

BEHAVIORAL NEUROSCIENCE

Orexin/hypocretin signaling at the orexin 1 receptor regulates cue-elicited cocaine-seeking

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Abstract

The orexin/hypocretin system has recently been implicated in reward-processing and addiction. We examined the involvement of the orexin system in cue-induced reinstatement of extinguished cocaine-seeking by administering the orexin 1 receptor antagonist SB-334867 (SB) or the orexin 2 receptor antagonist 4-pyridylmethyl (*S*)-*tert*-leucyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (4PT) prior to reinstatement testing. Male Sprague Dawley rats self-administered cocaine in 2-h sessions for 10 days, followed by extinction training. Reinstatement of cocaine-seeking was elicited by presentation of tone + light cues previously paired with cocaine infusions. SB (10, 20 and 30 mg/kg) dose-dependently decreased cue-induced reinstatement of cocaine-seeking without significantly affecting responding during late extinction. 4PT (10 and 30 mg/kg) did not significantly alter cue-induced reinstatement. In separate experiments, the highest doses of SB and 4PT had no significant effect on established cocaine self-administration, and 4PT reduced spontaneous activity in a locomotor test to a greater extent than SB. Finally, SB (30 mg/kg) had no effect on the acquisition of cocaine-paired cues during a Pavlovian cocaine-stimulus conditioning session in the operant chamber. Pretreatment with SB prior to the Pavlovian acquisition session had no effect on subsequent cue-induced reinstatement of cocaine-seeking elicited by those cues. However, pretreatment with SB prior to a second reinstatement session in the same animals significantly attenuated the expression of cue-induced reinstatement. These results show that orexin transmission at the orexin 1 receptor, but not the orexin 2 receptor, is necessary for the reinstatement of cocaine-seeking elicited by drug-paired cues and that orexin signaling is not critical for cocaine reinforcement or cocaine-stimulus conditioning.

Introduction

Relapse prevention is a difficult aspect of addiction treatment and pharmacotherapies for reducing cocaine relapse are very limited (O'Brien, 2005; Vocci *et al.*, 2005; Karila *et al.*, 2008). Drug-associated cues are powerful triggers for drug desire and relapse in cocaine addicts (Wallace, 1989; Childress *et al.*, 1992; Sinha *et al.*, 2003), and for reinstatement of an extinguished drug-seeking response in rodent models (Davis & Smith, 1976; Meil & See, 1996; for review see Shaham *et al.*, 2003). Recently, we and others have examined whether the orexin system is involved in relapse to drug-seeking.

The orexins (or hypocretins) are two recently discovered hypothalamic neuropeptides that act at two G protein-coupled receptors, orexin 1 receptor (OX₁R) and orexin 2 receptor (OX₂R) (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). Orexins have been extensively implicated in the maintenance of arousal states and narcolepsy with cataplexy (for review see Siegel, 2004; Nishino & Kanbayashi, 2005). Recent studies have shown that orexins are also involved in reward-seeking and neuroplasticity associated with drugs of abuse (Harris *et al.*, 2005, 2007; Borgland *et al.*, 2006; Narita *et al.*, 2006). The

OX₁R antagonist SB-334867 (SB) reduced stress-induced reinstatement of cocaine-seeking as well as yohimbine- and cue-induced reinstatement of ethanol-seeking (Boutrel *et al.*, 2005; Lawrence *et al.*, 2006; Richards *et al.*, 2008). However, the underlying neural circuitries are specific for the reinstatement modality and the drug being studied (Kalivas & McFarland, 2003; Shaham *et al.*, 2003; Rogers *et al.*, 2008), so these studies do not predict the potential involvement of orexin in cue-induced cocaine-seeking.

No studies to date have investigated the effects of OX₂R antagonists on drug-seeking behavior. OX₁R and OX₂R differ in several ways. First, OX₁R has 10-fold selectivity for orexin A, whereas OX₂R is non-selective for orexin A and B (Sakurai *et al.*, 1998). Second, OX₁R is coupled exclusively to a G_q subclass of G proteins, whereas OX₂R is coupled to both G_{i/o} and G_q proteins (Zhu *et al.*, 2003). Third, the distributions of OX₁R and OX₂R differ substantially in most brain regions (Trivedi *et al.*, 1998; Kilduff & de Lecea, 2001; Marcus *et al.*, 2001). Finally, an OX₂R mutation is associated with canine narcolepsy (Lin *et al.*, 1999), linking this receptor with arousal and sleep functions. Thus, it is important to determine the relative contribution of orexin transmission at OX₁ and OX₂ receptors in addiction-related behaviors to find appropriate pharmacological targets.

We hypothesized that orexin signaling at OX₁R is involved in cue-induced reinstatement of cocaine-seeking. We tested the effects of the

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OX₁R antagonist SB (Porter *et al.*, 2001) and the OX₂R antagonist 4-pyridylmethyl (*S*)-*tert*-leucyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (4PT) (Hirose *et al.*, 2003) on cue-induced reinstatement of cocaine-seeking following cocaine self-administration. We also determined whether orexin is necessary for the acquisition of cocaine-paired cues during self-administration, as it is involved in the acquisition of morphine-paired cues in a place preference paradigm (Narita *et al.*, 2006; Harris *et al.*, 2007). To further differentiate the effects of the two antagonists and to determine the specificity of behavioral effects, we tested antagonist effects on cocaine self-administration and spontaneous locomotion.

Materials and methods

Animals

Male Sprague Dawley rats (initial weight 250–300 g; Charles River, Wilmington, MA, USA) were single- or pair-housed under a reversed 12-h light/dark cycle (lights off at 06:00 h) and had *ad libitum* access to food and water. Animals were housed in a temperature- and humidity-controlled animal facility at the Medical University of South Carolina (AAALAC-accredited). All experiments were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and conducted according to specifications of the National Institutes of Health as outlined in their Guide for the Care and Use of Laboratory Animals.

Intravenous catheter surgery

Following acclimation to the animal facility, rats were implanted with chronic indwelling intravenous catheters while under ketamine/xylazine (56.5/8.7 mg/kg) anesthesia (+ equithesin in some cases: 66 mg/kg ketamine + 1.33 mg/kg xylazine + 0.5 ml/kg equithesin). A non-steroidal anti-inflammatory was administered as an analgesic prior to surgery. The catheters were constructed of silastic tubing (Dow Corning, Midland, MI, USA) connected to a modified guide cannula (C313G-5UP-SPC12, Plastics One, Roanoke, VA, USA), which was mounted on ProLite polypropylene monofilament mesh (Atrium, Hudson, NH, USA) using Ortho-Jet acrylic (Lang Dental, Wheeling, IL, USA); a small silicone bubble was placed 3.8 cm from the end of the silastic tubing. Briefly, the free end of silastic tubing was inserted into (and secured to) the right jugular vein, whereas the other end passed subcutaneously over the shoulder to the cannula, which was mounted on the back and exited via a biopsy hole. Beginning at 3 days after surgery, catheters were flushed once daily with 0.1 mL each of the antibiotic cefazolin (100 mg/mL) and heparin (100 U/mL). For each self-administration session, catheters were flushed with 0.1 mL saline to ensure patency prior to attachment to the cocaine infusion line (PE-50 tubing) and spring tether in the self-administration chamber, and flushed with 0.1 mL each of cefazolin and heparin following the session. Self-administration sessions began after 1 week of recovery from surgery.

Cocaine self-administration and extinction

Self-administration sessions were carried out in operant chambers housed in sound-attenuating cubicles and controlled via a MED-PC IV program (Med-Associates, St Albans, VT, USA). Rats learned to lever-press for intravenous cocaine (fixed ratio-1; 0.2 mg/50 μ L infusion via motorized pump; 20-s time-out after each infusion) in 2-h daily sessions. Presses on an inactive lever had no programmed

consequences. Except as noted, rats were given 10 self-administration sessions in which they earned ≥ 10 infusions. Rats then underwent daily extinction sessions, during which presses on either lever had no consequences (no drug or cues), until they met the criteria of two consecutive sessions with < 25 active lever presses (minimum of seven sessions prior to the first reinstatement test; minimum of two sessions between subsequent reinstatement tests).

Experiment 1: cue-induced reinstatement

During self-administration sessions, cocaine infusions were paired with discrete tone + light cues (78 dB, 2900 Hz; white stimulus light above the active lever). The red house light (on the wall opposite the levers) was turned off during cocaine infusions and time-outs. Rats then underwent extinction training prior to reinstatement testing. During cue-induced reinstatement of cocaine-seeking, active lever presses once again resulted in delivery of tone + light cues but no drug infusions.

To test the effects of the OX₁R antagonist SB (10, 20 or 30 mg/kg, i.p.) or the OX₂R antagonist 4PT (10 or 30 mg/kg, i.p.) on cue-induced reinstatement of cocaine-seeking, animals were given up to four test sessions: two cue-induced reinstatement sessions with separate vehicle and antagonist pretreatment and two late extinction sessions (no cues) with separate vehicle and antagonist pretreatment. In other words, all animals received both vehicle and antagonist pretreatment prior to separate sessions (two extinction and two reinstatement) in a within-subjects design. The order of these sessions was counterbalanced within groups so that the four test sessions were presented in different orders to different animals. An animal received the same dose of antagonist for all tests (i.e. the same dose of antagonist was given prior to a cue-induced reinstatement session and extinction session, and vehicle was given prior to a cue-induced reinstatement session and extinction session).

Animals in SB experiments were given 10 days of cocaine self-administration followed by extinction, as described above. For cue-induced reinstatement testing, animals pretreated (30 min) with either 20 mg/kg ($n = 9$) or 30 mg/kg ($n = 8$) SB received all four test sessions described above. Animals pretreated with 10 mg/kg SB ($n = 8$) received only the two cue-induced reinstatement sessions (with separate vehicle and SB pretreatment) because preliminary studies indicated this dose to be ineffective, eliminating the need for control sessions during late extinction.

Animals in 4PT experiments were given 12 days of cocaine self-administration (due to 4PT pretreatment on the 10th day of self-administration for Experiment 3) followed by extinction. For cue-induced reinstatement testing, animals pretreated (15 min) with either 10 mg/kg ($n = 9$) or 30 mg/kg ($n = 13$) 4PT received all four test sessions.

Animals were excluded from all analyses (vehicle and antagonist sessions) if they did not receive some or all of the reinstatement tests due to insufficient extinction or if they made < 20 presses during the cue-induced reinstatement session with vehicle pretreatment (i.e. no reinstatement in response to cues). One animal in the 4PT group was excluded from all analyses due to high responding on the active lever during reinstatement (more than 2.5 SDs different from group mean).

Experiment 2: locomotion

All animals used to test the effects of SB or 4PT on locomotor activity had previously been part of a cocaine self-administration experiment. At least 5 days after the last self-administration (or extinction/reinstatement) session, animals were tested for spontaneous locomotion in

clear acrylic chambers (approximately 40 × 40 × 30 cm) equipped with Digiscan monitors (AccuScan Instruments, Inc., formerly Omnitech Electronics, Columbus, OH, USA) containing a 16 × 16 photobeam array for the *x/y* axes (horizontal activity) and 16 photobeams for the *z* axis (vertical activity). Photobeam breaks were detected by a Digiscan analyser and recorded by DIGIPRO software (Version 1.4). Animals were pretreated with antagonist (30 min for SB, 15 min for 4PT) prior to a 60-min test session and data were collected in 5-min bins. Animals were tested twice for SB (0 and 30 mg/kg; *n* = 12) or 4PT (0 and 30 mg/kg; *n* = 12) in a counterbalanced fashion, with tests at least 2 days apart.

Experiment 3: established self-administration

During self-administration sessions, cocaine infusions were paired with discrete tone + light cues (78 dB, 2900 Hz; white stimulus light above the active lever). The red house light (on the wall opposite the levers) was turned off during cocaine infusions and time-outs.

To test the effects of SB (30 mg/kg, i.p.; *n* = 12) or 4PT (30 mg/kg, i.p.; *n* = 10) on established cocaine self-administration, animals were pretreated with either SB or 4PT (30 min for SB, 15 min for 4PT) prior to the 10th self-administration session (animals had received nine previous self-administration sessions in which they earned ≥ 10 infusions). Animals were subsequently given two additional self-administration sessions.

One animal in the 4PT group was excluded from all analyses due to extremely high responding on the active lever during self-administration (more than 3 SDs different from group mean).

Experiment 4: acquisition and expression of Pavlovian-conditioned cues

To test the role of orexin in the acquisition of cocaine-paired cues during self-administration, we utilized a Pavlovian-conditioned cue paradigm (See, 2005). This paradigm affords the opportunity to study the acquisition of cocaine-cue associations in a single conditioning session. Briefly, animals are trained to self-administer cocaine in the absence of programmed stimuli and are then given a single Pavlovian conditioning session during which they receive passive pairings of cocaine infusions with discrete stimulus cues (tone + light). Animals are then returned to daily cocaine self-administration in the absence of stimuli. Following extinction of responding in the absence of cocaine and cues, animals are given a reinstatement session where cues alone are presented in response to active lever presses, as described above.

More specifically, after 5 days of self-administration in the absence of cues (no discrete tone or light cues), animals were exposed to a single 2-h Pavlovian conditioning session in the operant chamber, in which no levers were extended and the animals received passive infusions of cocaine paired with discrete tone + light cues. The time between infusions was fixed. The number of infusions was individually based on the average number of infusions during the two prior self-administration sessions for that animal. The animals then received five more days of self-administration in the absence of cues and extinction training. During reinstatement of cocaine-seeking elicited by the cocaine-associated cues, active lever presses resulted in delivery of tone + light cues, with no drug infusions.

To test the effects of SB (30 mg/kg, i.p.) on the acquisition of cocaine-associated cues, animals were pretreated (30 min) with either SB (*n* = 16) or vehicle (*n* = 11) prior to the Pavlovian conditioning session and then tested drug-free during a subsequent cue-induced reinstatement. To test the effects of SB on the expression of

reinstatement induced by Pavlovian-conditioned cues, animals were pretreated (30 min) with either SB (*n* = 15) or vehicle (*n* = 12) prior to a second cue-induced reinstatement session (only a 1-h session so that animals could be used for Fos analysis, not included here). Animals were counterbalanced within this second reinstatement session so that some animals received the same pretreatment for both the acquisition and expression sessions, and some animals received different pretreatment for the two sessions.

Drugs

Cocaine HCl (NIDA, Research Triangle Park, NC, USA) was dissolved in 0.9% saline. SB [1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl urea hydrochloride; purchased from Tocris, Ellisville, MO, USA or generously donated by Eli Lilly, Indianapolis, IN, USA] was suspended in 2% dimethylsulfoxide and 10% 2-hydroxypropyl-β-cyclodextrin (Sigma) in sterile water; 0, 10, 20 or 30 mg/kg was given in a volume of 4 mL/kg (i.p.) 30 min prior to testing. 4PT (generously donated by Eli Lilly) was suspended in either: (i) 5% Solutol HS 15 (BASF, Ludwigshafen, Germany) and 10% 2-hydroxypropyl-β-cyclodextrin in sterile water or (ii) 2% dimethylsulfoxide and 10% 2-hydroxypropyl-β-cyclodextrin in sterile water; 0, 10 or 30 mg/kg was given in a volume of 4 mL/kg (i.p.) 15 min prior to testing. SB has 50-fold selectivity for OX₁R over OX₂R and 100-fold selectivity over approximately 50 other molecular targets (Porter *et al.*, 2001; Smart *et al.*, 2001). 4PT has 250-fold selectivity for OX₂R over OX₁R, as well as over 50 other receptors and targets (Hirose *et al.*, 2003).

Data analyses

One-way or mixed-model ANOVAs were utilized for most analyses, with test session or time as a repeated measure when appropriate. Posthoc analyses were computed with the Tukey-Kramer test. To evaluate the effects of SB or 4PT on cue-induced reinstatement, the four test sessions for the groups given 20 or 30 mg/kg SB, or 10 or 30 mg/kg 4PT (vehicle and antagonist pretreatment prior to two separate cue-induced reinstatement sessions, and vehicle and antagonist pretreatment prior to two separate extinction sessions), were analysed with one-way repeated-measures ANOVAs within each group. The two tests for the group given 10 mg/kg SB (vehicle and SB pretreatment prior to two separate cue-induced reinstatement sessions) were analysed with a paired *t*-test. To evaluate the effects of SB or 4PT on locomotion, the 60-min test sessions were divided into 5-min bins and analysed with mixed-model ANOVAs (two-way ANOVA with session time as a repeated measure); when overall ANOVAs were significant, individual paired *t*-tests were used to evaluate treatment differences within each 5-min bin. To evaluate the effects of SB or 4PT on self-administration, the antagonist-pretreated self-administration session was compared with the preceding and following sessions using one-way repeated-measures ANOVAs. For Pavlovian acquisition and expression, *t*-tests were used to evaluate differences between vehicle and SB pretreatment for reinstatement. Data are presented as means ± SEM throughout.

Results

Experiment 1: effects of SB-334867 or 4PT on cue-induced reinstatement of cocaine-seeking

Figure 1 shows the mean numbers of lever presses during cue-induced reinstatement when animals were pretreated with SB (Fig. 1a and b) or

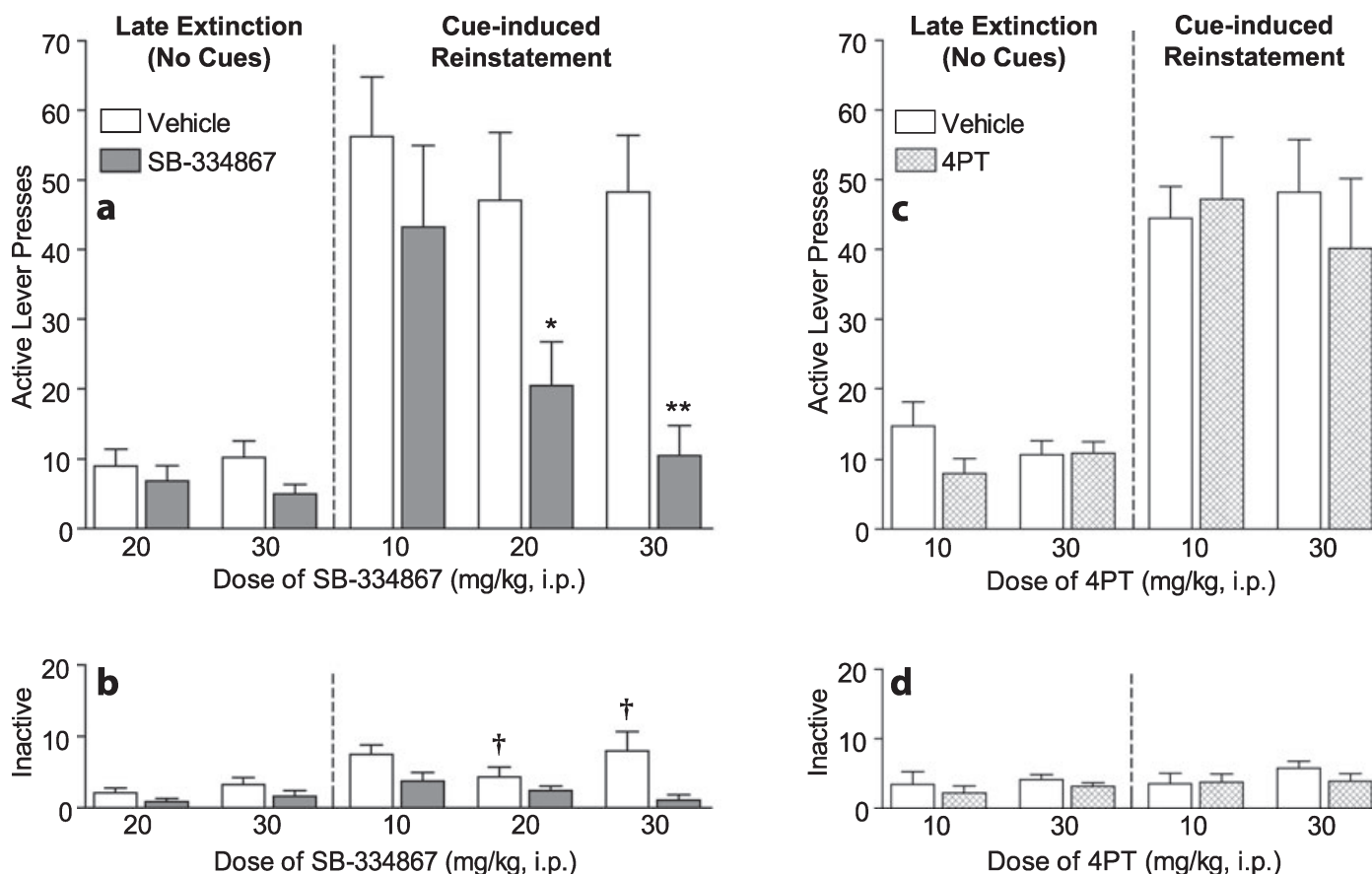


FIG. 1. Attenuation of cue-induced reinstatement of cocaine-seeking by the OX₁R antagonist SB-334867 (SB) but not the OX₂R antagonist 4PT. In a within-subjects design, rats were pretreated with a single dose of antagonist (10, 20 or 30 mg/kg, i.p.) or vehicle prior to late extinction sessions (no cues or cocaine) and cue-induced reinstatement sessions (tone + light cues) in a counterbalanced fashion. Mean (+ SEM) numbers of presses on the active and inactive levers in 2-h sessions in the self-administration chamber are shown. (a) Active lever responding during cue-induced reinstatement sessions was significantly attenuated by SB at 20 mg/kg ($n = 9$) and 30 mg/kg ($n = 8$) but not 10 mg/kg ($n = 8$), with no significant effect on late extinction session responding ($*P < 0.01$, $**P < 0.001$). (b) Inactive lever responding was significantly reduced during late extinction and cue-induced reinstatement sessions by 20 and 30 mg/kg SB, as compared with cue-induced reinstatement with vehicle pretreatment ($†P < 0.05$). (c) Active lever responding during late extinction and cue-induced reinstatement sessions was not significantly affected by 4PT at 10 mg/kg ($n = 9$) or 30 mg/kg ($n = 13$). (d) Inactive lever responding was also not significantly affected by 4PT.

4PT (Fig. 1c and d). Active lever (Fig. 1a and c) and inactive lever (Fig. 1b and d) presses are shown for late extinction sessions and cue-induced reinstatement sessions following vehicle or antagonist pretreatment.

Orexin 1 receptor antagonist: SB-334867

SB-334867 effects on active lever responding (Figs 1a and 2). For the 30 mg/kg SB group ($n = 8$), there was a significant effect of test session ($F_{3,4} = 16.90$; $P < 0.0001$) and posthoc analyses revealed that active lever responding during the cue-induced reinstatement session with vehicle pretreatment was significantly different from the other three sessions (i.e. cue-induced reinstatement with SB pretreatment, and extinction sessions with either vehicle or SB pretreatment, $P < 0.001$). For the 20 mg/kg group ($n = 9$), there was a significant effect of test session ($F_{3,5} = 14.57$; $P < 0.001$), and posthoc analyses revealed that active lever responding during the cue-induced reinstatement session with vehicle pretreatment was significantly different from the other three sessions (i.e. cue-induced reinstatement with SB pretreatment, $P < 0.01$; extinction sessions with either vehicle or SB pretreatment, $P < 0.001$). For the 10 mg/kg SB group ($n = 8$), active lever responding during cue-induced reinstatement sessions with either vehicle or SB pretreatment was not significantly different ($T_7 = 1.11$; $P = 0.30$). When the SB groups were compared across

doses (between-group analyses), there were no significant differences among SB groups for cue-induced reinstatement sessions with vehicle pretreatment but there was a significant difference among SB groups for cue-induced reinstatement sessions with SB pretreatment ($F_{2,22} = 4.32$; $P < 0.05$). Posthoc analyses revealed a significant difference between the 10 and 30 mg/kg SB groups only ($P < 0.05$). These data show that 20 or 30 mg/kg, but not 10 mg/kg, SB attenuated active lever responding during cue-induced reinstatement to extinction levels.

Figure 2 shows the mean numbers of active lever presses for each 30-min bin during cue-induced reinstatement sessions in animals pretreated with 30 mg/kg SB and vehicle ($n = 8$). When SB-pretreated reinstatement was compared with vehicle-pretreated reinstatement in the same animals, there was a significant effect of pretreatment ($F_{1,14} = 8.66$; $P = 0.01$) but no significant effect of time and no significant interaction.

SB-334867 effects on inactive lever responding (Fig. 1b). For the 30 mg/kg SB group, there was a significant effect of test session ($F_{3,4} = 4.65$; $P < 0.05$), and posthoc analyses revealed that inactive lever responding during cue-induced reinstatement with vehicle pretreatment was significantly different from cue-induced reinstatement and extinction with SB pretreatment ($P < 0.05$). For the

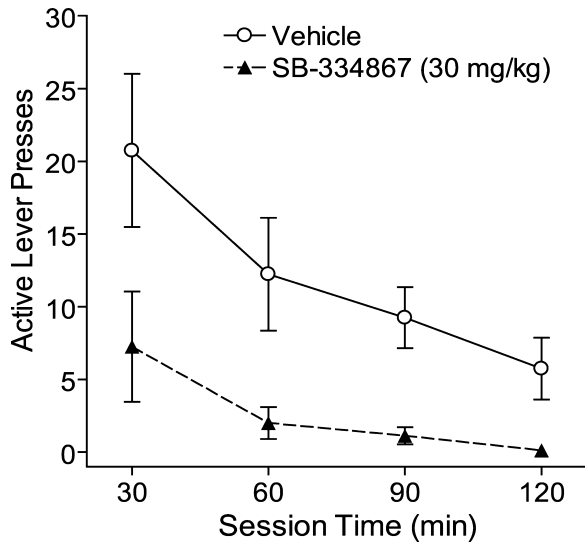


FIG. 2. Attenuation of cue-induced reinstatement of cocaine-seeking by the OX₁R antagonist SB-334867 (SB) across the 2-h session. Mean (\pm SEM) numbers of active lever presses for each 30-min time bin during cue-induced reinstatement sessions in animals pretreated with 30 mg/kg SB (i.p.) and vehicle ($n = 8$). Active lever responding across the 30-min time bins was significantly attenuated by SB ($P = 0.01$).

20 mg/kg SB group, there was a significant effect of test session ($F_{3,5} = 3.25$; $P < 0.05$), and posthoc analyses revealed that inactive lever responding during cue-induced reinstatement with vehicle pretreatment was significantly different from extinction with SB pretreatment ($P < 0.05$). For the 10 mg/kg SB group, inactive lever responding during the cue-induced reinstatement sessions with either vehicle or SB pretreatment was not significantly different ($T_7 = 1.90$; $P = 0.10$). When the SB groups were compared across doses, there were no significant differences among SB groups for cue-induced reinstatement sessions with either vehicle or SB pretreatment. These data indicate a minimal but significant effect of 20 or 30 mg/kg SB on inactive lever responding.

Baseline responding. Self-administration data were analysed to assess differences in responding among SB groups before SB administration. No significant differences were observed among groups for cocaine intake or active lever responding during the last two self-administration sessions, for active lever responding during the first 7 days of extinction or for active lever responding during cue-induced reinstatement following vehicle pretreatment. On the last two self-administration days, the across-group ($n = 25$ total) mean for cocaine infusions was 34.4 ± 2.1 and 32.6 ± 1.8 per session (18.8 ± 1.2 and 17.5 ± 0.9 mg/kg) and for active lever presses was 47.2 ± 4.4 and 42.0 ± 2.9 per session. During extinction, there was a significant main effect for extinction session ($F_{6,22} = 30.14$; $P < 0.001$), indicating that animals showed a significant decrease in active lever responding across extinction sessions. The across-group mean for active lever presses on days 1 and 7 of extinction was 79.0 ± 9.8 and 13.5 ± 1.8 . Active lever responding during cue-induced reinstatement following vehicle pretreatment as compared with the prior extinction session was not significantly different among SB groups but there was a significant main effect for cue-induced reinstatement ($F_{1,22} = 88.83$; $P < 0.001$), and posthoc analyses showed significant cue-induced reinstatement of active lever responding for all groups ($P < 0.001$). There were no significant differences for inactive lever responding among SB groups during cue-induced reinstatement in this analysis.

Orexin 2 receptor antagonist: 4PT

Lack of effect of 4PT on active lever responding (Fig. 1c). For the 30 mg/kg 4PT group ($n = 13$), there was a significant effect of test session ($F_{3,9} = 10.50$; $P < 0.0001$), and posthoc analyses revealed that active lever responding during the cue-induced reinstatement session with vehicle pretreatment was significantly different from the late extinction sessions (vehicle or 4PT pretreatment; $P < 0.001$). Similarly, responding during the cue-induced reinstatement session with 4PT pretreatment was significantly different from extinction sessions (vehicle or 4PT pretreatment; $P < 0.01$). For the 10 mg/kg 4PT group ($n = 9$), there was a significant effect of test session ($F_{3,5} = 16.22$; $P < 0.0001$), and posthoc analyses revealed that active lever responding during the cue-induced reinstatement session with vehicle pretreatment was significantly different from the late extinction sessions (vehicle or 4PT pretreatment; $P < 0.01$). Also, active lever responding during the cue-induced reinstatement session with 4PT pretreatment was significantly different from extinction sessions (vehicle or 4PT pretreatment; $P < 0.001$). Within the 10 and 30 mg/kg 4PT groups, cue-induced reinstatement sessions with vehicle or 4PT pretreatment were not significantly different from each other. When the 4PT groups were compared across doses (between-group analyses), there were no significant differences for cue-induced reinstatement sessions with vehicle pretreatment or sessions with 4PT pretreatment. These data show that 10 or 30 mg/kg 4PT had no significant effects on active lever responding during cue-induced reinstatement.

Lack of effect of 4PT on inactive lever responding (Fig. 1d). For 10 or 30 mg/kg 4PT groups, there were no significant effects of test session for inactive lever responding during cue-induced reinstatement or extinction sessions.

Baseline responding. Self-administration data were analysed to assess differences in baseline responding between 4PT groups before 4PT administration. No significant differences were observed between groups for cocaine intake or active lever responding during the last two self-administration sessions, for active lever responding during the first 7 days of extinction or for active lever responding during cue-induced reinstatement (following vehicle pretreatment). On the last 2 days of self-administration, the across-group ($n = 22$ total) mean for cocaine infusions was 44.3 ± 2.4 and 41.6 ± 2.1 per session (23.4 ± 1.3 and 21.8 ± 1.1 mg/kg), and for active lever presses was 66.7 ± 9.5 and 85.3 ± 26.0 per session. During extinction, there was a significant main effect of extinction session ($F_{6,20} = 17.64$; $P < 0.0001$), indicating that animals showed a significant decrease in active lever responding across extinction sessions. The across-group mean for active lever presses on days 1 and 7 of extinction was 58.0 ± 7.6 and 17.0 ± 2.5 . Active lever responding during cue-induced reinstatement (following vehicle pretreatment) as compared with the prior extinction session was not significantly different between 4PT groups but there was a significant main effect for cue-induced reinstatement ($F_{1,20} = 49.21$; $P < 0.0001$), and posthoc analyses showed significant cue-induced reinstatement of active lever responding for all groups ($P < 0.005$). No significant differences were observed for the inactive lever during cue-induced reinstatement in this analysis.

Experiment 2: effects of SB-334867 or 4PT on locomotion

Figure 3 shows the horizontal activity (number of photobeam breaks; Fig. 3a and c) and total distance traveled (cm; Fig. 3b and d) during a 60-min locomotion test following pretreatment with either SB (0 or 30 mg/kg; $n = 12$) or 4PT (0 or 30 mg/kg; $n = 12$). In all analyses,

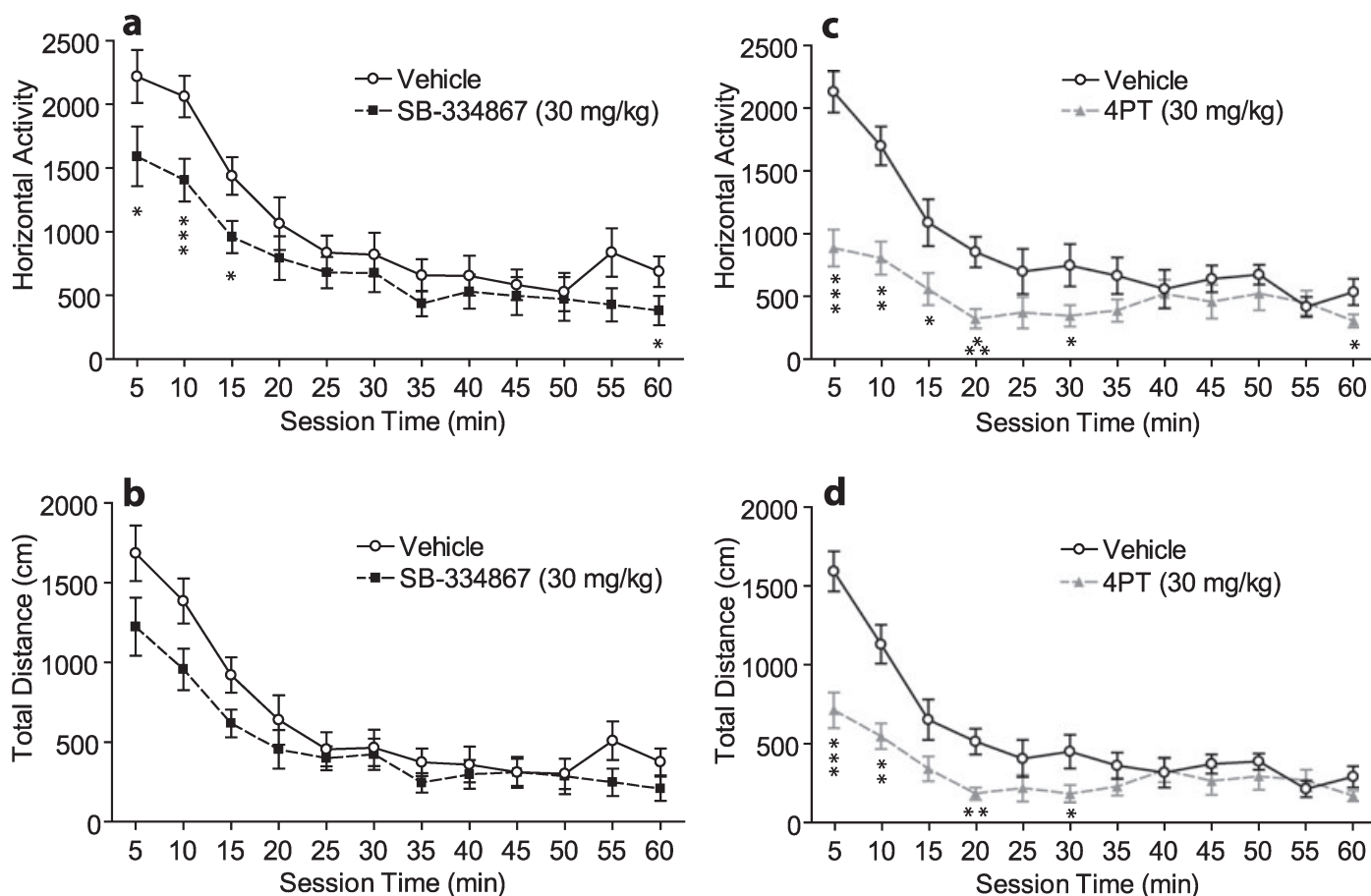


FIG. 3. Reduction in spontaneous locomotor activity by the OX_1R antagonist SB-334867 (SB) and OX_2R antagonist 4PT. In a within-subjects design, rats were pretreated with either SB, 4PT (30 mg/kg, i.p. each) or vehicle prior to two separate locomotor tests (60 min). Mean (\pm SEM) horizontal activity (number of photobeam breaks in the x/y axes) and total distance traveled (cm) are shown for each 5-min time bin. SB ($n = 12$) significantly affected overall horizontal activity ($P < 0.05$) (a) but not overall total distance (b). 4PT ($n = 12$) significantly affected overall horizontal activity ($P < 0.0005$) (c) and overall total distance ($P < 0.0005$) (d). Individual paired t -tests within each 5-min bin revealed specific time points for significant differences (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$).

there was a significant effect of session time during the 60-min test ($P < 0.0001$).

Orexin 1 receptor antagonist: SB-334867

There was a significant effect of SB treatment for horizontal activity ($F_{1,22} = 5.16$; $P < 0.05$) but not for total distance. There were no significant interactions between treatment and time for either locomotor measure. Individual paired t -tests for each 5-min bin of horizontal activity revealed that 30 mg/kg SB was significantly different from vehicle only during the first three time bins ($P < 0.05$, 0.0001 and 0.01, respectively) and the last time bin ($P < 0.05$).

Orexin 2 receptor antagonist: 4PT

There was a significant effect of 4PT treatment for horizontal activity ($F_{1,22} = 18.24$; $P < 0.0005$) and total distance ($F_{1,22} = 16.55$; $P = 0.0005$), and significant interactions between treatment and session time for horizontal activity ($F_{11,242} = 5.72$; $P < 0.0001$) and total distance ($F_{11,242} = 6.45$; $P < 0.0001$). Individual paired t -tests for each 5-min bin of horizontal activity revealed that 30 mg/kg 4PT was significantly different from vehicle during the first four time bins ($P < 0.0001$, 0.001, 0.05 and 0.0005, respectively), sixth bin ($P < 0.05$) and last bin ($P < 0.05$). Similar analyses of total distance revealed that 30 mg/kg 4PT was significantly different from vehicle

during the first two time bins ($P < 0.0001$ and 0.005), fourth bin ($P < 0.001$) and sixth bin ($P < 0.05$).

When SB and 4PT groups were compared, there were significant differences between the groups during antagonist-pretreated sessions for both horizontal activity ($F_{1,22} = 5.59$; $P < 0.05$) and total distance ($F_{1,22} = 6.72$; $P < 0.05$), and significant interactions between group and time for horizontal activity ($F_{11,242} = 2.80$; $P < 0.005$) and total distance ($F_{11,242} = 2.68$; $P < 0.005$). There were no significant differences between SB and 4PT groups during vehicle-pretreated sessions for either horizontal activity or total distance ($F_{1,22} < 1.3$; $P > 0.25$), and no significant interactions between group and time. These analyses indicate that 4PT caused significantly greater reductions in locomotor activity than SB.

Experiment 3: no effect of SB-334867 or 4PT on cocaine self-administration

Figure 4 shows the number of active lever presses (Fig. 4a and c) and cocaine infusions (Fig. 4b and d) for individual animals on sessions 8–12 of self-administration (means for each session are represented by horizontal line segments). Animals were pretreated with either SB (30 mg/kg; $n = 12$) or 4PT (30 mg/kg; $n = 10$) prior to session 10 of self-administration.

Orexin 1 receptor antagonist: SB-334867

Analyses comparing session 10 (with SB pretreatment) with sessions 9 and 11 (no SB pretreatment) revealed no significant differences in responding on the active (Fig. 3a; $F_{2,9} = 2.30$; $P > 0.1$) or inactive ($F_{2,9} = 2.20$; $P > 0.1$; not shown) lever across the self-administration sessions, as well as no significant differences for cocaine infusions (Fig. 3b; $F_{2,9} = 2.90$; $P > 0.05$) across the sessions.

Orexin 2 receptor antagonist: 4PT

Analyses comparing session 10 (with 4PT pretreatment) with sessions 9 and 11 (no 4PT pretreatment) revealed no significant differences in active lever responding (Fig. 3c; $F_{2,7} = 1.80$; $P > 0.1$) or cocaine infusions (Fig. 3d; $F_{2,7} = 3.06$; $P > 0.05$). There was a significant effect of session on inactive lever responding ($F_{2,7} = 3.97$;

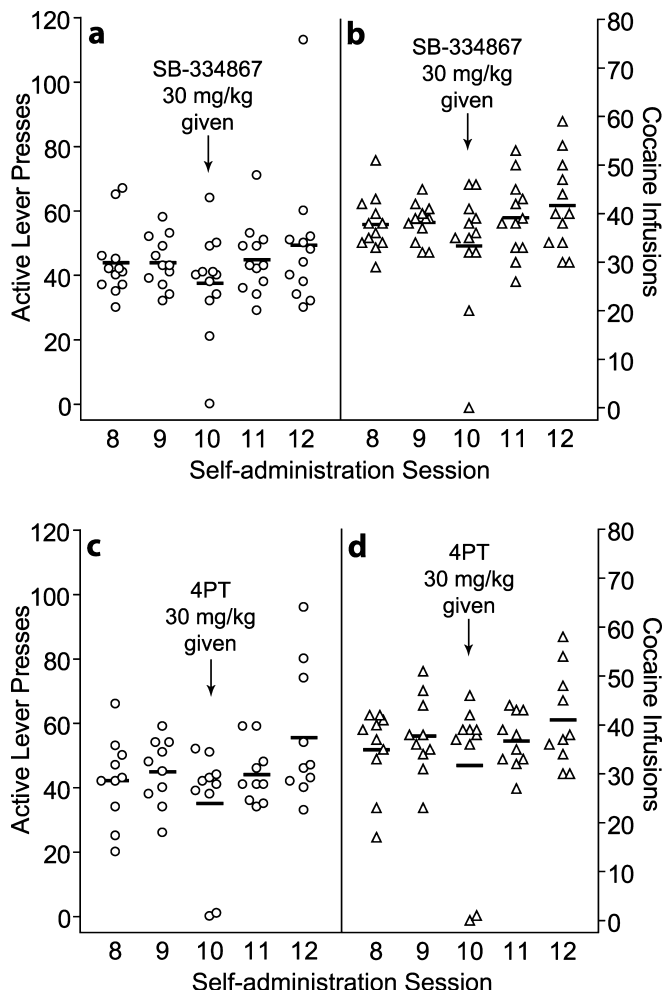


FIG. 4. No effects of the OX₁R antagonist SB-334867 (SB) or the OX₂R antagonist 4PT on cocaine self-administration. Rats were pretreated with either SB or 4PT (30 mg/kg, i.p.) prior to their 10th cocaine self-administration session (0.2 mg/infusion; fixed ratio-1; 20-s time-out; 2-h sessions). Number of active lever presses (left y-axis) and cocaine infusions (right y-axis) for individual animals across self-administration sessions 8–12 are shown. Means for each session are represented by horizontal line segments. Analyses of sessions 9–11 revealed that SB ($n = 12$) caused no significant effect on active lever responding (a) or number of cocaine infusions (b) across the sessions. 4PT ($n = 10$) also caused no significant effect on active lever responding (c) or number of cocaine infusions (d) across the sessions.

$P < 0.05$; not shown); posthoc analyses revealed a significant difference between sessions 10 and 11 ($P < 0.05$).

Experiment 4: effects of SB-334867 on acquisition and expression of Pavlovian-conditioned cues

Figure 5 shows the mean numbers of active lever presses during cue-induced reinstatement when animals were pretreated with SB or vehicle prior to the acquisition of the Pavlovian-conditioned cues used for reinstatement (Fig. 5a) or the expression of reinstatement elicited by those cues (Fig. 5b).

Pretreatment with 30 mg/kg SB ($n = 16$) prior to the acquisition of Pavlovian-conditioned cues had no significant effect on active lever responding during the expression of cue-induced reinstatement, as compared with pretreatment with vehicle in separate animals ($n = 11$) (unpaired *t*-test; $T_{1,25} = 0.86$; $P = 0.40$), indicating that SB did not affect the acquisition of cocaine-stimulus conditioning. In contrast, pretreatment with 30 mg/kg SB ($n = 15$) prior to the expression of cue-induced reinstatement significantly reduced active lever responding during that reinstatement session, as compared with pretreatment with vehicle in separate animals ($n = 12$) ($T_{1,25} = 3.28$; $P < 0.005$).

Pretreatment with 30 mg/kg SB had no significant effects on inactive lever responding during cue-induced reinstatement sessions.

Self-administration data were analysed to assess differences in baseline responding between test groups (e.g. vehicle vs. SB groups for acquisition, vehicle vs. SB groups for expression). No significant differences were observed between groups for the number of cocaine infusions received during the Pavlovian conditioning sessions, for cocaine intake or active lever responding during the last two self-administration sessions, or for active lever responding during the first 7 days of extinction. The across-group ($n = 27$ total) mean for the number of infusions during the Pavlovian session was 27 ± 1.8 . On the last two self-administration days, the across-group mean for cocaine infusions was 32.8 ± 2.4 and 34.6 ± 3.3 per session, and for active lever presses was 46.1 ± 5.4 and 51.4 ± 10.3 per session. During extinction, there was a significant main effect for extinction session ($F_{6,23} = 12.94$; $P < 0.001$), and the across-group mean for active lever presses on days 1 and 7 of extinction was 54.8 ± 5.0 and 16.7 ± 4.7 .

Discussion

The present studies indicate that orexin signaling at OX₁R, but not OX₂R, is critical for cue-induced reinstatement of extinguished cocaine-seeking in the self-administration paradigm. The OX₁R antagonist SB dose-dependently attenuated cue-induced reinstatement of cocaine-seeking, whereas the OX₂R antagonist 4PT did not attenuate reinstatement of extinguished responding (Fig. 1). This indicates an important functional difference between orexin signaling at OX₁R and OX₂R. Locomotor studies showed that 4PT caused more dramatic reductions in locomotor activity than SB (Fig. 3), which confirms that 4PT was administered at a behaviorally effective dose, and suggests that reduced locomotor activity does not account for the observed attenuation of cue-induced reinstatement following SB administration. Neither antagonist significantly attenuated established cocaine-taking behavior during self-administration (Fig. 4), indicating that orexin signaling is not critical for cocaine reinforcement. This was further supported by the finding that SB had no effect on the acquisition of cocaine-stimulus conditioning in the Pavlovian condi-

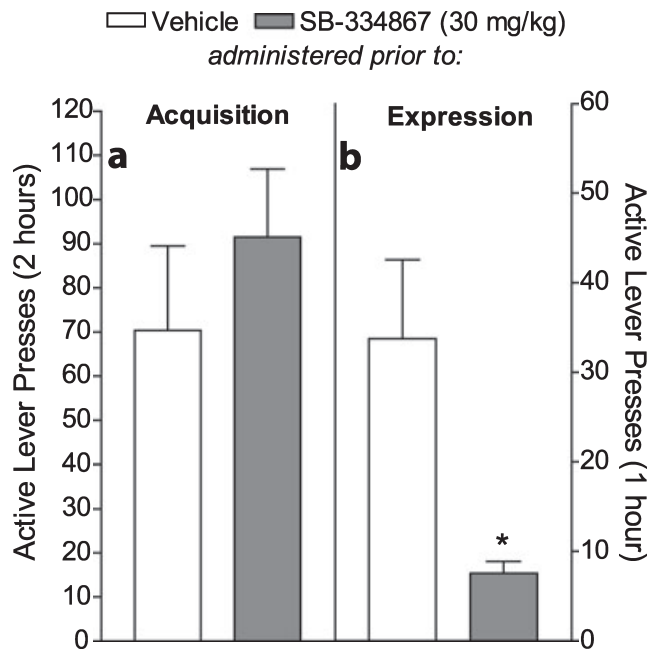


Fig. 5. Attenuation of the expression, but not acquisition, of Pavlovian-conditioned cocaine-associated cues by the OX_1R antagonist SB-334867 (SB). After 5 days of cocaine self-administration in the absence of cues, rats were exposed to a single 2-h Pavlovian conditioning session in the operant chamber, in which passive cocaine infusions were paired with discrete tone + light cues. Animals then received 5 more days of self-administration in the absence of cues and then extinction training. Animals were pretreated with either SB (30 mg/kg, i.p.) or vehicle prior to the Pavlovian conditioning session and then tested for reinstatement elicited by the Pavlovian cues. Rats were then pretreated with SB or vehicle prior to a second reinstatement session. Mean (+ SEM) number of presses on the active lever in the self-administration chamber is shown. (a) Active lever responding during a 2-h cue-induced reinstatement session was not significantly different between animals pretreated with SB ($n = 16$) or vehicle ($n = 11$) prior to the Pavlovian conditioning session (acquisition of cues). (b) Active lever responding during a 1-h cue-induced reinstatement session was significantly different between animals pretreated with SB ($n = 15$) or vehicle ($n = 12$) prior to the reinstatement session (expression of cues) (* $P < 0.005$).

tioned-cue paradigm (Fig. 5), which indicates that signaling at OX_1R is not necessary for cocaine reinforcement or learning cocaine-stimulus associations.

These results are consistent with previous findings that the orexin system is involved in stimulus-induced responding for other drugs. SB blocked olfactory discriminative cue-induced reinstatement of ethanol-seeking in a self-administration paradigm and reduced expression of preference for a morphine-paired environment in a conditioned place-preference paradigm (Harris *et al.*, 2005; Lawrence *et al.*, 2006). The current data also correspond with findings that orexin neurons are activated by stimuli associated with rewards such as morphine, cocaine, ethanol and food (Harris *et al.*, 2005; Harris & Aston-Jones, 2006; Dayas *et al.*, 2008). However, Hamlin *et al.* (2008) found that only non-orexin lateral hypothalamic neurons were Fos activated by re-exposure to a self-administration context during renewal-associated reinstatement of cocaine-seeking. This is in contrast to the present data showing involvement of orexin signaling at OX_1R in cue-induced reinstatement of cocaine-seeking, and may indicate that orexin is differently involved in cocaine-seeking elicited by discrete cues vs. context or that Fos may not accurately reflect the role of orexin in stimulus-induced cocaine-seeking.

Taken together with the results of Boutrel *et al.* (2005), these findings indicate that orexin signaling at OX_1R is necessary for

cocaine-seeking elicited by either previously drug-paired cues or stress. However, the current studies also show that orexin transmission at OX_1R is not necessary for established cocaine self-administration on a fixed ratio schedule, whereas previous studies have shown a role for orexin in self-administration of ethanol, nicotine and high-fat food but not self-administration of sucrose (Lawrence *et al.*, 2006; Hollander *et al.*, 2008; Nair *et al.*, 2008; Richards *et al.*, 2008). Similarly, other studies have shown that orexin is necessary for the acquisition of morphine place preference (Narita *et al.*, 2006; Harris *et al.*, 2007), whereas the current findings show that SB does not affect the acquisition of Pavlovian-conditioned stimuli associated with cocaine. These findings may reflect important differences in the involvement of orexin in the reinforcing properties of different types of rewards (e.g. food, cocaine, opiates and ethanol) (Aston-Jones *et al.*, 2009).

Orexin function in arousal

In the current studies, there were no overt signs of motor impairment or other obvious behavioral side effects following 30 mg/kg SB injections. Animals generally appeared active and non-sedated. However, motor impairments and slight sedation were apparent in several animals following injections of 30 mg/kg 4PT. 30 mg/kg SB significantly reduced horizontal activity during a spontaneous locomotor activity test; however, 4PT caused significantly greater reductions in measures of horizontal activity as well as total distance traveled, which suggests that the effect of SB on cue-induced reinstatement cannot be fully explained by locomotor reductions, and that signaling at OX_1R vs. OX_2R may be associated with different functions of the orexin system (e.g. conditioned motivation vs. general arousal). Other groups have reported slight reductions in spontaneous locomotor activity following 30 mg/kg, but not 20 mg/kg, administration of the OX_1R antagonists SB-334867 or SB-408124 (Rodgers *et al.*, 2001; Richards *et al.*, 2008; Dugovic *et al.*, in press) but there were no significant changes to sleep/wake states following administration of 30 mg/kg of SB-334867 or SB-408124 alone (Smith *et al.*, 2003; Dugovic *et al.*, in press). In contrast, the OX_2R antagonists JNJ-10397049 or *N*-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulphonyl)-amino]-*N*-pyridin-3-ylmethyl-acetamide (EMPA) reduced spontaneous locomotion (Dugovic *et al.*, in press; Malherbe *et al.*, in press) and JNJ-10397049 also caused significant decreases in the latency to non-rapid eye movement sleep and increases in sleep time (Dugovic *et al.*, in press). These studies indicate that orexin signaling at OX_2Rs , and not OX_1Rs , is involved in sleep/wake architecture and arousal.

The data presented here also indicate that attenuation of cue-induced reinstatement by SB is unlikely to be due to a generalized locomotor effect. First, SB had no significant effect on active or inactive lever responding during a late extinction session (Fig. 1a and b). Although significant effects on inactive lever responding were observed during cue-induced reinstatement with SB pretreatment, inactive lever responding may reflect changes in drug-seeking under reinstatement conditions (e.g. a decrease in drug-seeking may be reflected in decreased responding on the inactive lever as well as the active lever) and is not necessarily an indicator of impaired motor activity. Second, 30 mg/kg SB had no significant effect on active lever responding or cocaine intake during a self-administration session (Fig. 4a and b) and 20 mg/kg SB has previously been shown to have no effect on operant self-administration of 5% sucrose (Richards *et al.*, 2008), showing that SB does not impair lever-pressing behavior in general. Reductions in cocaine self-administration following SB or

4PT pretreatment in a small number of rats may have been due to adverse reactions to the antagonist injection itself in those animals (4 mL/kg suspension), and were not comparable to the magnitude of the effect observed during cue-induced reinstatement. Third, 30 mg/kg SB reduced spontaneous horizontal activity in a locomotor test predominantly in the first 30 min only (Fig. 3a and b), whereas SB reduced reinstatement behavior across the full 2-h session (Fig. 2). 30 mg/kg SB reaches peak brain levels at 30 min and has a terminal elimination half-life of approximately 4 h (Ishii *et al.*, 2005), which indicates that SB should be fully active for the entire duration of these tests. Reductions predominantly in initial locomotor activity might be explained by anxiolytic effects of either SB or 4PT in a novel environment (Chang *et al.*, 2007; Samson *et al.*, 2007). Lastly, 30 mg/kg 4PT caused greater reductions in spontaneous locomotion than SB (both horizontal activity and total distance traveled; Fig. 3c and d) but did not attenuate cue-induced reinstatement (Fig. 1c). Thus, self-administration behavior can proceed unabated despite substantially decreased locomotor activity. This last result also indicates that 30 mg/kg 4PT is a behaviorally active dose. Further evidence that 4PT and SB should be active at similar doses comes from *in vitro* studies that show that similar concentrations of SB and 4PT inhibited orexin-mediated responses at OX₁R and OX₂R, respectively (Smart *et al.*, 2001; Hirose *et al.*, 2003). Additionally, 4PT has been shown to have *in vivo* efficacy as an orexin antagonist (Chang *et al.*, 2007).

We hypothesize that signaling at OX₂R, as opposed to OX₁R, is primarily related to the arousal-associated functions of orexin, as others have also discussed (Marcus *et al.*, 2001; Willie *et al.*, 2003; Akanmu & Honda, 2005). Dysfunction in the orexin system has been closely associated with narcolepsy that co-occurs with cataplexy in humans and animals (Chemelli *et al.*, 1999; Lin *et al.*, 1999; Nishino *et al.*, 2000; Beuckmann *et al.*, 2004), and orexin-null or orexin-deficient mice show slight decreases in locomotor activity (Mochizuki *et al.*, 2004; Zhang *et al.*, 2007). This hypothesis of OX₂R function is based in part on findings by Lin *et al.* (1999) that familial canine narcolepsy is caused by a mutation in the OX₂R gene that renders the receptor non-functional, as well as findings by Willie *et al.* (2003) that OX₂R- and orexin-null mice had similar abnormalities in the transition between wake and non-rapid eye movement states, whereas OX₁R-knockout mice did not display overt behavioral abnormalities and had only mild fragmentation of sleep states. However, OX₂R-null mice exhibited only mild cataplexy attacks of rapid eye movement sleep, indicating that disruption at both OX₁ and OX₂ receptors may be necessary to achieve the full narcolepsy with cataplexy syndrome (Willie *et al.*, 2003). Recent studies using specific antagonists for the OX₁R and OX₂R also support a role for signaling at OX₂R in the sleep/wake functions of the orexin system (as discussed above).

Orexin function in drug-seeking

The current data show that orexin transmission at OX₁R is necessary for cue-elicited drug-seeking but not for cocaine reinforcement. Further, orexin is necessary for reinstatement driven by discrete cocaine-associated cues regardless of whether the cues are present throughout all self-administration sessions or during a single Pavlovian conditioned session with non-contingent cocaine. Orexin signaling at OX₁R may be necessary for the reinforcing properties of conditioned stimuli or the conditioned motivation (craving) triggered by drug-associated stimuli. In response to the renewed availability of conditioned stimuli, animals typically show increased stimulus-driven behavior (i.e. reinstatement of extinguished responding) despite drug

unavailability. Blockade of OX₁R via SB specifically reduced the responding elicited by the conditioned stimuli. However, SB did not significantly affect cocaine-taking behavior, indicating that orexin signaling is not necessary for the unconditioned reinforcing properties of cocaine. Animals given 20–30 mg/kg SB prior to a cue-induced reinstatement session showed active lever responding similar to late extinction sessions and not a complete blockade of responding; therefore, it appears that once animals were unrewarded by their lever pressing (i.e. no cocaine), they attenuated drug-seeking behavior just as they would during a late extinction session when no cues were available (Fig. 2).

The mechanism of action for orexin during reinstatement of drug-seeking is unknown. Orexin may be acting in the ventral tegmental area (VTA) during cue-induced reinstatement of cocaine-seeking. Orexin has projections to and actions in the VTA (Peyron *et al.*, 1998; Fadel & Deutch, 2002; Korotkova *et al.*, 2003; Vittoz *et al.*, 2008), and intra-VTA injections of SB attenuated behavioral responses associated with cocaine and morphine (Harris *et al.*, 2005, 2007; Borgland *et al.*, 2006; Narita *et al.*, 2006). Cocaine-seeking was reinstated by intra-VTA injections of orexin A (non-selective agonist for OX₁R and OX₂R) but not orexin B (selective agonist for OX₂R), supporting the hypothesis that signaling at OX₁R in particular is involved in drug-seeking (Wang *et al.*, 2009). Importantly, SB does not affect basal dopamine function in VTA, which is an important consideration therapeutically (Borgland *et al.*, 2006; Rasmussen *et al.*, 2007). Given the divergent projections of the orexin system, however, several addiction-associated brain regions may be involved in SB effects, such as nucleus accumbens, amygdala and prefrontal cortex, all of which have been previously implicated in cue-induced reinstatement of cocaine-seeking (Feltenstein & See, 2008). Recent self-administration studies suggest that orexin might play a role in drug-seeking via interactions with norepinephrine and corticotropin-releasing factor systems in the brain (Boutrel *et al.*, 2005; Richards *et al.*, 2008). Experiments are currently underway to test these hypotheses.

Conclusions

The current results indicate that orexin signaling at OX₁R, but not OX₂R, is necessary for reinstatement of cocaine-seeking elicited by discrete drug-paired stimuli in the self-administration paradigm, and that orexin signaling is not necessary for the reinforcing properties of cocaine or the maintenance of established drug-taking. Together with previous studies, these findings support a critical role for orexin in cue- and stress-induced reinstatement of cocaine- and ethanol-seeking (Boutrel *et al.*, 2005; Lawrence *et al.*, 2006; Richards *et al.*, 2008), and provide evidence that the orexin system may be an important therapeutic target for future addiction treatments. Signaling at OX₁R may be a particularly important target for combating relapse elicited by drug-associated stimuli.

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Abbreviations

4PT, 4-pyridylmethyl (*S*)-*tert*-leucyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline; OX₁R, orexin 1 receptor; OX₂R, orexin 2 receptor; SB, SB-334867; VTA, ventral tegmental area.

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