Orexin-A/Hypocretin-1 Mediates Cocaine-Seeking Behavior in the Posterior Paraventricular Nucleus of the Thalamus via Orexin/Hypocretin Receptor-2

Alessandra Matzeu, Tony M. Kerr, Friedbert Weiss, and Rémi Martin-Fardon

Molecular and Cellular Neuroscience Department, The Scripps Research Institute, La Jolla, California

ABSTRACT

Orexin/hypocretin (Orx/Hcrt) projections from the lateral hypothalamus to the paraventricular nucleus of the thalamus (PVT) are implicated in drug addiction. Specifically, the posterior section of the PVT (pPVT) innervates brain structures that modulate motivated behavior. This study investigated the role of pPVT-Orx/Hcrt transmission in cocaine-seeking behavior. Because the effects of Orx/Hcrt are mediated by two Orx/Hcrt receptors (Hcrt-r1 and Hcrt-r2), we examined the extent to which Hcrt-r1 and Hcrt-r2 are involved in Orx/Hcrt-induced cocaine seeking. Male Wistar rats were made cocaine dependent by self-administering cocaine 6 hours/day (long access) for 21 days. After self-administration training, the rats underwent daily extinction training, during which cocaine was withheld. After extinction, the rats were injected into the pPVT with Orx-A/Hcrt-1 (0–2 μg) alone or, using a single dose of 0.5 μg, in combination with an Hcrt-r1 antagonist (SB334867; 0–15 μg) or an Hcrt-r2 antagonist (TCSOX229; 0–15 μg). Orx-A/Hcrt-1 alone reinstated (primed) cocaine seeking. Unexpectedly, coadministration of Orx-A/Hcrt-1 with SB334867 did not have any effects on Orx-A/Hcrt-1-induced reinstatement, whereas when coadministered with Orx-A/Hcrt-1, TCSOX229 prevented cocaine-seeking behavior. These results indicate that Hcrt-r2 in the pPVT mediates the reinstating effect of Orx-A/Hcrt-1 in animals with a history of cocaine dependence and further identify Hcrt-r2 as a possible molecular target that can guide future therapeutic approaches for the prevention of drug-seeking behavior.

Introduction

A growing amount of evidence supports the significant implication of the paraventricular nucleus of the thalamus (PVT) in drug addiction because of its projections to limbic and cortical structures and involvement in the neurocircuitry that mediates craving and drug seeking (Everitt et al., 2001; McFarland and Kalivas, 2001; Ito et al., 2002; Kalivas and Volkow, 2005; Belin and Everitt, 2008; Steketee and Kalivas, 2011). Indeed, excitotoxic lesions of the PVT blocked beer-seeking behavior (Hamlin et al., 2009), attenuated reinstatement of cocaine seeking induced by a prime injection (James et al., 2010), and prevented the expression of cocaine conditioned place preference (Browning et al., 2014). Moreover, PVT neurons are activated by an acute dose of cocaine (Brown et al., 1992) and contextual stimuli that were conditioned to alcohol (Wedzony et al., 2003; Dayas et al., 2008; Perry and McNally, 2013), cocaine (Brown et al., 1992; Franklin and Druhan, 2000), and methamphetamine availability (Rhodes et al., 2005). Additionally, a correlation was observed between cocaine-seeking behavior and PVT activation and not during a highly palatable food-seeking behavior (Matzeu et al., 2015a), whereas presentation of stimuli previously paired with sucrose availability did not produce PVT activation (Wedzony et al., 2003).

Although frequently acknowledged as a homogeneous nucleus, the PVT consists of two structural portions—anterior (aPVT) and posterior (pPVT) (Vertes and Hoover, 2008)—that mediate distinct functions associated with reward-seeking behavior. For example, injection of TCSOX229, a hypocretin receptor 2 (Hcrt-r2) antagonist, into the aPVT decreased ethanol drinking but had no effect when injected into the pPVT (Barson et al., 2015). Additionally, voluntary ethanol drinking has been shown to activate the aPVT, but not the pPVT, to elevate Hcrt-r2 gene expression, whereas intra-gastrically administered ethanol increased Fos and Hcrt-r2 expression in the aPVT but not in the pPVT (Barson et al., 2015), suggestive of a role of Hcrt-r2 in the aPVT in modulating ethanol intake. On the contrary, temporary inactivation of the pPVT totally and selectively blocked the reinstatement of cocaine seeking (Matzeu et al., 2015b), suggesting that the pPVT is a key structure that regulates cocaine-seeking behavior.

It is known that the PVT receives abundant projections from the hypothalamus that contains numerous peptides, including orexin/hypocretin (Orx/Hcrt; Freedman and Cassell, 1994; Otake, 2005; Parsons et al., 2006; Baimel et al., 2015). The

ABBREVIATIONS: aPVT, anterior paraventricular nucleus of thalamus; Hcrt, hypocretin; LH, lateral hypothalamus; Orx, orexin; PVT, paraventricular nucleus of thalamus; pPVT, posterior paraventricular nucleus of thalamus; SB334867, *N*-(2-methyl-6-benzoxazolyl)-*N* '-1,5-ne th-aphthyridin-4-yl urea; TCSOX229, (2*S*)-1-(3,4-dihydro-6,7-dimethoxy-2(1*H*)-isoquinolinyl)-3,3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butanone hydrochloride.

Orx/Hcrt peptides orexin A and B (Orx-A and Orx-B, or Hcrt-1 and Hcrt-2) are synthetized only in neurons from the dorsal tuberal hypothalamic nuclei, including the lateral hypothalamus (LH), perifornical nucleus, and dorsomedial hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998). Although Orx/Hcrt neurons localization is restricted to hypothalamic nuclei, they send projections across the entire brain (de Lecea et al., 1998; Baldo et al., 2003) and are involved in many biologic functions, such as energy homeostasis and feeding, neuroendocrine and autonomic functions, arousal and wakefulness (Sutcliffe and de Lecea, 2000; Mieda and Yanagisawa, 2002; de Lecea, 2012). More recently, Orx/Hcrt has been described to be involved in drug addiction and drug seeking (Harris et al., 2005; Dayas et al., 2008; Martin-Fardon et al., 2010; Jupp et al., 2011), but what remains unclear is the participation of the pPVT Orx/Hcrt transmission in drug-seeking behavior after dependence.

Extended access to cocaine self-administration results in an increased (escalation) cocaine intake (Johanson et al., 1976; Bozarth and Wise, 1985). Specifically, rats with extended access to the drug exhibit a gradual daily increase in cocaine intake and an upward shift in the cocaine dose-effect function indicative of the need to increase the amount of cocaine intake to reach the desire effects (Ahmed and Koob, 1998; Ahmed and Koob, 1999). During abstinence, cocaine-escalated rats show persistent reward deficiency (Ahmed et al., 2002) very similar to depression and dysphoria during withdrawal reported by cocaine addicts (Markou and Koob, 1991). Thus, extended access to cocaine offers a unique animal model of cocaine dependence to investigate the consequence of chronic cocaine abuse in cocaine addicts.

Therefore, to shed light on the importance of Orx/Hcrt neurotransmission in the pPVT during cocaine seeking after dependence, the present study examined whether discrete administration of Orx-A/Hcrt-1 in the pPVT reinstates cocaine seeking in animals subjected to extended daily availability of cocaine. Knowing that Orx-A/Hcrt-1 binds to both Hcrt-r1 and Hcrt-r2 and that both receptors are expressed in the pPVT (Trivedi et al., 1998; Marcus et al., 2001), the present study also investigated the extent to which Hcrt-r1 and Hcrt-r2 are involved in Orx-A/Hcrt-1-induced cocaine seeking. A selective antagonist of either Hcrt-r1 or Hcrt-r2 was coadministered with Orx-A/Hcrt-1 and tested for its ability to block Orx-A/Hcrt-1–induced cocaine-seeking behavior.

Materials and Methods

Animals. One hundred sixteen male Wistar rats (Charles River, Wilmington, MA), weighing 200–225 g upon arrival, were housed two per cage in a temperature- and humidity-controlled vivarium on a reverse 12 hour/12 hour light/dark cycle with ad libitum access to food and water throughout the duration of the experiment. The animals were separated only when placed in the operant chambers (one animal per operant chamber). All the procedures were conducted in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Self-Administration and Extinction Training. Rats that were designated for cocaine self-administration were surgically prepared with jugular catheters under 1.5%–2% isoflurane anesthesia 7 days before starting cocaine self-administration training in daily 6-hour sessions and treated with flunixin (2.5 mg/kg, s.c.) twice per day for postsurgery pain. Catheters were flushed daily with 0.2 ml of sterile antibiotic solution containing cefazolin (100 mg/ml; Hospira, San Diego, CA) until the test day. Every session was initiated by the extension of two retractable levers into the operant conditioning chambers (29 × 24 × 19.5 cm, Med Associates, St. Albans, VT). Responses at the active lever were reinforced on a fixed-ratio 1 (FR1) schedule by i.v. cocaine (National Institute on Drug Abuse, Bethesda, MD; 0.25 mg/0.1 ml per infusion), dissolved in 0.9% sodium chloride (Hospira, Lake Forest, IL) and infused over 4 seconds. Each reinforced response was followed by a 20-second timeout (TO20) period that was signaled by the illumination of a cue light above the active lever. Responses at the inactive lever were recorded but had no scheduled consequences.

Fourteen days (Fig. 1) after beginning self-administration training, the rats were implanted with a guide cannula (23-gauge, 15-mm, Plastics One, Roanoke, VA) aimed at the pPVT (anterior/posterior, −3.3 mm; medial/lateral, ±2.72 mm from bregma; dorsal/ventral, −2.96 mm from dura, at an angle of 25°) (Paxinos and Watson, 1997) and positioned 3.5 mm above the target injection site (Fig. 2). After 7 days of recovery, the animals resumed self-administration training for an additional 7 days.

Twenty-four hours after the last self-administration session, the rats were placed under extinction conditions until an extinction criterion of ≤10 lever presses over the last three sessions was reached (usually between 14 and 21 days, Fig. 1). All the extinction sessions lasted 2 hous and began with extension of the levers into the operant chambers. The extinction sessions followed the same schedule as the self-administration training (including the TO20 signaled by the illumination of a cue light above the active lever) but without reward (cocaine) delivery when the active lever was depressed. Responses at the inactive lever were recorded as well but had no scheduled consequences.

Intra-PVT Microinjection. On the last day of extinction training, each rat received a sham injection for habituation to the microinjection (Fig. 1), and the rats were then divided into separate groups that were designated to receive either an intra-pPVT injection of Orx-A/Hcrt-1 or a combination of Orx-A/Hcrt-1 with an antagonist (between-subjects design). Twenty-four hours later, each animal received an intra-PVT microinjection of Orx-A/Hcrt-1 (0, 0.25, 0.5, 1, or 2 µg; American Peptide, Sunnyvale, CA) (Li et al., 2009; Li et al.,
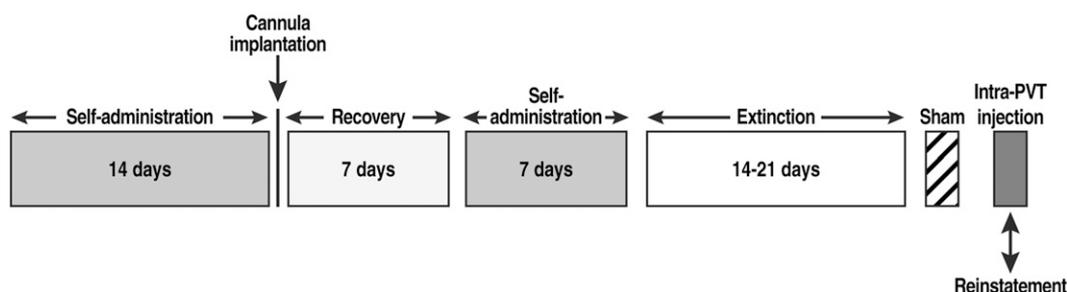

Fig. 1. Behavioral procedure.

2010b) in 0.9% sodium chloride (Hospira, Lake Forest, IL) or a combination of 0.5 μg Orx-A/Hcrt-1 (i.e., the minimal dose able to induce cocaine-seeking behavior) and the Hcrt-r1 antagonist SB334867 (0, 5, 10, or 15 μg; Lilly Research Laboratories, Indianapolis, IN) (Li et al., 2011) in ≥99.5% dimethylsulfoxide (DMSO; Sigma Aldrich, St. Louis, MO) or the Hcrt-r2 antagonist TCSOX229 (Tocris Biosciences, Bristol, UK; 0, 5, 10, or 15 μg; Li et al., 2011) in 0.9% sodium chloride (saline; Hospira, Lake Forest, IL). Intra-pPVT microinjections were made using a microinfusion pump (Harvard 22 Syringe Pump, Holliston, MA) with injectors extending 3.5 mm beyond the guide cannula. The injection volume was 0.5 μl. The injections were made over 1 minute, and the injector was left in place for an additional minute before being removed. Following the injections, the rats were returned to their home cages for 2 minutes and then transferred to the operant chambers and tested under extinction conditions for 2 hours (Fig. 1).

Histology. Upon completion of the reinstatement test (Fig. 1), the rats were euthanized by CO_2 inhalation, and their brains were collected and snap frozen. The brains were then sliced in 40 μm coronal sections, and injector placements within the pPVT were verified (Fig. 2).

Statistical Analysis. Cocaine intake (mg/kg) during the self-administration was analyzed with one-way repeated-measures analysis of variance (ANOVA) followed by Tukey post hoc tests. The dose-effect profile of Orx-A/Hcrt-1 or reversal effect profiles of SB334867 and TCSOX229 were analyzed using one-way ANOVA followed by Dunnett's post hoc tests. The magnitude of the increase in responding (percent increase) that was induced by Orx-A/Hcrt-1 and the magnitude of the inhibitory effects of SB334867 or TCSOX229 (percent decrease) were analyzed using one-way ANOVA, followed by Dunnett's post hoc tests to confirm significance.

Results

Six rats were lost (three to health complications, one for failure to acquire cocaine self-administration, and two because of off-target injections), reducing the total number of animals to 110 (n = 38 for Orx-A/Hcrt-1 dose effect; n = 38 for SB334867 test; n = 34 for TCSOX229 test).

Orx-A/Hcrt-1–Induced Reinstatement

Self-Administration and Extinction Training. Cocaine intake gradually increased from day 1 (14.1 ± 2.2 mg/kg) to day 11 (63.0 ± 3.6 mg/kg) and then stabilized (repeated measures ANOVA; $F_{37,\ 740}$ = 20.55, P < 0.001; Fig. 3A). At the end of extinction training (17 ± 1 days), the rats reached a 3-day average (± S.E.M.) number of responses of 7.5 ± 0.6 (Fig. 3B). The mean (± S.E.M.) number of responses during the extinction, sham, and vehicle sessions were statistically identical ($F_{2,14}$ = 0.076, P > 0.05; Fig. 3B).

Intra-PVT Microinjection of Orx-A/Hcrt-1. After saline injection (0 μg), the mean (± S.E.M.) number of responses was negligible (9.2 ± 1.6; Fig. 3B), whereas increased doses of Orx-A/Hcrt-1 induced cocaine-seeking behavior. This observation was confirmed by a main effect of Orx-A/Hcrt-1 doses (one-way ANOVA; $F_{4,33}$ = 3.626, P < 0.05). Dunnett's post hoc tests showed that Orx-A/Hcrt-1 reinstated cocaine seeking at 0.5, 1, and 2 μg (P < 0.05, vs. 0 μg; Fig. 3B). Transformation of the data into percent increase in responding (Fig. 3B) induced by Orx-A/Hcrt-1 also confirmed that Orx-A/Hcrt-1 reinstated cocaine-seeking behavior at 0.5, 1, and 2 μg (Dunnett's post hoc test after one-way ANOVA, $F_{4,33}$ = 3.626, P < 0.05; Fig. 3B). Responses at the inactive lever were negligible and unaltered by Orx-A/Hcrt-1 (one-way ANOVA; $F_{4,33}$ = 0.3367, P > 0.05) (Fig. 3B).

Blockade of Hcrt-r1 with SB334867

Self-Administration and Extinction Training. Cocaine intake gradually increased from day 1 (20 ± 3.3 mg/kg)
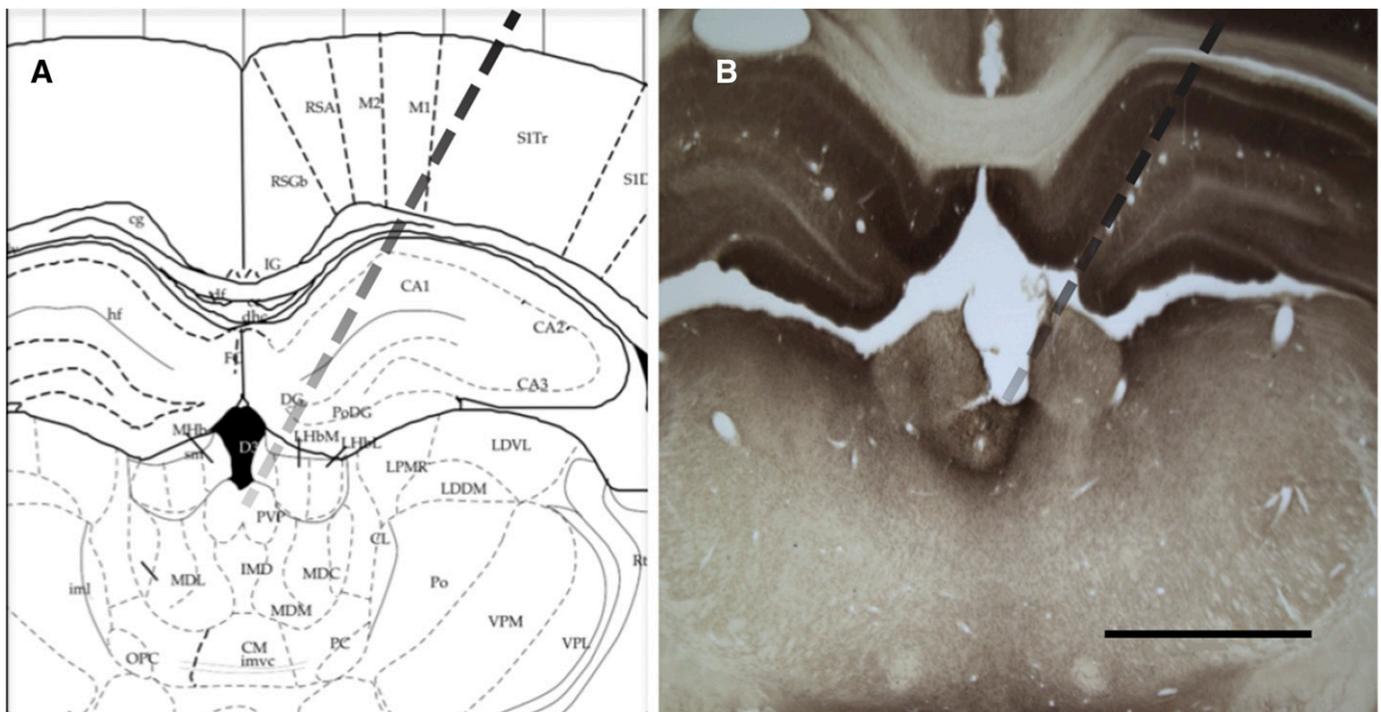
Fig. 2. Injector placement in the pPVT (Paxinos and Watson, 1997). (A) Schematic representation. (B) Representative image of injector placement. Scale bar: 2000 μm.
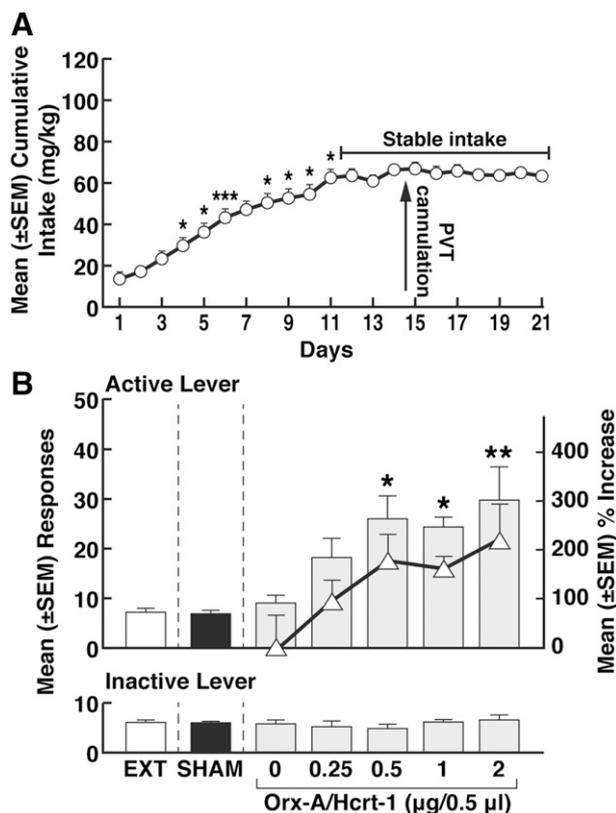
Fig. 3. Priming effect of Orx-A/Hcrt-1. (A) Time course of cocaine self-administration over the 21 days of training. Tukey post hoc tests *$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001 vs. respective previous day (data shown depict the mean $\pm$ S.E.M.; $n =$ 38). Intra-pPVT administration of Orx-A/Hcrt-1 induces cocaine-seeking behavior. *$P <$ 0.05; **$P <$ 0.01; vs. 0 μg (saline). Δ, % increase in responding): *$P <$ 0.05; **$P <$ 0.01 vs. 0 μg (saline). Asterisks (*, **) are referring to both the responses and the % increase. EXT, extinction; SHAM, sham injection (data shown depict the mean $\pm$ S.E.M; $n =$ 38 for EXT and SHAM; $n =$ 7–9 for Orx-A/Hcrt-1 doses).

to day 10 (62.4 $\pm$ 4.0 mg/kg) and then stabilized (repeated-measures ANOVA; $F_{37, 740} =$ 19.00, $P <$ 0.001) (Fig. 4A). By the end of extinction training (17 $\pm$ 2 days), the rats reached a 3-day average ($\pm$ S.E.M.) number of responses of 9.1 $\pm$ 0.4 (Fig. 4B). The mean ($\pm$ S.E.M.) number of responses during the extinction, sham, and vehicle sessions were statistically similar ($F_{2,16} =$ 1.236, $P >$ 0.05) (Fig. 4B).

Coadministration of SB334867 with Orx-A/Hcrt-1. After vehicle (DMSO) injection, the mean ($\pm$S.E.M.) number of responses was negligible (8.2 $\pm$ 1.0) (Fig. 4B). Coadministration of SB334867 (5, 10, and 15 μg) with Orx-A/Hcrt-1 (0.5 μg) did not prevent reinstatement (one-way ANOVA, $F_{4,33} =$ 3.490, $P <$ 0.05) (Fig. 4B). Transformation of the data into percent decrease of responding versus Orx-A/Hcrt-1 (Fig. 4B) confirmed the lack of effect of SB334867 on the reinstating effects of Orx-A/Hcrt-1 (one-way ANOVA, $F_{4,33} =$ 3.490, $P <$ 0.05) (Fig. 4B). Responses at the inactive lever were negligible and unaltered by SB334867 (one-way ANOVA; $F_{4,33} =$ 0.1038, $P >$ 0.05, Fig. 4B).

Blockade of Hcrt-r2 with TCSOX229

Self-Administration and Extinction Training. Cocaine intake gradually increased from day 1 (11.9 $\pm$ 1.7 mg/kg) to day 9 (62.4 $\pm$ 3.8 mg/kg) and then stabilized (repeated measures ANOVA; $F_{32,640} =$ 17.63, $P <$ 0.001) (Fig. 5A). At the

end of extinction training (16 $\pm$ 2 days), the rats reached a mean ($\pm$S.E.M.) 3-day average number of responses of 8.5 $\pm$ 0.7 (Fig. 5B). The mean ($\pm$S.E.M.) number of responses during the extinction, sham, and vehicle sessions were statistically comparable ($F_{2,16} =$ 1.413, $P >$ 0.05) (Fig. 5B).

Coadministration of TCSOX229 with Orx-A/Hcrt-1. After vehicle (saline) injection, the mean ($\pm$S.E.M.) number of responses remained low (8.3 $\pm$ 1.0) (Fig. 5B). Coadministration of TCSOX229 (5, 10, and 15 μg) with Orx-A/Hcrt-1 (0.5 μg) prevented cocaine-seeking behavior at 15 μg ($P <$ 0.05, Dunnett's post hoc test after one-way ANOVA, $F_{4,29} =$ 2.844; $P <$ 0.05) (Fig. 5B). Transformation of the data into percent decrease of responding (% decrease vs. Orx-A/Hcrt-1) confirmed that TCSOX229 completely blocked the effect of Orx-A/Hcrt-1 at 15 μg (one-way ANOVA followed by Dunnett's post hoc test, $F_{4,29} =$ 2.844, $P <$ 0.05) (Fig. 5B). Responses at the inactive lever were negligible and unaltered by TCSOX229 (one-way ANOVA; $F_{4,29} =$ 0.3701, $P >$ 0.05) (Fig. 5B).

Discussion

The present findings indicate that a microinjection of Orx-A/Hcrt-1 into the pPVT primed cocaine seeking in animals with a history of cocaine dependence. Surprisingly, however, coadministration of SB334867 with Orx-A/Hcrt-1 did not
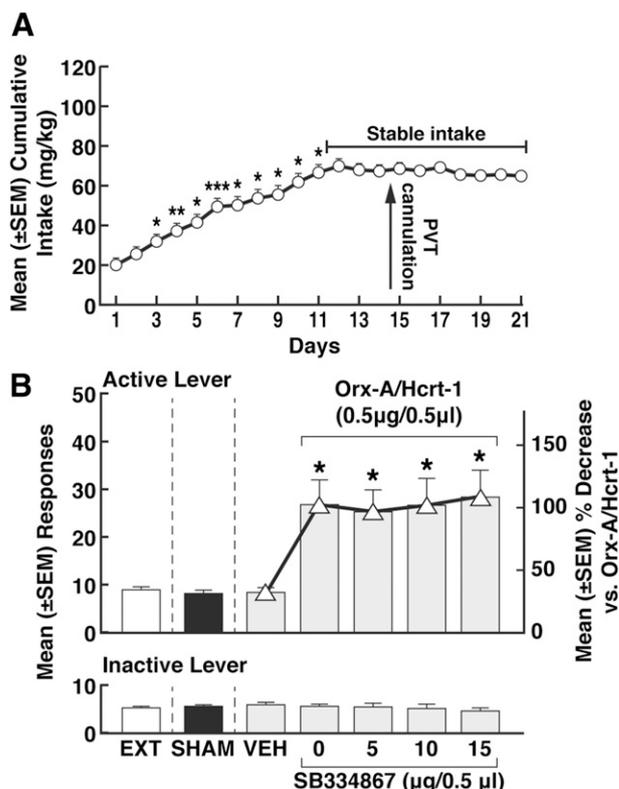

Fig. 4. Effect of SB334867 Orx-A/Hcrt-1's priming effect. (A) Time course of cocaine self-administration over the 21 days of training. Tukey post hoc tests *$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001 vs. respective previous day (data shown depict the mean $\pm$ S.E.M; $n =$ 38). (B) Lack of effect of SB334867 on Orx-A/Hcrt-1 (0.5 μg) priming effect: *$P <$ 0.05, vs. VEH (DMSO): Δ: % decrease in responding: *$P <$ 0.05, vs. VEH (DMSO). Asterisks (*) refer to both the responses and the % decrease. EXT, extinction; SHAM, sham injection; VEH, vehicle (data shown depict the mean $\pm$ S.E.M; $n =$ 38 for EXT and SHAM; $n =$ 9 for VEH; $n =$ 7–9 for SB334867 doses).
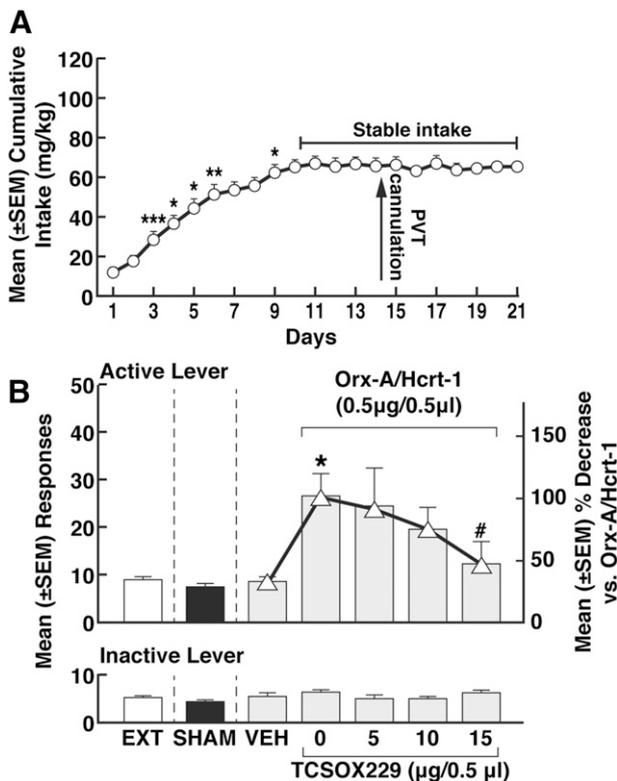
Fig. 5. Effect of TCSOX229 Orx-A/Hcrt-1's priming effect (A) Cocaine intake over the self-administration days. Tukey post hoc tests: *$P < 0.05$; **$P < 0.01$ vs. respective previous day (data shown depict the mean ± S.E.M; $n = 34$). (B) Reversal of Orx-A/Hcrt-1 (0.5 μg) priming effect by TCSOX229. *$P < 0.05$; **$P < 0.01$ vs. VEH (saline); #$P < 0.05$, vs. 0 μg (Orx-A/Hcrt-1 alone). Δ: % decrease responding: *$P < 0.05$, vs. VEH (saline); #$P < 0.05$, vs. 0 μg (Orx-A/Hcrt-1 alone). Symbols (*, #) refers to both the responses and the % decrease. EXT, extinction; SHAM, sham injection; VEH, vehicle (data shown depict the mean ± S.E.M; $n = 34$ for EXT and SHAM; $n = 9$ for VEH; $n = 6$ to 7 for TCSOX229 doses).

interfere with cocaine seeking, whereas TCSOX229 fully prevented Orx-A/Hcrt-1-induced cocaine seeking. The data indicate that the priming effects of an Orx-A/Hcrt-1 injection into the pPVT are mediated by Hcrt-r2, making this receptor subtype a potential target for cocaine relapse prevention.

Before considering the implications of these findings, it is important to discuss the possibility of nonspecific locomotor effects from the injection of Orx-A/Hcrt-1, SB334867, and TCSOX229 into the pPVT. For instance, one can argue that the priming effect of Orx-A/Hcrt-1 is due to a general non-specific locomotor activation rather than specific cocaine-seeking behavior. This is inconsistent with the inactive lever data showing no increased activity (Fig. 3B). The same observation was made with the administration of SB334867 and TCSOX229 (Figs. 4B and 5B) where the responses on the inactive lever stayed low and unaffected. Therefore the present data, consistent with earlier published findings, suggest that injection of Orx-A/Hcrt-1, SB334867, and TCSOX229 into the pPVT at the doses range used do not induce any nonspecific locomotor effect and further suggest that Orx-A/Hcrt-1 act on the PVT to produce arousal independent of general locomotor activation (Li et al., 2009, 2010b, 2011; Barson et al., 2015).

Another possible behavioral confound after microinjection into the pPVT could be due to the close position of the PVT to the third ventricle and therefore the possibility of Orx-A/

Hcrt-1, SB334867, and TCSOX229 to diffuse through the ventricles and act nonspecifically at other brain regions. This interpretation is inconsistent with the data obtained with SB334867. If nonspecific diffusion in other brain regions occurred, a reduction in cocaine seeking by SB334867 should have been measured similar to what was observed when SB334867 was injected peripherally (Martin-Fardon and Weiss, 2014), but this was not the case. Further, the absence of effect of SB334867 in the pPVT confirmed the observation by others (James et al., 2011) that intra-PVT administration of SB334867 did not affect cocaine-conditioned reinstatement (another animal model of relapse), suggesting a specific role for pPVT Hcrt-r2 signaling in cocaine-seeking behavior.

Demonstration that discrete injection of Orx-A/Hcrt-1 into the pPVT primed cocaine-seeking behavior strongly supports the implication of the Orx/Hcrt projection to the PVT in the control of drug craving and relapse. It has been hypothesized that LH Orx/Hcrt neurons may regulate the activation of the ventral striatum via a relay through the PVT (Kelley et al., 2005a,b; Parsons et al., 2006). This interpretation is consistent with earlier findings showing that the PVT is heavily innervated by Orx/Hcrt fibers that originate from the LH and perifornical nucleus, with the densest innervation in the pPVT (Kirouac et al., 2005). Moreover, a previous study has shown that both Orx/Hcrt neurons in the LH and PVT were activated by alcohol-related stimuli (Dayas et al., 2008), suggesting that LH Orx/Hcrt projections to the PVT are involved in drug-seeking behavior. Further anatomic evidence supports this hypothesis, as it was shown that Orx/Hcrt fibers juxtapose to PVT neurons and project to the nucleus accumbens shell (NACsh; Parsons et al., 2006), a brain region known to be involved in the reinstatement of ethanol-, cocaine-, and sucrose-seeking behavior (Dayas et al., 2007; Cruz et al., 2014; Guercio et al., 2015).

A potential explanation of Orx-A/Hcrt-1's priming effects could be related to the role of the Orx/Hcrt system in arousal states. In fact, it is known that Orx/Hcrt controls general arousal (Sakurai et al., 2010); for instance, it has been described that expectation of food rewards induces the activation of neurons that contain Orx/Hcrt receptors within the PVT (Choi et al., 2010). Most neurons in the PVT are sensitive to Orx-A/Hcrt-1 and Orx-B/Hcrt-2, and the pre-frontal cortex is an important target of Orx/Hcrt-activated PVT neurons (Ishibashi et al., 2005; Huang et al., 2006). The present results suggest that Orx/Hcrt inputs to the PVT facilitate cortical activation that is linked to general arousal (Sato-Suzuki et al., 2002), which could explain the reinstatement of cocaine seeking. Moreover, Orx-A/Hcrt-1 administration in the PVT significantly increased dopamine levels in the nucleus accumbens (Choi et al., 2012), suggesting that the PVT is a key relay for Orx/Hcrt's effects on the mesolimbic dopamine system and reward-seeking behavior.

Another possible mechanism by which injection of Orx-A/Hcrt-1 into the PVT induces cocaine-seeking behavior could be related to PVT's function in mediating anxiety- and stress-like responses, which are known factors to precipitate cocaine seeking in abstinent individuals. For example, the PVT sends projections to the dorsolateral bed nucleus of the stria terminalis and central nucleus of the amygdala that contains neurons densely expressing dynorphin and corticotropin releasing factor (Li and Kirouac, 2008), which are peptides associated with the manifestation of negative emotional states

and response to stress (Davis, 1998; Heinrichs and Koob, 2004; Shirayama and Chaki, 2006; Davis et al., 2010). In addition, Orx/Hcrt neurons become activated (i.e., increased Fos) after exposure to stress (Espana et al., 2003; Furlong et al., 2009), which indicates that the Orx/Hcrt system is involved in the expression of behavioral and physiologic responses to stressful events. Supporting this hypothesis, both Orx-A/Hcrt-1 and Orx-B/Hcrt-2, when injected in the PVT, produce anxiety-like behavior in rats observed in the open field (Li et al., 2010a) or in the elevated plus maze (Li et al., 2010b), suggesting that Orx/Hcrt can act as a stressor and can therefore precipitate cocaine-seeking behavior.

Surprisingly, Hcrt-r1 blockade did not prevent Orx-A/Hcrt-1 prime-induced reinstatement. This was unexpected considering the large number of studies that reported efficient blockade of drug-seeking behavior when SB334867 was administered peripherally (Richards et al., 2008; Zhou et al., 2012; Martin-Fardon and Weiss, 2014; Bentzley and Aston-Jones, 2015) and the significant expression of Hcrt-r1 in the pPVT (Trivedi et al., 1998; Marcus et al., 2001). One possible explanation of a lack of SB334867effect may be that the doses used were not behaviorally relevant. This interpretation is unlikely, however, because it has been shown by others that SB334867 at doses even lower than the ones tested in the present study attenuated physical symptoms of morphine withdrawal as well as cocaine seeking-behavior when injected in the locus coeruleus and in the ventral tegmental area, respectively (Azizi et al., 2010; James et al., 2011). Another more acceptable hypothesis is that the effect of a peripheral administration (i.e., not site specific) of SB334867 may be attributable to its action in brain regions other than the PVT. Supporting this hypothesis, it has been shown that SB334867 decreased conditioned reinstatement of cocaine-seeking behavior when injected into the ventral tegmental area but not PVT (James et al., 2011).

TCSOX229, in contrast to SB334867, prevented the priming effects of Orx-A/Hcrt-1. It is important to note that most studies in the drug addiction field have prioritized on investigating the role of Hcrt-r1, with only a few reports implicating Hcrt-r2 in drug-seeking behavior. As described for Hctrt-r1, Hcrt-r2 is highly expressed in the PVT (Trivedi et al., 1998; Marcus et al., 2001), and the literature suggests that Hcrt-r2 blockade can attenuate drug intake and drug seeking when Hcrt-r2 antagonists are administered peripherally. For example, peripheral injection of an Hcrt-r2 antagonist attenuated ethanol self-administration, reduced the acquisition of ethanol-induced CPP, prevented ethanol-induced expression and reinstatement of CPP (Shoblock et al., 2011), reduced cue-induced reinstatement of nicotine seeking (Uslaner et al., 2014), and attenuated heroin self-administration in escalated animals (Schmeichel et al., 2015). Further, when administered into the PVT, TCSOX229 prevented footshock-elicited anxiety-like behavior (Li et al., 2010b), implying that Hcrt-r2 signaling in the PVT is important in the mediation of stress-related behavior such as cocaine-seeking behavior.

In summary, the data showed that discrete administration of Orx-A/Hcrt-1 in the pPVT elicited a priming effect that reinstated cocaine-seeking behavior in dependent animals. The blockade of Hcrt-r1 did not prevent reinstatement induced by Orx-A/Hcrt-1, whereas Hcrt-r2 blockade reversed Orx-A/Hcrt-1-induced reinstatement, suggesting that Hcrt-r2

in the pPVT mediates Orx-A/Hcrt-1-induced cocaine seeking. Future studies are guaranteed to assess molecular changes (i.e., Orx/Hcrt and Hcrt-r production) attributable to cocaine dependence and long periods of abstinence. Such findings may shed light on valuable targets for the treatment of persistent vulnerability to relapse associated with cocaine dependence.

Authorship Contributions

Participated in research design: Matzeu, Kerr, Weiss, Martin-Fardon.

Conducted experiments: Matzeu, Kerr, Martin-Fardon.

Performed data analysis: Matzeu, Martin-Fardon.

Wrote or contributed to writing the manuscript: Matzeu, Martin-Fardon.

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Address correspondence to: Dr. A. Matzeu, Molecular and Cellular Neuroscience Department, The Scripps Research Institute, 10550 North Torrey Pines Road, SP30-2120, La Jolla, CA 92037. E-mail: amatzeu@scripps.edu