as compared with baseline (extinction) responding (†P < .01). Self-administration S' cue: average number of active lever responses over final three S' cocaine conditioning sessions. Self-administration S': average number of active lever responses over final three S' cocaine conditioning sessions. Self-administration EXT: average number of active lever responses over final three extinction sessions. Animals that fell below the median score were allocated to the ‘Low-Reinstaters’ (LR) group, whereas those that fell above the median score were allocated to the ‘High-Reinstaters’ group. Separation of high-reinstating animals from low-reinstating animals. To assess differences in animals exhibiting a high propensity for reinstatement versus those with a low propensity to reinstatement, a median split was performed on reinstatement scores. Animals that fell below the median score were allocated to the ‘Low-Reinstaters’ (LR) group, whereas those that fell above the median score were allocated to the ‘High-Reinstaters’ group. Reinstatement scores were significantly higher in the HR group than the LR group (*P < .05). Interestingly, the LR group did not differ significantly to the S' control group in terms of their reinstatement scores (n.s. P > .05).

Fig. 2.
(A) Self-administration and reinstatement testing. Throughout self-administration training, animals exhibited significantly higher levels of active lever responding during S' cocaine sessions as compared with S' saline sessions (†P < .01). Following extinction, presentation of the S' cue resulted in active lever responding during S' cocaine sessions as compared with saline sessions (†P < .01). Following extinction, presentation of the S' cue resulted in active lever responding during S' cocaine sessions as compared with saline sessions (†P < .01). Following extinction, presentation of the S' cue resulted in active lever responding during S' cocaine sessions as compared with saline sessions (†P < .01). Following extinction, presentation of the S' cue resulted in active lever responding during S' cocaine sessions as compared with saline sessions (†P < .01). Following extinction, presentation of the S' cue resulted in active lever responding during S' cocaine sessions as compared with saline sessions (†P < .01).

Patterns of neuronal activation—Fos-protein expression

Dorsal midline thalamic nuclei. When comparing numbers of Fos counts in the PVT and IMD between HR, LR and S' groups, there was a significant main effect of group (F(2,19) = 20.14, P < .05; Fig. 2b). Interestingly, the LR group did not differ from the S' control group in terms of their reinstatement scores (F(2,19) = 20.14, P > .05, Tukey’s post hoc test). Importantly, S', LR and HR groups did not differ in terms of time taken to reach stable responding during self-administration training or time taken to extinguish (P > .05). All subsequent analyses of Fos counts were conducted by comparing these groups (S', LR and HR groups).

Mediodorsal thalamus. A significant main effect of group was observed across the medial and central/lateral divisions of the MD (F(2,14) = 6.85, P < .01). In these regions, a significant difference was observed between HR and S' control groups (P < .01, Fig. 3b). Fos counts did not differ significantly between the LR and S' groups (P > .05, as well as the HR and LR groups (P > .10). Interestingly, significantly positive correlations were observed between relapse scores and Fos counts within both the medial and lateral/central (r² = .59, P < .04) and the lateral/central (r² = .59, P < .04) divisions of the MD of HR animals (data not shown).

Habenula. A main effect of group was observed across the habenula complex (F(2,24) = 6.75, P < .01). Across both regions, significantly greater Fos counts were observed in the HR group as compared with both the LR and S' control groups (P < .05 and P < .01, respectively, see Fig. 3c), whereas Fos counts were statistically indistinguishable between the S' and LR groups (P > .50). No significant correlations were observed between relapse scores and Fos counts in either the lateral habenula (LHB) or mHB in the HR group (data not shown).

DISCUSSION

Recent reports have highlighted the importance of studying individual differences in addiction and relapse vulnerability (Belin et al., 2009; Brown et al., 2011). Therefore, in the present study we have taken a novel approach to...
characterizing the cocaine cue-induced recruitment patterns of thalamic and epithalamic nuclei. We exploited our observation that in a cohort of cocaine self-administering animals, significant variability in reinstatement (relapse-like) behaviour in response to a cocaine-associated S$^+$ cue was exhibited. Specifically, a subgroup of animals showed a robust reinstatement following S$^+$ cue presentation, whereas another group of identically treated rats failed to display reinstatement responding, that is, behaviour responding in this group was indistinguishable from the S$^-$ control group or lever pressing observed over the final three extinction sessions. This observation is similar to that of a recent study that reported significant variability in reinstatement propensity using cocaine-primed CPP (Dayas et al., 2007, 2008; Zhao et al., 2006). Further, similar patterns of behaviour and Fos expression were observed between S$^-$ and LR animals in regions such as the PVT, suggesting that despite this difference in treatment, this experimental design is unlikely to have affected Fos expression between groups.

Fig. 3. Fos-protein expression in low-reinstating (LR) versus high-reinstating (HR) animals and S$^-$ control animals. Data are expressed as mean (±SEM) of the number of Fos-positive nuclei for that group. Fos-protein expression was assessed across bregma levels −2.16 to −3.24 for all regions. Fos protein was quantified in: (A) the dorsal midline thalamic group (paraventricular thalamus and intermediodorsal thalamus). There was a strong correlation between the number of Fos-positive nuclei within the PVT and reinstatement (B); $R^2=0.61$, $P<.05$ (Pearson’s correlational analysis). (C) Fos protein was also assessed in the mediodorsal thalamus and (D) the habenula complex. † $P<.05$ compared with S$^-$ control group. * $P<.05$ compared with LR group (two-way ANOVA with Tukey’s post hoc test).
We have recently shown that inactivation of the PVT using the sodium channel blocker tetrodotoxin, or infusions of the neuropeptide, cocaine and amphetamine-regulated transcript (CART) directly into the PVT, suppresses cocaine-prime–evoked reinstatement following extinction (James et al., 2010). These data are consistent with previous findings that PVT neurons are activated by alcohol-paired contextual cues (Wedzony et al., 2003; Dayas et al., 2008), as well as evidence that alcohol-cue sensitive PVT neurons are closely apposed to CART immunoreactive terminals (Dayas et al., 2008). Moreover, Hamlin et al. demonstrated that PVT neurons that project to the nucleus accumbens are activated during context-elicited reinstatement of alcohol seeking, and that this form of reinstatement is suppressed by axon-sparing lesions of the PVT (Hamlin et al., 2009). In addition, intra-PVT infusions of a δ-opioid receptor agonist prevent context-induced reinstatement of beer seeking (Marchant et al., 2010), further implicating this region in reinstatement behaviour. Here we extend these findings and propose a likely role for the PVT in drug-related behaviours. Our data are also consistent with reports that Fos-protein expression is observed in the PVT after CPP and cocaine sensitization (Hamlin et al., 2008; Dayas et al., 2008). These data are consistent with previous studies that have explicitly tested whether the PVT mediates stress-induced drug-seeking behaviour. For example, Hamlin et al. showed that the PVT is required for the acquisition of sucrose CPP (McAlonan et al., 1993). The MD has also been shown to have a role in drug-related behaviours, as lesions of the MD attenuate cocaine self-administration (Weissenborn et al., 1998). Recently, Kuo et al. showed that the MD is required for expression of psychostimulant CPP, and increased Fos expression is seen within MD neurons after re-exposure to a morphine-paired environment (Kuo et al., 2011). Furthermore, neurons in the medial MD respond to reward predictive stimuli and alter their activity during extinction and relearning (Oyoshi et al., 1996). Although inactivation of the MD does not affect cocaine-prime reinstatement (McFarland and Kalivas, 2001), our observation that cue-induced reinstatement of cocaine-seeking is associated with an increase in Fos-protein expression within the MD is consistent with these previous studies. Importantly, although there was a positive correlation between MD Fos counts and reinstatement in HR rats, the tendency for the MD to discriminate between HR and LR animals was less pronounced than for the dorsal midline thalamic nuclei, that is, PVT and IMD.

The habenula is an epithalamic structure that lies immediately dorsal to the thalamus and is thought to be important for a number of addiction-relevant behaviours, including stress responsivity and reward-based decision making (Hikosaka, 2010). Anatomically, the habenula is

Fig. 4. Fos-protein expression in the PVT of HR animals versus S− control animals. Photomicrographs of Fos-positive neurons in a representative high-reinstating animal (A) and an S− control animal (B) from the paraventricular thalamus (bregma −2.64; scale bar 50 μm).
broadly divided into medial and lateral subdivisions (mHb and LHb), with the latter previously implicated in cue-induced 'relapse' (Zhang et al., 2005). In the present study, significantly higher numbers of Fos-positive LHb neurons were seen in the brains of all S'-exposed animals. Similar to the dorsal midline thalamic nuclei, this recruitment pattern also discriminated between high and low 'reinstating' animals. This finding is consistent with the recent study of Brown et al., showing that activation of LHb neurons is observed only in those animals that restate in a cocaine CPP model (Brown et al., 2010). Despite this, correlation analysis failed to reveal a strong relationship between Fos counts and relapse-like behaviour.

Exactly how the LHb could influence relapse-like behaviour is unclear from the present study. Neurons in the LHb are known to receive dense input from the basal ganglia and send numerous projections to the substantia nigra and VTA (Herkenham and Nauta, 1977). Stimulation of the LHb reduces midbrain dopamine (and serotonin) cell firing, supporting a relatively straightforward habenula to VTA circuitry whereby glutamatergic habenula efferents act on local VTA GABA neurons to suppress dopamine cell activity (Matsumoto and Hikosaka, 2007). In this way, our finding of increased Fos counts in the LHb of the HR group is somewhat unexpected. However, Omelchenko and colleagues (Omelchenko et al., 2009) have recently published evidence that habenula neurons form asymmetrical synapses onto VTA dopamine neurons, indicating the presence of a direct projection that could be involved in triggering drug-seeking. Like the LHb, cue-induced reinstatement behaviour increased mHb Fos-protein expression in S’ exposed animals generally, and HR animals showed greater activation in this region than LR and control animals. It is noteworthy that Fowler and colleagues recently reported that recruitment of an mHb circuit limits drug-taking and reinstatement behavior increased mHb Fos-protein expression in this region is also associated with cue-induced reinstatement of cocaine-seeking, and that Fos-protein expression within these regions and to elucidate how thalamic and epithalamic structures modulate cue-induced reinstatement to cocaine-seeking.

In summary, we show that re-exposure to cocaine-associated cues is associated with increased Fos-protein expression in thalamic and epithalamic nuclei. Furthermore, by examining individual differences in relapse propensity, we show that Fos expression within dorsal midline thalamic nuclei (PVT and IMD) and the habenula is significantly greater in high- versus low-reinstating animals. Our findings build on existing literature implicating the PVT in cocaine-primed reinstatement of conditioned place preference: a behaviour dissociable from sensitization. PLoS One 5:e15889.

REFERENCES


CHAPTER TWO

Cocaine- and amphetamine- transcript (CART) signaling within the paraventricular thalamus modulates cocaine-seeking behaviour
CHAPTER TWO

COCAINE- AND AMPHETAMINE-REGULATED TRANSCRIPT (CART)

SIGNALING WITHIN THE PARAVENTRICULAR THALAMUS MODULATES

COCAINE-SEEKING BEHAVIOUR

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Statement II: Author contribution to Chapter 2 manuscript

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Cocaine- and Amphetamine-Regulated Transcript (CART) Signaling within the Paraventricular Thalamus Modulates Cocaine-Seeking Behaviour

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Abstract

Background: Cocaine- and amphetamine-regulated transcript (CART) has been demonstrated to play a role in regulating the rewarding and reinforcing effects of various drugs of abuse. A recent study demonstrated that i.c.v. administration of CART negatively modulates reinstatement of alcohol seeking, however, the site(s) of action remains unclear. We investigated the paraventricular thalamus (PVT) as a potential site of relapse-relevant CART signaling, as this region is known to receive dense innervation from CART-containing hypothalamic cells and to project to a number of regions known to be involved in mediating reinstatement, including the nucleus accumbens (NAC), medial prefrontal cortex (mPFC) and basolateral amygdala (BLA).

Methodology/Principal Findings: Male rats were trained to self-administer cocaine before being extinguished to a set criterion. One day following extinction, animals received intra-PVT infusions of saline, tetrodotoxin (TTX; 2.5 ng), CART (0.625 μg or 2.5 μg) or no injection, followed by a cocaine prime (10 mg/kg, i.p.). Animals were then tested under extinction conditions for one hour. Treatment with either TTX or CART resulted in a significant attenuation of drug-seeking behaviour following cocaine-prime, with the 2.5 μg dose of CART having the greatest effect. This effect was specific to the PVT region, as misplaced injections of both TTX and CART resulted in responding that was identical to controls.

Conclusions/Significance: We show for the first time that CART signaling within the PVT acts to inhibit drug-primed reinstatement of cocaine seeking behaviour, presumably by negatively modulating PVT efferents that are important for drug seeking, including the NAC, mPFC and BLA. In this way, we identify a possible target for future pharmacological interventions designed to suppress drug seeking.


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Introduction

Cocaine- and amphetamine-regulated transcript (CART) is a neuropeptide that was originally identified in the striatum of animals following acute psychostimulant exposure [1]. Subsequent studies have identified that CART is expressed in a number of regions known to be involved in reward and reinforcement, including the ventral tegmental area (VTA), nucleus accumbens (NAC), amygdala and hypothalamus [2,3]. Early studies firmly established a role for CART in appetite control, with central administration of CART found to dose-dependently suppress feeding behaviour [4]. More recently however, there has been renewed interest in the role that CART might play in modulating the rewarding and reinforcing effects of drugs of abuse, and in particular, psychostimulants such as cocaine and amphetamine [5–10].

The effects of CART on the reinforcing and locomotor-activating properties of psychostimulants are complex and appear to be both brain region- and dose-dependent. For example, administration of the active CART55-102 peptide into the VTA results in a cocaine-like increase in locomotor activity and produces a conditioned place preference (CPP) similar to that induced by cocaine or amphetamine [11], indicating that CART signaling in this region is reinforcing. However, administration of CART55-102 into the NAC or the ventral pallidum (VP) significantly attenuates the locomotor effects of acute cocaine and amphetamine administration [12–14], and prevents the expression of conditioned hyperlocomotion in a cocaine-paired environment [15]. Together, these data suggest that CART signaling within the NAC and VP works to negatively regulate the effects of psychostimulants. Interestingly, when administered into the basolateral amygdala (BLA), lower doses of CART appear to...
be rewarding, whilst higher doses are aversive [6]. Taken together, these findings strongly implicate CART as a regulator of the reinforcing and rewarding effects of psychostimulants, however the effects appear to be highly regional and dose specific.

A recent study suggests that CART may also be involved in mediating the reinstatement of extinguished drug seeking, as intracerebroventricular (i.c.v.) administration of CART was found to prevent context-induced reinstatement of alcohol seeking in rats [16]. Importantly though, it remains unclear as to whether CART mediates reinstatement in animals that have a significant history of psychostimulant exposure. Further, it is important that the site(s) at which CART exerts its inhibitory effects in a reinstatement model be determined.

A region of particular interest with regards to the integration of CART peptide activity is the paraventricular thalamus (PVT). The PVT is a part of the midline and intralaminar thalamic group and is known to receive dense innervation from CART-containing neurons in the lateral hypothalamus [17–19]. Importantly, the PVT is known to project to a number of regions implicated in the reinforcing and rewarding effects of psychostimulants, however the effects appear to be highly regional and dose specific.

Results

Guide Cannulae Placement

As shown in Figure 1, guide cannulae from the majority of animals were localized to the PVT. Four TTX-treated animals were found to have guide cannulae that were misplaced; two were directed at the thalamus, another at the central medial thalamic nucleus (CM), and the other at the boundary of the intermediate thalamic nucleus (IMD) and the CM. The guide cannulae from one animal treated with 0.625 μg CART was directed at the stria medullaris of the thalamus, whilst one animal treated with 2.5 μg CART had its guide cannulae directed at the lateral boundary of the IMD and the CM.

Self-Administration Training and Extinction

Rats developed stable responding (±10% over 3 sessions) for cocaine within 8 days of training (±1.04 SEM). Over the last three days of cocaine self-administration training, the mean number of cocaine infusions per session across all animals was 29.32 (±1.63 SEM), which equated to approximately 19 mg/kg of cocaine per session. Importantly, levels of cocaine consumption did not differ across the four treatment groups. During cocaine self-administration, animals significantly favoured the active cocaine-paired lever compared to the inactive lever (F(1,37) = 130.84, p<.001) and this preference did not differ between the treatment groups (F(3,37) = 1.47, p = .24). Animals met the extinction criterion after an average of 29.63 (±1.67 SEM) days and the number of days taken to reach the extinction criterion was indistinguishable across all groups (F(3,30) = .95, p = .43).

Reinstatement Testing

After reaching the extinction criterion, all animals were subjected to a cocaine prime. Animals that received intra-PVT saline-injections prior to cocaine-priming injections displayed an identical reinstatement of responding on the active lever to animals that were prepared with PVT-guide cannulae but received no injection (F(1,37) = .60, p = .46). We therefore combined these two groups to form a single ‘Control’ group (n = 12). ANOVA revealed a significant main effect of ‘session’ (F(3,27) = 30.24, p<.001), indicating that the cocaine-prime brought about an overall increase in responding from extinction levels. A significant ‘session’ x ‘treatment’ interaction was also observed (F(3,27) = 8.26, p<.001) suggesting differences in the extent to which the treatments affected cocaine prime-induced reinstatement of responding. Planned, separate post-hoc analyses revealed that TTX-treated animals displayed on average a lower level of responding on the active lever compared to controls (p<.05). Similarly, animals treated with either 0.625 μg CART55-102 and 2.5 μg CART55-102 exhibited significantly reduced reinstatement responding compared to controls (p<.01), with the 2.5 μg CART dose having the greatest effect (see Figure 2A). Importantly, changes in lever pressing behaviour by all treatments was limited to the active lever with neither a significant main effect (F(3,27) = 1.97, p = .17) nor a significant interaction (F(3,27) = 1.47, p = .23) observed for left (inactive) lever responses during the test phase (see Figure 2C).

We also assessed differences in responding on the active lever at ten-minute intervals across the one-hour reinstatement session (see Figure 2B). Repeated measures ANOVA revealed a significant ‘time’ x ‘treatment’ interaction (F(27,27) = 4.91, p<.01, Huynh-Feldt correction). Planned post-hoc test comparisons revealed that the control group exhibited significantly greater levels of responding in the first ten minutes of reinstatement testing than the 2.5 μg CART group (p<.05, adjusted for multiple comparisons), but not the TTX- or 0.625 μg CART- treated groups. At all other time points, the control group exhibited significantly greater levels of responding than all other treatment groups (p<.05, adjusted for multiple comparisons).

We also examined the responding of animals that had misplaced guide cannulae to determine the specificity of the observed effects to the PVT. The four animals that received misplaced TTX injections (M = 49.25, SE = 27.86) did not differ in their active lever responses to the control group (M = 64, SE = 8.69, F(1,11) = 1.47, p = .28), but did respond on average at a higher rate to animals that received PVT-directed TTX (M = 34.57, SE = 6.25). Similarly, the two animals that received misplaced CART injections (M = 64.5, SE = 22.5) responded on the active lever at a level almost identical to controls, but higher than the PVT-directed 0.625 μg CART (M = 14.17, SE = 4.73) groups. As such, the effect of both CART and TTX treatment was most pronounced when injected into the PVT.

Discussion

The purpose of the present study was two-fold. Firstly, we aimed to investigate whether the role of the PVT in modulating reinstatement to cue and context-induced alcohol seeking...
extended to drug-primed cocaine seeking. To do this, we used TTX administration to functionally inactivate the PVT prior to cocaine-primed reinstatement testing. We report that TTX-treated animals exhibited significantly attenuated drug-seeking behaviour following drug prime, showing for the first time that the PVT plays an important role in mediating drug primed 'relapse' of cocaine-seeking behaviour. Our second aim was to determine whether CART signaling within the PVT might regulate drug-seeking behaviour, as we have previously shown CART-containing terminals within the PVT to be closely apposed to drug-cue activated PVT neurons [10]. Further, PVT cells project to a number of relapse-relevant brain regions including the NAC, mPFC and BLA [18–23]. To achieve these aims, animals were treated with either 0.625 mg or 2.5 mg of the CART55-102 peptide, based on previous studies that examined the role of CART in drug-motivated behaviours [6,11,13,15,16]. CART produced a significant attenuation of drug-seeking following drug-prime, with the 2.5 mg dose having the greatest effect. These findings build upon previous reports that i.c.v. CART inhibits reinstatement [16] by identifying a specific site at which CART acts to modulate relapse-like behaviour.

It is noteworthy that PVT-directed treatment did not alter responding on the inactive lever, suggesting that the effects we observed were specific to drug-seeking rather than a generalized reduction in arousal or locomotor activity as a result of CART or TTX infusion. Whilst it is possible that low levels of overall responding on the inactive lever may have masked a non-specific effect of CART or TTX, several pieces of evidence indicate that this is highly unlikely. Firstly, previous work studying the effects of ibotenic acid lesions of the PVT on renewal of beer seeking reported no alterations in inactive nose-poke responding [24]. Presumably, these lesions also disrupted CART signaling onto PVT cells as well as the actions of other inputs into this site. Secondly, i.c.v. [16] and intra-accumbal infusions [15] of CART resulted in a specific reduction of drug-related locomotor activity, and i.c.v. CART injections do not alter spontaneous locomotor activity [4]. It is also important to note that CART has been reported to produce some postural changes and motor tremors. For example, i.c.v. infusion of 1 mg or 2 mg of CART produced a modest movement-associated tremor, however this did not impair the ability of the animals to engage in reward-seeking behaviour, nor did it significantly alter locomotor activity [4]. Motor tremors

Figure 1. Location of microinfusion injector tips. The majority of injections were made directly into the PVT region. Four TTX-treated animals and two CART-treated animals had injections that fell beyond the PVT boundary, and the responding of these animals was assessed to determine the specificity of the observed effects to the PVT. Numbers represent the approximate rostrocaudal distance from bregma. Figures adapted from Paxinos and Watson [44]. Symbols represent different groups; ●: saline; ○: TTX; ◊: 0.625 μg CART; □: 2.5 μg CART, X: misplaced TTX; *: misplaced 0.625 μg CART injections; #: misplaced 2.5 μg CART injections, CM: central medial thalamic nucleus; D3V: dorsal third ventricle; IMD: intermediodorsal thalamic nucleus; MDC: mediodorsal thalamic nucleus, central part; MDM: mediodorsal thalamic nucleus, medial part; PVA: Paraventricular thalamic nucleus, anterior part; PVT: Paraventricular thalamic nucleus; PVP: Paraventricular thalamic nucleus, posterior part.

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level of active lever responding than 2.5 different to controls (A). Control animals exhibited significantly higher levels of responding than TTX- and both CART-treatment groups. †: Control group significantly different to 2.5 μg CART-treatment group; *: Control group significantly different to 2.5 μg CART-treatment group, all p<0.05 (B).

Importantly, TTX and CART treatment did not affect responding on the inactive lever, indicating that the observed effects were specific to drug-seeking behaviour rather than an overall reduction in arousal and/or locomotion (C). SA: Average number of active lever presses over last three days of self-administration training period. EXT: Average number of active lever presses over last three days of extinction training. Error bars represent + S.E.M.

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Figure 2. Microinfusion of TTX or CART into the PVT attenuates cocaine-primed reinstatement. All animals were tested following a cocaine prime (10 mg/kg, i.p.) preceded by a PVT-directed microinfusion of either saline, 2.5 ng TTX, 0.625 μg CART55-102 or 2.5 μg CART55-102 (n=6–7). A group of animals served as no-injection controls, and were grouped with saline-treated animals to form a ‘control’ group (CONT). Treatment with TTX or CART produced a significant attenuation of active lever responding following cocaine prime, as compared to controls (†: p<0.05, ††: p<0.01), significantly different to controls (A). Control animals exhibited a significantly higher level of active lever responding than 2.5 μg CART-treated animals in the first ten minutes of reinstatement testing. At all other time points, control animals exhibited significantly higher levels of responding than TTX- and both CART-treatment groups. †: Control group significantly different to TTX-treatment group; *: Control group significantly different to 0.625 μg CART-treatment group; ††: Control group significantly different to 2.5 μg CART-treatment group, all p<0.05 (B).

Importantly, TTX and CART treatment did not affect responding on the inactive lever, indicating that the observed effects were specific to drug-seeking behaviour rather than an overall reduction in arousal and/or locomotion (C). SA: Average number of active lever presses over last three days of self-administration training period. EXT: Average number of active lever presses over last three days of extinction training. Error bars represent + S.E.M.

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were also observed following intra-VTA infusion of a 5 μg dose of CART, but not a 1 μg dose [11]. Importantly, close observation of our animals revealed no evidence of motor effects at the relatively low doses of CART used in our study.

It should also be noted that a dorsal approach to the PVT for the placement of guide cannulae was employed in our study. Whilst this approach, as compared to an angled approach, may have marginally increased the possibility of the injectate spreading dorsoally into the D3V, we are confident that the observed effects were specific to the PVT region. This conclusion is based on the responding seen from animals with misplaced injections of TTX directed at the D3V that resulted in responding that was indistinguishable from control animals. Similarly, animals that received TTX and CART injections that were directed at thalamic structures surrounding the PVT and, indeed one case that was adjacent to the D3V, also exhibited responding that was identical to controls, again indicating PVT-specific effects. Importantly, CART and TTX infusions were made at various rostro-caudal levels of the PVT, and responding within each treatment group was consistent at all injection sites. This is consistent with anatomical tracing studies that suggest that the number of projections torelapse relevant brain regions is relatively consistent along the entire rostro-caudal length of the PVT [18].

The PVT is known to respond to noxious stimulation, and inputs from tactile and nociceptive pathways have been hypothesised to be integrated within intralaminar and midline thalamic nuclei [25,26]. Further, hypothalamic-pituitary-adrenal (HPA) axis [27] and central amygdala [28] responses to stress are altered by functional manipulations of the PVT. It has been inferred from these data that the PVT plays an important role in arousal and attentional control. The present findings build upon this literature and strongly support an emerging body of evidence that suggests that the PVT also plays a critical role in reward-related processing [29]. For example, PVT lesions blocked the conditioned locomotor response to a cocaine-paired environment in a sensitisation experiment [30], whilst PVT activation has been shown to be associated with increased locomotor responses entrained to regulated feeding schedules [31]. Particularly pertinent to the present study, the PVT has recently been implicated in modulating the reinstatement of alcohol seeking. For example, presentation of cues previously associated with alcohol availability increased c-fos expression within the PVT [10], whilst ibotenic acid lesions of the PVT prevented context-induced reinstatement of alcohol seeking [24]. Together, these data indicate a role for the PVT in reward-seeking behavior that we demonstrate here also includes reinstatement of drug-seeking behaviour precipitated by a cocaine prime.

It has recently been suggested that the PVT modulates reward-related behaviour by acting to integrate and relay hypothalamic activity to reward-relevant regions, including the NAC and perhaps the mPFC [29]. Indeed, anatomical tracing studies have
shown that the PVT receives dense innervation from the lateral hypothalamus [18,19,32,33] and in turn sends glutamatergic projections to the NAC, mPFC and BLA [20-23]. Further, stimulation of the PVT has been shown to modulate neuronal excitability in both the NAC and PFC [23,34,35]. Interestingly, a significant percentage of PVT neurons send collateral (i.e. branched) projections to both the NAC and mPFC, suggesting that the PVT is anatomically positioned to simultaneously influence both of these drug-relevant regions [20,22]. Furthermore, CART-positive terminal fibres in the PVT are closely apposed to neurons that project directly to the NAC shell [19].

Taken together with the data presented here, we propose that CART signaling within the PVT acts to inhibit drug-primed reinstatement by modulating the activity of PVT-efferents, including to the NAC and/or mPFC. Indeed, electrophysiological studies indicate that CART can produce inhibitory post-synaptic effects when applied to brain slices [36].

Consistent with this suggestion, anatomical studies show that glutamatergic efferents from the PVT are closely associated with dopamine immunoreactive terminals in the NAC shell, and this relationship may potentiate NAC activity [37]. Indeed, stimulation of the PVT increases dopamine efflux within the shell of the NAC [33]. The NAC has been extensively demonstrated to be critically involved in the reinstatement of drug-seeking behaviour. For example, NAC shell inactivation suppresses reinstatement of drug-seeking elicited by contextual cues [38] and NAC shell neurons show increased levels of activation after presentation of drug-paired discriminative stimuli measured using Fos [39] or single unit recordings [40]. In addition, dopamine antagonist injections aimed at the NAC shell block the reinstatement of cocaine seeking induced by a drug prime [41]. Taken together, these data indicate that CART might act to suppress drug-primed reinstatement by preventing PVT-glutamate efferents modulating the responsivity of NAC neurons to cocaine-induced dopamine release.

In summary, we demonstrate that the PVT plays an integral role in modulating drug-primed reinstatement of cocaine seeking and that CART signaling within this region negatively regulates relapse-like behaviour. Whilst not directly addressed here, CART signaling in the PVT may act to negatively modulate dopamine release within the NAC and other regions known to be involved in reinstatement. As such, CART signaling within the PVT may represent a potential target for pharmacological interventions designed to suppress drug-seeking behaviour. It will be for future studies to determine if CART signaling also suppresses drug seeking evoked by cues linked to drug taking and stress. Presumably however, a full evaluation of the potential role of CART as a therapeutic treatment target awaits the identification of the receptor system through which this peptide acts.

Materials and Methods

Ethics Statement

All procedures performed were approved by the University of Newcastle Animal Care and Ethics Committee (approval number 1006) and were carried out in accordance with the New South Wales Animal Research Act.

Animals

Male Sprague-Dawley rats (Central Animal Care House, University of Newcastle, NSW, AUS, weighing 200-250 g upon arrival) were housed two per cage on a reverse 12-hour light/dark cycle (lights off at 7:00 am) with ad libitum access to food and water.

Drugs

- Rat CART35-102 (Phoenix Pharmaceuticals, CA, USA) and TTX (Alomone, Israel) were dissolved in sterile saline and stored at 4°C until use. Both doses of CART35-102, TTX, and saline were microinjected at volumes of 0.25 pl.

Surgery

Catheterisation. Prior to surgery, rats were injected intramuscularly with 0.3 mL of a broad-spectrum antibiotic (150 mg/mL procaine penicillin, 112.5 mg/mL benzathine penicillin, Norbrook Laboratories, UK) and subcutaneously with 0.2 mL of a 50 mg/mL solution of carprofen (Norbrook Laboratories, UK). Rats (250-300 g) were anaesthetized with isoflurane (~3% with a flow rate of 2 L/min) and, using aseptic procedures, a Silastic catheter was surgically implanted into the right jugular vein as described in detail previously [42]. Post-surgery, jugular catheter lines were flushed with 0.3 mL of 50 mg/mL of carprofen (Norbrook Laboratories, FL, USA) and 0.2 mL of 50 unit heparinised saline.

PVT-Directed Guide Cannulae. Prior to surgery, animals were treated with procaine penicillin and carprofen as above. Animals were anaesthetized with sodium pentobarbital being placed in a stereotaxic frame (Stoelting, IL, USA). Cranial holes were made into the skull to facilitate the insertion of four stainless steel jeweller’s screws (Mann Optics, QLD, AUS), whilst a fifth craniotomy was made to allow the insertion of a stainless steel guide cannula (26 gauge, Small Parts, FL, USA) targeting the level of the PVT (−2.6 mm AP relative to bregma, −4.6 mm DV relative to skull surface). Guide cannulae were secured to the four screws with dental cement (Henry Schein, AUS). Cannulae were kept clear by using stainless steel stylets (33 gauge, Small Parts, FL, USA) of identical length to the guides.

Behavioural Training

Behavioural procedures were conducted in standard operant conditioning chambers located inside sound-attenuating, ventilat- ed cubicles (Med Associates, VT, USA). Chambers were equipped with two retractable levers (0 cm above the floor), white cue lights (one above each lever), two speakers to deliver auditory stimuli and a house light located at the top of the chamber wall opposing the levers. A syringe pump (5 rpm motor, Med Associates, VT, USA) located on the outside of the sound-attenuating cubicle delivered the IV cocaine/saline vehicle solution. Data acquisition and behavioural testing equipment were controlled by a Windows-based PC, using MED-PC IV (Med Associates, VT, USA).

Seven days after surgery, rats were trained to self-administer cocaine hydrochloride (Johnson Matthey, Edinburgh, UK), dissolved in sterile physiological saline 2.5 mg/mL intravenously in three-hour sessions conducted daily for 5 days a week. Responding on the active (right) lever resulted in a 4 second infusion of cocaine (0.1 mL) via the intravenous catheter and activation of a white cue light above the active lever that remained illuminated for 20 seconds signaling a time out period. The inactive (left) lever was not extended during the initial training sessions. Training was continued until stable responding for cocaine was achieved (±10% rewarded responses over 5 sessions) at which time animals were subjected to eight two-hour sessions whereby both the active (right) and inactive (left) levers were presented. In these sessions, responding on the active lever had similar consequences as in the initial training phase, whilst responding on the inactive lever was recorded but had no scheduled consequences.

CART Modulates Reinstatement
Three days following the final training session, animals were implanted with PVT-directed guide cannulae and were allowed one week for recovery. Lever responding was then extinguished by again presenting both the active and inactive levers, but with IV infusions withheld. Extinction trials continued until a criterion of ≤10 responses per session on the active lever over three consecutive days was achieved.

Reinstatement Testing

Over the final five to six days of extinction training, animals were gently restrained whilst their stylet was removed and replaced, in order to condition animals to the testing procedure. One day following the final extinction session, animals were again restrained whilst their stylet was removed and placed in 70% ethanol. Animals then received a microinfusion of either saline (n = 6), 2.5 μg TTX (n = 11), 0.625 μg CART (n = 7) or 2.5 μg CART (n = 7). A group of six animals were implanted with guide cannulae into the PVT but were not subjected to intra-PVT injections and served to control for the effect of intra-PVT drug infusion on cocaine prime-induced responding. Based on previous studies, the selected dose of TTX was expected to inactivate tissue within a ∼0.35–0.4 mm radius from the site of injection [13] and therefore have minimal effect on structures surrounding the PVT.

Further, the doses of CART were selected based on previous reports that these doses are sufficient to modulate the reinforcing effects of cocaine [13,15]. All microinjections were made through an injector cannulae (30 gauge, Small Parts, FL, USA), which protruded 1.5 mm below the tip of the guide cannula into the PVT region. Infusions were delivered using a Hamilton microsyringe mounted on a motorised pump (Stoeling, IL, USA) at a rate of 2 μl/min. The injector remained in the guide cannulae for a further minute following administration in order to allow adequate diffusion of the injectate into the PVT. Animals were then administered a cocaine drug-prime (10 mg/kg, i.p.) and placed into the operant chamber. Testing for reinstatement of responding began five minutes later under extinction conditions for one hour. Two hours following commencement of testing, animals were deeply anaesthetised with sodium pentobarbital (200 mg/kg IP) and transcardially perfused with 150 mLs of PBS (pH 7.4) followed by 500 mLs of 4% paraformaldehyde (pH 9.3) to allow for verification of injector sites.

Data Analysis

Separate one-factor ANOVAs were used to compare the experimental and control groups in terms of number of training days, overall cocaine intake, time taken to reach extinction, and responding on both the active and inactive levers over the last three days of extinction. Lever preference during training was assessed using a 2 'Lever' (active, inactive) x 4 'Treatment' (Control, TTX, 0.625 μg CART, 2.5 μg CART) mixed-model ANOVA, whilst reinstatement of responding on both the active and inactive levers was analysed using a 2 'Session' (extinction, reinstatement) x 4 'Treatment' (Control, TTX, 0.625 μg CART, 2.5 μg CART) mixed-model ANOVA. Where significant interactions were observed, planned Tukey post-hoc tests were used to assess differences between treatment groups. Differences in active lever responding at various timepoints across the one hour reinstatement session was assessed using a 4 'Treatment' (Control, TTX, 0.625 μg CART, 2.5 μg CART) x 6 'Time' (10 min, 20 min, 30 min, 40 min, 50 min, 60 min) repeated measures ANOVA. Where significant interactions were observed, planned t-tests analyses were used to compare treatment groups and Bonferroni α corrections were made to control for family-wise error. An alpha level of 0.05 was used for all statistical tests.

Author Contributions

Conceptualized and designed the experiments: MHJ JLC SJ EML JRF DWS CVD. Performed the experiments: MHJ JLC SJ EML JWY JRF. Analyzed the data: MHJ CVD. Wrote the paper: MHJ DWS CVD.

References


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CHAPTER THREE

Orexin-1 receptor signaling within the ventral tegmental area, but not the paraventricular thalamus, is critical to regulating cue-induced reinstatement of cocaine seeking
CHAPTER THREE

OREXIN-1 RECEPTOR SIGNALLING WITHIN THE VENTRAL TEGMENTAL AREA, BUT NOT THE PARAVENTRICULAR THALAMUS, IS CRITICAL TO REGULATING CUE-INDUCED REINSTATEMENT OF COCAINE-SEEKING

Morgan H. James, Janine L. Charnley, Emily M. Levi, Emma Jones, Jiann Wei Yeoh, Doug W. Smith & Christopher V. Dayas

International Journal of Neuropsychopharmacology (2011) pp 684-690

Statement III: Author contribution to Chapter 3 manuscript

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<th><strong>Morgan H. James</strong></th>
<th>Conceived and designed the experiments. Performed the experiments. Analysed and interpreted the data. Wrote and edited the manuscript.</th>
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<td><strong>Doug W. Smith</strong></td>
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<td><strong>Christopher V. Dayas</strong></td>
<td>Conceived and designed the experiment. Analysed and interpreted the data. Wrote and edited the manuscript.</td>
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CHAPTER ONE

PROPENSITY TO 'RELAPSE' FOLLOWING EXPOSURE TO COCAINE CUES IS ASSOCIATED WITH THE RECRUITMENT OF SPECIFIC THALAMIC AND EPITHALAMIC NUCLEI

Morgan H. James, Janine L. Charnley, Jamie R. Flynn, Doug W. Smith & Christopher V. Dayas
Neuroscience (2011) pp 235-242

Statement I: Author contribution to Chapter 1 manuscript

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25 September 2013

PROFESSOR ROBERT CALLISTER
Deputy Head of Faculty (Research and Research Training)
Orexin-1 receptor signalling within the ventral tegmental area, but not the paraventricular thalamus, is critical to regulating cue-induced reinstatement of cocaine-seeking

Morgan H. James, Janine L. Charnley, Emily M. Levi, Emma Jones, Jiann Wei Yeoh, Doug W. Smith and Christopher V. Dayas

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Abstract

Orexinergic signalling is critical to drug relapse-like behaviour; however, the CNS sites(s) of action remain unknown. Two candidate brain regions are the paraventricular thalamus (PVT) and ventral tegmental area (VTA). We assessed the effect of intra-PVT or -VTA administration of the orexin-1 receptor (OrxR1) antagonist SB-334867 on discriminative cue-induced cocaine-seeking. Animals received either PVT- or VTA-directed SB-334867 (0, 3 or 6 mg; 0, 1 or 3 mg, respectively) prior to reinstatement testing elicited by presenting cocaine-paired stimuli (S+). The effect of VTA-directed injections of SB-334867 (0 or 3 mg) on locomotor activity was also assessed. Intra-VTA, but not -PVT, SB-334867 dose-dependently attenuated S+-induced reinstatement (3 mg dose, \(p < 0.01\)). Intra-VTA SB-334867 had no effect on locomotor activity. We conclude that OrxR1 signalling within the VTA, but not the PVT, mediates cue-induced cocaine-seeking behaviour. We hypothesize that blockade of VTA OrxR1 signalling may reduce nucleus accumbens dopamine in response to drug cue presentation.

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Key words: Cocaine, orexin, paraventricular thalamus, relapse, ventral tegmental area.

Introduction

Orexin (hypocretin) is a peptide neurotransmitter that is expressed in a discrete population of cells within the posterior lateral hypothalamus (de Lecea et al. 1998; Sakurai et al. 1998). Neurons expressing orexin project to a number of reward-associated structures within the brain indicating a likely role for these neurons in reward function and motivation. Indeed, early studies implicated orexin in the regulation of appetite and food-seeking (Sakurai, 1999). More recently, orexin has been implicated in addiction (Aston-Jones et al. 2010) with several demonstrations that this neuropeptide plays a role in regulating the reinforcing and rewarding effects of highly salient rewards including high-fat food and some drugs of abuse (Borgland et al. 2009; Boutrel et al. 2005; Hollander et al. 2008; Lawrence et al. 2006).

With respect to drug-seeking, Harris and colleagues (2005) demonstrated that hypothalamic orexin cells are activated by cues previously associated with cocaine and morphine availability and that systemic administration of the orexin-1 receptor (OrxR1) antagonist, SB-334867, blocks reinstatement of morphine place preference. Systemic administration of SB-334867 also blocks both cue- and stress-induced reinstatement (Boutrel et al. 2005; Lawrence et al. 2006; Smith et al. 2009). At present, however, the specific site(s) of relapse-related orexin signalling within the brain remains unclear.

We, among others, have recently suggested that the paraventricular thalamus (PVT) might be a potential integrator of hypothalamic peptide signals and responsible for relaying reward-relevant information to brain regions that trigger drug-seeking (Dayas et al. 2008; Hamlin et al. 2009; Kelley et al. 2005). Indeed, the
PVT has been shown to receive input from hypothalamic orexin neurons (Parsons et al., 2006; Peyron et al., 1998) and PVT neurons send projections that innervate the nucleus accumbens (NAc) shell (Hamlin et al., 2009). Further, inactivation of the PVT blocks both cue- and drug-primed reinstatement (Hamlin et al., 2009; James et al., 2010) and presentation of cues previously associated with drug availability activate PVT neurons that are closely opposed to hypothalamic orexin-positive terminals (Duyas et al., 2008). At present, however, it is unclear whether disruption of orexin signalling within the PVT modulates reinstatement of drug-seeking behaviour.

Another brain region through which orexin might mediate its pro-reward-seeking effects is the ventral tegmental area (VTA). The VTA has been shown to express both orexin receptor subtypes (OrxR1 and OrxR2) (Marcus et al., 2001; Narita et al., 2006) and electrophysiological studies have shown that orexin applied to midbrain slices increases VTA dopamine neuron excitability (Korotkova et al., 2003). Although anatomical studies demonstrate that lateral hypothalamic orexin neurons do not make frequent synaptic contacts within the VTA (Balcita-Pedicino & Sesack, 2007), intra-VTA orexin stimulates local dopamine and glutamate release, which is suggested to indirectly activate dopamine neurons by local paracrine diffusion or volume transmission (Wang et al., 2009). Importantly, dopaminergic projections from the VTA to regions such as the NAc are known to play a key role in mediating reinstatement of drug-seeking behaviour (Bossert et al., 2007). Interestingly, intra-VTA infusion of orexin-A into the VTA reinstates extinguished preference for a morphine-paired environment (Harris et al., 2005); however, intra-VTA infusion of an OrxR1 antagonist does not block stress-induced reinstatement (Wang et al., 2009). It remains to be determined whether OrxR1 signalling within the VTA plays a role in mediating cue-induced relapse in a self-administration paradigm.

In the present study, we tested whether infusion of the OrxR1 antagonist SB-334867 into either the PVT or VTA modified reinstatement of drug-seeking elicited by discriminative cues previously associated with cocaine availability. Our results demonstrate a role for OrxR1 signalling in the VTA but not the PVT in cocaine-seeking behaviour that is not a consequence of non-specific effects on locomotor activity.

Methods

Ethical statement

All procedures performed were approved by the University of Newcastle Animal Care and Ethics Committee and were performed in accordance with the New South Wales Animal Research Act.

Drugs

Cocaine hydrochloride (Johnson Matthey, UK) was dissolved in sterile physiological saline (2.5 mg/ml). Similar to a number of previous studies (Li et al., 2010a,b, 2011), SB-334867 was dissolved in 100% DMSO due to difficulties dissolving this compound in lower DMSO concentrations. Importantly, avoidance memory studies report that 100% DMSO does not alter the antagonist properties of the SB compound, nor does it produce behavioural effects compared to saline (Akbari et al., 2006, 2007, 2008). Similarly, 100% DMSO has been used as a vehicle in the VTA without consequence (Pierce et al., 1999). In addition, pilot studies revealed no differences between vehicle (DMSO) and no-injection controls in terms of reinstatement behaviour in animals implanted with PVT-directed guide cannulae (see Fig. S1, online). As we and others (e.g., Hamlin et al., 2009; James et al., 2010) have previously demonstrated that the PVT is critical to reinstatement, any functional effects of 100% DMSO on reinstatement behaviour would be revealed by comparing PVT-DMSO injections with no-injection controls.

Animals and surgery

Male Sprague–Dawley rats weighing 200–250 g upon arrival (Animal Resources Centre, Australia) were housed two per cage on a reversed 12-h light/dark cycle (lights off 07:00 hours) with food and water available ad libitum. Rats were handled daily for 1 wk before undergoing intravenous catheter surgery as described previously (James et al., 2010). To facilitate the placement of PVT- or VTA-directed guide cannulae, animals were anaesthetized with isofluorane and then placed in a stereotaxic frame (Stoelting, USA). Craniotomies were made into the skull and stainless-steel guide cannulae (26-gauge, Small Parts, USA) lowered unilaterally to the level of either the VTA (AP −5.3, ML ±0.8, DV −8.0) or PVT (AP −2.6, ML 0.0, DV −4.6; Paxinos & Watson, 2007). Guide cannulae were secured to four stainless-steel jewellers’ screws (Mann Optics, Australia) with dental cement (Henry Schein, Australia) and were kept clear by stainless-steel stylets (33-gauge, Small Parts, USA).

Expt 1: effect of intra-PVT or -VTA SB-334867 administration on cue-induced reinstatement

Self-administration training

Seven days after catheter surgery, rats were trained to self-administer cocaine hydrochloride (0.25 mg/
injection over 4 s) intravenously (i.v.) in 3-h sessions, once daily/5 d a week. Training was continued until stable responding for cocaine was achieved (± 10% over three sessions) at which time animals were subjected to daily, 2-h randomized conditioning sessions for cocaine or saline infusions (FR1), in the presence of distinct discriminative stimuli. For cocaine, this involved a constant 70-dB white noise and illumination of the white cue-light (20 s) above the active lever (S+ ) and for saline, constant illumination of the white house-light and a 20-s intermittent beeping tone (S− ). Responding on the inactive lever was recorded but did not result in cocaine or saline infusions. After 16 d of conditioning (8 S+/8 S− sessions) lever responding was extinguished in daily, 1-h sessions until stable responding (≤ 6 responses per session over three consecutive days) was achieved. During extinction, discriminative stimuli were withheld along with i.v. cocaine infusions. Four animals were excluded from the study due to catheter failure during the training period.

Reinstatement testing

After extinction, animals were presented with the S− cue and tested for reinstatement. On the following day, animals received either PVT-directed SB-334867 (3 μg/0.5 μl, n = 7; 6 μg/0.5 μl, n = 8), VTA-directed SB-334867 (1 μg/0.5 μl, n = 6; 3 μg/0.5 μl, n = 7) or vehicle (100% DMSO; VTA, n = 6; PVT, n = 8). Fifteen minutes post-injection animals were tested for reinstatement under S+ conditions for 1 h. One hour following the end of testing, animals received either an intra-PVT or -VTA injection of Cresyl Violet (0.5 μl) using the same injector that was used to infuse either the SB-334867 or vehicle, before being perfused. Brains were removed and later sectioned on a freezing microscope (Leica SM 2000R, Leica Biosystems, Germany). Injection sites for each animal were verified by inspecting a series of 40-μm (PVT) or 50-μm (VTA) tissue slices surrounding the injection site under a light microscope (CX40, Japan) at 40x magnification.

Expt 2: effect of intra-VTA SB-334867 administration on locomotor activity

Animals (n = 8) were trained to self-administer cocaine in the manner described in expt 1. Following discriminative cue-training, animals were habituated to black circular arenas (40 cm diameter, 39 cm height) in 1-h sessions over 3 d. On the final day of habituation, locomotor activity was recorded to provide a baseline measure of activity. One day later, animals received either an intra-VTA infusion of SB-334867 (3 μg/0.5 μl; n = 4) or DMSO (n = 4) and locomotor activity was assessed for 1 h. Locomotor activity was subsequently analysed using EthoVision XT (Noldus Information Technology, USA).

Data analysis

Within both PVT- and VTA-guide implanted cohorts, single-factor ANOVAs were used to compare treatment groups for responding during conditioning, overall cocaine intake and time taken to reach extinction. For PVT-guide implanted animals, differences in reinstatement were assessed using a 2-session (extinction, reinstatement) ×3-treatment (vehicle, 3 μg SB-334867, 6 μg SB-334867) mixed-model ANOVA. Similarly, reinstatement was assessed in VTA-guide implanted animals using a 2-session (extinction, reinstatement) ×3-treatment (vehicle, 1 μg SB-334867, 3 μg SB-334867) mixed-model ANOVA. Where significant interactions were observed, Tukey’s post-hoc tests were used to assess differences between treatment groups. To assess differences in locomotor activity on both days of testing, 2-treatment (DMSO, 3 μg SB-334867) ×6-time (10, 20, 30, 40, 50, 60 min) mixed-model ANOVAs were performed. Animals that received misdirected guide cannulae placements into either the PVT or VTA were excluded from the analysis.

Results

One animal received a misdirected PVT injection of the 6 μg dose of the SB-334867 compound that was targeted at the medial dorsal thalamic nucleus and was excluded from the analysis (see Fig. S2, online). All other PVT-directed injectors were localized to the PVT or the boundary of the PVT and intermediodorsal thalamus. Importantly, reinstatement did not differ between centrally vs. ventrally placed PVT injections (see Fig. S2, online). All VTA injections were accurate, with the majority falling into the paranigral nucleus and parabrachial pigmented nucleus of the caudal extent of the VTA (see Fig. S2, online).

Within each cohort (i.e. either PVT- or VTA-guide implanted animals) vehicle- or SB-334867-treated animals did not differ in terms of overall cocaine consumption, responding under S− and S+ training conditions or time taken to reach extinction (see Training Statistics section, available online). It should be noted, however, that animals prepared with PVT-directed guide cannulae exhibited lower levels of cocaine-reinforced responding during conditioning compared to VTA-guide implanted animals. This was associated with an overall lower level of reinstatement.
following the presentation of the $S^+$ stimuli. This is likely to be due to a cohort effect, as animals were trained and tested separately and at different times of the year.

**Effect of PVT-directed SB-334867 on $S^+$-induced reinstatement**

A significant main effect of session revealed an overall increase in responding on the active lever following presentation of the $S^+$ cue ($F_{1,19}=43.02, p<0.001$). Importantly, no session $\times$ treatment interaction was observed ($p=0.73$) indicating that neither dose of SB-334867 administered into the PVT affected reinstatement compared to vehicle treatment (Fig. 1). Similarly, responding on the inactive lever did not differ between groups ($p=0.20$).

**Effect of VTA-directed SB-334867 on $S^+$-induced reinstatement**

A significant main effect of session indicated that presentation of the $S^+$ cue produced an overall reinstatement of responding on the active lever ($F_{1,16}=23.34, p<0.001$). A significant session $\times$ treatment interaction was also observed, suggesting differences in the extent to which treatment affected $S^+$-elicited reinstatement ($F_{2,16}=7.80, p<0.01$). Post-hoc analyses revealed that intra-VTA treatment with SB-334867 dose-dependently attenuated reinstatement, with the $3\ mg$ dose producing a significant reduction compared to controls ($p<0.01$, Fig. 1). Importantly, SB-334867 treatment had no effect on inactive lever responding ($p=0.22$).

**Effect of VTA-directed SB-334867 on locomotor activity**

There was no statistical difference between treatment groups at any time-point during baseline recording ($p=0.57$). On test day, intra-VTA infusion of SB-334867 had no effect on locomotor activity compared to DMSO-treated controls at all time-points tested ($p=0.77$, Fig. 2).
Discussion

The present findings demonstrate, for the first time, that infusion of the OrxR1 antagonist SB-334867 directly into the VTA attenuates reinstatement of cocaine-seeking behaviour elicited by a cocaine S+ previously associated with drug availability. In contrast, infusion of SB-334867 into the PVT had no effect on cue-induced reinstatement. These findings build upon previous evidence that central or peripheral administration of OrxR-1 antagonists block both cue- and stress-induced reinstatement of drug-seeking (Boutrel et al. 2005; Lawrence et al. 2006; Smith et al. 2009).

We have previously suggested that the PVT may influence reinstatement of drug-seeking; a hypothesis based on several experimental findings. First, inactivation of the PVT attenuates context- and drug-induced reinstatement of alcohol- and cocaine-seeking, respectively (Hamlin et al. 2009; James et al. 2010). Furthermore, Hamlin and colleagues (2009) demonstrated that the percentage of PVT neurons that demonstrate Fos-like immunoreactivity and project to the NAc shell increases after context-induced renewal of alcohol-seeking. There is evidence to suggest that these effects may be mediated by orexin signalling, as the PVT is densely innervated by orexin neurons (Peyron et al. 1998) and orexin terminals make putative contact with PVT neurons that project to the NAc shell (Parsons et al. 2006). Furthermore, drug cue-sensitive PVT neurons are closely apposed to orexin-positive neuronal terminals (Dayas et al. 2008). Thus, the present demonstration of a lack of involvement of PVT OrxR1 signalling in reinstatement-like behaviour is somewhat unexpected.

It should be noted that a role for PVT orexin release in drug-seeking cannot be completely ruled out, as the PVT is also known to strongly express OrxR2 (Marcus et al. 2001). Of relevance here is evidence that orexin-B produces stronger actions on PVT neurons than orexin-A (Huang et al. 2006). Furthermore, intra-PVT administration of OrxR2-specific antagonists have been recently shown to decrease footshock-evoked anxiety-like behaviour (Li et al. 2010b), suggesting a possible role for PVT OrxR2 signalling in stress-induced reinstatement. It will therefore be important for future experiments to determine whether PVT-directed injections of compounds that specifically modulate OrxR2 signalling alter relapse-like behaviour.

We also investigated whether VTA-directed injections of the OrxR1 antagonist alter cue-induced reinstatement and show an important role for this receptor subtype in cocaine-motivated responding. Early electrophysiological studies demonstrated that intra-VTA administration of orexin produces an increase in the firing frequency of VTA dopaminergic (and non-dopaminergic) neurons (Korotkova et al. 2003). While orexin terminals were later shown to make infrequent contact within the VTA (Balcita-Pedicino & Sesack, 2007), both OrxR1 and OrxR2 have been identified on the surface of VTA dopamine cells (Narita et al. 2006). Furthermore, in VTA neurons, orexin-mediated potentiation of NMDA receptor (NMDAR) excitatory post-synaptic currents was increased in animals trained to self-administer cocaine and high-fat food over animals trained to respond for regular food (Borgland et al. 2009). This change was linked to increased presynaptic glutamate release. An earlier study from this group identified that orexin can also potentiate NMDAR currents through protein kinase C-dependent trafficking of post-synaptic NMDARs (Borgland et al. 2006), indicating that orexin may alter the regulation of VTA dopamine neuron activity through both pre- and post-synaptic mechanisms. Finally, using microdialysis, Wang and colleagues (2009) showed that intra-VTA infusion of orexin increases local dopamine and glutamate levels.

Consistent with electrophysiological data, behavioural studies have reported that infusion of orexin directly into the VTA produces reinstatement of extinguished morphine and cocaine preference (Harris et al. 2005; Wang et al. 2009). In addition, unilateral intra-VTA SB-334867 (10 nmol) administration reduces...
In the present study we extend upon these findings and show for the first time that OrexR1 signalling within the VTA mediates cocaine S- induced relapse. Although not directly addressed in the present study, a parsimonious explanation for this effect is that antagonism of OrexR1 decreased dopamine release within the NAc (Narita et al. 2006). Indeed, intra-VTA orexin administration is associated with increased dopamine release in the NAc (Narita et al. 2006) and context-induced reinstatement is sensitive to dopamine receptor blockade in this brain region (Bossert et al. 2007).

Interestingly, the effective dose in the current study (3 μg or 9.4 nmol) was lower than that published in two other behavioral studies. Zheng et al. (2007) showed that bilateral intra-VTA infusion of SB-334867 (15 nmol/side) was required to reduce high-fat food consumption, while Hollander et al. (2008) report that bilateral intra-insular infusions of 1 μg and 5 μg (6.2 and 31.2 nmol, respectively) doses of SB-334867 attenuated nicotine self-administration. The robust effects observed in our study by unilateral VTA injections of a lower dose may indicate some spread of injection to the contralateral VTA and/or differences in the dose of antagonist that is required to suppress behaviour elicited by primary (Hollander et al. 2008; Narita et al. 2006; Zheng et al. 2007) vs. conditioned reinforcers.

Importantly, intra-VTA SB-334867 did not reduce general locomotor activity in animals that were trained to self-administer cocaine. This was despite an apparent, but statistically non-significant, decrease in inactive lever responses in SB-334867-treated animals. The lack of a non-specific effect of SB-334867 is consistent with other studies showing that intra-VTA infusions of the OrexR1 antagonist did not alter locomotor activity (Borgland et al. 2006). It is plausible that the trend towards decreased inactive lever responding is actually due to the effects of SB-334867 on drug-seeking behaviour.

In summary, we show for the first time that OrexR1 signalling within the VTA, but not the PVT, is critical in mediating cue-induced reinstatement. Taking previous findings into consideration, this effect is likely to be mediated by reduced activity of VTA dopamine neurons that target the NAc. Future studies will need to assess whether OrexR2 signalling within the PVT modulates drug-seeking. It is also important to note that orexin signalling may modulate reinstatement at other central sites including the insular (Hollander et al. 2008) and prefrontal (Jupp et al. 2010) cortex.


SUPPLEMENTARY MATERIAL

Available online at:

Figure S1. Levels of reinstatement displayed by animals that received intra-PVT administration of DMSO vehicle (0.5 µl) did not differ to animals prepared with a PVT-directed cannulae that received no injection in terms of active (F_{1,16} = 10.43, p = .82) or inactive (F_{1,16} = 1.97, p = .74) lever responses following presentation of the S^+ cue (n = 8-10). EXT: Average number of responses for both treatment groups over final three extinction sessions were pooled. Importantly, treatment groups did not differ in terms of final extinction responding on either the active (F_{1,17} = 2.81, p = .113) or the inactive lever F_{1,17} = .34, p = .566); data not shown.

Figure S1. James et al. (2011)
SUPPLEMENTARY MATERIAL: S2

Figure S2. (A) Location of the microinjection sites from PVT-treated animals included in the reinstatement experiment. Circles represent microinjections of 6µg SB-334867. Squares represent microinjections of 3µg SB-334867. Triangles represent microinjections of vehicle. The cross represents the single misplaced microinjection of 6µg SB-334867. CM: central medial thalamic nucleus; D3V: dorsal third ventricle;
**IMD**: intermediodorsal thalamic nucleus; **MDC**: mediodorsal thalamic nucleus, central part; **MDM**: mediodorsal thalamic nucleus, medial part; **PV**: paraventricular thalamic nucleus; **PVP**: paraventricular thalamic nucleus, posterior part. **(B)** Location of microinjection sites from VTA-treated animals included in the reinstatement experiment. *Circles* represent microinjections of 3µg SB-334867. *Squares* represent microinjections of 1µg SB-334867. *Triangles* represent microinjections of vehicle. **IF**: interfascicular nucleus; **PBP**: parabrachial pigmented nucleus of the VTA; **PN**: paranigral nucleus of the VTA; **RLi**: rostral linear nucleus of the raphe; **SNR**: substantia nigra, reticular part; **VTA**: ventral tegmental area; **Vtgx**: ventral tegmental decussation. **(C)** Location of microinjection sites from VTA-treated animals included in the locomotor experiment. *Circles* represent microinjections of 3µg SB-334867. *Triangles* represent microinjections of vehicle. Figures adapted from (Paxinos and Watson, 1997). Numbers indicate millimeters from bregma.

**REFERENCE**

SUPPLEMENTARY MATERIAL: S3

Training Statistics

PVT-Treated Animals

Animals received, on average, 316.41 (± 9.51 SEM) infusions throughout self-administration training trials (equivalent to 206 mg/kg cocaine). During training, animals exhibited a strong preference for the active lever (30.27 responses per session ± 0.97 SEM) as compared to the inactive lever (0.64 responses per session ± 0.21 SEM; F_{1,21} = 364.77, p < .001). Animals also exhibited a significantly greater number of responses on the active lever during S^+ trials (30.27 responses per session ± 1.54 SEM) as compared to S^- trials (10.88 responses per session ± 1.45 SEM; F_{1,21} = 128.22, p < .001). On average, 19.73 (± 3.15 SEM) extinction sessions were required to meet the extinction criterion. No differences were observed across treatment groups for all parameters.

VTA-Treated Animals

Animals received, on average, a total of 387.89 (± 15.13 SEM) infusions throughout self-administration training trials (equivalent to 258 mg/kg cocaine). During training, animals exhibited a strong preference for the active lever (46.37 responses per session ± 6.73 SEM) as compared to the inactive lever (0.26 responses per session ± 0.06 SEM; F_{1,18} = 47.32, p < .001). Animals also exhibited a significantly greater number of responses on the active lever during S^+ trials (46.37 ± 6.73 SEM) as compared to S^- trials (13.98 ± 2.01 SEM; F_{1,18} = 18.92, p < .001). On average, 17.42 (± 2.14 SEM) extinction sessions were required to meet the extinction criterion. No differences were observed across treatment groups for all parameters.
CHAPTER FOUR

*Insights for developing pharmacological treatments for psychostimulant relapse targeting hypothalamic peptide systems*
CHAPTER FOUR

INSIGHTS FOR DEVELOPING PHARMACOLOGICAL TREATMENTS FOR PSYCHOSTIMULANT RELAPSE TARGETING HYPOTHALAMIC PEPTIDE SYSTEMS

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Statement IV: Author contribution to Chapter 4 manuscript

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<tr>
<td>Morgan H. James</td>
<td>Conceived, designed and carried out behavioural and immunohistochemical experiments. Wrote the manuscript (original experiments and review).</td>
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<td>Jiann Wei Yeoh</td>
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<td>Brett. A Graham</td>
<td>Conceived and designed electrophysiological experiments included in manuscript. Edited the manuscript.</td>
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<td>Christopher V. Dayas</td>
<td>Conceived and designed all experiments. Wrote and edited the manuscript.</td>
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25 September 2013

PROFESSOR ROBERT CALLISTER
Deputy Head of Faculty (Research and Research Training)
Insights for Developing Pharmacological Treatments for Psychostimulant Relapse Targeting Hypothalamic Peptide Systems

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Abstract

Effective pharmacotherapeutic treatment options for psychostimulant addiction are lacking, in part due to an incomplete understanding of the complex neural circuitry involved in relapse. The lateral hypothalamus (LH) has received renewed interest with respect to its role in addiction-related behaviours, prompted largely by the identification of a number of hypothalamic neuropeptides shown to be important mediators of reward-seeking. In particular, orexin (hypocretin) and cocaine- and amphetamine-regulated transcript (CART) have been shown to play largely opposing roles in feeding behaviour and these roles have recently been shown to extend to drug-seeking behaviour. We have previously proposed that these two peptide systems may interact with the ‘classical’ reward circuitry via the ventral tegmental area (VTA), as this region is densely innervated by both orexin and CART-positive fibres and projects to a number of regions critical to drug-seeking [1]. The present review provides a comprehensive overview of the current literature implicating both orexin and CART in drug-seeking and relapse behaviour and presents a revised summary of the role of the PVT in mediating the actions of these two Creb-family members. In addition, we provide novel data demonstrating that blockade of orexin receptor 1 (OXR1) signalling within the ventral tegmental area (VTA) alters Per-3 protein expression in relapse-relevant regions, including the PVT and the nucleus accumbens shell (NacS). Further, whilst previous findings have shown that blockade of CART in the VTA prevents reinstatement of cocaine-seeking, we show here that this treatment does not affect natural reward seeking for sweetened condensed milk. In light of the reviewed body of literature, as well as the novel data presented, we discuss the considerations for future pharmacotherapies targeting the orexin and CART systems for relapse prevention.

Keywords: Hypothalamus; Orexin; Hypocretin; CART; Cocaine; Reinforcement; Relapse; Fox; Reward seeking; Drug seeking

Introduction

The last two decades have seen significant advances in our understanding of the brain circuitry and molecular changes that drive the addiction process. However, relapse to drug taking continues to represent a significant impediment to the successful treatment of addiction. Indeed, treatment options such as effective pharmacotherapies are lacking, particularly in the case of psychostimulant addiction [2]. Despite significant recent progress, it is likely that the lack of pharmaceutical options to treat this phase of the addiction cycle is in part due to the inadequate understanding of the complex interactions within the neural circuitry that triggers renewed drug-seeking and relapse. Accordingly, in the present review, we aim to highlight some of the recent progress in our understanding of the brain mechanisms underpinning psychostimulant relapse. In particular, we will focus upon the emerging evidence of an important role for neuropeptide systems expressed within the lateral hypothalamus (LH) in modulating drug-seeking and relapse to psychostimulant use. Of the multiple peptides expressed in the LH that appear to regulate reward-seeking, we will focus on two, orexin and cocaine- and amphetamine-regulated transcript (CART), which appear to have opposing effects on food-seeking behaviour. We outline current research that is investigating how these peptide systems might interact with the ‘classical’ neural circuitry implicated in the addiction and relapse process, as well as other regions that have ‘re-emerged’ as potential mediators of addiction-related behaviours [3].

Although imaging techniques for human research have dramatically improved, much of the progress in our understanding of the brain mechanisms involved in addiction and relapse continues to come from preclinical studies. Using experimental animals, researchers have attempted to model human relapse through the use of the experimental procedure known as REINSTATEMENT. Progress from such studies has led to the formulation of a putative ‘final common pathway’ comprised of a series of interconnected brain regions shown to be critical to reinstatement or relapse-like behaviour [4,5]. Briefly, this pathway is thought to involve glutamatergic projections from the prefrontal cortex (PFC) to the nucleus accumbens (NAC), which in turn results in the disinhibition of the ventral pallidum (VTA) [4,5] (Figure 1). Importantly, dopamine released in the PFC, amygdala and NAC is thought to be important for drug-motivated behaviours including relapse-like behavior [6-8]. Dopamine input to these regions arises primarily from the ventral tegmental area (VTA) A10 dopamine neurons [9-14]. Although activation of this pathway is clearly central to the drug-seeking response, other brain regions have recently been found to, or have ‘re-emerged’ as, important modulators of this ‘brain relapse network’ [3]. As such, there is a significant current focus on understanding how these regions of the brain may interface with the ‘classic relapse circuitry’ described above. In particular, significant recent attention has been given to the hypothalamus, as well as the paraventricular thalamus (PVT), the latter being a proposed integrator and relay of hypothalamic neuropeptide signals to higher centres.

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Behavioral Pharmacology
Renewed Interest in Hypothalamic and Dorsal Thalamic Circuitry in Mediating Drug ‘Relapse’

Hypothalamus

The hypothalamus has long been recognized as an important regulator of reward-seeking behavior [15,16]. For example, early studies implicated the LH as the brain’s ‘feeding centre’ following demonstrations that stimulation of the LH resulted in an increase in feeding behaviour and lesions of the LH promoted aphagia and weight loss [15]. In contrast, the ventromedial hypothalamus (VMH) was thought to function as a ‘satiety centre’, with lesions of this region reported to produce hyperphagia and obesity [16]. Although this ‘dual-centre’ hypothesis is undoubtedly oversimplified, this body of work has been influential in establishing that the LH is a key component of a ‘brain reward-seeking system’ [17,18]. Additional support for this proposal can be found in the work of Olds and Milner [19] showing that the lateral portion of the hypothalamus supports INTRACRANIAL SELF-STIMULATION (Table 1) as well as anatomical studies demonstrating that the LH is reciprocally connected with key nodes of the brain circuitry that control motivation and reward, including the PFC, NAC and VTA [20]. Surprisingly, despite this well-established role in reward seeking, only recently has significant attention returned to the LH in terms of its involvement in pathological rewarding-seeking behavior [21]. An important catalyst for this renewed interest in LH control of reward-seeking was the discovery of the hypothalamic neuropeptide transmitters cocaine- and amphetamine-regulated transcript (CART) and orexin (hypocretin). Interestingly, following their identification in the late nineties, studies have generally showed opposing roles for orexin and CART in feeding behaviour. Indeed, subsequent work has shown that a similar relationship may exist for drug-seeking behaviour. Thus, a role for orexin in promoting relapse-like behaviour is now well established and recent reports suggest that CART may negatively modulate relapse-like behaviour. These findings have driven speculation that modulators of LH neuropeptide systems may lead to new treatments for psychostimulant relapse and other neurological disorders [22].

Paraventricular thalamus

The PVT is another structure that has undergone a ‘renaissance’ of sorts in relation to its role in motivated behaviors. The PVT is a component of the dorsal midline thalamic group [23] and has been implicated in stress reactivity [24], reward [25] and general arousal [25]. Our interest in the PVT was stimulated by studies showing that the PVT is densely innervated by peptide expressing neurons originating from the hypothalamus [26-28], as well as midbrain dopamine and brainstem catecholamine neurons [29]. Compellingly, the PVT receives perhaps the strongest orexin and CART peptide input in the forebrain and anatomical evidence indicates that this region may relay hypothalamic neuropeptide activity to the striatal axis and other reward-related regions. For example, Parsons et al. [28] showed that NAC shell-projecting neurons in the PVT receive both orexin and CART terminals. These findings provide an anatomical basis for the influential proposal by Kelley and colleagues [25] that the PVT is as a key relay in a hypothalamic-thalamic-striatal axis that regulates motivation and reward-seeking [25]. Interestingly, the PVT also has strong projections to other important reward-related brain regions, including the PFC, NAC and basolateral amygdala [30-37]. Indeed, anatomical studies have shown that single PVT neurons send branched projections to both the NAC and PFC [30,33]. It is also noteworthy that early studies suggested an important role for the PVT in drug-related behaviours, with lesions of the PVT...

Behavioural sensitisation: A progressive increase in the locomotor-activating effects of drug following repeated exposure to a set drug dose (typically 5-10 days). The expression of behavioural sensitisation is typically assessed during withdrawal following an acute drug challenge at a dose equivalent to that administered during the sensitisation exposure.

Conditioned place preference (CPP): A classical (Pavlovian) conditioning paradigm whereby animals are administered either drug or saline (delivered intraperitoneally). After injection animals are immediately placed into one of two distinct contexts that differ in terms of their contextual cues (e.g. wall colour/pattern, floor texture). Following conditioning, the animals are tested for their preference for the drug- vs. saline-paired chamber, as measured by time spent in each chamber. An increase in preference for the drug-paired context is thought to indicate a positive reinforcing effect of the drug.

Conditioned place aversion (CPA): Similar to CPP, however, one context is associated with an aversive stimulus, whilst the other is associated with a neutral stimulus. Following conditioning, animals are tested for their preference for the contexts. An increase in preference for the neutral context indicates an aversive effect of the last stimulus.

Operant drug self-administration model: A procedure whereby animals are trained to respond (typically lever press or nose poke) for a drug reward. Psychostimulants such as cocaine and amphetamine are typically delivered to the animal via a chronic indwelling jugular catheter, whereas drugs such as alcohol are delivered in small volumes for oral consumption. This model can also be used to train animals to respond for natural rewards, such as a sucrose solution or sucrose/food pellets.

Extinction learning: A reduction in drug-seeking produced by breaking the contingency between drug-seeking behaviour or drug-predictive stimuli and delivery of drug reward. Importantly, extinction learning involves new learning, and is not a 'forgetting' of the previous association.

Intracranial self-stimulation (ICSS): A procedure whereby animals learn to respond for direct electrical stimulation via an electrode implanted into a specific region of the brain. Higher rates of responding for ICSS are thought to be caused by decreased ‘reward’ pathway sensitivity whereas lower rates of responding for ICSS are thought to result from increased ‘reward’ pathway sensitivity.

Progressive ratio schedule: A reinforcement schedule applied to the drug self-administration procedure to assess an animal’s motivation to obtain a natural or drug reward. Under this schedule, the number of responses required to obtain a drug reward is increased following every reward. The point at which the animals cease to press is referred to as the animals’ ‘break point’.

Operant reinstatement models of relapse-like behaviour: In the drug self-administration procedure, animals are trained to self-administer drug before their responding is extinguished to a set criterion e.g. numbers of days of extinction training or number of responses/session. After reaching the extinction criterion, ‘reinstatement’ of responding can be elicited by exposure to drug-associated stimuli, psychological stress or re-exposure to the drug itself (also known as a drug-prime) – analogous stimuli are thought to elicit drug-seeking and relapse in humans.

Reinstatement of a CPP: CPP is induced by drug administration as described above. The CPP is then extinguished by repeatedly placing the animal into the CPP apparatus without drug/saline injections. Reinstatement of a CPP can be induced by drug-prime injections or exposure to stress.

Renewal: Animals are trained to self-administer drug in Context A, before drug-seeking is extinguished in Context B. Context A and B differ from one another in terms of various contextual cues, including wall colour/pattern, floor texture. ‘Renewal’ of drug-seeking can then be elicited by placing the animal back into Context A.

Table 1: Glossary of terms.

The CART Neuropeptide System

In 1996, Douglass and colleagues [46] identified an mRNA that was upregulated in the striatum of rats following acute cocaine and amphetamine administration. Interestingly, this mRNA was found to encode a gene product that had been identified by Spiess et al. [47] in the ovine hypothalamus close to 15 years earlier. This peptide was subsequently named cocaine- and amphetamine-regulated transcript (CART). Human and rat CART mRNA share 91% sequence homology but despite this similarity the rat has long and short splice variants of the CART peptide whereas only the short form is present in humans [48]. Two active fragments of the rat long form (CART 55-102 and CART 61-102) have been identified, however, a CART receptor has yet to be fully characterized, meaning that a specific CART receptor antagonist is yet to be developed. CART cell bodies are expressed in a number of hypothalamic regions, including the LH, the paraventricular nucleus (PVN), supraoptic nucleus and the arcuate nucleus (ARC) [46-50]. In addition to this distribution, CART is co-expressed with a number of other neuropeptides including thyrotropin-releasing hormone (TRH) in the PVN, melanin concentrating hormone (MCH) in the DMH/LH and pro-opiomelanocortin (POMC) in the ARC nucleus and retrochiasmatic area (RCA). It is interesting and somewhat surprising that within the ARC nucleus, CART is co-expressed with the POMC-derived anti-feeding peptide a melanocyte stimulating hormone (α-MSH) whereas in the DMH/LH CART is expressed with the pro-feeding peptide MCH [18,49,51]. (Although not a focus of this review, it is interesting that MCH appears to have pro drug-seeking effects within the NAC [52]). These findings raise the possibility that different populations of CART neurons may subserve different functions. Beyond the hypothalamus, CART peptide-expressing neurons are found in the NAC, VTA and amygdala [50,53]. Interestingly, approximately 15% of CART terminals in the VTA contain MCH, whilst CART peptides also co-localise, albeit to a lesser extent, with GABA and dynorphin in both the VTA and substantia nigra [53]. CART-cell bodies are also shown to block BEHAVIOURAL SENSITISATION to cocaine [38]. Furthermore, Fos protein expression has been shown to increase in the PVT following re-exposure to cocaine-paired environments after experimenter administered drug injections [39,40].

Based on these observations, we recently proposed that the PVT is a site central to the integration of CART and orexin signalling in controlling drug-seeking [1]. Consistent with our hypothesis, we recently identified an interesting anatomical relationship involving CART and orexin terminals closely apposed to PVT neurons recruited by exposure to ethanol-linked discriminative cues in an operant reinstatement paradigm to model alcohol relapse [1]. Important studies have shown that a role for the PVT extends to renewal of alcohol-seeking [37,41], reinstatement of cocaine-seeking [42,43], as well as the expression of EXTINCTION behaviour [44,45]. Below, we review the evidence implicating both the CART and orexin peptides in psychostimulant relapse and discuss recent data demonstrating that CART signalling within the PVT appears to negatively modulate drug-seeking behavior. We also review recent findings from our laboratory demonstrating that, in contrast to our original hypothesis, orexin signalling in the PVT does not appear to be critical to drug-seeking behavior. We therefore discuss evidence that the VTA is a more likely target of relapse-relevant orexin signaling. In addition, we present new, unpublished, data showing that orexin receptor antagonism in the VTA is associated with changes in Fos-protein expression patterns in relapse-relevant regions. We also show that whilst VTA orexin receptor antagonism prevents cue-induced cocaine-seeking behavior, similar doses of this antagonist does not affect natural reward-seeking. These preliminary data raise the possibility that orexin antagonism for relapse prevention may be possible without producing significant off-target effects. Firstly, however, we review the historical evidence linking both CART and orexin with rewarding-seeking in general and subsequently their role in drug motivated behaviours.
for a CART-mediated inhibitory effect that attenuates cocaine-induced locomotion [73]. In contrast to this evidence, the VP, one of the main nuclei that receive accumbal dopamine [72], consistently showed no effect, injections of CART into the NAC reduces the cocaine-like locomotor-activating effects of these drugs [70] and prevents the expression of CPP [74]. Finally, Rademaker et al. [75] showed that at low doses (2µg/side), intra-basolateral amygdala injections of CART produced CPP, whilst higher doses (4µg/side) produced CONDITIONED PLACE AVERSION (CPA). Together, this work clearly establishes a role for CART in addiction-like behaviour, but also suggests that the underlying mechanisms are complex.

With respect to reinstatement of drug-seeking behaviour, a potential role for CART was first alluded to in the study of Matson and Morrell [76]. These authors showed that conditioned cues associated with passive cocaine administration increased the number of CART-positive neurons in the NAC. Since this report, a number of subsequent studies have provided evidence that hypothalamic CART cells in regulating drug-seeking behaviours. For example, Dayas et al. showed that exposure to drug-associated cues and ethanol-seeking behaviour were associated with an increase in Fos/CART-positive cells within the ARC nucleus, but not the DMP/H-LH [1]. Interestingly, Miilan et al. [41] showed that reinstatement of alcohol-seeking elicited by inactivation of the NAC shell is associated with an increase in PeF hypothalamic CART cells expressing c-Fos.

As discussed above, we previously proposed that the PVT might be a central site through which hypothalamic CART activity is relayed to critical reinstatement-related brain regions. Consistent with this suggestion, alcohol-associated cues activate cells within the PVT that are closely apposed to CART terminals [1]. We recently provided further support for this hypothesis by demonstrating that intra-PVT infusions of the CART 55-102 peptide fragment dose-dependently decreased drug-primed reinstatement of cocaine-seeking [43]. To date, only one other study has examined the functional effects of CNS CART gene in a Korean population [67] and acute administration of cocaine or amphetamine treatment attenuates the locomotor-activating effects of CNS CART and addiction-relevant behaviours [56]. Behavioral Pharmacology

CART gene in a Korean population [67] and acute administration of alcoholism has been shown to be associated with a mutation in the CART and dopamine efferents from the PVT are closely associated with dopamine immunoreactive terminals in the NAC shell (NACsh). Moreover, stimulation of the PVT increases dopamine release within this part of the ventral striatum [34]. The NAC is critically involved in the reinstatement of drug-seeking [86,81] and dopamine signalling in this region is necessary for drug primed [82] and context-elicited [81] reinforcement.
reinstatement. Taken together, CART signaling within the PVT may act to inhibit reinstatement by modulating the responsivity of NAC neurons to cocaine-induced dopamine release [43].

Further to its role in the PVT, CART neurons also project to the VTA and their interaction with dopamine neurons suggests they may influence reinstatement. In the VTA, nerve terminals that contain CART mRNA and peptide contact dopaminergic neurons as well as GABAergic interneurons [58,53]. It has been proposed that CART may therefore regulate dopamine release from the VTA either directly or via the disinhibition of GABA interneurons [83]. Further, CART is also expressed in NAC, particularly within the medial part of the shell [84], which corresponds with the region of the ventral striatum that receives the strongest innervation by dopaminergic neurons [85]. TH-positive terminals make contact with CART peptide-containing GABAergic neurons in the NAC [84], suggesting that a functional relationship exists between midbrain dopaminergic cells and CART-peptide containing striatal neurons. Finally, MCH neurons in the LH project to the NACs [86] and MCH terminals exist in the NACs [86]. Given that LH MCH neurons also express CART [49], it is probable that a direct CART projection from LH to NACs exists [Haemmerle, Bittencourt and Dayas, personal communication, 2012] although it is currently not known if CART is released from these MCH-expressing LH-projection neurons.

Work using an alcoholic beer RENEWAL model has also provided data supporting a role for CART signaling pathway in the expression of extinction through a circuit involving the PVT, the NACsh and hypothalamus [41]. Specifically, McNally and colleagues showed that extinction in this model appeared to recruit neurons in the NACsh and possibly the infralimbic prefrontal cortex (IL-PFc) that project to the mediodorsal hypothalamus [45]. Further, Marchant et al. [44] showed that expression of extinction modulated CART-sensitive neurons in the MDH that express dynorphin and project to the PVT. Consistent with this circuitry, infusing the ε-opioid receptor agonist US50488 into the PVT promoted the expression of extinction-i.e. prevented drug-seeking behaviour.

In summary, given the above literature, a role for CART in addiction is now well established, however, a number of questions remain to be answered before pharmacological manipulation of this system can be realistically evaluated. For example, it will be important for future studies to determine the role of CART in mediating both cue- and stress-induced reinstatement, as well as the central site(s) of action in these processes. Further, the role for CART during extinction versus reinstatement warrants further investigation. Moreover, a greater understanding of the role of CART in addiction-related behaviours and indeed reward-seeking in general, presumably awaits the characterization of the CART receptor(s).

The Orexin Neuropeptide System

Orexin-expressing neurons are located in the DMH, PF and LH areas and secrete two peptides (orexin A & orexin B) derived from the same precursor gene (prepro-orexin) [87]. It should be noted that two research groups independently identified these peptides almost concurrently, with one group naming these peptides orexins [87] and the other naming them hypocretins [88]. The orexin peptides are highly conserved between humans and rodents, with identical orexin-A sequences and just two amino acid substitutions in orexin-B. Unlike the CART system, where the search for an endogenous receptor continues, orexin peptides have been shown to interact with two G-protein-coupled receptors; Orexin receptor 1 (OX₁) and orexin-receptor 2 (OX₂). Orexin A binds to both OX₁ and OX₂ with equal affinity, whereas orexin B binds to OX₂ with a higher affinity than OX₁ [89]. OX₁ is found in the PFC, hippocampus, PVT, VTA, VMH, dorsal raphe nucleus and locus coeruleus. OX₂ are expressed in regions including the prefrontal and insular cortices septal nuclei, hippocampus, medial thalamic groups, VTA, raphe nuclei and hypothalamic nuclei including the tuberomammillary nucleus, dorsomediaal nucleus, paraventricular nucleus (PVN) and ventral premammillary nucleus [90-92]. Consistent with this distribution, the function of orexin peptides is diverse. Thus, in addition to reward-seeking, orexins have been implicated in the regulation of sleep [93], energy metabolism and the maintenance of arousal [94,95]. Interestingly, nearly all orexin neurons co-express hypocretin-1 [96] and glutamate [97]. It is also important to note that vagal and spinal primary afferent neurons, enteric neurons and endocrine cells in both the gut and pancreas display orexin- and orexin receptor-like immunoreactivity [98] and exogenous orexin administration has been shown to excite secretomotor neurons, modulate intestinal motility and secretion [98-100] and influence hormone release from pancreatic endocrine cells [101].

Orexin and feeding behaviour

Consistent with its anatomical location within the LH reward-seeking zone, central administration of both orexin A and B has been found to dose-dependently initiate food-seeking [87,102,103] while systemic administration of the OX₁ antagonist SB-334867 has the opposite effect [104-107]. Furthermore, prepro-orexin mRNA is upregulated following fasting [87], whereas obese mice (ob/ob and db/db) show decreased prepro-orexin gene expression [108]. It is important to recognize the possibility however, that the effects of orexin on feeding behaviour may reflect the role of orexin in the maintenance of arousal and locomotor activity, both of which are essential for food-seeking behaviour following periods of fasting [108] (but see [109]). In contrast, SB-334867 has no effect on reinstatement of high-fat food seeking elicited by i.c.v. administration of orexin A, pellet-priming or yohimbine, suggesting that the OX₁ plays a minimal role in reinstatement of food-seeking [110].

Orexin and addiction

An interesting series of observations highlighting the potential role for orexin in addiction includes the low CSF orexin-A levels in patients with the neurological disorder narcolepsy [111-113] and the lack of dependence in these patients despite receiving amphetamine treatment [114]. The first experiment directly implicating orexin in addiction was the demonstration that orexin knock-out mice display attenuated morphine dependence [115]. More recent studies have shown that blockade of the OX₁ reduces self-administration of alcohol [116-118], nicotine [119,120] and high-fat food [110]. With respect to psychostimulants, OX₁ blockade does not appear to reduce cocaine self-administration under normal conditions, but does reduce the extent to which rats are willing to work for cocaine reward under a PROGRESSIVE RATIO SCHEDULE [121]. These data suggest that orexin may not play a large role in the pro-drug-like rewarding effects of psychostimulants, but may modulate circuitry that drives the motivation of goal-directed behavior [121,122]. Interestingly however, orexin signaling does appear to modulate the rewarding properties of psychostimulants under some experimental conditions, as treatment with SB-334867 reduces the acquisition and expression of cocaine-conditioned reinforcement and the expression of amphetamine-induced CPP [123]. A clear role for OX₁ signaling has also been shown in the development of BEHAVIOURAL SENSITISATION.
to psychostimulants. For example, SB-334867 treatment prevents sensitization following repeated cocaine and amphetamine treatment [124,125]. In comparison, OX2 signaling appears to primarily mediate wakefulness and arousal and play less of a role in mediating reward seeking, however, infusions of orexin B (presumably acting on orexin-2 receptor) into the VTA increases preference for morphine [126] and repeated cocaine exposure produces an up-regulation of OX2 levels in the NAC [127]. It is also worth highlighting recent evidence that blockade of the OX2,2 can prevent ethanol self-administration, place preference and reinstatement [128].

With respect to drug-seeking behaviour, activation of orexin cells, as assessed by Fos-protein, has been associated with reinstatement of drug-seeking using different procedures. Importantly, Harris et al. [129] showed that re-exposure to cocaine- and morphine-associated contexts in a CPP paradigm increased the percentage of LH orexin neurons that express Fos. Further, the proportion of Fos-positive LH orexin neurons was strongly correlated with the propensity for reinstatement of CPP [129]. These authors also provided evidence that Fos-protein expression increased specifically within LH orexin neurons, as correlations between Fos expression and reinstatement of CPP was not observed with PeF or DMH orexin neuron populations, nor for non-orexin neurons within the LH [129]. Dayas et al. [1] showed in an operant self-administration reinstatement procedure that re-exposure to alcohol-associated cues increases the numbers of DMH and PeF/LH Fos-positive orexin neurons. Interestingly, Hamlin et al. [130] also implicated PeF orexin neurons in the processes that are required for drug-seeking but perhaps not the drug-seeking response itself. These findings demonstrate the importance of understanding the role of distinct subpopulations of orexin neurons within the DMH, PeF and LH. Indeed, recent evidence suggests that there are two distinct subgroups of orexin cells with unique firing properties and morphologies [131] and it will be important to further understand how these different populations may differentially influence drug-seeking behaviour.

In order to confirm a functional role for orexin cell activation in reinstatement of CPP, Harris and colleagues [129] microinjected the Y4 receptor agonist rat pancreatic polypeptide (rPP) directly into the LH. As predicted by the expression of Y4 receptors in orexin cells within LH, this manipulation induced Fos expression in LH orexin neurons and robustly reinstated extinguished morphine CPP. A functional role for orexin signaling in psychostimulant reinstatement has also been well established by experiments using operant based procedures. In a key study, Routel and colleagues [132] demonstrated that i.c.v. infusion of orexin-A reinstated extinguished cocaine-seeking and that the OX1 antagonist SB-334867 reduced reinstatement elicited by exposure to acute footshock stress. Similarly, Lawrence et al. [116] found that pretreatment with SB-334867 (i.p.) abolished olfactory cue-induced reinstatement of alcohol-seeking behaviour. Since these seminal studies, the orexin system has been shown to mediate various forms of reinstatement. For example, systemic administration of the OX1 antagonist SB-334867 (10-30mg/kg) blocks reinstatement of extinguished cocaine-seeking elicited by both discrete cues [133] and discriminative contexts previously associated with cocaine availability [134]. These findings extend to non-extinguished cocaine-seeking, as SB-334867 pretreatment (10-30mg/kg) attenuates drug-seeking in cocaine-associated contexts following two weeks of abstinence in the home cage [134].

The orexin system also appears to interact with stress pathways to regulate footshock-induced reinstatement [132]. Specifically, in this paradigm administration of the corticotropin-releasing factor (CRF) antagonist D-Fse-CRF [12-41] and the α2-noradrenergic agonist clonidine, prevents reinstatement induced by i.c.v. orexin, suggesting that orexins may mediate footshock-induced reinstatement by recruiting these stress systems. Importantly, antagonism of OX1 does not appear to block the priming effects of psychostimulants on reinstatement behaviour, as pretreatment with SB-334867 (10-30mg/kg, i.p.) had no effect on reinstatement of cocaine-seeking induced by an acute cocaine injection [135].

As discussed above, the PVT has been proposed as an important recipient site for hypothalamic orexin signaling in reward-seeking (eg. [25]). Indeed, we proposed that orexin signaling within the PVT might be critical to reinstatement of drug-seeking. Consistent with this suggestion, drug-associated cues activate PVT neurons that are closely apposed to orexin fibres [1] and orexin terminals make putative contact with PVT neurons that project to the NAC [34]. Somewhat surprisingly therefore, we recently reported that intra-PVT infusion of SB-334867 has no effect on cue-induced reinstatement of cocaine-seeking [136], suggesting that orexin signaling, at least through OX1, in this region is not critical to reinstatement behaviour. Consistent with these data, subsequent preliminary work from our laboratory suggest that infusions of orexin-A into the PVT does not reinitiate extinguished drug-seeking behavior. However recent evidence from another group has shown that at small doses, cocaine-seeking can be reinstated by intra-PVT infusions of orexin-A [137]. In addition to these recent findings, a role for PVT-based orexin signaling in reinstatement behaviour cannot be ruled out, as this region also strongly expresses OX2 [90] and orexin B has been shown to produce stronger actions of PVT neurons than orexin A [118]. Further, the PVT is known to play an important role in reactivity to psychological stress [24] and orexin signaling in the PVT has been shown to modulate anxiety-like behaviour through CRF and s-opioid receptors [139]. Finally, OX1 signaling in the PVT has been shown to be important...
for the ability to adapt to repeated stress [140]. Therefore, it will be for future studies to determine the function of OX₂ signaling in this region, as well as to investigate the potential role for the PVT in stress-induced drug seeking.

The VTA is another reward pathway structure that appears to be a possible target of addiction-relevant orexin signaling. Although very few ‘classic’ synaptic contacts between orexin axons and VTA dopamine neurons exist [141], both OX₁ and OX₂ have been identified in VTA dopamine cells [142]. Importantly, functional electrophysiological studies have repeatedly shown that orexins modulate dopaminergic neuronal activity within the VTA [143,144]. For example, Korotkova et al. [144] showed that orexins increase the firing rate and can also elic tit burst firing of VTA dopamine neurons. These authors also showed that subpopulations of dopamine neurons appear to respond to orexin A versus B and can express (OX₁, OX₂) or both receptor subtypes. In work that described a potential pathway for orexins to enhance VTA dopamine neuron activity, Börgland et al. [124] reported that orexin-A potentiates NMDAR currents in this population through protein-kinase C-dependent trafficking of NMDARs. Systemic SB-334867 has also been shown to block cocaine sensitization and to elevate the AMPA/NMDA ratio in VTA DA neurons – a surrogate measure of synaptic plasticity [145]. More recent studies have shown that the orexin-induced changes in VTA dopamine neuron activity are also thought to involve pre-synaptic mechanisms, as orexin potentiates excitatory inputs onto VTA neurons in cocaine-trained animals [121]. Interestingly, orexin-induced presynaptic changes were also seen in animals that self-administered high fat but not regular food. Consistent with these electrophysiological findings, in vivo studies using microdialysis have shown that local infusion of orexin-A increases extrasynaptic VTA dopamine and glutamate levels [146] and enhances the effects of cocaine on VTA dopamine signaling [147]. Similarly, using in vivo recordings in rats, Moorman & Aston-Jones [145] demonstrated that orexin application in the VTA increases baseline DA neuron activity and augmented DA neuron activity evoked by mPFC stimulation, a region known to play a key role in mediating reinstatement.

Supporting these anatomical and physiological data, there is now a significant body of behavioural evidence to suggest that orexin signaling in the VTA plays a key role in mediating drug-seeking. For example, intra-VTA injections of orexin-A reinstated morphine preference in a CPP model [129] and extinguished cocaine-seeking behaviour in a self-administration paradigm [147], whereas intra-VTA SB-334867 administration suppressed morphine CPP [145]. Consistent with these findings, work from our laboratory has implicated VTA orexin signaling in cue-induced reinstatement of cocaine seeking [148]. In this report, we showed that unilateral infusions of SB-334867 (7µg) into the VTA prior to reinstatement testing suppressed reinstatement of drug-seeking elicited by discriminative cues previously associated with cocaine availability and that this effect was independent of any effects on locomotor activity [136]. Preliminary evidence suggests that this attenuation of drug-seeking is associated with a reduction in the number of Fos-positive neurons within the PVT (Figure 2). It is plausible that OX₁ antagonism reduced the activity of VTA dopamine neurons that project to the PVT. This is consistent with previous studies showing dopamine-immunoreactive fibers in the PVT [149-151]. Further, PVT neurons express D₁, receptor mRNA [152] and the DA transporter has been shown to be present in the midline thalamic nuclei [153]. However, a recent retrograde tracing study indicates that VTA dopamine projections to the PVT may not be as strong as previously thought [154]. Future studies will need to investigate the specific pathways via which VTA OX₁ signaling in the VTA modulates PVT activity in response to drug-related cues. Alternatively, it is possible that OX₂ signaling in the VTA may influence PVT activity via an indirect route. Blockade of the VTA OX₂ signaling also produced an increase in the number of Fos-positive cells in the NAcSh, an interesting finding in light of recent reports that the NAcSh mediates the expression of extinction via projections to the hypothalamus [41].

More recently, we sought to investigate whether the dose of SB-334867, effective in reducing cocaine-seeking, also reduced natural reward-seeking behaviour. To achieve this aim, rats were trained to self-administer sweetened condensed milk (SCM) in the presence of...
discriminative cues using identical procedures as for cocaine-trained animals. While presentation of SCM-associated cues following extinction elicited reward-seeking, intra-VTA administration of SB-334867 did not alter this behavior, even at a dose higher than what was effective in suppressing cocaine-seeking (Figure 3). These data are in keeping with a report that higher systemic doses of SB-334867 are required to reduce SCM compared to cocaine-seeking [137] and are in keeping with a report that higher systemic doses of SB-334867 did not alter this behavior, even at a dose higher than what was effective in suppressing cocaine-seeking, intra-VTA administration of SB-334867 following abstinence (Figure 3). This study demonstrates that the hypothalamus itself, which might contribute to the recruitment of orexin signaling, is postulate raises the possibility of drug-seeking elicited by stress [155-158] and a brain region densely innervated by hypothalamic orexin neurons. To date, most of the work aimed at understanding orexin function in addiction has been focused on downstream projection targets of the hypothalamus, which might affect the recruitment of orexin neurons by stress, cues or drug. A study by Ahmed and colleagues [139] first alluded to this possibility by demonstrating by microarray that the hypothalamus is the most transcriptionally responsive to extended cocaine exposure compared to brain regions such as the prefrontal cortex or accumbens. Interestingly, many of the changes that were identified in this study were for genes encoding molecules implicated in pre- and post-synaptic plasticity. Our group has recently addressed whether cocaine modulates hypothalamic orexin circuitry by using anatomical and electrophysiological techniques. Rats given seven days of passive cocaine exposure exhibited increased excitatory vesicular glutamate transported 2 (VGLUT2)–positive puncta closely apposed to orexin neurons, whereas vesicular γ-aminobutyric acid (GABA) transporter (VGAT)–positive inputs were unchanged [136]. This finding was reinforced by electrophysiological studies that identified an increased frequency, but not amplitude, of miniature excitatory post-synaptic currents (mEPSCs) in PF/LH of cocaine-trained rats compared to saline-treated animals. Importantly, similar electrophysiological differences were observed between animals trained to self-administer cocaine for two weeks versus food-trained rats. In addition, we found that the AMPA/NMDA ratio of evoked excitatory post-synaptic currents was unchanged in PF/LH and orexin neurons from cocaine-trained rats whereas the paired-pulse ratio of these inputs was reduced in the cocaine-trained group. Together these data provide evidence that passive or self-administered cocaine increased pre but not post-synaptic plasticity in hypothalamic circuitry that may control orexin neuron excitability. One limitation of this work was that most recordings were made from unidentiﬁed PF/LH neurons. Furthermore, these assessments of synaptic function were made at a single time point in the self-administration paradigm. Thus, it will be important to repeat these studies in mice expressing GFP in orexin neurons, as well as to determine whether these changes persist into withdrawal. Our study also did not determine the mechanisms responsible for this pre-synaptic plasticity. Although this work has not yet determined the mechanisms underlying plasticity at excitatory inputs to orexin neurons, a potential candidate is cocaine-induced changes to Group III metabotropic glutamate receptor (mGluR) function. Previous work has shown that this system maintains background levels of tonic inhibition of excitatory synaptic input onto hypothalamic orexin neurons [160]. Accordingly, a loss in function of these receptors may contribute to plasticity changes that would enhance the activity of excitatory inputs. This postulate raises the possibility that mGluR receptor modulators, already showing promise in pre-clinical studies [161], may prove to be beneficial in reversing drug-induced plasticity that affects orexin cell excitability.

**Considerations for Future Pharmacotherapies Targeting the Orexin and CART Systems for Relapse Prevention**

Although pre-clinical studies support the potential therapeutic benefits of targeting orexin and CART to reduce relapse risk following psychostimulant abuse, it is important to acknowledge the challenges and limitations that such an approach faces [22]. In the case of orexin, the importance of this peptide in mediating a number of important physiological processes, including arousal, raises the possibility of...
of-target effects. Despite this, and consistent with the differential roles of the orexin receptors, blockade of the \( \text{OX}_1 \) does appear to specifically attenuate reward-seeking behaviour while not affecting overall arousal levels. Thus, doses of SB-334867 that attenuate both cue- and stress-induced reinstatement do not affect extinction responding [133] or locomotor activity in a drug free state [136,162].

Regarding \( \text{OX}_2 \) antagonism, although a recent study reported that the selective \( \text{OX}_2 \) antagonist JNJ-10397049 attenuates reinstatement of alcohol CPP [128], the \( \text{OX}_2 \) antagonist 4-pyridylmethyl (S)-tert-leucyl 6.7-dimethylx-1,2,3,4-tetrahydropyridosquinline [4PT] had no effect on cue-induced reinstatement of cocaine-seeking and produced marked impairments in locomotor activity [133]. Additionally, while JNJ-10397049 significantly alters sleep-wake states, the \( \text{OX}_1 \) antagonists SB-334867 and SB-469124 do not affect these parameters [163,164]. Importantly however, the orexin system has recently been implicated in vestibular motor control through effects on the lateral vestibular nucleus and there is some evidence that SB-334867 attenuates motor performance in tasks that involve a motor challenge [165]. Therefore, only after a comprehensive analysis of the CNS effects of \( \text{OX}_1 \) antagonists will it become clear if targeting the orexin system has the clinical efficacy and target selectivity required to be a realistic pharmacotherapy.

With respect to CART, a number of reports have described disrupted locomotor activity as a result of CART administration. For example, i.c.v. CART 55-102 peptide (1-2µg) causes a flattened body posture and movement-associated tremor [58,59,166,167]. Central administration of CART (2µg) has also been reported to reduce water intake [167] and alter licking patterns [166]. Importantly however, these effects appear to be less pronounced when CART is administered into discrete brain regions. For example, injections of CART (2.5µg) into the PVT has no noticeable effect on locomotor activity [43]. Further, intra-VTA infusions of CART have no effect on movement, except at high doses (bilateral/5µg/side) [74]. Thus, the challenge for a pharmacotherapeutic approach based on the CART system may center on the ability to selectively manipulate specific components of this system and avoid off target effects.

Another important consideration with respect to targeting both the orexin and CART systems is the effects on feeding behaviour and the potential for weight loss. Relatively high doses of SB-334867 reduce progressive ratio responding for both cocaine and natural rewards such as food [110,121,168,169]. There is some evidence however, that SB-334867 can reduce drug-seeking without affecting feeding behaviour. For example, Hollander et al. [119] showed that SB-334867 (4mg/kg, i.p.) blocks fixed ratio responding for nicotine but not food, whilst Ipp et al. [118] showed that SB-334867 (5mg/kg) reduces progressive ratio responding for alcohol without affecting responding for sucrose pellets. Moreover, our data presented earlier in this review, along with that of Martin-Fardon et al. [137], suggests that it is possible to administer SB-334867 either systemically or directly into the VTA at doses that attenuate cue-induced reinstatement of cocaine-seeking but not natural reward-seeking.

At present it is less clear whether ‘therapeutic doses’ of CART that reduce drug-seeking would be free of anorectic effects. For example, a 1.25µg dose of CART administered i.c.v. reduces progressive ratio responding and context-induced reinstatement for alcohol [77], whereas small doses (1µg) injected i.c.v. significantly inhibit feeding [58]. Given that substance abuse is often associated with weight loss and has a high comorbidity with eating disorders [170], it will be important for future studies to determine whether it is possible to administer CART at a dose that will suppress drug-seeking without affecting feeding behaviour.

**Summary**

The results reviewed above indicate critical but generally opposing roles for orexin and CART peptides in the regulation of psychostimulant-motivated behaviours, including reinstatement. Although the exact mechanisms through which orexin and CART mediate reinstatement behaviour are not completely understood, it appears that both peptides exert their influence through a number of key reward-related regions. With respect to CART, the PVT appears to be a central site involved in the anti-drug seeking effects of this peptide. Indeed, we have shown that CART signaling in this region is important for anti-drug seeking and preliminary evidence suggests that this might also be true for cue-induced reinstatement. Future studies are required to determine whether CART signaling in the PVT is also critical to stress-induced reinstatement and whether CART may also exert its inhibitory effects on drug-seeking through other ‘classic’ reward regions, such as the VTA and NAC.

We had previously proposed that the PVT might also be critical to reinstatement relevant orexin signaling. Contrary to this suggestion, we recently reported that \( \text{OX}_1 \) signaling in this region is not necessary for cue-induced reinstatement of cocaine-seeking. Future studies should address whether \( \text{OX}_2 \) signaling in the PVT is involved in this form of reinstatement, as well as whether orexin signaling at either \( \text{OX}_1 \) or \( \text{OX}_2 \) may contribute to stress-induced drug-seeking.

In contrast, the VTA appears to be an important site responsible for mediating the pro-drug-seeking effects of orexin. Considerable anatomical and electrophysiological evidence suggests that orexin might mediate drug-seeking via VTA dopamine neuron activity and we have recently shown that blockade of the \( \text{OX}_1 \) in this region blocks cue-induced reinstatement of cocaine-seeking, but not natural reward-seeking. It will be important to determine whether other orexin receptors in brain regions implicated by Fos-mapping can also regulate drug-seeking [117]. Importantly, orexin signaling in the VTA does not appear to be critical to stress-induced reinstatement and it will therefore be important that future studies investigate alternative loci that might be involved in the known role of orexin in this form of reinstatement. Finally, recent evidence from our laboratory suggests that cocaine induces pre-synaptic plasticity within the hypothalamic circuitry that may affect orexin cell recruitment by relapse-evoking stimuli. A more detailed characterization of these changes may lead to new insights for developing pharmacological agents to reduce psychostimulant relapse.

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**References**


CHAPTER FIVE

Orexin cell hypo-responsiveness to stress in adult rats subjected to early life stress: Gender-specific effects of exercise
CHAPTER FIVE

OREXIN CELL HYPO-RESPONSIVENESS TO STRESS IN ADULT RATS
SUBJECTED TO EARLY LIFE STRESS: GENDER-SPECIFIC EFFECTS OF
EXERCISE

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Statement V: Author contribution to Chapter 5 manuscript

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CHAPTER ONE

PROPENSITY TO 'RELAPSE' FOLLOWING EXPOSURE TO COCAINE CUES IS ASSOCIATED WITH THE RECRUITMENT OF SPECIFIC THALAMIC AND EPITHALAMIC NUCLEI

Morgan H. James, Janine L. Charnley, Jamie R. Flynn, Doug W. Smith & Christopher V. Dayas

Neuroscience (2011) pp 235-242

Statement I: Author contribution to Chapter 1 manuscript

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OREXIN CELL HYPO-RESPONSIVENESS TO STRESS IN ADULT RATS SUBJECT TO EARLY LIFE STRESS: GENDER-SPECIFIC EFFECTS OF EXERCISE

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ABSTRACT

**Background:** Exposure to early life stress (ELS) alters behavioural and neuroendocrine responses to stress in adulthood. Lateral hypothalamus (LH) orexin (hypocretin) cells play an important role in stress responsivity and recent studies suggest that hypoactivity of orexin function is associated with depressive-like symptomology. Despite the strong link between ELS and the development of mood disorders, the effect of ELS on orexin neuron function in adulthood has not been directly tested. Further, whilst the antidepressant and anxiolytic effects of exercise are well characterised, the effects of physical activity on orexin system function are unknown.

**Methods:** Male and female rats were subjected to 0 or 3hrs of maternal separation from postnatal days (PND) 2-14. A subgroup of ELS animals was given access to running wheels for 1hr/day from PND 40-70. All rats were exposed to 30mins restraint stress in adulthood (PND 75-79) before being tested on the open field (OF) and elevated plus maze (EPM). Brains were processed for Fos-protein immunohistochemistry and co-labelling for orexin or TH.

**Results:** Male and female rats exposed to ELS exhibited a reduced percentage of Fos-positive orexin cells. Reduced Fos-like immunoreactivity was observed in the medial parvocellular PVN and paraventricular thalamus. ELS was also associated with reduced exploratoriation in the OF but no behavioural changes were observed on the EPM. Interestingly, wheel running protected against ELS-induced neural and behavioural deficits in male but not female rats.

**Conclusions:** We report that exposure to ELS results in a hypoactive LH orexin cell response to stress in adulthood. These changes are likely to increase susceptibility to depressive-like-behaviours. Interestingly, wheel running protects against ELS-induced neural and behavioural deficits in male but not female rats. Our findings identify that maladaptive rewiring of orexin circuits can be reversed by exercise and we highlight a need to better understand the gender-specific effects of this intervention on these parameters.
INTRODUCTION

Early life trauma or stress is a major risk factor for the emergence of mood related disorders such as depression and anxiety in adulthood (Moffitt et al., 2007; Danese et al., 2008; Danese et al., 2009). A typical feature accompanying this increased risk is altered neuroendocrine function (hypothalamic-pituitary-adrenal [HPA] axis) responses to stress (Carpenter et al., 2011; Ouellet-Morin et al., 2011; Goldman-Mellor et al., 2012). Pre-clinical studies show that separation of rat pups from their mother during the neonatal period (known as maternal separation) also increases vulnerability to anxiety- and depression-like behaviour in adulthood (Winslow and Insel, 1991; Matthews et al., 1996; Marais et al., 2008; Marais et al., 2009). Indeed, seminal studies employing this approach have shown that the impact of ELS on the brain is dramatic and includes maladaptations to the neuroendocrine hypothalamus (i.e. the paraventricular nucleus, PVN) and associated feedback circuits that manifest in adulthood (Meaney et al., 1996; Meaney et al., 2007; Murgatroyd and Spengler, 2011). Importantly however, other hypothalamic systems are known to influence autonomic, neuroendocrine and behavioural responses to stress, but there have been few studies addressing the impact of ELS on these non-PVN cell groups. For example, cell groups within the lateral hypothalamus (LH) have the capacity to influence a number of stress relevant behavioural adaptations including changes in arousal and reward status (Furlong et al., 2009; Harris and Aston-Jones, 2006). Dysregulation of these LH systems by ELS could significantly increase the risk for development of anxiety and depression in later life.

Of particular interest in this context are the orexin (hypocretin) neurons that are now known to be central to LH-mediated changes in arousal and motivational states (Harris and Aston-Jones, 2006; Johnson et al., 2012). Orexin activity is diurnally regulated, reaching maximal activity during waking and being minimally active during sleep (Yoshida et al., 2001; Zeitzer et al., 2003; Grady et al., 2006). Consistent with these findings, orexin antagonists have been shown to be effective hypnotics (Brisbare-Roch et al., 2007). A number of studies have indicated that the activity of the orexin system is sensitive to both acute and chronic stressors. Acute stress robustly increases activation of orexin neurons (Ida et al., 2000; Furlong et al., 2009), whereas chronic stress appears to have an opposite effect (Lutter et al., 2008; Nocjar et al., 2012). The ability of
chronic stress to restrict orexin activity is particularly interesting, as chronic stressors are also strongly associated with the emergence of mood disorders including depression (Berton et al., 2006; Tsankova et al., 2006; Bortolato et al., 2007; Tynan et al., 2010), a condition characterised by low arousal, anhedonia and HPA axis dysfunction (Bao and Swaab, 2010; O'Keane et al., 2012). Further, strong evidence has recently emerged linking low orexin system function with depressive symptoms in humans (Brundin et al., 2007; Brundin et al., 2009). As highlighted above, ELS is strongly associated with the emergence of mood disorders in adulthood, however, the effect of ELS on orexin neuron function in adulthood has not been directly tested. Therefore, the primary aim of this study was to investigate the effects of maternal separation on orexin system function following stress exposure in adulthood.

Non-pharmacological approaches to prevent or augment antidepressant/anxiolytic action have significant clinical relevance and appeal. One of the most effective non-pharmacological interventions to improve mood state is increased physical activity or exercise (Bridle et al., 2012). Preclinical studies using rats also show that voluntary exercise can produce antidepressant-like effects. Of particular relevance to the present study is evidence that voluntary wheel running reversed maladaptive behavioural and molecular changes induced by maternal separation (Maniam and Morris, 2010; Daniels et al., 2012; Dimatelas et al., 2013). However, it is unclear whether any of the antidepressant or anxiolytic effects of physical activity might be linked with improvement in LH-orexin system function. Thus, a secondary aim of the present study was to investigate the possible preventative effects of physical activity on ELS-induced maladaptive orexin cell responses to stress in adulthood.

To achieve our aims we mapped Fos-protein in orexin cells in ELS versus control animals following restraint stress in adulthood. Fos-mapping was also carried out in stress-sensitive brain sites that receive orexinergic innervation, including the ventral tegmental area (VTA), paraventricular thalamus (PVT) and the PVN. We found that male and female animals exposed to ELS exhibited a ‘hypoactive’ orexin cell response to restraint stress. Notably, the activation pattern in orexin cells was associated with reduced exploratory behaviour on the open field but normal behaviour in the elevated
plus maze test. Interestingly, access to running wheels appeared to prevent this pattern of orexin and behavioural hypoactivity only in male rats.
METHODS

Ethics Statement
All procedures performed were approved by the University of Newcastle Animal Care and Ethics Committee (approval number 1071), and were carried out in accordance with the New South Wales Animal Research Act.

Animals
Ten experimentally naïve Wistar dams were obtained from the University of Newcastle Animal house and bred with two experimentally naïve males in the University of Newcastle vivarium. A total of 34 male and 39 female offspring were included in the study. On postnatal day 1 (PND 1), animals from each litter were randomly allocated to the ELS or control (no ELS) condition. ELS allocated litters underwent maternal separation procedures (detailed below) between PND 2-14. On PND 21, animals were weaned and separated into same-sex housing, with two to three animals per cage (41.5cm x 28cm x 22cm cages; Mascot Wire Works, Sydney). Food (Rat and Mouse Pellets, Glen Forest, Western Australia) and water were available ad libitum and rats were maintained on a 12 hour light (0600-1800): 12 hour dark cycle. Temperature was maintained at 20 ± 2°C and humidity was kept at 34 ± 2%.

Early Life Stress
The maternal separation procedure was performed as per our previously published procedures (Nakamura et al., 2011) that were based on earlier studies (Plotsky and Meaney, 1993). Briefly, from PND 2 - 14, litters in the maternal separation condition were removed from their home cage and individually placed in clear separation containers (13cm x 13cm x 7cm) in an alternate temperature controlled room (30-34°C) for three hours each day, from 0900h – 1200h. Pups in the control condition remained undisturbed during this period except for weekly weighing.

Exercise
A subgroup of animals exposed to ELS (males n=6; females n=9) was allowed access to a running wheel between PND 40 – 70 (85cm x 7.5cm, 94cm x 12cm; Transoniq; for 1 hour per day, 5 days a week between 1800h and 2100h). A rotation counter attached to
each wheel quantified distance travelled. Food intake was also measured across the experimental period. Importantly, preliminary studies indicated that wheel running had no effect on non-ELS animals in terms of stress-related behaviour in the open field (data not shown). Therefore, a non-ELS + Exercise group was not included in this study.

Adult stress exposure
Between PND 75-79, all animals were exposed to 30 minutes restraint stress prior to behavioural testing. Animals were removed from their home cage and were placed inside a soft wire mesh restrainer (25cm x 20cm) that was folded around the animal and secured with butterfly clips. This procedure has been previously demonstrated to produce a pattern of Fos-activity centred on amygdaloid and brainstem catecholamine nuclei that is distinct from physical stressors (Dayas, Buller, & Day, 1999). Females were tested only in the diestrous phase, monitored using a rat vaginal impedance device (Muromachi Kikai, Tokyo, Osaka).

Behavioural testing
Both open field (OF) and elevated plus maze (EPM) testing was conducted in complete darkness. Time and event data for both apparatuses was recorded using a computer-automated behavioural tracking system (Motion Mensura Ltd., Australia). Immediately following restraint stress, animals were placed in a square 1m x 1m open field task apparatus enclosed by 40cm high walls for ten minutes. Exploratory variables measured were total distance travelled and time spent in locomotion. Animals were then transferred to a separate room where they were placed on an EPM apparatus. The EPM was painted black, and consisted of two open and two closed arms (45.0cm length x 10cm width) as well as a central square (10.0cm x 10.0cm). Anxiety-like behaviours were assessed, including number of open and closed arm entries as well as locomotor activity.

Sacrifice, tissue harvesting and immunohistochemistry
Two hours following restraint stress, rats were deeply anaesthetised with sodium pentobarbitone (1ml intraperitoneal, Virbac, Australia). Animals were then perfused with 200mL of 0.1M Phosphate Buffered Saline (PBS) followed by 500mL of 4%
paraformaldehyde (pH 9.5). Brains were removed and postfixed in 4% paraformaldehyde at 4°C overnight and then stored in 12.5% sucrose until sectioning. Serial rostral forebrain (40-µm) and caudal midbrain (50-µm) sections were cut using a freezing microtome (Leica Microsystems, SM2000R) and a 1-in-4 series of all sections were processed for immunohistochemical detection of Fos-protein (72 h, 1:5000, rabbit polyclonal, Santa Cruz Biotechnology, CA, USA) as described previously in detail (Smith and Day, 1993; Dayas et al., 2008). Hypothalamic sections were dual-labeled for orexin A (48h, 1:15000, Orexin A antibody, goat polyclonal, Santa Cruz Biotechnology) or in the case of VTA sections, tyrosine hydroxylase (TH; 48 hours, 1:10000, TH antibody, mouse polyclonal, Millipore). An equal number of animals from each treatment group were included in each individual run.

Bilateral counts of single-labelled Fos-positive cells were made in the perifornical area (PFA) and lateral hypothalamus (LH; bregma -2.28 to -3.24), paraventricular thalamus (PVT; -2.28 to -3.24) and medial parvocellular PVN (mpPVN; -1.46 to -1.94). Fos only cell counts in the PVN and PVT were quantified using Metamorph Imaging System Software (Version 7.5; Molecular Devices Analytical Technologies) at 10x total magnification (Olympus CX40). The number of Fos-positive cells was determined by creating a region of interest around each structure and a thresholding procedure was used to quantify Fos expression. Counts of Fos-positive orexin neurons in the LH and Fos-positive TH cells in the VTA (bregma -5.30 to -5.94) were made by one observer blind to treatment using a 20x objective (Olympus CX40). In the LH, cell counts were made in the perifornical area (PFA) and the lateral hypothalamus (LH) divisions, as these sections have previously been shown to contain the highest concentration of orexin neurons (Dayas et al., 2008). The PFA was defined as the area surrounding the fornix and the LH was defined as the area from the lateral side of the PFA to the optic tract (Laorden et al., 2012). Cells in the VTA were quantified in the parabrachial pigmented nucleus (PBP) region of the VTA.
**Data Analysis**

All statistical analyses were conducted using IBM SPSS version 19. Male and female animals were analysed separately. Body weight of treatment groups was compared on PND 72 using a one-way between subjects ANOVA. Food intake and behavioural data were compared across treatment groups using a one-way between-subjects ANOVA and subsequent least significant differences (LSD) post-hoc analyses where appropriate. For immunohistochemical analyses, all cell counts were averaged across each animal for each rostrocaudal level of each brain region examined. To minimise the effects of variability across multiple immunohistochemistry runs, counts for each treatment group were calculated as a fold change relative to control animals processed in the same run. These fold changes were averaged across the rostral-caudal extent of each brain region and were compared across groups using one-way ANOVAs. These analyses were followed by least LSD post-hoc analyses where appropriate. An alpha value of 0.05 was adopted for all statistical tests. All figures depict means and standard errors.

**Figure 1.** A schematic illustration of the experimental design. Neonatal treatment consisted of either early life stress (maternal separation for 3hrs/day from postnatal days (PND) 2-14), or no early life stress. A subset of animals was given access to running wheels for 1hr/day, 5 days/week from PND 40-70. All animals were subjected to restraint stress in adulthood (PND 75-79) for 30mins. Immediately following restraint, animals underwent behavioural testing in the open field test (10mins) and elevated plus maze (5mins). Ninety minutes following restraint stress, animals were sacrificed and brains collected.
RESULTS

Effect of ELS on body weight and food intake

On PND72, male animals from each treatment group did not differ significantly in terms of their body weight ($F_{2,44} = 2.366, p = 0.106$), or food intake across the experiment ($F_{2,14} = 2.554, p = 0.113$). Similarly, body weight of females was indistinguishable between treatment groups ($F_{2,67} = 0.026, p = 0.975$) as was their food intake ($F_{2,20} = 0.302, p = 0.743$). Interestingly, female rats ran on average approximately three times further than male animals in each exercise session ($F_{1,16} = 19.429, p < 0.001$; Figure 2).

ELS is associated with a reduced percentage of Fos-positive orexin cells after restraint: Protective effect of exercise in male but not female rats

In male animals there was no effect of ELS on the number of orexin cells in either the PFA or LH subdivisions of the hypothalamus ($F_{2,18} = 0.292, p = 0.750$; $F_{2,18} = 1.648, p = 0.220$ respectively). To assess the effect of ELS on the reactivity of orexin neurons to stress in adulthood, we quantified the percentage of orexin cells expressing Fos protein following restraint. ANOVA revealed a significant effect of ELS on the percentage of orexin cells expressing Fos protein in the PFA ($F_{2,18} = 17.646, p < 0.001$), and a trend

![Figure 2. Effect of early life stress (ELS) on body weight and food intake. There was no effect of ELS on body weight at postnatal day 72 in both males and female rats (A). Similarly, ELS had no effect on food intake (B). Female rats engaged in significantly greater amounts of wheel running per day compared to male rats (C). Males: No ELS: n=19; ELS: n=22; ELS + ex: n=6. Females: No ELS: n=30; ELS: n=28; ELS + Ex: n=12. ***p<0.001.](image-url)
towards significance in the LH ($F_{2,18} = 3.248, p = 0.062$). Post-hoc analyses revealed that ELS animals displayed a significantly lower percentage of orexin neurons that expressed Fos-protein after restraint compared to controls in the PFA ($p = 0.002$). Interestingly, ELS animals given access to running wheels displayed a pattern of Fos/orexin immunoreactivity similar to controls in the PFA ($p = 0.042$ compared to controls, $p < 0.001$ compared to ELS; Figure 3A).

Figure 3. Early life stress (ELS) was associated with a decrease in the percentage of Fos-positive orexin cells in both male and female rats: Wheel running was protective against these deficits in male but not female rats. The percentage of Fos-positive orexin cells in the PFA of the hypothalamus was significantly lower in male ELS rats compared to controls. Wheel running resulted in a protective effect of ELS. This trend was also observed in the LH but failed to reach significance (A; $p=.06$; no ELS: n=7, ELS: n=9, ELS + ex: n=6). As in male animals, ELS-exposed females exhibited a reduced percentage of Fos-positive orexin cells in the PFA. In contrast to males, wheel running exacerbated these effects in female rats (B; no ELS: n=7, ELS: n=8, ELS + ex: n=7). A similar trend was observed in the LH (B). Photomicrographs of coronal sections of the PFA of the hypothalamus immunolabelled for Fos-protein and orexin (C, D). *p<.05 vs. No ELS, **p<.01 vs. No ELS, +p<.05 vs. No ELS, +++p<.001 vs. No ELS, ##p<.01 vs. ELS, ###p<.001 vs. ELS, scale bar, 20µm.
Similar to males, orexin cell numbers did not differ across treatment groups in female rats in both the PFA and LH ($F_{2,18} = 0.141$, $p = 0.87$; $F_{2,18} = 0.166$, $p = 0.849$ respectively). There was a significant main effect of ELS on the percentage of orexin cells that displayed Fos-like immunoreactivity in response to restraint stress in the PFA ($F_{2,18} = 26.907$, $p < 0.001$) and LH ($F_{2,18} = 14.292$, $p < 0.001$). Consistent with male animals, post-hoc analyses showed that ELS females exhibited a significantly lower percentage of Fos-positive orexin cells compared to control animals in the PFA ($p = 0.018$) and a similar trend in the LH ($p = 0.094$). In contrast to males however, access to running wheels tended to exacerbate the effect of ELS on orexin cell reactivity as assessed by Fos-labelling in the PFA ($p < 0.001$ compared to controls and ELS) and LH ($p < 0.001$ compared to controls, $p < 0.01$ compared to ELS; Figure 3B).

**ELS is associated with a reduction in Fos-protein expression in VTA TH-positive, PVN and PVT neurons following restraint: Protective effect of exercise in male but not female rats**

In addition to orexin neurons, we assessed the level of Fos-like immunoreactivity in the VTA, PVN and PVT following restraint stress in adulthood. In males, despite there being a trend towards an ELS-induced reduction in the percentage of Fos-positive TH cells in the VTA, treatment groups did not differ significantly on this index ($F_{2,15} = 1.369$, $p = 0.284$; Figure 4A). There was a significant main effect of ELS on Fos-immunoreactivity in the PVN ($F_{2,15} = 9.316$, $p = 0.002$), with post-hoc analyses revealing a significant reduction in Fos-positive cells in ELS animals compared to controls ($p = 0.008$). Access to voluntary exercise significantly increased the number of Fos-positive PVN cells compared to ELS-exposed animals ($p < 0.001$). There was no significant difference between exercised males and controls in this region ($p = 0.287$; Figure 4B). In the PVT, there was a significant main effect of ELS on Fos-positive cells ($F_{2,15} = 5.248$, $p = 0.015$). Post-hoc analyses revealed a significant increase in Fos immunoreactivity in the PVT of animals given access to running wheels ($p = 0.023$ compared to controls, and $p = 0.006$ compared to ELS; Figure 4C). No significant difference was found in the number of Fos-positive cells in the ELS group compared to controls in this region ($p = 0.602$).
In females, there was no significant main effect of ELS on the number of TH-positive cells that expressed Fos-protein ($F_{2,18} = 1.415, p = 0.269$; Figure 4A). In the PVN, ANOVA revealed a significant main effect of ELS on the number of Fos-positive cells in this region ($F_{2,19} = 8.27, p = 0.003$). Post-hoc analyses revealed no significant difference between controls and ELS ($p = 0.152$). However, access to running wheels

![Graphs and images related to Fos-positive cells in VTA, mpPVN, and PVT regions under different conditions (No ELS, ELS, ELS + Exercise).](image)

**Figure 4.** Early life stress (ELS) was associated with a decrease in Fos-like immunoreactivity in VTA TH-positive neurons and mpPVN and PVT neurons: Protective effect of exercise in male but not female rats. ELS resulted in a trend towards a reduction in the percentage of Fos-positive TH cells in the VTA in male rats. Wheel running appeared to be protective against these effects. No ELS: n=6, ELS: n=6, ELS + Ex: n=6 (A). In females, there was a trend towards a reduction in the percentage of Fos-positive TH cells in the VTA, and these effects were exacerbated by wheel running (A; no ELS: n=7, ELS: n=7, ELS + ex: n=7). In the PVN and PVT, there was an ELS-induced reduction in Fos-positive cells in males and this effect was reversed by wheel running. No ELS: n=7; ELS n=9; ELS + Ex: n=6 (B, C). In females, there was a trend towards an ELS-induced reduction in Fos-positive cells in the PVN and PVT and wheel running appeared to exacerbate these effects. No ELS: n=7; ELS: n=8; ELS + Ex: n=7 (B, C). Photomicrographs of coronal sections taken through the VTA and immunolabelled for Fos-protein and TH (D), scale bar 20µm. Coronal sections of the PVN and PVT immunolabelled for Fos-protein (E, F), scale bar 100µm. **p<0.01 vs. No ELS, +p<0.05 vs. No ELS, ++p<0.01 vs. No ELS, +++p<0.001 vs. No ELS, #p<0.05 vs. ELS, ##p<0.01 vs. ELS, ###p<0.001 vs. ELS.

significantly reduced the number of Fos-positive PVN cells ($p < 0.001$ compared to controls, $p = 0.016$ compared to ELS; Figure 4B). In the PVT, there was a significant main effect of ELS on Fos-protein expression ($F_{2,19} = 6.409, p = 0.008$) with post-hoc analyses revealing a trend towards a reduced number of Fos-positive cells in ELS.
animals compared to controls ($p = 0.156$). This reduction in Fos-positive PVT cells was exaggerated in rats given access to running wheels ($p = 0.002$ compared to controls, $p = 0.041$ compared to ELS; Figure 4C).

**ELS animals exhibit hypoactivity in the open field following restraint stress in adulthood: Protective effect of exercise in males but not female rats**

In males, one-way ANOVA revealed a main effect of ELS on the distance travelled in the OF ($F_{2,31} = 2.66, p = 0.043$). Post-hoc comparisons revealed that ELS-exposed animals travelled significantly less distance compared to controls ($p = 0.026$). This effect was reversed when ELS animals were given access to voluntary exercise throughout adolescence ($p = 0.044$, compared to ELS group; Figure 5A). Analyses also showed a trend towards the ELS group exhibiting increased time spent in immobility as compared to the control group, with this effect again being reversed by exercise intervention ($F_{2,31} = 2.21, p = 0.063$, data not shown).

**Figure 5. Early life stress (ELS) is associated with reduced locomotor activity in the open field task: Protective effects of exercise in males but not in females.** In both male and females, ELS was associated with a significant reduction in the distance travelled in the open field. Wheel running protected against this effect in male rats but exacerbated this effect in female rats (A). In both male and female rats, there was no effect of ELS or wheel running on the number of open (B) or closed (C) arm entries across in the elevated plus maze. *p<.05 vs. No ELS, **p<.01 vs. No ELS, ++p<.01 vs. No ELS, #p<.05 vs. ELS.
In females, there was a significant main effect of ELS in terms of distance travelled (F_{2,36} = 7.13, p = 0.001). Similarly to males, maternally separated animals exhibited locomotor hypoactivity in the open field when compared to controls (p = 0.007). In contrast to males however, this effect was not reversed by voluntary exercise, but in fact was exaggerated (p < 0.001, compared to controls; Figure 5A). This same trend was observed in terms of time spent in immobility (F_{2,36} = 7.44, p = 0.001) with ELS females spending significantly more time in immobility compared to controls (p = 0.01). Time spent in immobility was exaggerated in exercised females (p < 0.001 compared to controls).

With respect to the EPM, there was no effect of ELS on the number of open (F_{2,15} = 0.382, p = 0.689) or closed (F_{2,15} = 1.624, p = 0.230) arm entries in males. Similarly, in females there was no difference between treatment groups on open (F_{2,21} = 0.617, p = 0.549) or closed arm entries (F_{2,21} = 0.040, p = 0.961; Figure 5B,C).
DISCUSSION

The primary aim of the present study was to characterise the effect of ELS on the responsiveness of the orexin system to a psychological stress experienced in adulthood. To this end, we report that regardless of gender, ELS exposed animals exhibited a ‘hypoactive’ orexin cell response to restraint stress as assessed by Fos-like immunoreactivity in orexin neurons, particularly in the PFA subpopulation. Notably, both male and female animals exposed to ELS displayed reduced exploratory behaviour on the OF test following restraint stress. With respect to the ability of exercise to ameliorate ELS-induced deficits, the effects were strongly gender dependent. While wheel running in males rescued the impairment in orexin activity in adulthood, this did not occur in females. A similar level of gender specificity was also seen in brain regions that are known to respond to orexin innervation. Together these results not only highlight the profound effect that ELS has on orexin function in adulthood but that strategies directed at reversing this deficit are not equal across genders.

In the current study, we examined the degree to which orexin cells had become activated following a brief stressor in adulthood, by quantifying the number of orexin that were also Fos-positive. Using this highly standard strategy, orexin function in ELS-exposed animals was substantially lower than what was observed in the non-ELS controls. Aligning with this result, we found that ELS-exposed animals exhibited significantly lower activity in the open field in adulthood. While this is the first study to clearly demonstrate that ELS is capable of promoting significant functional alterations within the PFA/LH orexin cell populations, other research groups have demonstrated that chronic stress can produce similar results. For instance, both Lutter et al. (2008) and Nocjar et al. (2008) demonstrated that exposure to chronic social defeat also induces significant hypoactivity of orexin cell function. Interestingly, these authors also reported that these changes to orexin function were associated with increased depressive-like behaviour, a finding in line with recent human data showing an inverse relationship between CSF orexin peptide levels and symptoms of depression (Brundin et al., 2007; Brundin et al., 2009). It is interesting that in the present study, the reduction in orexin neuron activation after ELS was accompanied by reduced activity in the OF – a test of novelty-induced exploratory behaviour - whereas ELS-exposed animals exhibited
normal open arm entries on the EPM, a standard test of anxiety-like behaviour. One interpretation of these findings is that reduced orexin activity induced by ELS produced a depressive-like behavioural state that manifested as reduced exploratory behaviour.

We examined ELS-induced changes in orexin function in two key subpopulations, the PFA and LH. These regions are commonly assumed to be responsible for distinct functions, with the PFA tightly linked to the regulation of stress responses whereas the LH is associated with reward mechanisms. Illustrating this distinction, foot shock stress increases Fos-protein expression in PFA, but not LH orexin cells, whereas food and drug associated cues activate the LH but not PFA (Harris and Aston-Jones, 2006). In our study, the effects of ELS on orexin cell reactivity to stress were most pronounced in the PFA, possibly supporting the hypothesis that this population of cells is more important in stress regulation. However, ELS also tended to result in hyporesponsivity of orexin cells in the LH. A deficit in the functioning of this ‘reward-related’ population of cells would be consistent with a behavioural phenotype of reduced motivated arousal – possibly reflected as reduced exploratory behaviour in the OF in our study. Further behavioural tests are required to clarify this issue. For example it would be interesting to test whether these changes in LH orexin cell function might manifest as deficits in motivated behaviour on behavioural assays such as the sucrose preference test.

We also investigated the effect of ELS on the responsivity of a number of key orexinergic targets. Interestingly, the pattern of Fos-like immunoreactivity we observed in the mpPVN, PVT and VTA dopamine neurons generally paralleled that observed for the orexin cells. While we did not directly assess the functional relationship between reduced orexin system function and the responsiveness of these downstream innervation targets, there is considerable evidence that orexin signalling can modulate activity in these structures. For example, central administration of orexin-A induced Fos-protein expression in corticotropin releasing factor (CRF)-expressing cells in the mpPVN (Sakamoto et al., 2004) and increased plasma corticosterone (CORT) and adrenocorticotropic hormone (ACTH) levels (Ida et al., 2000; Kuru et al., 2000). As reduced mpPVN Fos-like immunoreactivity is a reliable index of HPA axis activity after stress (Dayas et al., 1999; Dayas et al., 2000), these findings are consistent with
studies showing that ELS impairs mpPVN and HPA-axis responsivity to stress in adulthood (Plotsky and Meaney, 1993; Ladd et al., 2000; Daniels et al., 2004; Roman et al., 2006; Desbonnet et al., 2008). Of particular relevance to the present finding are reports from clinical studies suggesting that plasma cortisol and ACTH levels are significantly reduced in depressed patients that report one or more ‘atypical’ symptoms, including an extreme lack of energy, or anergia (Geracioti et al., 1992; Kasckow et al., 2001). This evidence would appear to be consistent with our observation that ELS produces a hypoactivity of the mpPVN and consequently HPA-axis responses to stress.

The PVT is a midline thalamic structure that is important for neuroendocrine and behavioural responses to stress (Spencer et al., 2004; Heydendael et al., 2012). The PVT is densely innervated by orexinergic terminals (Kirouac et al., 2005; Parsons et al., 2006) and contains moderate to high densities of both orexin receptors (Marcus et al., 2001; Cluderay et al., 2002). Microinjections of either orexin-A or B into the PVT increase anxiety-like behaviour on both the open field and EPM (Li et al., 2010b; Li et al., 2010a), and HPA axis responses to a novel stressor can be blocked by intra-PVT injections of the orexin receptor-1 antagonist SB-334867 (Heydendael et al., 2012). It is also interesting to note that stimulation of the PVT can modulate dopamine release in the nucleus accumbens (NAC) (Jones et al., 1989; Pinto et al., 2003; Parsons et al., 2007). Reduced PVT signaling in ELS animals may therefore contribute to reduced striatal dopamine release, an outcome consistent with previous studies showing that hypoactivity of the VTA dopamine→NAC projection is causally linked to depressive-like behaviour following social defeat (Berton et al., 2006).

We also observed a non-significant trend towards reduced activity of VTA TH-positive cells in ELS-exposed animals. This finding is interesting in light of a study by Jahng and colleagues, who reported reduced stress-induced TH mRNA expression in the VTA in animals previously exposed to ELS (Jahng et al., 2010). Hypofunction of the VTA has been associated with anhedonic behaviour in stress-induced depression models and depressed patients (Di Chiara and Tanda, 1997; Klimek et al., 2002; Miczek et al., 2011). Orexin cells directly innervate the midbrain and activate VTA dopamine cells (Narita et al., 2006; Balcita-Pedicino and Sesack, 2007; Vittoz et al., 2008; Espana et
Further, stimulation of this pathway promotes effort and motivation (Muschamp et al., 2007; Aston-Jones et al., 2010), and we and others have shown that orexin signalling in the VTA is critical for drug-seeking behaviour (Borgland et al., 2006; James et al., 2011). Directly related to the possibility that loss of orexin signalling in the VTA might contribute to depression-like behaviour, Nocjar and colleagues reported reduced orexin peptide levels in the VTA of animals exhibiting depressive-like behaviour including sexual disinterest following chronic social defeat stress (Nocjar et al., 2012). It will be important that further studies directly test the role of this orexinergic pathway in controlling stress-related vulnerability to depression-like behaviours.

Perhaps the most striking observation of the present study was that access to voluntary exercise was protective against both the neural and behavioural deficits associated with ELS in male but not female animals. These findings are consistent with previous studies demonstrating that voluntary wheel running protects against the expression of anxiety behaviour in adult male rats exposed to maternal separation stress (Maniam and Morris, 2010) or footshock stress in adulthood (Greenwood et al., 2013). With respect to female animals, our findings are in line with those of Brocardo et al. who showed that voluntary exercise had no effect on the expression of anxiety- and depression-like behaviours in female rats exposed to ethanol in early life, despite this intervention having protective effects in males (Brocardo et al., 2012). Similarly, findings from the addiction field have yielded differential effects of voluntary exercise on drug-related behaviours in males and females, despite being exposed to identical exercise regimes (Smith et al., 2008; Ehringer et al., 2009; Thanos et al., 2010). Our findings that exercise actually tended to exacerbate ELS-induced orexin and behavioural changes in females perhaps points to the possibility that our ‘exercise’ regime was stress provoking, particularly given that female animals ran significantly further than males. These findings point to the need for a greater understanding of how optimum exercise conditions (type, intensity, duration) can be modified to produce beneficial effects in both genders.
One candidate mechanism via which ELS might produce stress-induced changes in orexin neuron gene expression is through post-translational modifications of histone proteins – known as the histone code (e.g. Wang et al. 2008). Specific histone modifications can remodel the structure of chromatin, promoting transcriptionally permissive or repressive states. Such modifications have already been implicated in lasting changes in neuroendocrine hypothalamic and hippocampal function after ELS (Weaver et al., 2004; Murgatroyd et al., 2009). Importantly, while there are no studies investigating the ELS-induced epigenetic modifications in the LH, evidence does exist that histone modifications associated with orexin gene expression do occur in response to chronic stress. For example, in the study described above by Lutter et al., decreased orexin mRNA expression after social defeat stress was associated with histone modifications known to repress gene expression (Tsankova et al., 2006; Lutter et al., 2008). Future studies will need to determine whether exercise can reverse repressive histone marks and increase orexin mRNA expression. In this regard, it is noteworthy that voluntary wheel running can increase brain derived neurotrophic factor (BDNF) expression through acetylation and demethylation of the BDNF promoter (Gomez-Pinilla et al., 2011).

In the present study we provide the first direct evidence the orexin system’s response to adult stress is altered by ELS. Identical effects of ELS on orexin cell activity in stressed adults were observed in male and female rats. These data are consistent with recent clinical evidence, which taken together with the present data, suggest that vulnerability to stress-related mood disorders is linked with orexin neuron hypofunction. We also show that exercise was protective against both the behavioural and neural effects of ELS in males, suggesting that the beneficial effects of exercise on stress-related behaviour is associated with a 'normalization' of orexin function. Indeed, not only did exercise normalise orexin neuron activity, it normalised activity in brain regions known to receive orexinergic input, indicating orexinergic signaling was normalised as a whole. Exercise, however, was not uniformly positive, with females exhibiting significantly greater deficits in orexin function following wheel running. Collectively, these findings highlight that while the effects of ELS on orexin function are similar
across genders, future studies may need to consider alternative exercise regimens to recover orexin function in female rats.
REFERENCES


APPENDICES
APPENDIX I

WHAT ABOUT ME…? THE PVT: A ROLE FOR THE PARAVENTRICULAR THALAMUS (PVT) IN DRUG-SEEKING BEHAVIOUR

Morgan H. James & Christopher V. Dayas

What about me...? The PVT: a role for the paraventricular thalamus (PVT) in drug-seeking behavior

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A commentary on

The lack of effective pharmacotherapies to prevent relapse to drug taking emphasizes the importance of fully characterizing the brain pathways responsible for this behavior (Kalivas and McFarland, 2003). Recently, there have been attempts to more fully understand the brain circuitry responsible for drug-seeking behavior, beyond the well-characterized nodes such as the prefrontal cortex (PFC), nucleus accumbens (NAC), and ventral tegmental area (VTA). In this respect, the review of Martin-Fardon and Boutrel (2012) is important and timely and should serve to stimulate continued focus on the paraventricular thalamus (PVT) in the addiction field. Indeed, their review is an appropriate addition to the recent article “Emerging, re-emerging, and forgotten areas of the reward-circuit” (McGinty et al., 2011). The first purpose of this commentary is to reiterate this point, but to perhaps go one step further. Thus, in response to the authors’ first question (i.e., whether the PVT should be considered part of the drug-seeking circuitry), we argue that there is sufficient anatomical and functional evidence to support this suggestion. For example, the PVT sends glutamatergic projections to the NAC and PFC (Christie et al., 1987; Bulber and Deutch, 1998; Vertes and Hoover, 2008), and a large percentage of these projections are branched, suggesting that a single PVT neuron can influence these areas simultaneously (Otake and Nakamura, 1998). PVT neurons also project to medial, central, and basal nuclei of the amygdala as well as the bed nucleus of stria terminalis (Vertes and Hoover, 2008). Importantly, glutamatergic efferents from the PVT are closely apposed to dopamine fibers in the NAC shell (Pinto et al., 2003) and stimulation of the PVT produces an efflux of dopamine in this brain region (Jones et al., 1989; Parsons et al., 2007). Earlier, lesion and Fos-mapping studies were the first to implicate the PVT as a reward-responsive site. For example, acute psychostimulant administration was found to activate the PVT (Deutch et al., 1998) and lesions of the PVT block the conditioned locomotor response to a cocaine-paired environment (Young and Deutch, 1998). More recent studies have extended these initial findings. Work by McNally’s group, and ours, has demonstrated that lesions or chemical inactivation of the PVT suppresses drug-seeking behavior. For example, Hamlin et al. (2009) showed that lesions of the PVT prevent context-induced reinstatement of alcohol-seeking and Marchant et al. (2010) showed that intra-PVT infusion of a κ-opioid receptor agonist also inhibits this behavior. Our group has also shown that inactivation of the PVT using TTX or intra-PVT injections of the inhibitory peptide cocaine- and amphetamine-regulated transcript (CART) attenuates cocaine-primed reinstatement (James et al., 2010). This role likely extends to cue-induced cocaine-seeking, as the magnitude of reinstatement behavior is strongly correlated with Fos-activation in the PVT (Dayas et al., 2008; James et al., 2011a). Together, these data strongly support a functional role for the PVT in drug-seeking, however, it will be important for future studies to apply electrophysiological or optogenetic techniques to dissect the circuit-level changes involving PVT efferents onto reward-relevant brain regions (Cao et al., 2011). Designer receptors exclusively activated by designer drugs (DREADDs) may also be useful in allowing for selective activation/inactivation of the PVT during reinstatement testing (Dong et al., 2010). The second question the authors’ raise in their review is whether orexin (hypocretin) input within the PVT modulates reinstatement behavior. We agree with the authors that there is strong anatomical evidence implicating the PVT as a site of integration for drug-related hypothalamic signaling. However, the answer to this question appears less straightforward than their more general question regarding the PVT, and we believe that this second issue requires further study—a point acknowledged by Martin-Fardon and Boutrel. The authors cite recent data from their laboratory supporting a role for PVT orexin in reinstatement behavior. PVT infusions of orexin-A reinstated both extinguished cocaine- and sweetened condensed milk-seeking (SCM) behavior. Interestingly, moderate...
doses of orexin-A produced a stronger reinstatement of cocaine-seeking than for SCM, indicating drug-induced adaptation to orexin receptor expression/function in the PVT (Martin-Fardon et al., 2011). We recently tested the effect of intra-PVT administration of SB-334867, an orexin receptor 1 antagonist, on cue-induced cocaine-seeking. Given our previous demonstration that drug-cue sensitive PVT neurons are closely apposed by orexin terminals (Dayas et al., 2008), it was surprising that microinjections of SB-334867, at a dose also likely to block orexin receptor 2, had no effect on cue-induced reinstatement of cocaine-seeking (James et al., 2011b). In contrast, intra-VTA SB-334867 suppressed drug-seeking behavior (James et al., 2011b), consistent with studies showing that infusions of orexin peptide into the VTA enhance dopamine release in the NAC (Narita et al., 2006; Espona et al., 2010) and reinstate drug-seeking (Wang et al., 2009). Interestingly, we found reduced PVT Fos-expression after intra-VTA SB-334867 infusion, but increased Fos-protein in the NAC shell (James et al., 2012). Previous reports indicate that the NAC shell can exert an inhibitory influence over drug-seeking through its projections to the LH (Millan et al., 2010). Thus, it is possible that intra-VTA SB-334867 reduced PVT recruitment and increased NAC shell inhibitory output to the LH, resulting in attenuated drug-seeking behavior. How can these apparent contradictory findings relating to orexin signaling in the PVT be reconciled? One plausible explanation is that infusion of orexin-A into PVT may have engendered a stress-like response that evoked drug-seeking. Indeed, Martin-Fardon and Boutrel discussed recent evidence that orexin signaling in this region is important in regulating negative emotional states. For example, Li and colleagues report that intra-PVT infusion of TGNVSX229, an orexin receptor-2 antagonist, attenuates the expression of anxiety-like behaviors produced by prior footshock stress (Li et al., 2010) as well as conditioned place aversion produced by precipitated morphine withdrawal (Li et al., 2011). Further, Heydendael and colleagues recently showed that in rats exposed to daily swim stress, orexin-A application increases the responsivity of PVT cells, and intra-PVT infusions of SB-334867 prior to daily swim stress inhibit the ACTH secretion in response to novel stress (Heydendael et al., 2011). Thus, it is possible that the disparate findings regarding orexin signaling in PVT may reflect a preferential role for orexin signaling in stress-induced reinstatement. Future studies using more sophisticated techniques may help resolve this issue.

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APPENDIX II

COCAINE POTENTIATES EXCITATORY DRIVE IN THE PERIFORNICAL/LATERAL HYPOTHALAMUS

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Cocaine potentiates excitatory drive in the perifornical/lateral hypothalamus

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Key points
• Drugs of addiction are well-established in their capacity to alter brain reward pathways.
• The perifornical/lateral hypothalamus has previously been shown to be drug responsive, participate in relapse to drug taking, and project to key reward pathway structures.
• This study demonstrates that cocaine enhances excitatory drive to perifornical/lateral hypothalamic neurones, and these changes involve altered presynaptic function. Orexin-positive neurones were among the populations that underwent these presynaptic changes.
• The results indicate that a greater understanding of the drug-induced synaptic changes in perifornical/lateral hypothalamus may instruct future pharmacotherapies aimed at preventing drug relapse.

Abstract The hypothalamus is a critical controller of homeostatic responses and plays a fundamental role in reward-seeking behaviour. Recently, hypothalamic neurones in the perifornical/lateral hypothalamic area (PF/LHA) have also been implicated in drug-seeking behaviour through projections to extra-hypothalamic sites such as the ventral tegmental area. For example, a population of neurones that expresses the peptide orexin has been strongly implicated in addiction-relevant behaviours. To date, the effect of addictive drugs on synaptic properties in the hypothalamus remains largely unexplored. Previous studies focusing on the PF/LHA neurones, however, have shown that the orexin system exhibits significant plasticity in response to food or sleep restriction. This neuroadaptive ability suggests that PF/LHA neurones could be highly susceptible to modifications by drug exposure. Here, we sought to determine whether cocaine produces synaptic plasticity in PF/LHA neurones. Whole-cell patch-clamp techniques were used to examine the effects of experimenter-administered (passive) or self-administered (SA) cocaine on glutamatergic synaptic transmission in PF/LHA neurones. These experiments demonstrate that both passive and SA cocaine exposure increases miniature excitatory postsynaptic current (mEPSC) frequency in PF/LHA neurones. In addition, SA cocaine reduced the paired-pulse ratio but the AMPA/NMDA ratio of evoked excitatory inputs was unchanged, indicative of a presynaptic locus for synaptic plasticity. Dual-labelling for orexin and excitatory inputs using the vesicular glutamate transporter (VGLUT2), showed that passive cocaine exposure increased VGLUT2-positive appositions onto orexin neurones. Further, a population of recorded neurones that were filled with neurobiotin and immunolabelled for orexin confirmed that increased excitatory drive occurs in this PF/LHA population. Given the importance of the PF/LHA and the orexin system in modulating drug addiction, we suggest that these cocaine-induced...
Introduction

The perifornical/lateral hypothalamic area (PF/LHA) regulates reward-seeking behaviour. Seminal studies first alluded to this role by demonstrating that lesions of the lateral hypothalamus affected feeding behaviour, and that rats would press a lever to obtain electrical stimulation of this region (Anand & Brobeck, 1952; Olds & Milner, 1954). More recent anatomical studies have established that the PF/LHA strongly projects to important brain regions responsible for controlling motivation and reward-seeking behaviour, including the prefrontal cortex (PFC), nucleus accumbens (NAC) and ventral tegmental area (VTA) (Petrovich et al. 2005; Borgland et al. 2006; Gonzalez et al. 2012). Despite these earlier studies describing a key role for the lateral hypothalamus in natural reward-seeking behaviour (e.g. for food), it has only been in the last decade that significant progress has been made in understanding the specific neurotransmitter systems responsible for these behaviours (DiLeone et al. 2003). Research has now begun to address how lateral hypothalamic ‘feeding peptides’ interface with brain regions known to drive reward-seeking behaviours and how these hypothalamic systems might be ‘hijacked’ by highly potent non-conventional chemical rewards such as drugs of abuse.

It is now well established that in addition to triggering food-seeking behaviour, PF/LHA neurones are also recruited by stimuli associated with addictive drugs (Dayas et al. 2008; Hamlin et al. 2008). Furthermore, drug-seeking is abolished by inactivation of the PF/LHA (Marchant et al. 2010) indicating that the neurones in this region contribute to the control of ‘motivational action’ and produce both conventional and ‘pathological’ reward-seeking behaviour. A number of neuropeptide transmitters have been implicated in both natural and drug-seeking behaviours. Of these, neurones expressing the neuropeptide orexin (hypocretin) found within the PF/LHA have received significant attention. Specifically, Harris & Aston-Jones (2006) showed that an orexin-1 receptor (OrxR-1) antagonist SB-334867 reduced preference for a morphine-paired environment (Harris & Aston-Jones, 2006). Further, in rodent models of relapse, systemic SB-334867 administration reduces drug-seeking behaviour (Boutrel et al. 2005; Harris & Aston-Jones, 2006; Lawrence et al. 2006). Recent data suggest that orexins are likely to influence drug-seeking through their projections to the VTA (Wang et al. 2009; James et al. 2011, 2012; Mahler et al. 2012). Importantly, orexins may also influence drug-seeking through projections to cortical regions (Hollander et al. 2008; Jupp & Lawrence, 2009).

Despite this evidence of PF/LHA involvement in drug-seeking, very few studies have addressed how the functional properties of hypothalamic neurones are altered by drug exposure. Thus, while drug-induced synaptic plasticity is well characterized in both the VTA and NAC (Borgland et al. 2006; Mameli et al. 2009), it is not known whether similar modifications occur at synaptic inputs onto PF/LHA neurones. There is, however, some evidence to suggest that hypothalamic neurones are likely to be susceptible to drug-induced plasticity. For example, Ahmed et al. (2005) showed that the lateral hypothalamus exhibits substantial gene expression changes for markers of both pre- and post-synaptic signalling molecules in animals with escalated cocaine intake. Evidence from other fields also supports a hypothesis that the PF/LHA orexin neurones are easily ‘rewired’ and likely to be susceptible to drug-induced plasticity (Horvath & Gao, 2005). For example, overnight food restriction increased the frequency of miniature excitatory postsynaptic currents (mEPSCs) in orexin neurones and promoted the formation of new excitatory, vesicular glutamate transporter 2 (VGLUT2)-positive inputs onto these cells (Horvath & Gao, 2005). Similarly, sleep deprivation also promotes plasticity at hypothalamic glutamatergic synapses, increasing both the frequency and amplitude of mEPSCs in orexin neurones (Kao et al. 2007). These data indicate that hypothalamic circuits may be ‘soft-wired’, a characteristic that could endow a vulnerability to repeated drug exposure and confer increased addiction and relapse vulnerability. Whether similar changes occur within PF/LHA circuitry that could affect orexin neurone recruitment (or other hypothalamic cell populations that regulate drug-seeking) in response to drug exposure is yet to be determined (Chung et al. 2009).

The purpose of the present study was therefore to provide evidence that the PF/LHA itself is subject to synaptic modifications in response to drug exposure. Accordingly, we used experimenter-administered (i.e.
passive) and self-administration (SA) drug exposure procedures in rats and tested whether cocaine altered excitatory drive to PF/LHA using patch-clamp recording techniques. To determine if cocaine-induced changes in excitatory drive in the PF/LHA were likely to affect the recruitment of orexin neurones, we used immunohistochemistry for vesicular glutamate transporter 2 (VGLUT2) and vesicular GABA transporter (VGAT), markers of excitatory versus inhibitory inputs, respectively. Further, a population of recorded neurones was filled with neurobiotin and immunolabelled to identify orexin neurones post hoc.

**Methods**

**Animals**

All experimental procedures were approved by the University of Newcastle Animal Care and Ethics Committee and performed in accordance with the New South Wales Animal Research Act. Male Sprague–Dawley rats (n = 58, Animal Resource Centre, Perth, Australia) aged 3–5 weeks were housed two per cage in a temperature- and humidity-controlled room on a reversed 12 h–12 h light–dark cycle (lights off at 10:00 am) with ad libitum access to food and water. All the experimental procedures (i.e electrophysiological recordings and immunohistochemistry) were carried out 24 h after the last passive cocaine or cocaine self-administration session.

**Cocaine exposure procedures**

Animals were subjected to one of two methods of drug exposure: passive or SA cocaine. In passive cocaine experiments, animals were weighed and separated into two groups: one group received cocaine injections (n = 14), and the other group received saline injections (vehicle, n = 15). Prior to treatment with cocaine, 6-week-old animals were conditioned to daily handling (1 h per day for 3 days) and were subjected to single-daily sham injections and placed in an enclosed arena (50 cm x 50 cm) for 1 h. Over the next 7 days, animals received an injection of either saline or cocaine hydrochloride (15 mg kg⁻¹ i.p.) and placed in the enclosed arena for an hour before they were returned to their home cage.

For cocaine self-administration experiments, animals (n = 15) underwent catheter surgery as previously described (James et al. 2011). Briefly, a Silastic catheter was surgically implanted into the right jugular vein under isoflurane anaesthesia (1–3%). Cocaine self-administration was conducted in standard operant conditioning chambers equipped with two retractable levers controlled by a Windows-based PC, using MED-PC IV software (Med Associates, St Albans, VT, USA). Cocaine was delivered via a syringe pump (5 rpm motor, Med Associates) located on the outside of the cubicle (dose of 0.25 mg per infusion i.v.). At 6 weeks of age animals were trained (2 h sessions, 7 days) to press on the right lever for a cocaine reward on a fixed ratio 1 (FR1) schedule. Infusions were followed by a white cue light above the active lever signalling a 20 s time-out period and rewards were limited to 20 infusions per session. Following training, animals self-administered cocaine for an additional 7 days with the cocaine infusion limit of 20 rewards removed.

In control experiments, a food dispenser mounted to the exterior of the operant chambers delivered food rewards directly into a food hopper inside the operant chamber. During the first 7 days of self-administration training, animals (n = 14) were food restricted in their home cage to 20 g of Purina rat chow per day. Animals were trained to press the right lever (FR1) to receive a 45 mg Noyes food pellet (TestDiet, Richmond, IN, USA). This was followed by a 20 s time-out period as above, but food rewards were limited to 200 pellets per sessions. Following the 7 day training protocol, animals were returned to unrestricted feeding conditions in their home cage and allowed to self-administer food-pellets for an additional 7 days. Importantly, this avoided any possible role for food restriction-related alterations in the PF/LHA as previous work has demonstrated that neuronal properties, which can be altered by food restriction, normalize within 24 h of re-feeding (Horvath & Gao, 2005).

**Electrophysiology**

Rats were deeply anaesthetized with ketamine (1 ml kg⁻¹) and decapitated. Brains were rapidly removed and immersed in ice-cold oxygenated (95% O₂, 5% CO₂) sucrose substituted artificial cerebrospinal fluid (S-ACSF, containing in molar: 236.2 sucrose, 25 NaHCO₃, 13.6 glucose, 2.3 KCl, 2.5 CaCl₂, 1 NaH₂PO₄ and 1 MgCl₂). Transverse slices (300 µm) containing PF/LHA were obtained using a vibrating-blade microtome (Leica, VT-1000S, Heidelberg, Germany), then transferred to an oxygenated chamber containing artificial cerebrospinal fluid (ACSF; 119.4 m NaCl substituted for sucrose) and incubated for 1 h at room temperature. Slices were transferred to a recording chamber and continually superfused with oxygenated ACSF (~32°C). Neurones were visualized using infrared differential interference contrast microscopy. Whole-cell recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), digitized at 10 kHz, via an ITC-18 computer interface (Instrutech, Long Island, NY, USA) and recorded onto a Macintosh computer running Axograph X software (Axograph, Berkeley, CA, USA). All recordings were restricted to the
PF/LHA region. After obtaining the whole-cell recording configuration, series resistance and input resistance were calculated based on the response to a −5 mV voltage step from a holding potential of −70 mV. These values were monitored at the beginning and end of each recording and data were rejected if values changed by more than 20%. Miniature excitatory postsynaptic currents (mEPSCs) were recorded in voltage clamp at a holding potential of −70 mV with series resistance of <25 MΩ, in the presence of tetrodotoxin (1 μM) and picrotoxin (50 μM). Recording pipettes (2–4 MΩ) were filled with an internal solution containing (in mM): 135 KCl, 6 NaCl, 2 MgCl₂, 10 HEPES, 0.1 EGTA, 2 MgATP, 0.3 NaGTP (pH 7.3 with KOH) and in some cases 0.1% Neurobiotin/orexin co-labelling. In the course of the experiments a bipolar stimulating electrode was positioned immediately medial and dorsal to the PF/LHA to stimulate excitatory inputs (0.1 ms pulse duration, 1.2 × threshold stimulus intensity, range = 120–140 μA). Evoked EPSCs (eEPSCs) were recorded with an internal solution containing (in mM): 120 cesium methanesulphonate, 20 CaCl₂, 10 HEPES, 4 Mg-ATP, 0.3 Na₂-GTP, 0.2 EGTA, 10 sodium phosphocreatine, 5 QX-314 (pH 7.3 with CsOH). These recordings assessed both the paired-pulse, and AMPA/NMDA ratio of eEPSCs. Neurones were voltage clamped at −70 mV, in the presence of picrotoxin (50 μM), to record the paired pulse ratio (50 ms interstimulus interval) of AMPAR-mediated EPSCs. NMDAR-mediated EPSCs were recorded at a holding potential of −40 mV with the addition of CNQX (10 μM). A minimum of 5–10 eEPSCs was collected for analysis, with a 30 s interval between protocols. In a subset of experiments we assessed the ability of picrotoxin (50 μM) to abolish inhibitory postsynaptic currents (IPSCs) evoked by a bipolar stimulating electrode. This confirmed that bath application of picrotoxin provided a full block of evoked IPSCs (n = 6, data not shown). Following recording, some slices were fixed (PFA, 1 h, at 4°C), and sections containing the PF/LHA were cut on a freezing microtome (Leica), and sections containing the PF/LHA were obtained and processed for immunohistochemistry.

Data analysis

mEPSCs were detected and captured using a sliding template method, along with a minimum amplitude threshold criterion of 10 pA (Axograph software). Under these conditions, extremely small mEPSCs could escape detection because they could not be resolved from background recording noise. This could potentially introduce errors when assessing mEPSC amplitude and frequency across conditions. In order to assess the effect of these detection parameters, we constructed histograms from 5 ms of baseline data preceding each mEPSC in all recordings. In all cases the noise distributions were Gaussian with means clustered around 0 pA. Importantly, the mean value for peak to peak noise was not different across recording conditions (passive cocaine vs. saline, 10.13 ± 0.79 pA vs. 11.48 ± 0.55 pA, P = 0.27; self administration cocaine vs. food, 10.09 ± 0.47 pA vs. 9.75 ± 0.43 pA; P = 0.6), suggesting that the effect of noise was the same across our dataset. We cannot, however, exclude the possibility that very small mEPSCs may have gone undetected and were more frequent in one particular condition.

Captured mEPSCs were individually inspected and excluded from the analysis if they included overlapping events or had an unstable baseline before the rise or after the decay phase of the mEPSC. Data were rejected if a significant trend was evident in either mEPSC amplitude or instantaneous frequency over the course of the experiment. Analyses were performed on averaged mEPSCs, generated by aligning the rising phase of all accepted events. Peak amplitude, rise-time (calculated over 90–100% of peak amplitude) and decay time constant (calculated over 20–80% of the decay phase) were obtained using automated procedures (Axograph software). Average mEPSC frequency was obtained by dividing the number of captured events by the analysis duration in seconds. Peak amplitude, rise-time and decay time constant were also evaluated for eEPSCs. Paired-pulse ratio (PPR) was calculated by dividing the mean peak amplitude of the second eEPSC by the mean peak amplitude of the first. AMPA/NMDA ratios were calculated by dividing the mean peak amplitude of evoked AMPA EPSCs (recorded at −70 mV) by the mean peak amplitude of evoked NMDA EPSCs (recorded in CNQX at −40 mV). For statistical analyses, the Shapiro–Wilk test of normality was initially used to determine if data were normally distributed. Normally distributed data were compared using Student’s t tests. In cases where the assumption of normality was violated, data were analysed using non-parametric Mann–Whitney U tests (indicated in text). All data are expressed as means ± SEM.

Immunohistochemistry

Animals were deeply anaesthetized and transectially perfused with 0.1 M phosphate buffered saline, followed by 4% paraformaldehyde (PFA). Brains were removed, post-fixed (PFA, 1 h, at 4°C), and cryoprotected (15% sucrose in 0.1 M phosphate buffer, pH 7.4 at 4°C). Serial 25 μm coronal sections of the forebrain were cut on a freezing microtome (Leica), and sections containing the PF/LHA were obtained and processed for immunohistochemistry.
Free floating brain sections were incubated in the following series of antibodies: primary orexin antibody (24 h, 1:1000, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) with either vesicular glutamate transporter (VGLUT2) antibody (1:5000, rabbit polyclonal, Synaptic Systems Goettingen, Germany) or vesicular GABA transporter (VGAT) antibody (1:250, rabbit polyclonal; Millipore, Temecula, CA, USA). Sections were then incubated in a cocktail of secondary antibodies (3 h, 1:500, AMCA anti-goat, Jackson Immunoresearch, and 1:500, Alexa Fluor 488 donkey anti-rabbit, Molecular Probes). Sections were mounted onto gelatin-coated slides and coverslipped with gelvatol.

In a separate experiment, dual-immunolabelling for orexin and neuronal specific nuclear protein (NeuN) was carried out to assess the proportion of orexin-positive neurones in the PF/LHA region where the majority of recordings were made. Brain sections were incubated with primary orexin antibody (as above) and NeuN antibody (1:200, mouse polyclonal, Millipore, Temecula, CA, USA). Sections were then incubated in AMCA anti-goat and Alexa Fluor 488 anti-mouse (1:500 Molecular Probes, Invitrogen). Controls for antibody specificity and cross reactivity were performed for all experiments by primary antibody omission.

Labelled PF/LHA sections were examined using confocal microscopy (Nikon Eclipse 80i Microscope attached to Eclipse C1 confocal system). Z-series were taken (60× objective) and images were imported into ImageJ version 1.37 (National Institute of Health, USA) for analysis. Images were background subtracted and the z-series reconstructed. Counts of VGLUT2 or VGAT puncta in close apposition to orexin neurones (<1 µm) were made within the PF/LHA. Data were first analysed using the Shapiro–Wilk test to determine if the data were normally distributed, followed by Student’s t test. All data are expressed as means ± SEM.

Drugs
Cocaine hydrochloride (GlaxoSmithKline, Victoria, Australia) was dissolved in sterile physiological saline: 15 mg ml⁻¹ for intraperitoneal injection, or 2.5 mg ml⁻¹ for self-administration. Drugs for electrophysiology were made at 1000 times stock concentrations and then diluted to the final concentration in bath superfusate. Picrotoxin and CNQX were purchased from Sigma-Aldrich (St Louis, MO, USA), and TTX was obtained from Alomone Laboratories (Jerusalem, Israel).

Results
This study includes two sets of experiments designed to assess the effect of cocaine on excitatory drive to neurones in the PF/LHA. In experiment 1 we exposed animals to passive cocaine injections (Fig. 1A). In experiment 2 we used a self-administration procedure to expose animals to cocaine (Fig. 1B) and as a control, a group of animals were trained to lever press for food rewards. Food-trained...
rats exhibited higher levels of rewarded lever pressing than cocaine rats (69.3 ± 9.3 vs. 30.3 ± 2.0). Similarly, inactive (unrewarded) lever presses were higher in the food versus cocaine groups (7.2 ± 2.8 vs. 2.5 ± 1.8). Differences between the pharmacological effects of cocaine versus the shorter satiating actions of a food reward are likely to account for these behavioural differences and are consistent with previous studies (Borgland et al. 2009).

**Experimenter-administered cocaine potentiates excitatory synaptic input to PF/LHA neurones**

To determine whether experimenter-administered cocaine potentiates excitatory synaptic input to PF/LHA neurones, we recorded AMPAR-mediated mEPSCs from PF/LHA neurones in saline- and cocaine-exposed rats (Fig. 2). Cocaine treatment had no effect on the input resistance (cocaine vs. saline, 182.19 ± 19.22 MΩ vs. 227.94 ± 34.30 MΩ; P > 0.05) and the series resistance of recorded neurones (cocaine vs. saline, 9.70 ± 0.96 MΩ vs. 11.87 ± 1.77 MΩ; P > 0.05). mEPSC frequency, however, was significantly increased in cocaine-compared to saline-exposed rats (21.90 ± 5.13 Hz versus 8.31 ± 1.95 Hz, P < 0.05, Mann–Whitney U test; Fig. 2A and B). In contrast, mEPSC amplitude (22.48 ± 2.17 pA vs. 28.74 ± 4.82 pA), rise time (0.53 ± 0.04 ms vs. 0.53 ± 0.07 ms), and decay time constant (2.39 ± 0.32 ms vs. 2.57 ± 0.43 ms) were similar in cocaine and saline groups. Together, these results suggest that experimenter-administered cocaine may preferentially alter presynaptic function onto PF/LHA neurones without affecting postsynaptic receptor density or kinetic properties.

**Cocaine self-administration substantially potentiates excitatory synaptic input to PF/LHA neurones**

Previous work has shown that passive cocaine exposure can have differential effects on synaptic plasticity within some brain regions compared to self-administered cocaine (Chen et al. 2008). Therefore, in a second series of experiments we recorded mEPSCs from PF/LHA neurones in animals following 2 weeks of cocaine self-administration (Fig. 3). Similar to the experimenter-administered cocaine effects (above), cocaine self-administration had no effect on the input resistance (cocaine vs. food, 160.58 ± 17.01 MΩ vs. 147.75 ± 11.39 MΩ; P > 0.05) and the series resistance of recorded neurones (cocaine vs. saline, 7.94 ± 0.56 MΩ vs. 7.12 ± 0.39 MΩ; P > 0.05), but mEPSC frequency was substantially elevated in cocaine-trained rats compared to food-trained controls (22.46 ± 2.30 Hz vs. 10.46 ± 1.77 Hz, P < 0.01, Mann–Whitney U test, Fig. 3A and B). In contrast, mEPSC amplitude (24.32 ± 0.99 pA vs. 23.27 ± 2.06 pA), rise time (0.51 ± 0.02 ms vs. 0.56 ± 0.04 ms), and decay time constant (1.51 ± 0.09 ms vs. 1.72 ± 0.10 ms) remained similar between cocaine- and food-trained rats. Thus, in our hands the cocaine-induced effects on excitatory drive to PF/LHA neurones are similar under passive and self-administered drug exposure regimens. Interestingly, two-way analysis

![Figure 2. Presynaptic plasticity in PF/LHA after passive cocaine injections](image)
of variance (ANOVA) revealed a significant main effect of treatment (i.e. passive vs. self-administration) on input resistance (205.61 ± 14.33 MΩ vs. 154.42 ± 10.34 MΩ, \( F_{1,113} = 8.79, P < 0.01 \)). These data suggest that the act of training animals to self-administer drug and food may have altered the input resistance of PF/LHA neurones.

**Cocaine self-administration alters paired-pulse but not AMPA/NMDA ratio**

In order to further explore the effects of cocaine on excitatory synapses in PF/LHA neurones, a bipolar stimulating electrode was used to electrically activate synaptic inputs. These experiments compared synaptic function in cocaine- and food-trained rats. First, short-term synaptic plasticity and presynaptic release probability were assessed using a paired-pulse protocol (50 ms inter-stimulus interval) to determine paired-pulse ratios (Fig. 4). The outcome of these experiments showed that neurones from rats that self-administered cocaine had significantly lower paired-pulse ratios, i.e. paired-pulse depression, compared to food-rewarded controls (cocaine vs. food, 0.82 ± 0.07 vs. 1.05 ± 0.1, \( P < 0.05 \), Fig. 4A). We also recorded evoked EPSCs (eEPSCs) to assess whether cocaine self-administration causes postsynaptic changes to excitatory synapses in the PF/LHA. Responses were recorded at −70 mV or +40 mV holding potentials to isolate the AMPA- and NMDA-mediated components of eEPSCs, respectively (Fig. 4B). A comparison of the properties of AMPA and NMDA eEPSCs showed that amplitude (AMPA, 271.38 ± 53.95 pA vs. 260.08 ± 53.65 pA; NMDA, 102.97 ± 18.17 pA vs. 139.47 ± 40.57 pA), rise time (AMPA, 1.57 ± 0.22 ms vs. 1.64 ± 0.29 ms; NMDA, 4.45 ± 0.37 ms vs. 6.92 ± 2.17 ms), and decay time constant (AMPA, 5.61 ± 0.73 ms vs. 7.74 ± 1.05 ms; NMDA, 95.63 ± 10.46 ms vs. 140.62 ± 37.74 ms) were all similar in cocaine- versus food-trained animals. The peak amplitude of AMPA- and NMDA-mediated...
Anatomical evidence that experimenter-administered cocaine alters excitatory input to orexin neurones

To determine whether any of the cocaine-induced changes in excitatory drive to PF/LHA neurones may affect orexin neurones, we assessed the density of putative VGAT-positive puncta closely apposed to orexin-positive neuroglutameric (VGLUT2-apposition) and GABAergic (VGAT-apposition) synapses onto orexin-positive neurones (Fig. 5A). No differences were detected in VGAT-positive puncta closely apposed to orexin neurones in cocaine- versus saline-exposed rats (21.15 ± 1.52 vs. 22.64 ± 0.87, Fig. 5B). Rats exposed to cocaine, however, showed a significant increase in VGLUT2-positive puncta closely apposed to orexin neurones as compared to saline-treated rats (23.74 ± 0.68 vs. 19.77 ± 1.52, P < 0.01, Fig. 5B).

Functional evidence that self-administered cocaine alters excitatory input to orexin neurones

In order to directly assess whether orexin neurones in the lateral hypothalamus undergo enhanced excitatory drive, neurobiotin-filled recorded neurones were dual-immunolabelled for orexin/neurobiotin (Fig. 5C), and data from orexin/neurobiotin-positive neurones were grouped. A small proportion of recorded slices produced reliable orexin/neurobiotin immunostaining (control n = 9; cocaine n = 10), with only a subgroup of these confirmed as containing orexin/neurobiotin-positive (control n = 4, cocaine, n = 3) neurones that yielded data included in the final analysis (thus approximately 36% of PF/LHA neurones were orexin-positive). Despite these limited recoveries, mEPSC frequency in orexin/neurobiotin-positive neurones was increased by cocaine self-administration (28.63 ± 2.05 Hz vs. 7.34 ± 2.63 Hz, P < 0.01, Fig. 5E) while amplitude (−27.05 ± 2.13 pA vs. −18.80 ± 2.42 pA), rise time (0.49 ± 0.04 ms vs. 0.60 ± 0.05 ms) and decay time constant (1.72 ± 0.36 ms vs. 1.84 ± 0.16 ms) remained similar between cocaine- and food-trained rats (P > 0.05). To assess whether the proportion of neurones recovered in our recordings simply reflects the overall proportion of PH/LHA neurones that express orexin within the area we sampled, we combined immunolabelling for orexin and NeuN, a marker of post-mitotic neurones. This analysis revealed that orexin neurones accounted for approximately 43% (48 out of 112 cells counted in 6 sections) of the total population of PF/LHA neurones in the region we used for our electrophysiological analyses.

Discussion

In the present study we provide the first anatomical and functional evidence that cocaine exposure induces plasticity in the PF/LHA. Our data are consistent with evidence that plasticity occurs in PF/LHA neurones after acute non-drug environmental challenges and the emerging role of a number of hypothalamic neuropeptide systems in drug-motivated behaviours. Taken together we believe these data indicate that hypothalamic circuitry is highly 'plastic', and thus susceptible to cocaine-induced maladaptations. These changes in hypothalamic circuitry may contribute to increased addiction...
Figure 5. Cocaine treatment increases putative apposition of excitatory VGLUT2 puncta
A, dual-labeling for VGAT and orexin (left), or VGLUT2 and orexin (right). Upper panels show saline-treated animals and lower panels show cocaine-treated animals. B, cocaine exposure did not increase putative apposition of inhibitory VGAT puncta onto orexin neurones. In contrast, cocaine exposure significantly increased putative apposition of excitatory VGLUT2 puncta onto orexin neurones (**P < 0.01). C, image panels show immunolabelling for neurobiotin (left), or orexin (middle), and merged pictures (right) from a recorded neurone confirming that population of recordings were from orexin positive neurones. Upper panels are from food-trained controls and lower panels are from cocaine-trained rats. D, schematic diagram showing the collated locations of all recordings in cocaine-exposed and control animals, with red dots indicating confirmed orexin positive recordings (Bregma 2.76–Bregma 3.24). Note that recovery of neurobiotin and orexin labelling was only performed for cocaine self-administration experiments. E, cumulative probability of orexin neurones (from representative recordings, left) and bar plots (group data, right) of mEPSC frequency highlight a significant increase in events recorded from cocaine- compared to food-trained rats (**P < 0.01).
and relapse vulnerability. Interestingly, although we only recovered a small population of recorded neurones, it appears clear from our data that one of the PF/LHA populations that are vulnerable to drug-induced plasticity expresses the orexin neuropeptides.

The present findings indicate that cocaine specifically alters presynaptic inputs onto PF/LHA neurones. This conclusion was supported by our findings that both methods of cocaine exposure produced an identical signature of changes to mEPSCs, i.e. passive or self-administered cocaine increased the frequency of mEPSCs, but not amplitude. One caveat to this finding is that we cannot exclude the possibility that some very small mEPSCs (<10 pA) may have gone undetected in control or cocaine treatment groups. This has the potential to influence our measurements of mEPSC amplitude and frequency, but our capacity to resolve all events above 10 pA and the comparison of baseline noise amongst groups suggests this effect should be minimal. It is interesting to note that our data indicate that self-administration training may have reduced the input resistance of PF/LHA neurones. This is probably unsurprising given that acquiring the operant task is a learning process that could evoke changes in PF/LHA cells that are different from passive drug injections. Further studies will be required to understand the significance of this finding with respect to addiction and to identify the putative mechanisms responsible, e.g. potential training-induced changes in channel expression in PF/LHA neurones.

Consistent with cocaine having effects at hypothalamic synaptic terminals, we observed no change in the AMPA/NMDA ratio in animals trained to self-administer cocaine. The AMPA/NMDA ratio is a common measure of changes in postsynaptic receptor composition and increases in this ratio have been shown to occur in other addiction relevant brain regions such as the NAC and VTA (Kauer & Malenka, 2007; Mameli et al. 2009; Moussawi et al. 2011). Importantly, other studies have shown that PF/LHA neurones do demonstrate postsynaptic changes in the AMPA/NMDA ratio in response to pharmacological (modafinil) and environmental (sleep deprivation) challenges (Horvath & Gao, 2005; Rao et al. 2007). These data indicate that our failure to detect a change in this parameter is not due to an inability of this cell population to undergo measurable changes in postsynaptic strength. Presynaptic plasticity was also supported by our paired-pulse experiments, which demonstrated a cocaine-induced paired-pulse depression. This finding is widely interpreted as indicative of increased release probability (Debanne et al. 1996), and consistent with a cocaine-induced increase in mEPSC frequency.

With respect to the likely identity of the PF/LHA neurones that undergo cocaine-induced presynaptic plasticity, increased numbers of VGLUT2-positive puncta were observed closely apposed to orexin neurones. Further supporting this finding was the observation that mEPSC frequency increased in a small group of neurobiotin-positive neurones confirmed as immunopositive for orexin. While we did not functionally test cocaine-induced changes to inhibitory GABAergic inputs onto PF/LHA neurones, we did not observe a change in the number of VGAT-positive inputs onto orexin neurones after cocaine exposure, making it unlikely that the effects we report are due to decreased inhibitory inputs from local GABAergic neurones. It is noteworthy that the recovery of orexin neurones in the PF/LHA using neurobiotin filling and orexin immunohistochemistry was somewhat lower than we expected (approximately 36%). Therefore, to determine if we are likely to have underestimated the proportion of orexin neurones in our recordings, we assessed the proportion of orexin neurones relative to the total number of neurones by labelling PF/LHA tissue for orexin and NeuN. We found that orexin neurones account for approximately 43% of the total neuronal population in the PF/LHA region. These data suggest that our recordings did in fact include a representative proportion of orexin neurones and reinforces our conclusion that cocaine-induced synaptic plasticity in PF/LHA does include orexin neurones.

The identity of non-orexin PF/LHA neurones that presumably also undergo cocaine-induced plasticity was not pursued in this study. Melanin concentrating hormone (MCH)-positive neurones are a separate, non-overlapping population of cells that are anatomically adjacent to orexin neurones in the PF/LHA (Elias et al. 2001). Importantly, like orexin neurones, this population has recently been implicated in drug-motivated behaviours and thus may also participate in cocaine-induced synaptic remodelling in the PF/LHA. Chung et al. (2009) recently showed that MCH-1 receptor knockout mice display reduced place preference and sensitization for cocaine and MCH1-R antagonism reduced cue-, and cocaine-elicited drug-seeking behaviour (Chung et al. 2009). These effects appear to be mediated by a direct projection to the NAC shell (Georgescu et al. 2005; Bittencourt, 2011). It will be interesting for future studies to determine whether cocaine-induced presynaptic plasticity occurs in MCH PF/LHA neurones.

It is also worth noting the evidence of an important contribution for local, intra-hypothalamic glutamate neurones in providing positive feedback that exists in the PF/LHA (Acuna-Goycolea et al. 2004). In light of this circuitry, it is possible that the increased presynaptic plasticity we observed was caused by an increase in both the number of release sites and the probability of release from local glutamate interneurones. As the PF/LHA also receives a significant glutamatergic projection from other addiction-relevant brain regions, e.g. prefrontal...
cortex and basolateral amygdala (BLA), the possibility that cocaine-induced plasticity occurred at excitatory terminals originating from these sites cannot be ruled out (Henny & Jones, 2006; Morshedi & Meredith, 2008). For example, Morshedi & Meredith (2008) have recently shown that repeated injections of amphetamine up-regulate Fos immunoreactivity in medial PFc (mPFc) neurones that project to the LHA. (Morshedi & Meredith, 2008). Further, Petrovich et al. (2002, 2005) demonstrated that a BLA to PF/LHA circuit controls cue-potentiated feeding behaviour (Petrovich et al. 2002, 2005). The NAC shell a part of the brain ‘reward’ circuitry may also influence the recruitment of hypothalamic neurones in response to drug-relevant stimuli (Marchant et al. 2009; Millan et al. 2010). Because the NAC projection neurones are presumably GABAergic, any influence over hypothalamic circuits with relevance to the present findings would presumably involve a polysynaptic pathway leading to the disinhibition of glutamatergic interneurones. It will be important for future studies to identify the source of these glutamatergic inputs that are altered by cocaine exposure.

With regard to potential mechanisms for the cocaine-induced increase to excitatory input, previous studies have demonstrated that Group III metabotropic glutamate receptors (mGluRs) maintain tonic inhibition of excitatory synaptic input onto hypothalamic orexin neurones (Acuna-Goycolea et al. 2004). Group III mGluRs normally reduce neurotransmitter release either by inhibiting presynaptic voltage-dependent calcium channels or through direct effects on release machinery (Kuzmiski & Bains, 2010). Cocaine-induced loss of mGluR function has been observed in other brain regions such as the NAC (Mousawi et al. 2011) and it is therefore tempting to speculate that mGluR dysfunction may contribute to the elevated synaptic input we observed onto hypothalamic neurones. Future experiments that employ selective mGluR antagonists will be required to further test this possibility.

In summary, we show for the first time that cocaine induces presynaptic plasticity in hypothalamic neurones, and that orexin neurones are one population of PF/LHA neurones that are susceptible to these changes. These findings are particularly relevant to the accumulating evidence that lateral hypothalamic orexin neurones play an important role in the expression of addiction-like behaviours including drug-seeking (Harris & Aston-Jones, 2006; Lawrence et al 2006; James et al. 2011). Orexins appear to mediate these effects, at least in part, through projections to the VTA. For example, systemic SB-334867, an orexin-1 receptor antagonist, prevented cocaine-induced increases in the AMPA/NMDA ratio in VTA dopamine neurones and blocked cocaine sensitization (Borgland et al. 2006). Further, we recently showed that intra-VTA SB-334867 attenuates cue-induced cocaine-seeking (James et al. 2011). Enhanced excitatory drive to the VTA from the over-excited PF/LHA orexin neurones could initiate and contribute to the persistent plasticity within dopamine neurones following long-term cocaine self-administration and increase relapse vulnerability (Chen et al. 2008).

The present findings are interesting in light of the recent studies demonstrating that food restriction and sleep deprivation and now cocaine increases evidence of pre-synaptic plasticity in PF/LHA and orexin neurones. These data indicate an intrinsic capacity of PF/LHA circuits to rapidly rewire in response to acute challenges to homeostasis. In the case of natural stimuli, e.g. food, this presynaptic plasticity is reversed within 24 h after re-feeding (Horvath & Gao, 2005). Thus, increases in excitatory inputs may be an important mechanism via which PF/LHA circuits respond to changes in energy status and/or levels of arousal to appropriately regulate behavioural output. Presumably cocaine ‘hijacks’ these same homeostatic mechanisms. Future studies will need to determine how these effects are conferred and whether cocaine persistently corrupts these pre-synaptic plasticity mechanisms, or might also produce postsynaptic effects. In addition to the obvious implications for addiction, these studies will advance our understanding of ‘normal’ homeostatic plasticity within the PF/LHA. Understanding these processes may provide new insight into the development of pharmacological treatment options that reduce pathological reward-seeking behaviour.

References

**Author contributions**

C.V.D., B.A.G and J.W.Y. conceived and designed the experiments. J.W.Y. collected the data. J.W.Y., C.V.D., B.A.G., M.H.J. and P.J. analysed the data. J.W.Y., C.V.D., B.A.G., M.H.J., P.I. and J.S.B. drafted the article. Experiments were performed in the laboratories of C.V.D. and B.A.G. at the University of Newcastle. All authors approved the final version of this manuscript.

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