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 renewed drug-seeking and 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 paraventricular thalamus (PVT), 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 peptides. In addition, we provide novel data demonstrating that blockade of orexin receptor 1 (OX1R) signaling within the ventral tegmental area (VTA) alters Fos-protein expression in relapse-relevant regions, including the PVT and the nucleus accumbens shell (NacS). Further, whilst previous findings have shown that blockade of OX1R 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; Reinstatement; Relapse; Fos; 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 [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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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]. 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].

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

Figure 1: Diagram of how CART (A) and orexin (B) -containing cells within the lateral hypothalamus may influence structures involved in the reinstatement of drug-seeking behaviour. The classical ‘final common pathway’ is highlighted in red. Solid lines denote established projections based on functional studies, whilst dotted lines represent projections that are yet to be confirmed. LH: Lateral hypothalamus. NAC: Nucleus accumbens. PFC: Prefrontal cortex. PVT: Paraventricular thalamus. VP: Ventral pallidum. VTA: Ventral tegmental area.
showed 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 signaling 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 drug-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 signaling 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 signaling 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 antagonist 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 reward-seeking in general and subsequently their role in drug-motivated behaviours.

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-enkephalocortin (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 antifeeding peptide α-melanocyt 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

| Table 1: Glossary of terms. |
|----------------------------------|

| 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. |
| 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. |
| Reinforcement 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 animal ceases to press is referred to as the animal’s ‘break point’. |
| Intracraniial 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 decreased ‘reward’ pathway sensitivity whereas lower rates of responding for ICSS are thought to result from increased ‘reward’ pathway sensitivity. |
| 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. |

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found in the peripheral nervous system, including myenteric neurons in the gastrointestinal tract, sympathetic preganglionic neurons and in the adrenal glands [54,55]. 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 [56].

**CART and feeding behaviour**

Early studies investigating the effects of CART on reward-seeking firmly established a role for this peptide in the regulation of feeding behaviour. Acute i.c.v. CART administration dose-dependently suppresses feeding behaviour in rats [57,58], whilst chronic CART treatment prevents body weight gain in both lean and obese animals [48]. 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. Thus, central administration of CART antisera decreases feeding, corroborating findings that CART peptide injections exert an inhibitory influence on feeding behaviour [57,58]. Consistent with these functional effects, 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 signalling [58]. Somewhat surprisingly though, intra-DMH infusions of CART have been shown to increase feeding [59], which may be attributed to a disinhibitory effect of CART in the DMH [60].

**CART and addiction-relevant behaviours**

A role for CART in drug-motivated behaviours has been evident since the original report by Douglass and colleagues [46] who showed that acute cocaine and amphetamine administration increases CART mRNA in the striatum of the rat brain. This finding has been difficult to replicate [61] and some studies have suggested that binge/repeated, rather than acute, cocaine administration is required to reliably increase CART transcript expression in the brain [62,63]. Despite these technical caveats, acute cocaine has been shown increase the proportion of Fos-immunoreactive CART neurons in the NAC, even under conditions that do not produce changes in CART mRNA levels [64]. 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 cases [65,66]. In addition, alcoholism has been shown to be associated with a mutation in the CART gene in a Korean population [67] and acute administration of ethanol increases CART mRNA and peptide expression in the rat NAC [68].

Pre-clinical studies have demonstrated a clear, but complicated, role for CART in mediating the effects of psychostimulants. Administration of CART into the VTA attenuates cocaine-induced locomotor activation, especially at higher doses [69], whereas infusions of CART into the NAC have no effect on locomotor activity [70]. However, NAC injections of CART immediately prior to systemic cocaine or amphetamine treatment attenuates the locomotor-activating effects of these drugs [70] and prevents the expression of conditioned hyper-locomotion [71].

Further, direct co-administration of CART and dopamine into NAC reduces the cocaine-like locomotor-activating effects of dopamine [72]. Consistent with this effect, injections of CART into the VP, one of the main nuclei that receive accumbal efferents, also attenuate cocaine-induced locomotion [73]. In contrast to this evidence for a CART-mediated inhibitory effect on addiction-like behaviors, infusion of CART 55-102 peptide fragment into the VTA causes an efflux of dopamine in the NAC resulting in a cocaine-like increase in locomotor activity and can even produce a CONDITIONED PLACED PREFERENCE (CPP) [74]. Finally, Rademacher 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 Mattson 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 for the role of 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 DMH/LH [1]. Interestingly, Millan 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 infusions of CART on reinstatement behaviour. Consistent with our findings, King and colleagues [77] showed that i.c.v. infusion of CART 55-102 prevents context-elicited renewal of alcohol-seeking. While it is unclear from this study where CART acts to inhibit context-elicited reinstatement, the PVT is considered a potential candidate given its known reactivity to drug-associated cues and contexts [37,42]. Indeed, preliminary findings from our laboratory suggest that intra-PVT infusions of CART 55-102 attenuate cue-induced reinstatement of cocaine-seeking. Importantly, there also exists a possible role for CART in stress-induced reinstatement. CART mRNA and peptide is expressed throughout the hypothalamic-pituitary-adrenal (HPA) axis and other regions of the brain known to regulate the HPA axis, including the prefrontal cortex and amygdala [46,50,78], while i.c.v. and intra-PVN administration of CART increases circulating ACTH and corticosterone levels [79]. Future studies must therefore determine how CART signaling within the PVT and other stress-related regions may influence stress-induced reinstatement of drug-seeking, as based on the literature above, it seems plausible that CART-peptide injections into the PVT or stress-responsive regions may have pro-drug-seeking effects.

With respect to how CART within the PVT may modulate the brain relapse circuitry, anatomical studies show that glutamatergic 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 [80,81] and dopamine signaling in this region is necessary for drug-primed [82] and context-elicited [8]
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 dopamine neurons as well as GABAergic interneurons [50,53]. It has been proposed that CART may therefore regulate dopamine release from the VTA either directly or via the disinhibition of GABA interneurons [83].

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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 (OXR1) and orexin-receptor 2 (OXR2). Orexin A binds to both OX1 and OX2 with equal affinity, whereas orexin B binds to OX2 with a higher affinity than OX1 [89]. OX1s are found in the PFC, hippocampus, PVT, VTA, VMH, dorsal raphe nucleus and locus coeruleus. OX2s 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, dorsomedial 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 dynorphin-A [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 [18] (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 receptor 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 showed 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 primary 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 rewarding 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 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 PF/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, PFA 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] microinfused 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, Boutrel 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-Phe-CRF (12-41) and the a2-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) did not have an 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 reinstate extinguished drug-seeking behavior. However recent evidence from another group has shown that at small doses, cocaine-seeking can be reinstated by intra-PVT infusion 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 [138]. 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 k-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 elicit 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, Borgland 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 NA 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 [143]. 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 (3µg) 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 D₂ 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 OX₁ signaling in the VTA may influence PVT activity via an indirect route. Blockade of the VTA OX₁ 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

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**Figure 3:** Intra-VTA SB-334867 (3 and 6µg) fails to affect reinstatement elicited by presentation of cues predictive of sweetened condensed milk (SCM) availability. Male (n=20) Sprague Dawley rats were trained to respond for SCM in the presence of discriminative stimuli predictive of reward availability or non-availability. A constant 70dB white noise (S+) indicated availability of SCM, whilst constant illumination of a 2.8W house light (S-) in the top corner of the operant chamber indicated non-availability of SCM (i.e. lever responses resulted in no consequence). Sessions were initiated by extending two retractable levers into the chamber that coincided with the presentation of either the S+ or S- discriminative cues. During S+ conditioning sessions, responses on the active lever (under a fixed ratio 1 schedule of reinforcement) resulted in a delivery of (0.1 ml) SCM into a receptacle positioned between the two levers. Rewarded responses were followed by a 20-sec timeout period signaled by the illumination of a cue light above the lever. During S- conditioning sessions, responses on the active lever resulted in no reward and was followed by an intermittent beeping tone that lasted for 20 sec. In both S+ and S- sessions, responses on the left (inactive) lever were recorded but responses were inconsequential as per training conditions. Following lever training, animals were exposed to S+ conditioning sessions and S- conditioning sessions over eight days (2 sessions per day, in random sequence, 20 minutes/session). Lever pressing was then extinguished in 1-hour sessions during which reinforcers and discriminative stimuli were withheld, until a criterion of 5 active lever responses over three consecutive days was reached. One day following extinction, animals were exposed to the S+ cue and lever responding was recorded for two hours. The following day, animals received unilateral intra-VTA injections of either SB-334867 (3µg or 6µg/0.5µl) or vehicle (equivolume) 15 minutes before being presented with the S+ cue in the absence of SCM reward, and reinstatement responding was recorded over two hours. Presentation of S+ stimuli resulted in a robust reinstatement of responding on the active lever in all animals but had no effect on inactive lever responding. A one-way ANOVA revealed that there were no significant differences in active lever responding between treatment groups (p>0.05). No differences were observed across groups in terms of inactive lever responding (data not shown). Together, these data indicate that intra-VTA SB-334867 (3 and 6µg) has no affect on cue-induced reinstatement of SCM-seeking.

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OXR1 antagonist SB-408124 has no effect on footshock-elicited seeking without altering natural reward-seeking. Together these data provide evidence that passive or self-administered 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 did not alter this behavior, even at a dose higher than what was identified an increased frequency, but not amplitude, of miniature excitatory post-synaptic currents (mEPSCs) in PF/LHA of cocaine-treated 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/LHA 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/LHA 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

| Reinforcer | Animals | Treatment | Reinstatement Stimuli | Effect | Reference |
|------------|---------|-----------|----------------------|--------|-----------|
| Cocaine (SA) | Male, SD | Intra-PVT administration CART 55-102 (0.625µg and 2.5µg) | Cocaine Prime (10mg/kg) | 2.5µg dose significantly attenuated reinstatement | James et al. (2010) |
| Cocaine (SA) | Male, SD rat | SB-334867 (10, 20, 30mg/kg; i.p.) 4PT (10, 30mg/kg; i.p.) | Tone + cue lights paired with cocaine infusions | SB-334867. Dose-dependent decrease in reinstatement. 4PT: No effect on reinstatement | Smith et al. (2009) |
| Cocaine (SA) | Male, SD, rat | SB-334867 (10, 20, 30mg/kg; i.p.) | Re-exposure to SA environment following abstinence Context (visual, auditory, olfactory and tactile cues) | Dose-dependently decreased reinstatement following abstinence and context-induced reinstatement | Smith et al. (2010) |
| Cocaine (SA) | Male, SD rat | Intra-VTA SB-334867 (1µg and 3µg/0.5µl, unilaterally) | S+ (Background white noise) | Decreased reinstatement (3µg) | James et al. (2010) |
| Cocaine (SA) | Male, Wistar rat | 1. SB-334867 (15, 30mg/kg; i.p.) 2. Orexin-A (0.3, 0.75, 1.5nmol; i.c.v.) | 1. Footshock (15mins, 0.5mA, variable interval 40s) 2. None | 1. Dose-dependent decrease in reinstatement 2. 0.75 and 1.5nmol doses induced reinstatement (dose-dependently) | Boudrel et al. (2005) |
| Cocaine (SA) | Male, LE rat | 1. Intra-VTA Hcrt-1 and Hcrt-2 (10µm) 2. Intra-VTA SB-408124 | 1. None 2. Footshock (20mins, 0.4-0.6mA, variable interval 40 + 30s) | 1. Hcrt-1: induced reinstatement 2. Hcrt-2: No effect 2. No effect | Wang et al. (2009) |

* Only one other study has investigated the functional role of CART on reinstatement behaviour. King et al. (2010) showed that i.c.v. administration of CART 55-102 (0.625µg and 2.5µg), but not CART 1-27 (1.25µg), dose-dependently decreased reinstatement alcoholic beer-seeking in a renewal model. * SA: Self-administration, SB-334867 and SB-408124 are selective OX1 antagonists. 4PT is a selective OX2 antagonist.

Table 2: Summary of studies investigating the functional effects of CART and orexin on reinstatement of psychostimulant-seeking behaviour.
‘off-target’ effects. Despite this, and consistent with the differential roles of the orexin receptors, blockade of the OX1 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 OX2 antagonism, although a recent study reported that the selective OXR2 antagonist JNJ-10397049 attenuates reinstatement of alcohol CPP [128], the OX2 antagonist 4-pyridylmethyl (S)-tert-leucyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline [4PT] had no effect on cue-induced reinstatement of cocaine-seeking and produced marked impairments in locomotor activity [133]. Additionally, while [N]-10397049 significantly alters sleep-wake states, the OX1 antagonists SB-334867 and SB-408124 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 OX1 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 have no noticeable effect on locomotor activity [43]. Further, intra-VTA infusions of CART have no effect on movement, except at high doses (bilateral/5ug/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 Jupp 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 drug-primed reinstatement of cocaine-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. Further, it will also be important to investigate 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 OX1 signaling in this region is not necessary for cue-induced reinstatement of cocaine-seeking. Future studies should address whether OX2 signaling in the PVT is involved in this form of reinstatement, as well as whether orexin signaling at either OX1 or OX2 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 OX1 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-induced 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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