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Differential role of hypothalamic orexin/hypocretin neurons in reward seeking motivated by cocaine vs. palatable food

Rémi Martin-Fardon, Gabrielle Cauvi, Tony M. Kerr, and Friedbert Weiss

The Scripps Research Institute, Molecular and Cellular Neuroscience Department, La Jolla CA 92037, USA

Abstract

Hypothalamic orexin/hypocretin (Orx/Hcrt) neurons are thought to mediate both food-reinforced behaviors and behavior motivated by drugs of abuse. However, the relative role of the Orx/Hcrt system in behavior motivated by food vs. drugs of abuse remains unclear. This investigation addressed this question by contrasting hypothalamic Orx/Hcrt neuronal activation associated with reinstatement of reward seeking induced by stimuli conditioned to cocaine (COC) vs. highly palatable food reward, sweetened condensed milk (SCM). Orx/Hcrt neuronal activation in the lateral hypothalamus (LH), dorsomedial hypothalamus (DMH), and perifornical area (PFA), determined by dual *c-fos/orx* immunocytochemistry, was quantified in rat brains following reinstatement of reward seeking induced by a discriminative stimulus (S^+) conditioned to COC or SCM. The COC S^+ and SCM S^+ initially produced the same magnitude of reward seeking. However, over four subsequent tests, behavior induced by the SCM S^+ decreased to extinction levels, whereas reinstatement induced by the COC S^+ perseverated at undiminished levels. Following both the first and fourth tests, the percentage of Orx/Hcrt cells expressing Fos was significantly increased in all hypothalamic subregions in rats tested with the COC S^+ , but not rats tested with the SCM S^+ . These findings point toward a role for the Orx/Hcrt system in perseverating, compulsive-like cocaine seeking but not behavior motivated by palatable food. Moreover, analysis of the Orx/Hcrt recruitment patterns suggests that failure of Orx/Hcrt neurons in the LH to respond to inhibitory inputs from Orx/Hcrt neurons in the DMH/PFA may contribute to the perseverating nature of cocaine seeking.

Keywords

orexin; hypocretin; hypothalamus; cocaine; natural reward; relapse

Correspondence: Dr. Remi Martin-Fardon, The Scripps Research Institute, SP30-2120, 10550 North Torrey Pines Rd, La Jolla, CA 92037. rmartinf@scripps.edu; Tel: 858-784-7154; Fax: 858-784-7146.

DISCLOSURES

The authors report no biomedical financial interests or potential conflicts of interest.

AUTHORS CONTRIBUTION

R.M.-F. and F.W. were responsible for the study concept and design. R.M.-F, G.C., and T.M.K. performed the experiments, collected all data, performed the statistical analyses, interpreted the findings, and drafted the manuscript. All authors critically reviewed the content and approved the final version for publication.

INTRODUCTION

Orexin/hypocretin (Orx/Hcrt) peptides are neuropeptides expressed exclusively in neurons of dorsal tuberal hypothalamic nuclei: lateral hypothalamus (LH), perifornical area (PFA), and dorsomedial hypothalamus (DMH; de Lecea et al., 1998; Sakurai et al., 1998). The hypothalamic Orx/Hcrt system has multiple behaviorally relevant functions that include regulation of feeding, arousal, sleep/wake states, behavioral responses to stress, energy homeostasis, and reward (for review, see Sakurai, 2014).

With regard to reward seeking and reinforcement, Orx/Hcrt has been widely implicated in the mediation of food-reinforced behaviors, particularly operant responses for palatable food (Borgland et al., 2009; Cason and Aston-Jones, 2013a, b; Cason et al., 2010; Choi et al., 2010; Thorpe et al., 2005). However, mounting evidence supports a major role for the Orx/Hcrt system in behavior motivated by drugs of abuse (for review, see Mahler et al., 2012). Orx/Hcrt neurons in the lateral hypothalamus are activated by stimuli that are associated not only with palatable food (e.g., sweet-flavored cereals) but also drugs of abuse (e.g., morphine, alcohol, and cocaine; Days et al., 2008; Harris et al., 2005). Moreover, some reports suggest that pharmacological blockade of Orx/Hcrt receptors preferentially reduces both the self-administration of drugs of abuse (e.g., alcohol) over sucrose (Brown et al., 2013; Jupp et al., 2011a; Shoblock et al., 2011) and context-induced reinstatement motivated by drugs of abuse (e.g., cocaine and alcohol) compared with reinstatement that is motivated by palatable food reinforcers (Martin-Fardon and Weiss, 2014a, b).

Despite scattered evidence suggestive of a preferential role of the Orx/Hcrt system in the behavioral effects of drugs of abuse, the precise function of the Orx/Hcrt system in controlling food *vs.* drug seeking remains unclear. The purpose of this investigation was to provide a better understanding of the regulatory role of the Orx/Hcrt system in the rewarding effects of drugs of abuse *vs.* food reward, with a particular focus on reward-seeking behavior motivated by environmental stimuli conditioned to drugs of abuse *vs.* palatable food. Recent findings suggest that environmental stimuli associated with drugs of abuse produce a distinctly different reward seeking profile over repeated tests of reinstatement than stimuli conditioned to food reinforcers with high hedonic value, such as sweetened condensed milk (SCM; see Martin-Fardon and Weiss, 2016). Specifically, with repeated testing, stimuli that were conditioned to drugs induced reward seeking that perseverated, while stimuli that were associated with food reinforcers with high hedonic value did not produce perseveration; rather the behavior decreased to extinction levels rapidly (Martin-Fardon and Weiss, 2016). To more conclusively establish the function of Orx/Hcrt neurons in reward seeking and permit dissociation of a possibly distinct role in regulating drug *vs.* food motivation, reward-seeking (i.e., reinstatement) sessions were conducted repeatedly for a total of four tests at 3-day intervals, using a model of perseverating reinstatement employed previously to characterize the persistent nature of cocaine and alcohol seeking (Martin-Fardon and Weiss, 2016). Following each test, the pattern of Orx/Hcrt neuronal activation was determined by dual *c-fos/orx* immunocytochemistry within the three major Orx/Hcrt-containing hypothalamic subdivisions; the LH, DMH, and PFA. Effects that were associated with the reinstatement of reward seeking induced by stimuli conditioned to cocaine were contrasted with those of a motivationally equivalent palatable food reinforcer, SCM.

MATERIALS AND METHODS

Subjects

Male Wistar rats (Charles River, Wilmington, MA, USA; 200–250 g upon arrival) were housed 2–3/cage in a temperature- and humidity-controlled vivarium on a reverse 12 h/12 h light/dark cycle with *ad libitum* access to food and water. All of the procedures were conducted in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Self-administration and conditioning

Rats were trained and tested in operant conditioning chambers (Med Associates, St. Albans, VT, USA) located inside ventilated sound-attenuating cubicles as described previously (e.g., Martin-Fardon et al., 2009). Intravenous and oral reinforcers were delivered using a syringe pump (Razel Scientific Instruments, Stamford, CT, USA) located outside the sound-attenuating cubicles. Cocaine (National Institute on Drug Abuse, Bethesda, MD, USA; 0.25 mg/0.1 ml in sterile physiological saline) was infused intravenously (IV) over 4 s. Sweetened condensed milk (Carnation, Nestlé, Vevey, Switzerland; 2:1 v/v in dH₂O) was dispensed in a volume of 0.1 ml into a drinking reservoir located in the chamber's front panel. Rats designated for cocaine self-administration were surgically prepared with jugular catheters, followed by 7 days of recovery.

Cocaine and SCM self-administration training was conducted in separate groups of rats on a fixed-ratio 1 (FR1) schedule of reinforcement (2 h/day for cocaine and 40 min/day for SCM to prevent satiation by excessive ingestion). Reinforced lever responses were followed by a 20-s timeout (TO) period signaled by illumination of a cue light, during which time the lever remained inactive. Responses at a second, inactive lever had no scheduled consequences. Following acquisition of stable cocaine or SCM-maintained responding, a contingency was introduced in which responses at the active lever were differentially reinforced in the presence of discriminative stimuli (S^D) that signaled availability *vs.* non-availability of the reinforcer. A constant 70 dB white noise served as the S^D (S^+) for availability of the reinforcer (cocaine or SCM), whereas illumination of a 2.8 W house light served as the S^D (S^-) signaling non-availability of the reinforcer. Sessions were initiated by extension of the levers and onset of the respective S^D , which remained present until termination of the session. In the presence of the S^+ , responses at the active lever were reinforced by cocaine or SCM, followed by the signaled 20-s TO period. In the presence of the S^- , depression of the active lever remained non-reinforced and followed by 20-s TO period signaled by an intermittent tone (Sonalert, Med Associates, St. Albans, VT, USA). Three daily sessions (each lasting 1 h for cocaine and 20 min for SCM) separated by 30-min intervals were conducted, with two “reward” sessions and one “non-reward” session sequenced in random order. After 8 training days (i.e., 16 reward and 8 non-reward sessions; Fig. 1), the rats were placed on extinction (EXT) conditions in daily 30-min sessions, during which the reinforcers and S^D were withheld until a criterion of 5 responses/session for 3 consecutive days was reached (Fig. 1).

Reinstatement test procedure

The reinstatement test phase began 1 day after the extinction criterion was reached. The rats were divided into two cohorts. Cohort 1 was subjected to a single reinstatement test and subsequent euthanasia for immunocytochemistry (Test 1; Fig. 1a); cohort 2 was tested repeatedly every third day (with 2 days off between reinstatement tests; Fig. 1b) and euthanized following the fourth reinstatement session (Test 4). To provide necessary comparison conditions for evaluation of the Fos/Orx immunohistochemical data, both cohorts were subdivided into three groups ($n = 8/\text{group}$). Group 1 was tested for reinstatement in the presence of the S^+ , group 2 was tested for reinstatement in the presence of the S^- and group 3 was tested under EXT conditions without reintroduction of the S^D (Fig. 1a, b). The reinstatement tests lasted 30 min.

Dual *c-fos/orx* immunocytochemistry

Following the completion of behavioral testing, rats were returned to the vivarium for 1 h. They were then deeply anesthetized by CO_2 and transcardially perfused with cold saline followed by 4% paraformaldehyde in 0.1 mM sodium tetraborate (pH 9.5). Brains were postfixed in 4% paraformaldehyde overnight (12 h) and stored in a 30% (w/v) sucrose, 0.1% (w/v) sodium azide, 0.01 mM KPBS solution. Brains were coronally sectioned (40 μm) on a cryostat that was maintained at -20°C . Sections were blocked for 1 h using 3% bovine serum albumin/1% Triton-X/PBS, followed by 48 h incubation at 4°C with anti-*c-fos* antibody (rabbit, 1:20000, sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-orexin-A antibody (goat, 1:20000, sc-8070, Santa Cruz Biotechnology). Tissue sections were processed for Fos detection by incubation with biotinylated secondary donkey anti-rabbit antibody (1:300, 711-065-152, Jackson Immuno-Research, West Grove, PA, USA) for 2 h, followed by ABC-Elite (Vector Laboratories). Nuclear *c-fos* immunostaining was visualized using DAB-Ni. The sections were then processed for Orx-A detection by incubation with biotinylated secondary donkey anti-goat antibody (1:400, 705-065-003, Jackson Immuno-Research) for 2 h, followed by ABC-Elite. Cytoplasmic staining was developed using DAB (Fig. 1d–f). Counts of Fos⁺ cells, Orx⁺ cells, and Fos⁺/Orx⁺ cells were made using sections that incorporated the LH, DMH, and PFA (range, -2.1 and -3.6 mm from bregma; Fig. 1c). The number of Orx/Hcrt cells that were Fos-immunopositive was expressed as a percentage of the number of Orx⁺ cells ($\% \text{ Fos}^+/\text{Orx}^+ \text{ cells} = [\text{Orx}^+/\text{Fos}^+ \text{ cells} \div \text{Orx}^+ \text{ cells}] \times 100$) for each individual rat. The counting criterion for identifying Fos⁺/Orx⁺ cells, as well as individual counting, was based on standard morphology, and only neurons with a clearly defined black round nucleus surrounded by brown cytoplasm where the membrane was visible (i.e., the cells were anatomically intact) were counted (Fig. 1d–f). To control for the possibility that differences in Fos expression or the number of Fos⁺/Orx⁺ neurons between the COC and SCM groups resulted from their differential history of drug exposure, all histochemical data were compared with those from age-matched experimentally naive rats ($n = 8$) that were handled daily for 5 min.

STATISTICAL ANALYSIS

The data were analyzed by one-way (histochemical data) or two-way (behavioral data) analysis of variance (ANOVA). Significant main effects or interactions were followed by the

Protected Least Significant Difference (PLSD) *post hoc* tests when appropriate. Differences in responding at the active lever between the respective reward and non-reward conditions during the last day of the training/conditioning phase were analyzed by paired *t*-tests.

RESULTS

Reinstatement of cocaine and SCM seeking

Test 1 (cohort 1)—All rats acquired cocaine (COC)- or SCM-reinforced responding and maintained stable self-administration in S^+ -signaled sessions during the conditioning phase, accompanied by significantly decreased responding in non-reward (S^-) sessions (paired *t*-test; COC: $t_{23} = 11.6$, $p < 0.001$; SCM: $t_{23} = 70.9$, $p < 0.001$; Fig. 2a, left panel). Following extinction (EXT), re-introduction of the COC S^+ or SCM S^+ produced strong recovery of responding ($p < 0.001$ vs. EXT and S^- , PLSD *post hoc* test following ANOVA: group [COC, SCM], $F_{1,47} = 0.9$, $p > 0.05$; group $\times$ reinstatement phase interaction [EXT, S^- , and S^+], $F_{2,42} = 0.07$, $p > 0.05$; reinstatement phase, $F_{2,42} = 29.3$, $p < 0.001$), whereas the non-reward S^- was without effect (Fig. 2a, right panel). Moreover, the reinstating effects of the COC S^+ and SCM S^+ were statistically identical (Fig. 2a, right panel). Inactive lever responses remained negligible throughout the experiment.

Test 4 (cohort 2)—As in cohort 1 (tested only once, Test 1), all rats acquired COC- or SCM-reinforced responding, continued to show stable S^+ -signaled responding during the conditioning phase, and developed negligible responding during S^- sessions (paired *t*-test; COC: $t_{23} = 13.9$, $p < 0.001$; SCM: $t_{23} = 95.3$, $p < 0.001$; Fig. 2b, left panel). Replicating the findings in cohort 1, the initial re-introduction of the respective S^+ (but not S^-) produced strong recovery of responding at identical levels in the COC S^+ and SCM S^+ groups ($p < 0.01$ vs. EXT and S^- , PLSD *post hoc* test following ANOVA: group [COC, SCM], $F_{1,42} = 1.6$, $p > 0.05$; group $\times$ reinstatement phase interaction [EXT, S^- , and S^+], $F_{2,42} = 0.4$, $p > 0.05$; reinstatement phase, $F_{2,42} = 30.3$, $p < 0.001$). Over the subsequent four repeated tests, the COC S^+ continued to produce reinstatement at undiminished levels ($p < 0.01$ vs. EXT and S^- , PLSD *post hoc* test following ANOVA: group [COC, SCM], $F_{1,42} = 13.6$, $p < 0.001$; group $\times$ reinstatement phase interaction [EXT, S^- , and S^+], $F_{2,42} = 7.6$, $p < 0.001$; reinstatement phase, $F_{2,42} = 9.1$, $p < 0.001$), whereas the SCM S^+ ceased to elicit behavior different from extinction performance ($p > 0.05$ PLSD *post hoc* test; Fig. 2b, right panel and Fig. 2c). Responses in the EXT and S^- groups remained at extinction levels, and inactive lever responses were negligible (data not shown).

Activation of Orx/Hcrt neurons

Reinstatement elicited by the COC S^+ or SCM S^+ during the first test was identical (Test 1, Fig. 2a, right panel), but these effects were accompanied by differential activation of Orx/Hcrt neurons in the COC S^+ vs. SCM S^+ groups (Fig. 3). In the LH (Fig. 3a), a higher percentage of Fos⁺ Orx/Hcrt neurons was recorded following Test 1 in the COC S^+ group vs. all other groups ($p < 0.01$). Following Test 4 (Fig. 2b, right panel), the percentage of Fos⁺ Orx/Hcrt neurons in the COC S^+ group also was higher than in all other groups ($p < 0.05$, Fig. 3a), but lower than in Test 1 COC S^+ group (PLSD *post hoc* test following ANOVA: $F_{12,82} = 6.8$, $p < 0.001$). In the DMH (Fig. 3b), the percentage of Fos⁺ Orx/Hcrt neurons was

higher in the COC S⁺ groups in both Tests 1 and 4 ($p < 0.01$) vs. all other groups (PLSD *post hoc* test following ANOVA: $F_{12,85} = 6.6$; $p < 0.001$; Fig. 3b). In the PFA (Fig. 3c), a higher percentage ($p < 0.05$) of Fos⁺ Orx/Hcrt neurons was observed in the COC S⁺ group vs. the SCM S⁺ group in both Tests 1 and 4 (PLSD *post hoc* test following ANOVA: $F_{12,88} = 4.2$, $p < 0.01$).

Number of Orx⁺/Hcrt⁺ cells (Table 1)

In the LH, no differences were found between the number of Orx⁺ cells across groups (PLSD *post hoc* tests, $p > 0.05$ following significant overall group effect in the ANOVA: $F_{12,82} = 1.9$, $p < 0.05$). No overall group effect was observed in the DMH (ANOVA: $F_{12,90} = 1.5$, $p > 0.05$) or PFA (ANOVA: $F_{12,90} = 1.6$, $p > 0.05$).

DISCUSSION

To provide a systematic evaluation of the role of hypothalamic Orx/Hcrt neurons in appetitive behavior motivated by cocaine vs. a highly palatable food substance (SCM), rats were exposed to the respective reward-predictive stimulus contexts over four repeated tests, with the goal of establishing the recruitment pattern of Orx/Hcrt neurons associated with the acute response-reinstating actions of these stimuli, as well as the perseveration of responsiveness to these stimuli over time. At the behavioral level, the reward-predictive stimulus context (S⁺) produced robust reinstatement during the first testing occasion, regardless of whether it had been conditioned to cocaine or SCM availability. However, across the remaining three tests, reward seeking induced by these stimuli decreased to extinction levels when motivated by palatable food (SCM), but perseverated without decline when motivated by cocaine. This time-dependent differential profile of reinstatement presumably reflects the perseverative, compulsive nature of drug addiction (e.g., Ciccocioppo et al., 2004; Weiss et al., 2001) vs. the “normal” course of extinction of behavior motivated by non-addictive reward (Martin-Fardon and Weiss, 2016).

The behavioral effects of the cocaine- and SCM-predictive stimulus context (S⁺) during the first reinstatement test were statistically identical. Nonetheless, only the cocaine S⁺ produced significant elevations in the percentage of Fos⁺/Orx⁺ cells and did so in all three targeted hypothalamic regions. A similar pattern of selective activation of Fos⁺/Orx⁺ cells by the cocaine S⁺ was observed during the final (i.e., fourth) reinstatement test, except that the magnitude of activation in the LH was statistically smaller than during the first test, even though S⁺-induced reinstatement in the two tests was identical. There was no difference among groups in the total number of Orx⁺/Hcrt⁺ cells in any hypothalamic region in brains that were obtained after the first and fourth tests (Table 1). Therefore, the cocaine S⁺-induced increases in the proportion of activated Orx/Hcrt neurons in the LH, DMH, and PFA were specific to the S⁺ presentation and not the result of changes in the number of Orx/Hcrt cells, which could have possibly occurred as a result of the drug self-administration history in the cocaine S⁺ group.

Prior to discussing the implications of these findings, examination of the difference in baseline levels of responding during conditioning is necessary, which were substantially higher with SCM than with cocaine, even though the length of the conditioning sessions was

shorter for SCM (20 min) *vs.* cocaine (1 h). Despite these differences, the level of reinstatement induced by the cocaine- and SCM-associated stimuli during the first reinstatement test was similar, suggesting that the present dose of cocaine and concentration of SCM used, as well as the different lengths of the conditioning sessions between cocaine and SCM, resulted in a reliable and comparable conditioning effects, which is consistent with previous reports (e.g., Baptista et al., 2004; Hodos, 1961; Martin-Fardon et al., 2009; Martin-Fardon and Weiss, 2014a; Matzeu et al., 2015; Martin-Fardon and Weiss, 2016). It is important to note that reinforcing efficacy and the rate of responding on FR schedules are not necessarily correlated (Richardson and Roberts, 1996; Stafford et al., 1998). In fact, the concentration of SCM that was used in the present study has been shown to maintain breakpoints under a progressive-ratio schedule of reinforcement that are comparable to those that are measured with the dose of cocaine that was used in the present study (Hodos, 1961; Richardson and Roberts, 1996; Stafford et al., 1998). Moreover, the magnitude of the response that was induced by the cocaine and SCM S^+ during the reinstatement tests was statistically identical, which was the goal of the present paradigm.

It is known that S^+ -induced reinstatement of alcohol seeking (Dayas et al., 2008) and context-induced reinstatement of sucrose (Hamlin et al., 2006), alcoholic beer (Hamlin et al., 2007), and cocaine (Hamlin et al., 2008) seeking are all associated with increases in Fos expression in LH neurons. Additionally, transient inactivation of the LH prevents renewal of sucrose and alcoholic beer seeking (Marchant et al., 2009), suggesting that the LH, as a whole, is critical for reinstatement induced by reward-predictive stimulus contexts. However, evidence exists to implicate a specific role of LH Orx/Hcrt neurons in drug addiction, in which case Orx/Hcrt neurons in the LH are activated by exposure to stimuli that are associated with cocaine, alcohol, and morphine (Dayas et al., 2008; Harris et al., 2005; Jupp et al., 2011b). Although the present data do not reveal the precise functional role for Orx/Hcrt neurons in COC seeking *vs.* SCM seeking, they clearly show a differential recruitment pattern of Orx/Hcrt neurons by the COC S^+ *vs.* SCM S^+ during the first reinstatement test or over time with distinct differences in the three hypothalamic subregions. One possible explanation that only the COC S^+ increased the percentage of activated Orx/Hcrt neurons in the LH is that the COC S^+ and not the SCM S^+ specifically recruited Orx/Hcrt neuronal populations within the LH, thus possibly maintaining the perseveration of drug seeking. The recruitment of different neural ensembles by drug *vs.* non-drug reward has previously been described in the nucleus accumbens (NAC), where neurons differentially encode information about natural rewards *vs.* cocaine or alcohol (Carelli et al., 2000; Carelli and Wondolowski, 2006; Robinson and Carelli, 2008). One may speculate that a similar scenario exists within the LH, such that distinct neuronal populations process information about drug *vs.* non-drug stimuli (i.e., Orx/Hcrt neurons that process S^+ information for cocaine and non-Orx/Hcrt neurons that process S^+ information for SCM). This possibility remains to be tested by, for example, selective inactivation of neuronal ensembles that are activated by COC- *vs.* SCM-associated stimuli using the Daun02 inactivation method in *c-fos-lacZ* transgenic rats (Koya et al., 2009), followed by subsequent tests for selective reversal of SCM S^+ - *vs.* COC S^+ -induced reinstatement.

Earlier studies using conditioned place preference have reported that activation of the LH Orx/Hcrt neurons was strongly associated with drug- and palatable food-paired

environments (Harris and Aston-Jones, 2006; Harris et al., 2005), an observation somewhat different from that reported here with presentation of a SCM S⁺. The reason for this inconsistency is likely due to procedural discrepancies (i.e., conditioned place preference *vs.* voluntary operant response; self- *vs.* experimenter-administration). Nonetheless, a role for LH orexin neurons specifically in drug seeking has been reported with similar experimental approaches (e.g., ABA renewal) as those used in the current study. Increased Fos expression in LH Orx/Hcrt neurons was associated with context-induced reinstatement of alcoholic beer seeking (Hamlin et al., 2007), but no specific activation of LH Orx/Hcrt neurons was observed during context-induced reinstatement of sucrose (also a palatable sweet solution) seeking (Hamlin et al., 2006). Similar results have been recently reported by others with context-induced reinstatement of sucrose seeking, again, failing to induce specific LH Orx/Hcrt neurons activation (Cason and Aston-Jones, 2013b; Hamlin et al., 2006), consistent with the data reported here during context-induced reinstatement of SCM seeking. On the other hand, context-induced reinstatement of cocaine seeking also failed to activate LH Orx/Hcrt neurons in an earlier study (Hamlin et al., 2008), while the cocaine conditioning time lasted 2 h/day, a finding that contrasts with the effects of the cocaine S⁺ that were observed here. The reasons for this discrepancy are unclear.

Orx/Hcrt transmission in the LH has been implicated in the mediation of the primary reinforcing effects of palatable food (i.e., food-maintained operant responding; Borgland et al., 2009; Cason and Aston-Jones, 2013a, b; Cason et al., 2010; Choi et al., 2010; Thorpe et al., 2005). Therefore, LH Orx/Hcrt neurons may be preferentially recruited during food intake (i.e., consummatory behavior) and not food seeking (i.e., appetitive behavior), whereas LH Orx/Hcrt activation induced by drug-related stimuli controls appetitive and approach aspects of behavior in the case of drug seeking. This interpretation is consistent with findings that Hcrt-r1 blockade selectively prevented reinstatement induced by a COC or alcohol S⁺ but not reinstatement induced by an S⁺ conditioned to palatable food (Martin-Fardon and Weiss, 2014a, b). Lastly, Orx/Hcrt promotes motivation during specific environmental challenges and opportunities. Orx/Hcrt neurons in the LH are recruited by homeostatic imbalance and/or exposure to salient environmental stimuli, especially those that are associated with particular physiological needs (e.g., hunger; for review, see Mahler et al., 2014). Thus, the specific activation of LH Orx/Hcrt cells by the COC S⁺, and not the SCM S⁺, may point to the possibility that “deprivation” associated with drug abstinence may engage mechanisms that overlap with those that have evolved to promote alleviation of physiological deprivation states (Martin-Fardon and Weiss, 2016). By this account, stimuli conditioned to food reinforcers would be predicted to engage LH Orx/Hcrt neurons when rats are in a nutritionally restricted state. This hypothesis requires further testing.

The strong activation of Orx/Hcrt neurons in the DMH and PFA by the cocaine S⁺ was unexpected when considering the reported dichotomy between hypothalamic subregions, in which Orx/Hcrt neurons in the LH participate in the regulation of reward processes, and Orx/Hcrt neurons in the DMH and PFA mediate responses to stressful events (Harris and Aston-Jones, 2006; Harris et al., 2005; Plaza-Zabala et al., 2010). One interpretation for these findings is that the activation of Orx/Hcrt neurons in the DMH and PFA by the cocaine S⁺ reflects arousal or stress associated with exposure to the drug-associated stimulus context (Fox et al., 2005; Sinha et al., 2003), and may be the result of drug memory associated with

stress that occurred during cocaine conditioning, but not during SCM conditioning. Another possibility is that white noise itself, a stimulus that can produce stress-like responses (increased respiratory rates) in rats when presented briefly (Bondarenko et al., 2014a, b), produced activation of Orx/Hcrt neurons in the DMH/PFA. Transient inactivation of the DMH/PFA has been shown to inhibit increased respiratory rates induced by brief white noise presentation (Bondarenko et al., 2015) suggesting that when presented acutely, white noise can induce respiratory responses that are controlled by the DMH/PFA. The present study was designed differently and the rats were highly habituated to the white noise because of the long exposure to this stimulus during the conditioning phase. This scenario is supported by the data obtained in the SCM groups. If white noise by itself was stressful under the present contingencies, and produced partial activation of Orx/Hcrt cells in the DMH/PFA, substantial activation of Orx/Hcrt cells in the DMH/PFA of the SCM groups should have been measured in both Test 1 and Test 4, but this was not the case. An alternative interpretation for the activation of Orx/Hcrt neurons within the DMH and PFA by the cocaine S^+ is related to findings that the DMH and PFA are activated during extinction of drug seeking (e.g., Millan et al., 2011). Thus, the activation of Orx/Hcrt neurons in these regions may have occurred as a result of the repeated non-reinforced exposure to drug-predictive stimulus contexts that resembled an extinction-like paradigm (i.e., where the contingency between drug-predictive stimuli and the delivery of the drug reward was interrupted, and the drug-predictive stimuli were repeatedly presented). This interpretation is consistent with the observation that under extinction conditions (EXT, see Fig. 3) no activation of Orx/Hcrt neurons in the DMH/PFA was observed because the reinforcers and the reward-predictive stimuli were absent.

Viewed from this perspective, the present findings may reflect the presumed behavioral specialization among hypothalamic subregions, with a central role for the LH in the promotion (reinstatement) of drug seeking (e.g., Marchant et al., 2009) and a major role for the DMH/PFA in the inhibition of this behavior (Marchant et al., 2012). In support of this possibility, concurrent self-stimulation of the DMH and LH decreases the reinforcing actions of LH self-stimulation (Porrino et al., 1983), and administration of the inhibitory peptide cocaine- and amphetamine-regulated transcript (CART) in the DMH/PFA prevents the expression of extinction in a rat model of alcoholic beer seeking (Marchant et al., 2010). Considering the evidence that the DMH/PFA regulates extinction and decreases LH activity (Porrino et al., 1983), one may speculate that presentation of the cocaine S^+ that activated the DMH/PFA would, under normal circumstances, initiate the expression of extinction by inhibiting the LH (Millan et al., 2011; Porrino et al., 1983). In fact, all Orx/Hcrt neurons co-express dynorphin at both the mRNA and protein levels (Chou et al., 2001; Li and van den Pol, 2006), and dynorphin's actions at κ opioid receptors mediate the expression of extinction (e.g., Marchant et al., 2012). Possibly, neuroplasticity that developed during cocaine self-administration or conditioning may interfere with negative feedback from the DMH/PFA Orx/Hcrt neurons (via the dynorphin system), such that LH neurons, which are known to express κ opioid receptors (for review, see Mansour et al., 1995), were no longer inhibited. This possibility is tentatively supported by the lack of full inhibition of Orx/Hcrt neurons in the LH during the fourth reinstatement test despite the strong activation of DMH/PFA Orx/Hcrt neurons.

In summary, the purpose of the present study was to advance understanding of the regulatory role of the Orx/Hcrt system in behavior motivated by drugs of abuse *vs.* food, with a focus on its role in reward seeking controlled by environmental stimuli conditioned to drug and food reinforcers. Recent findings show that environmental stimuli associated with drugs of abuse produce a distinctly different reward-seeking profile than stimuli conditioned to food reinforcers with high hedonic value, characterized by rapid extinction of behavior when motivated by palatable food substances contrasting with perseverating, extinction-resistant reward seeking when motivated by drugs of abuse (see, Martin-Fardon and Weiss, 2016). This profile is thought to reflect the expected normal decay of non-reinforced behavior in the case of natural reward as opposed to the compulsive nature of addiction with drug seeking that appears impervious to extinction. The present findings suggest that Orx/Hcrt signaling in the hypothalamus may be a neurobiological mechanism underlying perseverating cocaine seeking produced by cocaine-predictive contextual stimuli. In contrast, the lack of Orx/Hcrt neuronal activation by response-reinstating exposure to contextual stimuli conditioned to SCM suggests that the Orx/Hcrt system may not participate in the control of reward seeking motivated by palatable food. Lastly, the findings tentatively suggest that cocaine-induced disruption of inhibitory inputs to the LH from the DMH/PFA regions interferes with extinction of non-reinforced behavior and contributes to the perseveration of cocaine seeking.

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Figure 1. Experimental design for (a) the single (Test 1) and (b) repeated reinstatement test sequence that terminated with Test 4. (c) Schematic illustrating the rostrocaudal level from which Fos⁺/Orx⁺ cells were counted. (d–f) Representative photomicrographs of Fos⁺ Orx⁺ cells in the LH following presentation of the COC S⁺ (e) and SCM S⁺ (f) vs. naïve (a). Scale bars = 100 μm.

Figure 2.

Differential perseveration of reinstatement by stimuli conditioned to cocaine (COC) vs. natural reward (SCM). **(a) Test 1** (single test). Conditioning: Mean ($\pm$ SEM) responses averaged across the final three self-administration/conditioning sessions. Responding was differentially reinforced in the presence of discriminative stimuli that signaled the availability (Reward + S⁺) or nonavailability (Non-reward + S⁻) of COC or SCM. * $p < 0.001$ vs. Non-reward + S⁻. Reinstatement: reintroduction of the COC S⁺ or SCM S⁺ produced similar, strong recovery of responding. + $p < 0.001$ vs. EXT and S⁻. **(b) Test 4** (repeated reinstatement tests). Conditioning: Mean ($\pm$ SEM) responses during reinforcer availability

(Reward + S⁺) vs. nonavailability (Non-reward + S⁻) averaged across the final three self-administration/conditioning sessions. * $p < 0.001$ vs. non-reward + S⁻. Reinstatement: on the 4th reinstatement session, reintroduction of the COC S⁺ produced a strong recovery of responding, while reintroduction of the SCM S⁺ did not produce any recovery of responding. + $p < 0.001$, vs. EXT and S⁻ for COC only. (c) **Reinstatement profile over all four sessions in the Test 4 group**. Reinstatement: + $p < 0.01$, vs. S⁻ and EXT; * $p < 0.01$ vs. SCM S⁺; ANOVA: group effect ($F_{1,14} = 17.7$, $p < 0.01$), reinstatement effect ($F_{5,70} = 38.9$, $p < 0.001$), group $\times$ reinstatement interaction ($F_{5,70} = 10.9$, $p < 0.001$). $n = 8/\text{group}$.

Figure 3.

Percentage of Orx/Hcrt neurons that were Fos⁺ following single (Test 1) or multiple (Test 4) reinstatement tests in the (a) lateral hypothalamus (LH), (b) dorsomedial hypothalamus (DMH), and (c) perifornical area (PFA). (a) **LH**: * $p < 0.01$ vs. all groups (Test 1 and 4); + $p < 0.05$ vs. all Test 4 groups; # $p < 0.05$ vs. COC S⁻, COC S⁺, SCM EXT, and SCM S⁺ for Test 1 and COC S⁺ for Test 4. (b) **DMH**: * $p < 0.001$ vs. all groups except COC S⁺ for Test 4; + $p < 0.01$ vs. all groups except COC S⁺ for Test 1; # $p < 0.05$ vs. all groups, except COC S⁻ for Test 1, and SCM EXT and SCM S⁻ for Test 4. (c) **PFA**: * $p < 0.05$ vs. COC EXT, SCM EXT,

and SCM S⁺ for Test 1, and COC EXT, COC S⁻, SCM EXT, SCM S⁻, and SCM S⁺ for Test 4; ⁺ $p < 0.05$ vs. COC S⁻ and COC EXT for Test 4; [#] $p < 0.05$ vs. all groups except SCM EXT for Test 1 and COC EXT, SCM EXT, and SCM S⁻ for Test 4. $n = 5-8/\text{group}$.

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Table 1 Number of Orx⁺/Hcrt⁺ cells.

Group		
Naive		
LH	424.5 ± 83.3	
DMH	123.8 ± 38.1	
PFA	70.5 ± 16.4	
	<i>TEST 1</i>	<i>TEST 4</i>
COC EXT		
LH	371.5 ± 93.7	171.9 ± 51.6
DMH	183.6 ± 37.6	126.0 ± 38.1
PFA	80.9 ± 18.4	71.8 ± 19.5
COC S⁻		
LH	376.6 ± 83.6	347.7 ± 60.3
DMH	177.4 ± 40.4	228.9 ± 51.0
PFA	68.5 ± 13.2	112.3 ± 20.0
COC S⁺		
LH	353.7 ± 51.7	377.0 ± 51.3
DMH	121.4 ± 17.6	183.6 ± 47.6
PFA	55.1 ± 10.8	76.8 ± 18.3
SCM EXT		
LH	361.8 ± 74.3	180.3 ± 55.2
DMH	178.4 ± 43.5	69.9 ± 36.1
PFA	77.3 ± 20.0	43.8 ± 16.7
SCM S⁻		
LH	247.9 ± 60.9	245.0 ± 56.7
DMH	132.9 ± 28.8	109.6 ± 27.8
PFA	57.0 ± 12.7	58.9 ± 8.0
SCM S⁺		
LH	482.6 ± 47.2	399.7 ± 56.6
DMH	195.9 ± 20.9	193.2 ± 21.2
PFA	93.4 ± 7.2	99.8 ± 7.2