Central orexin (hypocretin) 2 receptor antagonism reduces ethanol self-administration, but not cue-conditioned ethanol-seeking, in ethanol-preferring rats

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Abstract

Orexins are hypothalamic neuropeptides which bind to two G-protein-coupled receptors, orexin-1 (OX1R) and orexin-2 (OX2R) receptor. While a role for OX1R has been established in both ethanol reinforcement and ethanol-seeking behaviour, the role of OX2R in these behaviours is relatively less-studied. The aim of this study was to determine the role of central OX2R in ethanol-taking and ethanol-seeking behaviour. Indiana ethanol-preferring rats were trained to self-administer ethanol (10% w/v) or sucrose (0.7–1% w/v) in the presence of reward-associated cues before being implanted with indwelling guide cannulae. The selective OX2R antagonist TCS-OX2-29 was administered i.c.v. to assess its effect on operant self-administration and cue-induced reinstatement following extinction. Following i.c.v. injection TCS-OX2-29 reduced self-administration of ethanol, but not sucrose. Despite reducing ethanol self-administration, TCS-OX2-29 had no impact on cue-induced reinstatement of ethanol seeking. To determine where in the brain OX2R were acting to modulate ethanol self-administration, TCS-OX2-29 was microinjected into either the shell or core of the nucleus accumbens (NAc). Intra-NAc core, but not shell, infusions of TCS-OX2-29 decreased responding for ethanol. Importantly, the doses of TCS-OX2-029 used were non-sedating. Collectively, these findings implicate OX2R in the NAc in mediating the reinforcing effects of ethanol. This effect appears to be drug-specific as antagonism of central OX2R had no impact on sucrose self-administration. Thus, OX2R in addition to OX1R may represent a potential therapeutic target for the treatment of ethanol-use disorders. However, unlike OX1R, no impact of OX2R antagonism was observed on cue-induced reinstatement, suggesting a more prominent role for OX2R in ethanol self-administration compared to cue-conditioned ethanol-seeking.

Introduction

Orexins (A and B), or hypocretins (1 and 2) are a recently characterized family of neuropeptides which are proteolytically cleaved from the same precursor, prepro-orexin (prepro-hypocretin; de Lecea et al., 1998; Sakurai et al., 1998). Orexin neurons originate exclusively in the lateral, dorsomedial and perifornical areas of the hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998) yet project widely throughout the brain, innervating numerous regions including components of the mesocorticolimbic system (Peyron et al., 1998; Baldo et al., 2003). The widespread nature of these efferent projections suggests that the orexin system is involved in the regulation of multiple brain functions. Indeed, orexins have been shown to influence multiple physiological processes including energy homeostasis and feeding, neuroendocrine and autonomic functions as well as arousal and maintaining wakefulness (Sutcliffe and de Lecea, 2000; Mieda and Yanagisawa, 2002; de Lecea, 2012; Girault et al., 2012;
Nattie and Li, 2012). In addition, substantial evidence implicates the orexins in reward and motivational processes as well as drug-seeking behaviour (Boutrel et al., 2005; Harris et al., 2005; Scammell and Saper, 2007; Mahler et al., 2012b).

Orexins elicit their effects via two G-protein-coupled receptors: the orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R). OX1R appears to exclusively couple via the Gq subclass of G proteins whereas OX2R has been shown to couple to Gq, Gi and Gq/o subclasses (Sakurai et al., 1998; Zhu et al., 2003; Tang et al., 2008). Furthermore, both orexin A and orexin B display equal affinity for OX1R whereas orexin A preferentially binds to OX1R (Sakurai et al., 1998). Consistent with the diffuse pattern of expression displayed by orexin-containing neurons, OX1R and OX2R are widely distributed within the brain (Trivedi et al., 1998; Hervieu et al., 2001; Marcus et al., 2001; Chuderay et al., 2002) showing overlapping yet also divergent patterns of expression. OX1Rs are highly expressed in cortical regions, the bed nucleus of the stria terminalis and brainstem regions such as the locus coeruleus. In contrast, OX2Rs are comparatively more densely expressed in nucleus accumbens (NAc), hypothalamic regions and medial thalamic groups. The ventral tegmental area (VTA) contains moderate levels of both receptors (Trivedi et al., 1998; Marcus et al., 2001). Collectively, these differences in signalling, distribution and binding affinity for the orexin ligands indicate a possible divergence of function between the two orexin receptors. Indeed, the majority of evidence to date indicates arousal is most closely associated with activation of OX2R and reward with OX1R activation (for review see Mahler et al., 2012b).

As such, OX1R has been shown to be involved in mediating the reinforcing and motivational properties of multiple drugs of abuse as well as drug-induced plasticity and drug-seeking behaviour (Harris et al., 2005; Borgland et al., 2006; James et al., 2007; Hollander et al., 2008; for review see Mahler et al., 2012b). Antagonism of OX1R prevents reinstatement of extinguished drug-seeking and self-administration of some drugs of abuse and it appears the VTA is a locus for this effect (España et al., 2010; James et al., 2011; Mahler et al., 2012a; Srinivasan et al., 2012). Findings from our laboratory provided the first evidence for a role for OX1R in the reinforcing properties of ethanol. The OX1R-selective antagonist SB 334867 attenuated operant self-administration of ethanol by ethanol-preferring (iP) rats. In addition, SB 334867 attenuated cue-induced reinstatement of ethanol-seeking following extinction (Lawrence et al., 2006; Jupp et al., 2011a). Subsequently, similar effects have been demonstrated in Long-Evans (Richards et al., 2008) and Wistar (Martin-Fardon and Weiss, 2012) rats in a manner which does not extend to natural reinforcers (see Jupp et al. 2011b; Cason and Aston-Jones, 2012; Srinivasan et al., 2012). Recent research suggests that OX1R are recruited selectively when levels of ethanol consumption or motivation to consume ethanol are high (Moorman and Aston-Jones, 2009; Jupp et al., 2011b; Shoblock et al., 2011; Kim et al., 2012).

In contrast to OX1R there has been a relatively less investigation of OX2R as a potential therapeutic target for drug addiction, despite the fact that OX2R are heavily expressed in the NAc, a region of the brain critically involved in reward-related behaviour (Trivedi et al., 1998). The first study to examine OX2R in this context found no effect on cocaine self-administration or cue-induced reinstatement of cocaine-seeking (Smith et al., 2009). However, non-contingent chronic cocaine up-regulates OX2R in the NAc, an effect that persists for at least 60 d after withdrawal (Zhang et al., 2007). Furthermore, the selective OX2R antagonist, TCS-OX2-29, was recently shown to prevent the acquisition and expression of a morphine conditioned place preference (CPP) in both morphine naive and dependent mice (Tabaeizadeh et al., 2013). A recent study also implicates OX2R in ethanol reward. The OX2R antagonist INJ-10397049 reduced ethanol, but not saccharin, self-administration in rats after systemic administration (Shoblock et al., 2011). This same antagonist also decreased the acquisition, expression and primed reinstatement of ethanol CPP in mice (Shoblock et al., 2011). The precise anatomical loci for this effect are unknown at this stage, although a recent study utilizing the dual OX1/OX2R antagonist Almorexant found intra-VTA administration of this drug reduced self-administration of ethanol (Srinivasan et al., 2012). Furthermore, whether or not these findings extend to ethanol-seeking behaviour in an operant paradigm is yet to be determined.

Thus, in order to extend our understanding of the role of OX2R in the centrally mediated effects of ethanol, as well further its assessment as a potential therapeutic target for the treatment of ethanol abuse, the effects of i.c.v. administration of the selective OX2R antagonist TCS-OX2-29 was tested on two processes central to ethanol abuse: ethanol reinforcement; cue-induced reinstatement of ethanol-seeking iP rats. The NAc core and shell were chosen as putative loci due to their well-established involvement in mediating the rewarding properties of ethanol (Gonzales et al., 2004) and relatively high expression of OX2R (Trivedi et al., 1998). We also sought to determine if any observed reductions in ethanol self-administration by
OX2R antagonism could be attributable to locomotor impairments, and whether any such effects were selective for ethanol by assessing effects of i.c.v. administration of TCS-OX2-29 on responding for the natural reinforcer sucrose.

Method

Animals

Male inbred iP rats (n = 75) were obtained aged 6–8 wk from the breeding colony housed at the Florey Institute of Neuroscience and Mental Health. Parental stock had previously been obtained from Professor T. K. Li (while at Indiana University, USA). Animals were housed in groups of two or three and experienced a 12 h light/dark cycle (lights on 07:00 hours) and ad libitum access to food and water. All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986, under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Prior approval was obtained from the Animal Ethics Committee of the Florey Institute of Neuroscience and Mental Health, University of Melbourne.

Drugs

TCS-OX2-29 (C_{23}H_{31}N_{3}O_{3}.HCl.3H_{2}O, molecular weight = 488.02), a selective OX2R antagonist (Hirose et al., 2003) was obtained from Tocris Bioscience (UK) and dissolved in artificial cerebrospinal fluid for i.c.v. microinjections or 0.9% saline for microinjections targeting the NAc core or shell. Differences in vehicles were due to differences in injection volume and solubility of TCS-OX2-29.

Operant responding for ethanol or sucrose

The iP rats were trained to self-administer 10% ethanol (v/v) or sucrose 5 d/wk in operant chambers supplied by Med Associates (USA) in a procedure adapted from Cowen et al. (2005b). Each operant chamber featured a house light; two sides had a stimulus light and an adjacent receptacle. During the 20 min operant sessions retractable levers located under each stimulus light were extended and a fixed ratio of three lever presses was required for each delivery of 0.1 ml solution (ethanol, sucrose or water) and activation of the stimulus light (1 s). The active lever was associated with ethanol/sucrose delivery while the inactive lever was associated with the delivery of water. A cap containing two to three drops of vanilla essence was placed on the bedding under the active lever to provide an olfactory cue indicating drug availability and the receptacle adjacent to the active lever was primed with two drops of ethanol/sucrose solution before each session. Behaviour was recorded by a computer running Med-PC IV software (Med Associates, USA).

Training of rats in ethanol self-administration followed an 8 d sucrose-fade protocol beginning with an overnight (15 h) session using a 5% ethanol/5% sucrose solution and then increasing the ethanol and decreasing the sucrose concentration. Rats were trained on 10% ethanol for 25 d and excluded if they did not self-administer at least 0.25 g/kg ethanol per session in the final week of training or if active lever presses accounted for < 70% of total lever presses.

Training of rats in sucrose self-administration began with an overnight (15 h) session with 10% sucrose (w/v), followed by 2 d 10% sucrose. Similarly to Jupp et al. (2011b), every 2–4 d the concentration of sucrose was modified (to 5, 2.5, 1 or 0.7%) in order to match the number of responses to rats trained for ethanol and to control the variance within the sample while preserving a minimum of 80 lever presses for sucrose per session.

Stereotaxic surgery

After completing 5 wk self-administration training, rats were anaesthetised using isoflurane (Delvet, Australia), administered 3 mg/kg meloxicam i.p. (Troy Laboratories, Australia), placed in a stereotaxic frame (Stoelting, USA) and surgically implanted with chronically indwelling guide cannulae obtained from PlasticsOne (USA). Guide cannulae inserted i.c.v. (26 ga, projecting 2.4 mm below pedes tal) were aimed at coordinates (mm from Bregma) based on a standard rat atlas (Paxinos and Watson, 1998); −0.7 AP, +1.4 ML and −2.4 DV. The NAc was targeted using 26 ga bilateral cannulae 1.5 or 3 mm apart (targeting shell and core respectively) and projecting 6.5 mm below pedes tal implanted at +1.7 AP, ±0.75–1.5 ML and −6.5 DV.

After surgery, rats were singly housed and allowed to recover. For the first 4 d recovery, rats were administered 0.2 ml/kg Tribactral® S i.p. (40 ng/ml trimethoprim, 200 mg/ml sulfadoxine; Jurox Pty Ltd, Australia), topical Tricin® powder (250 iu/g bacitracin zinc, 5 mg/g neomycin sulphate, 5000 iu/g polymxin B sulphate; Jurox Pty Ltd) was applied and a ‘mash’ (standard chow dissolved in sucrose solution 10% w/v) was provided. Meloxicam (3 mg/kg i.p.) was administered as required. Following recovery, rats were given at least 4 d operant reacquisition before any behavioural testing commenced.
Microinjections

Injections were performed i.c.v. using a 10 μl syringe (Hamilton Company, USA) and injectors projecting 2.6–3.1 mm below the guide cannula. Injections were performed manually over 15 s, waiting a further 10 s before withdrawing the injector. Injection volumes were 5 μl. Injections of 1 ng human angiotensin II (Auspep, Australia) were used to confirm delivery to the ventricles via rapid dipsogenesis (McKinley et al., 2003).

Intra-parenchymal microinjections were performed using a Pico Plus infusion pump (Harvard Apparatus, USA) equipped with 1 μl syringes (SGE Analytical Science, Australia) connected to injectors projecting 2 mm below the guide cannula. Microinjections of 500 nl per side were conducted over 130 s in a separate chamber and the injectors were left in place for an additional 130 s to allow for diffusion throughout the target region. Correct cannula placement was determined post-mortem following an injection of Methylene Blue (500 nl per side) and cryo-sectioning at 40 μm by a person blind to data.

Habituation to microinjections was achieved with four mock microinjections where rats were handled according to microinjection protocols except chronically indwelling dummies (identical in length to injectors) were simply removed and replaced to simulate injector insertion.

Self-administration

After surgery, recovery and reacquisition of stable self-administration, rats first received a microinjection of TCS-OX2-29 prior to the operant session. Rats receiving microinjections to the NAc were immediately placed in the operant chamber whereas rats receiving i.c.v. injections were returned to their home cage for 10 min to allow the solution time to circulate throughout the brain before being placed in the operant chamber. Operant chamber and session conditions were identical to training. A within-subjects design was employed whereby if responding on vehicle was within the range of responses from the previous four sessions, on the following day TCS-OX2-29 was administered. Rats whose responding on vehicle was not consistent were excluded (n = 2).

Extinction and reinstatement

Following self-administration testing rats underwent a series of extinction sessions, during which time there were no cues present in the operant chamber and no programmed response subsequent to task completion. Rats were given daily extinction sessions until their mean responding per session on the active lever was < 15 for at least 3 d consecutively, which was achieved with 10 d extinction training.

Reinstatement was triggered by representation of olfactory, visual and auditory cues (vanilla, stimulus light and the sound of dispenser activation respectively). Ethanol was not delivered upon task completion despite the presence of cues. Prior to the reinstatement session the left dispenser was flushed with 10% ethanol and the reservoir was removed, allowing a residual 80 μl to provide an olfactory/gustatory cue (Bäckström and Hyytiä, 2004; Vengeliene et al., 2007). Rats were randomly allocated to vehicle or drug-treatment groups. If a rat (n = 6) did not press the active lever within 200 s, the stimulus light was automatically illuminated (a single 1 s pulse) to precipitate reinstatement.

Locomotor activity

To test for possible sedative effects rats which had completed operant behavioural experiments described earlier were placed in locomotor cells connected to a computer running TruScan 2.01 (TruScan Photobeam, Coulbourn Instruments, USA) consisting of a 40.64 cm wide × 40.64 cm deep × 40.64 cm high arena encased by twin photo-optic beam break monitors (Liang et al., 2006). Testing was performed under dimmed light conditions, but during the regular light phase in the vivarium. Rats were placed in the cells for 15 min to habituate, then removed, given a microinjection of vehicle or drug (based on random allocation) and immediately returned to the locomotor cell for 30–40 min. Locomotor activity was measured as distance moved (cm).

Data analysis

Statistical analysis was conducted using SPSS 20.0.0 (IBM, USA). Lever press data were analysed using mixed analysis of variance (ANOVA), with treatment or cue presentation as a within-subjects factor and group received as a between-subjects factor, or two-way repeated-measures ANOVA with treatment and lever as factors. Locomotor results were analysed using independent two-tailed t tests or one-way ANOVA as appropriate. Post hoc testing used Bonferroni’s corrections. Data are presented as means±S.E.M.
Results

Central OX2R antagonism reduces ethanol, but not sucrose self-administration

The effect of centrally administered TCS-OX2-29 was tested on rats trained to self-administer ethanol. Following injections of vehicle, the mean number of lever presses was 147±15 for ethanol and 6±1 for water, which corresponds to a 1.1 g/kg mean ethanol intake during an operant session. The following day rats were randomly assigned to receive administration of 100 μg TCS-OX2-29 (n=9) or 300 μg TCS-OX2-29 (n=6) prior to a self-administration session. Both doses of TCS-OX2-29 were found to significantly reduce responding for ethanol (Fig. 1). Analysis by mixed ANOVA revealed a main effect of treatment (F1,13 = 40.0, p < 0.001, ηp2 = 0.76) but no significant treatment × group interaction (F1,13 = 0.39, p = 0.54, ηp2 = 0.029). This was not the case for water responding, where a significant treatment × group interaction was found (F1,13 = 5.44, p = 0.036, ηp2 = 0.34), but no significant effect of TCS-OX2-29 treatment (F1,13 = 4.17, p = 0.075, ηp2 = 0.036; Fig. 1b) or treatment × lever interaction (F1,8 = 4.75, p = 0.061, ηp2 = 0.37).

Central OX2R antagonism has no effect on cue-induced reinstatement of ethanol-seeking

After 10 d extinction the mean number of lever presses was 9±2 for the lever previously paired with ethanol and 4±1 for the water lever. Before the reinstatement session, the same rats used for the self-administration experiment were randomly assigned to receive either vehicle (n=5), 100 μg TCS-OX2-29 (n=6) or 300 μg TCS-OX2-29 (n=6). There was a significant effect of cue presentation, but no significant difference between vehicle and treatment groups. Data shown as means ± S.E.M. * p < 0.05 compared to extinction (mixed analysis of variance).

Fig. 1. Administration of i.c.v. TCS-OX2-29 reduced operant self-administration of ethanol (10% v/v), but not sucrose. 100 μg TCS-OX2-29 (TCS100; n=9) reduced responses for ethanol and 300 μg TCS-OX2-29 (TCS300; n=6) reduced responses for ethanol and water (a). In a separate cohort of rats trained to self-administer sucrose, i.c.v. administration of TCS100 was not significantly different to responding following administration of vehicle (b). Data shown as means ± S.E.M. * p < 0.05 compared to vehicle (mixed analysis of variance with Bonferroni-adjusted paired t tests).

Fig. 2. Administration of i.c.v. TCS-OX2-29 had no effect on cue-induced reinstatement of ethanol-seeking following extinction. After completing extinction training, rats were administered vehicle (n=5), 100 μg TCS-OX2-29 (TCS100; n=6) or 300 μg TCS-OX2-29 (TCS300; n=6). There was a significant effect of cue presentation, but no significant difference between vehicle and treatment groups. Data shown as means ± S.E.M. * p < 0.05 compared to extinction (mixed analysis of variance).
microinjections of TCS-OX2-29 (100 μg) into the core (n = 15) or the shell (n = 12) of the nucleus accumbens. Compared to responding after vehicle administration, there was a significant reduction in responding for ethanol and water following administration of TCS-OX2-29 into the core (a), but not into the shell (b). Data shown as means ± S.E.M. * p < 0.05 compared to vehicle (two-way analysis of variance with Bonferroni-adjusted paired t tests).

presentation had a significant effect, indicating reinstatement was robust in all groups (F_{2,14} = 31.4, p < 0.001, η_p^2 = 0.68).

**OX2R antagonism in the NAc core, but not the shell reduces ethanol self-administration**

Given the high expression of OX2R in the NAc (Marcus et al., 2001) and the key involvement of this brain region in mediating reward and reinforcement, targeted microinjections of TCS-OX2-29 (100 μg per side) had differential effects on ethanol self-administration in the core and shell of the NAc. As shown in Fig. 3a, two-way ANOVA revealed significant main effects of both treatment (F_{1,14} = 16.2, p = 0.001, η_p^2 = 0.54) and lever (F_{1,14} = 119.5, p < 0.001, η_p^2 = 0.9) and a significant treatment × lever interaction (F_{1,14} = 119.5, p < 0.001, η_p^2 = 0.9). Subsequent Bonferroni-adjusted paired t tests indicated that TCS-OX2-29 decreased responding for both ethanol (t_{14} = 3.59, p = 0.006) and water (t_{14} = 3.28, p = 0.011).

In contrast, injecting the same dose into the shell had no effect on ethanol self-administration. Two-way ANOVA showed there was no main effect of treatment (F_{1,11} = 0.22, p = 0.65, η_p^2 = 0.02) and no significant treatment × lever interaction (F_{1,11} = 0.25, p = 0.62, η_p^2 = 0.023). Rats continued to show a preference for the ethanol-paired lever with a main effect of lever (F_{1,11} = 148.4, p < 0.001, η_p^2 = 0.93). Injection sites were verified by microinjection of Methylene Blue and post-mortem histology. Figure 4 presents a map of microinjection sites.

**Central OX2R antagonism does not alter spontaneous locomotor activity**

In order to test for potential confounds from sedation, locomotor activity was measured in rats after they had completed operant behavioural experiments. Rats received central administration of vehicle (n = 9), 100 μg TCS-OX2-29 (n = 6), 300 μg TCS-OX2-29 (n = 6) or intra-accumbal microinjections of vehicle to the core (n = 4) and shell (n = 9) or 100 μg TCS-OX2-29 per side to the core (n = 5) and shell (n = 7). As shown in Table 1 there were no significant differences between the vehicle and treatment group(s) in any experiments.

**Discussion**

The current study provides the first evidence that OX2R in the NAc are implicated in the reinforcing properties of ethanol. However, where we and others have shown a clear role for OX1R in cue-conditioned ethanol-seeking, this does not appear to be the case for OX2R. Centrally administered TCS-OX2-29, a selective OX2R antagonist, reduced responding for ethanol in an operant paradigm in a manner independent of sedation, yet had no impact on cue-induced reinstatement of ethanol-seeking. This effect appears to be drug-specific as antagonism of central OX2R had no impact on sucrose consumption, although there may be some non-specific effects at higher centrally administered doses. The NAc, a region known to be critically involved in reward-related behaviour, is a possible locus for this effect, as microinjection of TCS-OX2-29 into NAc core (but not shell) also caused a reduction in ethanol responding. Collectively, these findings demonstrate that, unlike OX1R, an apparently dissociable role for OX2R exists in mediating the reinforcing effects of ethanol vs. cue-driven ethanol-seeking behaviour.
The finding that OX2R are involved in the reinforcing properties of ethanol is consistent with previous literature. Shoblock et al. (2011) also found a similar effect on ethanol self-administration in Wistar rats with a different OX2R antagonist, JNJ-10397049. Our study extends these findings in identifying the NAc as a possible locus for this effect. The NAc is known to mediate the reinforcing properties of drugs of abuse and expresses comparatively high levels of OX2R (Trivedi et al., 1998; Marcus et al., 2001). The NAc, in addition to VTA, receives substantial orexinergic input from the lateral hypothalamus, and significant overlap between dopamine and orexin innervation has been determined in these regions (Fadel and Deutch, 2002; Baldo et al., 2003). Orexin B, selective for OX2R, and dopamine can interact to modulate the firing of accumbal neurons (Mori et al., 2011). In addition, orexins dose-dependently depolarize and increase the firing rate of medium spiny neurons (MSNs) in slices (Mukai et al., 2009; Mori et al., 2011). This latter observation is particularly interesting given that the firing pattern of NAc MSNs is believed to encode goal-directed behaviour and reinforcement of both drug and natural rewards (Carelli, 2002; Robinson and Carelli, 2008). Thus, orexins acting at OX2R in the NAc are in a position to modulate accumbens signalling, thus potentially influencing reward-related behaviour.

It could be argued that the reduction in ethanol self-administration observed by administration of TCS-OX2-29 may actually reflect an increase in sensitivity to the centrally mediated effects of ethanol, thereby requiring less ethanol to be self-administered in order to achieve the same pharmacological effect. Although theoretically possible, we believe this is unlikely as OX2R antagonism also prevents the acquisition of an ethanol-CPP (Shoblock et al., 2011). This suggests that OX2R antagonism more likely decreased ethanol intake by directly modulating the rewarding value of ethanol. In addition, OX2R antagonism by JNJ-10397049 does not enhance the motor impairments produced by ethanol as measured by the rota-rod (Shoblock et al., 2011). Thus we hypothesize that intra-NAc blockade of OX2R produces a reduction in the reinforcing effects of ethanol.

Behavioural experiments consistently show that pharmacological manipulations of dopamine transmission in the NAc alter operant responding for ethanol (for review, see Gonzales et al., 2004). Hence the mechanism by which orexin is acting to mediate the primary reinforcing effects of ethanol is potentially via modulation of dopamine signalling in NAc. Until recently the bulk of literature has suggested that orexins modulate dopamine signalling in NAc indirectly via actions in VTA. For example, intra-VTA administration of orexins A and B increases dopamine release in NAc (Narita et al., 2006; Vittoz et al., 2008; Espana et al., 2011) and intra-VTA antagonism of OX1R reduces cocaine-induced dopamine overflow in NAc (Wang et al., 2009). Behaviourally, intra-VTA orexin A can induce a CPP which is dependent on NAc dopamine signalling (Taslimi et al., 2012). Indeed, retrograde tracing studies report that orexin neurons send only few projections into NAc core or dorsal NAc shell (Sharf et al., 2008). However, OX2R, and to a lesser degree OX1R, are densely expressed in NAc suggesting that orexins must also act directly on receptors located in the NAc (Trivedi et al., 1998; Hervieu et al., 2001; Marcus et al., 2001; Cluderay et al., 2002). The current finding that blockade of OX2R in NAc reduces self-administration of ethanol supports this hypothesis.
Recently, orexin A was shown to promote local dopamine release in NAc slices (Patyal et al., 2012), suggesting that direct projections of orexin neurons from the lateral hypothalamus can modulate dopamine release onto NAc MSNs (Patyal et al., 2012). Thus it is entirely possible that orexin signalling via OX2R is capable of influencing the reinforcing properties of ethanol via modulation of dopamine input into NAc. It is also possible that orexin acting in both VTA and NAc has the capacity to work in concert to regulate dopamine signalling in this mesolimbic pathway. However, it has been shown that OX2R antagonism has no impact on ethanol-induced dopamine release in NAc (Shoblock et al., 2011) which raises the possibility that NAc OX2R could also act via a non-dopaminergic mechanism to regulate ethanol reinforcement. A glutamatergic mechanism is one possibility since a role for glutamate has been established in the reinforcing properties of ethanol (Tzschentke and Schmidt, 2003; Cowen et al., 2005a; Hodge et al., 2006) and glutamate × orexin signalling interactions have been demonstrated in other brain regions such as VTA (Borgland et al., 2006, 2008). Direct glutamate × orexin signalling interactions in NAc are yet to be investigated, although recent evidence suggests orexin can modulate NAc dopamine transmission via glutamatergic mechanisms (Patyal et al., 2012).

The NAc consists of two anatomically, biochemically and behaviourally distinct subregions referred to as shell and core (Sesack and Grace, 2010). Our data support a role for OX2R in NAc core but not shell in mediating the reinforcing properties of ethanol, despite the fact that behavioural studies thus far examining orexin signalling in the NAc have focused on shell. For example, orexin A administered directly to NAc shell increases feeding but not ethanol intake as measured by the two bottle free-choice paradigm, and enhances locomotor activity as well as dopamine-dependent turning behaviour (Thorpe and Kotz, 2005; Schneider et al., 2007; Kotani et al., 2008). It appears that in the case of ethanol, OX2R specifically in the NAc core are responsible for modulating its reinforcing effects as measured in the operant self-administration paradigm. Critically, this does not discount a possible role for OX1R in the shell. To the best of our knowledge this is the first study to implicate OX2R signalling specifically in the NAc core in a particular behaviour, demonstrating a differential role for core/shell OX2R in terms of function. It should be noted that we made a deliberate effort to target our microinjections to the dorsomedial corner of the core as this is where

Table 1. Locomotor activity

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<thead>
<tr>
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<th>Move time (s)</th>
<th>Distance moved (cm)</th>
<th>Vertical plane time (s)</th>
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<tbody>
<tr>
<td>I.c.v.</td>
<td></td>
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<tr>
<td>Vehicle (n = 5)</td>
<td>519±54</td>
<td>2802±474</td>
<td>184±32</td>
</tr>
<tr>
<td>TCS100 (n = 6)</td>
<td>493±60</td>
<td>2552±368</td>
<td>130±24</td>
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<td>TCS300 (n = 6)</td>
<td>544±55</td>
<td>2914±387</td>
<td>116±15</td>
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<td>NAc core</td>
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<td></td>
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<tr>
<td>Vehicle (n = 4)</td>
<td>620±30</td>
<td>3863±222</td>
<td>354±55</td>
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<tr>
<td>TCS100 (n = 5)</td>
<td>636±18</td>
<td>4237±226</td>
<td>280±32</td>
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<td>NAc Shell</td>
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<td>Vehicle (n = 9)</td>
<td>507±34</td>
<td>2856±276</td>
<td>142±40</td>
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<tr>
<td>TCS100 (n = 7)</td>
<td>573±26</td>
<td>3337±226</td>
<td>160±28</td>
</tr>
</tbody>
</table>

TCS100, 100 μg TCS-OX2-29; TCS300, 300 μg TCS-OX2-29; NAc, nucleus accumbens. Rats were habituated to the locomotor cell for 15 min and then removed, given a microinjection and returned to the locomotor cell. In order to represent the locomotor activity during operant behavioural experiments, data are shown for between 10 and 30 min post-injection for rats receiving i.c.v. administration of vehicle, 100 μg or 300 μg TCS-OX2-29 and for 20 min post-injection for rats receiving intra-NAc administration of vehicle or 100 μg TCS-OX2-29. There were no significant differences between vehicle and treatment groups for time spent moving and distance moved in the floor plane or time spent in the vertical plane.

Data are shown as means±S.E.M.
OX2R expression and orexin innervation occurs (Trivedi et al., 1998; Marcus et al., 2001; Baldo et al., 2003). The differential effects, despite the close proximity of our injection sites, suggest that our results are not due to significant diffusion of drug away from the target area. Indeed NAc core mediates response-reinforcement learning in the acquisition of a lever-press task to obtain food reward (Kelley et al., 1997) and in terms of ethanol, stimulated dopamine overflow is higher in core of alko alcohol vs. alko non-alcohol rats selected for >100 generations for their differential ethanol preference. In contrast there is no difference in dopamine overflow in the shell between these strains (Pelkonen et al., 2010).

Given the substantial body of literature implicating OX2R in sleep and arousal (Brisbare-Roch et al., 2007; Scammell and Saper, 2007; Dugovic et al., 2009) and the observed non-specific effects on responding for water following i.c.v. or intra-NAc injections, it was important to ascertain that the reduction in operant responding for ethanol observed as a result of TCS-OX2-29 administration was not a result of sedation. Indeed, OX2R antagonism is known to promote sleep (Dugovic et al., 2009; Gozzi et al., 2011) and an OX2R mutation is associated with canine narcolepsy (Lin et al., 1999). Had the dose of TCS-OX2-29 used in the study been sedating we should have observed a similar reduction in responding for sucrose; however we saw no effect. Similarly, no impact on reinstatement was observed. Moreover, central and intra-NAc administration of TCS-OX2-29 had no impact on spontaneous locomotor activity. This is consistent with literature which similarly found an effect of OX2R antagonism on responding for ethanol and ethanol-induced CPP that was seemingly subthreshold to sedation (Shoblock et al., 2011). A previous study (Smith et al., 2009) has shown that systemic administration of the OX2R antagonist 4-PT reduces locomotor activity in rats tested in a manner similar to our study. Although it is difficult to compare systemic vs. i.c.v. studies, our data clearly demonstrate a lack of sedation and/or motor impairment with the doses of TCS-OX2-29 used.

Although the specific mechanism(s) by which OX2R antagonism decreased responding for ethanol requires delineation, there was no impact on responding for the natural reward, sucrose. These results are consistent with previous findings where OX2R antagonism had no impact on responding for the non-caloric natural reinforcer saccharin (Shoblock et al., 2011). Although we did observe non-specific effects on responding for water, it appears that at lower doses this does not occur suggesting that caution should be exercised with regard to antagonists at high doses which may diffuse from the NAc to other regions. It is difficult to determine their precise implications because in our paradigm the water reward is of minimal value and is often left unconsumed. On balance, our results further strengthen the hypothesis that OX2R has a substantially greater role in mediating the reinforcing effects of ethanol than non-drug rewarding substances in general. This parallels some of the literature regarding OX2R and responding for natural rewards (Jupp et al., 2011b; Cason and Aston-Jones, 2012). Nevertheless, a recent study showed a role for OX2R, but not OX2R, in binge eating of highly palatable food (Piccoli et al., 2012). This latter result concurs with our collective findings, whereby OX2R antagonism can reduce self-administration of both ethanol and sucrose, but with a greater effect on ethanol (Jupp et al., 2011b), while under the conditions of the present study we could demonstrate a reduction in ethanol, but not sucrose, responding by OX2R antagonism (at doses of TCS-OX2-29 devoid of non-specific effects).

A further important point of distinction between the orexin receptor subtypes OX2R and OX1R determined by the current study is their apparent differential role in ethanol-seeking behaviour. We, and others, have accumulated a body of evidence which suggests that OX2R is implicated in drug-seeking behaviour elicited by discrete and contextual cues, as well as stress (Boutrel et al., 2005; Lawrence et al., 2006; Richards et al., 2008; Smith et al., 2009, 2010), but not a priming injection of drug (Mahler et al., 2012a). Indeed, orexin neurons are activated by contextual cues previously associated with self-administration of ethanol (Dayas et al., 2008) and morphine-induced CPP (Harris et al., 2005). These findings indicate that orexin acting via OX2R may be involved in the learning of drug-cue associations and play a specific role in drug-seeking elicited by external stimuli. Moreover, the involvement of OX2R in ethanol-related cue associations appears to be long-lasting, persisting after protracted withdrawal (Jupp et al., 2011a). However, this does not seem to be the case for OX2R as antagonism of these receptors has no impact on cue-induced cocaine-seeking (Smith et al., 2009). The lack of an observed effect on ethanol-seeking in the present study is consistent with this, but should be interpreted with caution. It is possible that targeted microinjections into discrete brain nuclei may reduce cue-induced ethanol-seeking, which would require further study. Therefore, we cannot completely rule out a role for OX2R in cue-induced ethanol-seeking. However, given that we observed robust effects in the
self-administration experiments with a similar sample population, our data suggest overall that if there is an effect of OX2R antagonism on cue-conditioned ethanol-seeking then it is less powerful than its role in ethanol consumption. Therefore, under the conditions of our study, we find no evidence to support a prominent role for OX2R in cue-induced ethanol-seeking after extinction. Importantly, even using high doses of TCS-OX2-29 that showed non-specific reductions in responding during self-administration, we still saw no impact upon cue-driven ethanol-seeking. Clearly, it is possible that OX2R may be implicated in other forms of reward-seeking, such as stress-induced reinstatement, which remains to be established. Thus it is possible that, unlike OX1R, the role for OX2R is more related to the mediation of primary reward with respect to drugs of abuse. Future studies will undoubtedly pursue this issue.

In conclusion we report a prominent role for OX2R in mediating ethanol self-administration but not in cue-driven ethanol-seeking behaviour, as tested under our paradigm and even with high doses of TCS-OX2-29. This is in contrast to OX2R where a clear role in both behaviours has been established. The effect observed on self-administration was independent of sedation and could not be generalized to non-drug rewards such as sucrose, thus supporting the case for development of OX2R as a potential therapeutic target to reduce ethanol consumption. In terms of drug development it may be that drugs which target both orexin receptor subtypes may prove to be efficacious in treating ethanol-use disorders (see Srinivasan et al., 2012).

Acknowledgements

The intellectual input and technical assistance of Dr Jee Hyun Kim and Dr Andrezza Kim is acknowledged. The technical assistance of Ms Hanna Kastman is also acknowledged. These studies were supported by the National Health and Medical Research Council of Australia (project grant 1021227) of which A.J.L. is a Principal Research Fellow and R.M.B. is an Early Career Research Fellow. Financial support from the Besen Family Foundation and the Victorian Government’s Operational Infrastructure Support Program are acknowledged.

Statement of Interest

None.

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Title:
Central orexin (hypocretin) 2 receptor antagonism reduces ethanol self-administration, but not cue-conditioned ethanol-seeking, in ethanol-preferring rats

Date:
2013-10-27

Publication Status:
Accepted manuscript

Persistent Link:
http://hdl.handle.net/11343/41841