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EFFECTS OF COCAINE PLACE CONDITIONING, CHRONIC ESCALATING-DOSE "BINGE" PATTERN COCAINE ADMINISTRATION AND ACUTE WITHDRAWAL ON OREXIN/HYPOCRETIN AND PREPRODYNORPHIN GENE EXPRESSIONS IN LATERAL HYPOTHALAMUS OF FISCHER AND SPRAGUE–DAWLEY RATS

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Abstract—Recent evidence suggests an important role for hypothalamic orexins/hypocretins in modulation of drug reward and addiction-like behaviors in rodents. Our recent study has shown that the aversive state of arousal during acute morphine withdrawal is associated with increased orexin gene expression in lateral hypothalamus (LH) of Fischer 344 (F344) inbred rats, with no change in the expression of preprodynorphin (ppDyn), a gene co-expressed with LH orexin. Therefore, we determined whether orexin and ppDyn mRNA levels in LH or medial hypothalamus (including perifornical and dorsomedial areas) of F344 or Sprague–Dawley (SD) outbred rats, are altered following: 1) cocaine (10 mg/kg, i.p.) conditioned place preference (CPP); 2) chronic (14 days) cocaine exposure using both "binge" pattern administration in steady-dose (45 mg/kg/day) and escalating-dose (45–90 mg/kg/day) regimens; and 3) acute (1 day) and chronic (14 days) withdrawal from cocaine with opioid receptor antagonist naloxone treatment (1 mg/kg). We found that orexin mRNA levels were decreased after cocaine place conditioning in the LH of SD rats. A decreased LH orexin mRNA level was also observed after chronic escalating-dose cocaine (but not CPP pattern regimen without conditioning, or steady-dose regimen) in both strains. In F344 rats only, acute withdrawal from chronic escalating-dose cocaine administration resulted in increases in both LH orexin and ppDyn mRNA levels, which were unaltered by naloxone or after chronic withdrawal. Our results suggest that (1) alteration of LH orexin gene expression is region-specific after cocaine place conditioning in SD rats and dose-dependent after chronic exposure in both strains; and (2) increased LH orexin and ppDyn gene expressions in F344 rats may contribute to negative affective states in cocaine withdrawal.

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Abbreviations: ANOVA, analysis of variance; CPP, conditioned place preference; CRF, corticotropin-releasing factor; DMH, dorsomedial hypothalamus; EDTA, ethylenediaminetetraacetic acid; F344, Fischer 344; ICSS, intracranial self-stimulation; LH, lateral hypothalamus; MH, medial portion of the hypothalamus; MOP-r, mu opioid receptor; PFA, perifornical area; ppDyn, preprodynorphin; RNase, ribonuclease; SD, Sprague–Dawley; TCA, trichloroacetic acid; VTA, ventral tegmental area.

Key words: conditioned place preference, mRNA, naloxone, strain difference.

The orexins/hypocretins are neuropeptides mainly expressed in cells of the lateral hypothalamus (LH), perifornical area (PFA) and dorsomedial hypothalamus (DMH), with extensive CNS projections (de Lecea et al., 1998; Sakurai et al., 1998). It has been established that hypothalamic orexins (orexin A and orexin B) are involved in the regulation of sleep–wakefulness, arousal, feeding and stress (Saper et al., 2005). A growing body of evidence suggests that orexins may have an important role in the modulation of drug reward and drug-seeking behaviors. Studies of acute morphine administration have shown an attenuated increase in extracellular dopamine levels in the nucleus accumbens by orexin receptor blockade in the ventral tegmental area (VTA) (Narita et al., 2006). Orexin A in the VTA has been found to be critical for the development of cocaine-induced synaptic plasticity and behavioral sensitization (Borgland et al., 2006). Further, the interaction between orexins and their receptors has been found to underlie motivated behaviors (Harris et al., 2005; Boutrel et al., 2005; Narita et al., 2006; Dayas et al., 2008; Leri et al., 2006a). Of interest, aversive and arousal states during morphine withdrawal or stress are associated with increased LH orexin neuronal activity and gene expression, suggesting that the stress responsive LH orexin system may be a critical component of the neural circuitry involved in the aversive effects of drug withdrawal and stress-related drug seeking behavior (Georgescu et al., 2003; Boutrel et al., 2005; Zhou et al., 2006). In fact, i.c.v. infusion of orexin A (hypocretin-1) decreases brain reward function, as reflected by an elevated intracranial self-stimulation (ICSS) threshold in the LH, further supporting the hypothesis that orexin negatively regulates the activity of brain reward circuitry (Boutrel et al., 2005).

Cocaine exposure can alter the activity of the brain reward circuitry, resulting in heightened vulnerability to drug intake and relapse (Stewart et al., 1984; Koob et al., 2004). In fact, when trained to self-administer cocaine with long access to high doses of cocaine, rats display progressive escalations in contingent cocaine intake (Ahmed and Koob, 1998; Mutschler and Miczek, 1998; Mantsch et al., 2001, 2004; Kenny et al., 2003; Vanderschuren and Everitt, 2004; Ben-Shahar et al., 2004). Compared with short

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access to cocaine self-administration, rats with long access to cocaine self-administration or short access cocaine self-administration with long non-contingent drug exposure, show augmented reinstatement of cocaine seeking behavior, suggesting that enhanced susceptibility to cocaine relapse is associated with the increased amount of cocaine intake (Mantsch et al., 2001; Ahmed and Cador, 2006; Kippin et al., 2006; Knackstedt and Kalivas, 2007). After long access to cocaine, rats display a decrease in brain reward function during acute (1-day) withdrawal (as measured by ICSS in the LH). Notably, this brain reward deficit is correlated with the escalation in total cocaine intake (Ahmed et al., 2002).

Our laboratory has recently investigated the modulation of orexin gene expression in animal models of drug addiction. We have found that acute withdrawal from chronic escalating-dose morphine increases orexin gene expression in the LH of Fischer 344 (F344) rats (Zhou et al., 2006). To extend our research to the effects of cocaine, we here report a set of experiments addressing two research questions. The first was whether the orexin mRNA level in either the LH or PFA-DMH is altered after the development of cocaine conditioned place preference (CPP). On the basis of evidence implicating LH orexin neuronal activity (but not PFA-DMH) in the rewarding property of cocaine, morphine and food in Sprague–Dawley (SD) rats (Harris et al., 2005), we predicted an effect of cocaine CPP on LH orexin gene expression. Nearly all (94%) of LH orexin neurons express the preprodynorphin (ppDyn) gene (Chou et al., 2001), and activation of the kappa opioid receptor elevates ICSS threshold in the LH, reflecting a reduction in the rewarding impact of brain stimulation (Todtenkopf et al., 2004). Therefore, we examined the ppDyn mRNA level in the LH of the same animals after the expression of cocaine CPP. The SD rat chosen was also based on our pilot study, in which SD rats showed significant CPP for cocaine at 10 mg/kg, whereas F344 rats did not. This is consistent with previous reports by others (Guitart et al., 1992; Kosten et al., 1994; Harris et al., 2005).

The second research question was whether chronic cocaine exposure and its withdrawal alter the orexin and ppDyn gene expressions in the LH. To answer this question, we examined the effects of two regimens of chronic 14-day “binge” pattern cocaine administration: (1) chronic steady-dose “binge” pattern cocaine administration at a moderate dose (45 mg/kg/day) from day 1 to day 14 that mimics the dosing pattern often seen in human cocaine abusers, with respect to repeated administrations over several hours; and (2) chronic escalating-dose “binge” pattern cocaine administration beginning at 45 mg/kg/day on day 1 and escalating to a high dose of 90 mg/kg/day on day 10, that models the dose range found in long access to cocaine self-administration in rats (e.g., Ahmed and Koob, 1998; Mantsch et al., 2004) and the escalating pattern of intake utilized by human cocaine addicts to prevent symptoms of withdrawal (Koob and Kreek, 2007). It has been found that F344 rats self-administer cocaine at a higher level than either SD or Lewis inbred rats after cocaine self-administration behavior is established (Kosten et al., 1997, 2007; Haile et al., 2005). The enhanced maintenance of cocaine self-administration by F344 rats shows the values of examining the different strains when modeling vulnerability to develop addiction. Therefore, using F344 rats, we examined the effects of chronic (14 days) cocaine administration, and acute (24 h) and chronic (14 days) cocaine withdrawal on LH orexin and ppDyn mRNA levels in both steady-dose and escalating-dose procedures. Additionally, this inbred strain was compared with SD rats after chronic cocaine and acute withdrawal in escalating-dose procedure.

A related question was whether opioid receptors are involved in orexin or ppDyn mRNA modulation in acute cocaine withdrawal, since about 50% of LH orexin neurons express the mu opioid receptor (MOP-r) gene (Georgescu et al., 2003), and acute morphine withdrawal increases both orexin and MOP-r gene expression in the LH (Zhou et al., 2006). Therefore, we assessed the effect of the selective opioid receptor antagonist naloxone (1 mg/kg) on the LH orexin and ppDyn mRNA levels in response to acute cocaine withdrawal from chronic escalating-dose “binge” pattern cocaine in F344 rats only.

EXPERIMENTAL PROCEDURES

Experiment 1: Effects of cocaine-induced CPP expression on mRNA levels of orexin and ppDyn in the LH and medial hypothalamus of SD rats

Animals. Male SD rats (190–220 g, Charles River Laboratories, Kingston, NY, USA) were housed under a standard 12-h light/dark cycle (lights on from 07:00 h to 19:00 h) with free access to food and water for 7 days, and then habituated to the environment with daily handling for 5 days. The room temperature was maintained at 22±1 °C. The habituation, training, and testing were conducted during the light phase of the cycle. The experimental procedures were approved by the Committee on Animal Care and Use of the McLean Hospital at Harvard Medical School. We followed the Principles of Laboratory Animal Care (NIH publication no 86-23, 1996). During all procedures of experiments, the number of animals and their suffering by treatments were minimized.

Apparatus. The place conditioning apparatus (model ENV-013MD, Med Associates, VT, USA) was a PVC plastic rectangular chamber that consisted of three compartments. Two conditioning compartments were separated by a smaller middle one. One of the conditioning compartments had white walls and a stainless-steel mesh floor; the other had black walls and a “grid” floor, which consisted of stainless-steel rods. The middle compartment had gray walls and a plain gray floor. Animals could access the entire apparatus when the guillotine doors were removed. Through a computer interface, time spent in each compartment was recorded by sets of infrared beams located near the floor of each compartment.

Cocaine CPP procedure. The place conditioning procedure consisted of three phases: pre-conditioning, conditioning and post-conditioning tests, as previously described (Ren et al., 2002; Leri et al., 2006b). *Pre-conditioning*: Before the onset of the place conditioning, rats were placed in the middle compartment with the guillotine doors removed, allowing free access to two conditioning compartments for a 15 min period daily for three consecutive days. The time spent in each conditioning compartment was recorded on the third day. The conditioning compartments occupied for more and less time were designated as the preferred and non-preferred sides, respectively. The expression of any preference in this phase was considered as natural place preference. These data were used to assign

animals into groups with approximately equal biases for each conditioning compartment. Two groups of SD rats were trained in this CPP paradigm with cocaine conditioning at 10 mg/kg of cocaine ($n=11$) or with saline conditioning ($n=10$). *Conditioning*: The conditioning phase began on the day following the baseline testing. During conditioning, rats were injected i.p. with 10 mg/kg of cocaine (Sigma Chemical Co, St. Louis, MO, USA, dissolved in 0.9% saline) once, and immediately confined to one conditioning compartment for 30 min. The specific conditioning compartment chosen to be associated with cocaine was counterbalanced across rats. On alternate days, rats received saline injection and were confined to the other compartment for 30 min. The conditioning phase, with cocaine and saline on alternative days, lasted for 8 days. Control animals received saline injections in both conditioning compartments. *Post-conditioning test*: Four days after the last conditioning trial, animals were placed in the middle compartment with free access to both conditioning compartments for 15 min. The time spent in each side compartment was recorded.

Thirty minutes following this test, all rats were rapidly decapitated. The LH and medial portion of the hypothalamus (MH, including the PFA, DMH, paraventricular nucleus and arcuate nucleus), were collected for subsequent mRNA analyses. The data from one cocaine-treated and two saline-treated rats were removed because they were more than three standard deviations below the mean of all rats.

Experiment 2: Effects of cocaine administration in CPP pattern regimen without conditioning on mRNA levels of orexin and ppDyn in the LH of SD rats

Animals. A new cohort of SD rats identical to those used in experiment 1. Briefly, male SD rats (190–210 g, Charles River Laboratories) were adapted to a standard 12-h light/dark cycle (lights on from 9:00 h to 21:00 h) for 7 days prior to the beginning of the experiment. We followed the Principles of Laboratory Animal Care (NIH publication no 86–23, 1996). The specific protocol was approved by the Rockefeller University Animal Care and Use Committee.

Procedure of cocaine administration in CPP regimen. The dose of drug exposure (10 mg/kg of cocaine or saline), the pattern or route of injections and time points were identical to those of cocaine administration in experiment 1. The only difference from experiment 1 was that the SD rats received injections in the home cage, without conditioning procedures. Two groups of the SD rats were injected in this CPP pattern regimen with cocaine at 10 mg/kg ($n=8$) or with saline ($n=8$). Four days after the last injection, all SD rats were rapidly decapitated. The LH was collected for subsequent mRNA analyses. The data from one saline-treated rat was removed because it was more than three standard deviations below the mean of all rats.

Experiment 3: Effects of acute (1-day), chronic (14-day) steady-dose "binge" pattern cocaine administration and acute (1-day) withdrawal from chronic cocaine administration on mRNA levels of orexin and ppDyn in the LH of F344 rats

Animals. Male F344 rats (190–220 g, Charles River Laboratories) were housed individually in a stress-minimized facility with free access to food and water. Animals were adapted to a standard 12-h light/dark cycle (lights on from 09:00 h to 21:00 h) for 7 days prior to the beginning of the experiment. The specific protocol was approved by the Rockefeller University Animal Care and Use Committee.

Procedure for acute and chronic steady-dose "binge" pattern cocaine administration, and acute withdrawal. The "binge" pattern regimen of drug exposure (saline or cocaine) consisted of i.p. injections in the home cage three times daily with two 1-h intervals, beginning 30 min after the light cycle (09:30, 10:30, and 11:30 h) (Branch et al., 1992). For animals treated with cocaine, the dose was 3×15 mg/kg every day. This dosing schedule was chosen to mimic the pattern often observed in human cocaine abusers with respect to repeated administrations over several hours, with relation to the circadian rhythm of rest and activity.

In experiment 3.1 *acute (1-day) steady-dose "binge" cocaine*, rats received "binge" cocaine (3×15 mg/kg/day) or saline injections for 1 day ($n=8$) and were then sacrificed at 12:00 h on day 1, 30 min after the last "binge" cocaine or saline injection.

In experiment 3.2 *chronic (14 days) steady-dose "binge" cocaine*, rats received "binge" cocaine (3×15 mg/kg/day) or saline injections for 14 days ($n=8$) and were then sacrificed at 12:00 h on day 14, 30 min after the last "binge" cocaine or saline injection.

In experiment 3.3 *acute (1-day) cocaine withdrawal*, rats received "binge" cocaine (3×15 mg/kg/day) or saline injections for 14 days ($n=8$) and were then sacrificed at 12:00 h on day 15, 1 day after the last "binge" cocaine or saline injection.

All rats were rapidly decapitated after brief exposure to CO_2, and the LH was collected for subsequent mRNA analyses. The data from two saline-treated rats in experiment 3.2 were removed because they were more than three standard deviations below the mean of all rats.

Experiment 4: Effects of chronic (14-day) escalating-dose "binge" pattern cocaine administration, and its acute (1-day) and chronic (14-day) withdrawal with naloxone on mRNA levels of orexin and ppDyn in the LH of F344 and SD rats

Animals. Male F344 and SD rats (190–220 g, Charles River Laboratories) were housed individually in a stress-minimized facility with free access to food and water. Animals were adapted to a standard 12-h light/dark cycle (lights on from 9:00 h to 21:00 h) for 7 days prior to the beginning of the experiment. The specific protocol was approved by the Rockefeller University Animal Care and Use Committee.

Procedure of chronic escalating-dose "binge" pattern cocaine administration, and acute and chronic withdrawal with naloxone pretreatment. The "binge" pattern of drug exposure (saline or cocaine), injection route and time points were identical to those of steady-dose "binge" cocaine administration. For animals treated with cocaine, the doses were increased after every 3 days. Therefore, the cocaine-treated rats received initial dosing at 45 (3×15) mg/kg/day on days 1–3, 60 (3×20) mg/kg/day on days 4–6, 75 (3×25) mg/kg/day on days 7–9, and then 90 (3×30) mg/kg/day on days 10–14. As reported before (Schlussman et al., 2005), this dosing schedule was chosen to model the dose range self-administered by rats given long access (6–10 h) to cocaine (e.g., Ahmed and Koob, 1998; Mantsch et al., 2004).

In experiment 4.1 *chronic (14 days) escalating-dose "binge" cocaine in F344 and SD rats*, F344 rats received escalating-dose "binge" cocaine (from 3×15 up to 3×30 mg/kg/day) ($n=6$) or saline ($n=8$) injections for 14 days and were then sacrificed at 12:00 h on day 14, 30 min after the last "binge" cocaine or saline injection. The LH was collected for subsequent mRNA analyses. The data from one saline-treated rat were removed because it was more than three standard deviations below the mean of all rats.

Similar to F344 rats, SD rats received escalating-dose "binge" cocaine ($n=6$) or saline ($n=6$) for 14 days. The LH was collected on day 14, 30 min after the last injection for subsequent mRNA analyses.

In experiment 4.2 *acute (1-day) cocaine withdrawal with naloxone in F344 and SD rats,* F344 rats received "binge" cocaine (from 3×15 up to 3×30 mg/kg/day) or saline injections for 14 days and were then sacrificed at 12:00 h on day 15, 1 day after the last "binge" cocaine or saline injection. On day 14, an i.p. injection of

naloxone (1 mg/kg) or saline was administered 30 min after the last "binge" cocaine or saline injection. The naloxone dose chosen was based on a published study, in which a single 1 mg/kg dose was observed to moderately increase ACTH levels in cocaine naïve F344 rats (Zhou et al., 2005).

For this experiment, F344 rats were assigned to four groups for treatment: (1) *Acute cocaine withdrawal*: cocaine injections for 14 days with one saline injection 30 min after the last "binge" cocaine, followed by 1-day withdrawal, $n=6$; (2) *Saline*: saline injections for 14 days with one saline injection 30 min after the last "binge" saline, followed by 1-day withdrawal, $n=6$; (3) *Naloxone+ Acute cocaine withdrawal*: cocaine injections for 14 days with one naloxone injection (1 mg/kg) 30 min after the last "binge" cocaine, followed by 1-day withdrawal, $n=7$; and (4) *Naloxone*: saline injections for 14 days with one naloxone injection (1 mg/kg) 30 min after the last "binge" saline, followed by 1-day withdrawal, $n=6$. The LH was collected for subsequent mRNA analyses. The data from one naloxone-treated rat were removed because it was more than three standard deviations below the mean of all rats.

Different from F344 rats, SD rats were only assigned to two groups for cocaine treatment without naloxone: (1) *Acute cocaine withdrawal*: $n=6$; and (2) *Saline*: $n=6$. The LH was collected for subsequent mRNA analyses.

In experiment 4.3 *chronic (14-day) cocaine withdrawal in F344 rats*, F344 rats received "binge" cocaine ($n=6$) (from 3×15 up to 3×30 mg/kg/day) or saline ($n=6$) injections for 14 days and were then sacrificed at 12:00 h on day 28, 14 days after the last "binge" cocaine or saline injection. The LH was collected for subsequent mRNA analyses.

Orexin and ppDyn mRNA measurements

Preparation of RNA extracts. In each experiment, rats were sacrificed by decapitation after a brief exposure to CO_2 (within 15 s). Each rat brain was removed from the skull and placed in a chilled rat brain matrix (ASI Instruments, Houston, TX, USA). A coronal slice containing the hypothalamus was removed from the matrix and placed on a chilled Petri dish. Dissection was carried out using razor blades and forceps under a dissecting microscope. The brain regions of interest were identified according to *Rat Brain in Stereotaxic Coordinates* (Paxinos and Watson, 1986), as described in detail recently (Zhou et al., 2006). The LH and MH (including the PFA, DMH, paraventricular nucleus and arcuate nucleus) were dissected on ice, homogenized in guanidinium thiocyanate buffer and extracted with acidic phenol and chloroform. After the final ethanol precipitation step, each extract was resuspended in DEPC-treated H_2O and stored at −80 °C.

Solution hybridization ribonuclease (RNase) protection–trichloroacetic acid (TCA) precipitation assay. The solution hybridization RNase protection–TCA precipitation protocol has been described in detail in earlier reports (Branch et al., 1992; Zhou et al., 2006). A 531 bp fragment from rat hypocretin (or orexin) cDNA was cloned into the polylinker region of pBC SK+ (Stratagene, La Jolla, CA, USA). A 1700 bp fragment from the rat ppDyn cDNA was cloned into the polylinker region of pSP64 plasmids (Promega, Madison, WI, USA) in both the sense and antisense orientations. The plasmid pS/E (a pSP65 derivative) was used to synthesize riboprobe for the 18S rRNA to determine total RNA. ^{33}P-labeled cRNA antisense probes and unlabeled cRNA sense standards were synthesized using an SP6, T3 or T7 transcription system. A denaturing agarose gel containing 1.0 M formaldehyde showed that a single full-length transcript had been synthesized from each plasmid.

RNA extracts were dried in 1.5 ml Eppendorf tubes and resuspended in 30 μl of 2× TESS (10 mM N-Tris[hydroxy-methyl]methyl-2-aminoethane sulfonic acid, pH 7.4; 10 mM EDTA; 0.3 M NaCl; 0.5% sodium dodecyl sulfate [SDS]) that contained 150,000–300,000 cpm of a probe. Samples were covered with mineral oil and hybridized overnight at 75 °C. For RNase treatment, 250 μl of a buffer containing 0.3 M NaCl; 5 mM EDTA; 10 mM Tris–HCl (pH 7.5), 40 μg/ml RNase A (Worthington Biochemicals, Freehold, NJ, USA) and 2 μg/ml RNase T1 (Calbiochem, San Diego, CA, USA) was added and each sample was incubated at 30 °C for 1 h. TCA precipitation was effected by the addition of 1 ml of a solution that contained 5% TCA and 0.75% sodium pyrophosphate. Precipitates were collected onto a filter in sets of 24 using a cell harvester (Brandel, Gaithersburg, MD, USA) and were measured in a scintillation counter with liquid scintillant (Beckman, Palo Alto, CA, USA).

The procedure to measure mRNA levels involved a comparison of values obtained from experimental samples (brain extracts) to those obtained for a set of calibration standards. The calibration standards had known amounts of an *in vitro* sense transcript whose concentration was determined by optical absorbance at 260 nm. The set of calibration standards included those with no added sense transcript and those that contained between 1.25 and 80 pg of the sense transcript (Zhou et al., 2006). To determine the total picograms of each mRNA in each extract, the amounts calculated from the standard curves were multiplied by 1.3 for orexin or 1.4 for ppDyn to correct for the difference in length between the sense transcript (531 or 1700 bases for the orexin or ppDyn respectively) and the full-length mRNA (700 or 2400 base for the orexin or ppDyn). A new standard curve was generated each time experimental samples were analyzed and all extracts of a particular tissue were assayed for each mRNA as a group in a single assay.

Total cellular RNA concentrations were measured by hybridization of diluted extracts to a ^{33}P-labeled probe complementary to 18S rRNA at 75 °C. The calibration standards for this curve contained 10 μg of *E. coli* tRNA plus either 0.0, or from 2.5–40 ng of total RNA from rat brain whose concentration was determined by optical absorbance at 260 nm.

Data analysis

In experiment 1, phase differences in CPP scores were analyzed using a two-way analysis of variance (ANOVA) for drug side (cocaine, saline) and for phase (pre-conditioning, post-conditioning test) with repeated measures, followed by a planned comparison. Group differences in mRNA levels of both the orexin and ppDyn in the LH were analyzed together using a two-way ANOVA with repeated measures, followed by a planned comparison. In experiment 4.2, group differences in mRNA levels of the orexin and ppDyn were analyzed using two-way ANOVA for acute withdrawal (cocaine, saline) and for antagonist (naloxone, saline) followed by Newman-Keuls post hoc tests. In other experiments, differences between two groups were analyzed using a Student's *t*-test for each tissue. To determine correlation between measured variables, linear regression analysis was performed. The accepted level of significance for all tests was $P<0.05$. All statistical analyses were performed using Statistica (version 5.5, StatSoft Inc.).

RESULTS

Experiment 1: Effect of cocaine CPP on mRNA levels of orexin and ppDyn in the LH and MH of SD rats

Cocaine CPP expression. Two groups of SD rats were trained in a CPP paradigm with cocaine conditioning at 10 mg/kg of cocaine ($n=11$) or with saline conditioning ($n=10$). In the cocaine-treated group, the mean time spent in the cocaine-paired and saline-paired compartment on the pre-conditioning and post-conditioning test days are shown in Fig. 1A. Two-way ANOVA showed a significant effect of cocaine [$F(1,10)=5.09$, $P<0.05$] and a tendency

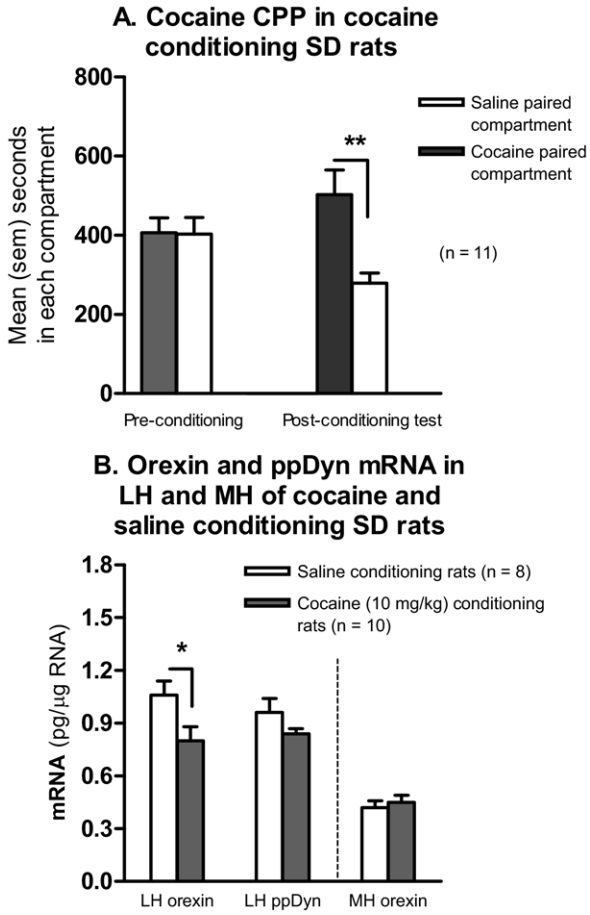

Fig. 1. (A) Expression of CPP induced by cocaine in SD rats (experiment 1). Mean (S.E.M.) seconds spent in the saline- and cocaine-paired compartments were recorded on a 15-min CPP test 4 days after the last cocaine conditioning test (right) and a 15-min pre-conditioning test 1 day before the cocaine conditioning phase (left). During 8 days of conditioning, animals received 0 or 10 mg/kg of cocaine on alternative days. In the post-conditioning test, rats (n=11) conditioned with cocaine displayed a preference for the cocaine-paired compartment. Significant difference is indicated: ** P<0.02, Cocaine-paired vs. Saline-paired compartment. (B) Effects of cocaine on orexin and ppDyn mRNA levels (pg/μg total RNA) in the LH and medial hypothalamus (MH) of cocaine conditioning (n=10) and saline conditioning SD rats (n=8) (experiment 1). For the orexin mRNA, there was a significant decrease after cocaine conditioning. Significant difference is indicated: * P<0.05, Cocaine conditioning vs. Saline conditioning rats. Note, an mRNA measurement from one cocaine-treated rat in Fig. 1A was removed because it was more than three standard deviations below the mean of all rats. Behavioral data from rats conditioned with saline are not shown in A.

toward the drug side×phase interaction, without reaching statistical significance [F(1,10)=4.38, P=0.06]. While rats spent similar time in each compartment in the pre-conditioning test, rats conditioned with cocaine displayed a preference for the cocaine-paired compartment in the post-conditioning test [planned comparison, F(1,10)=9.00, P<0.02]. Rats conditioned with saline showed no preference (data not shown).

Orexin and ppDyn mRNA levels in the LH and MH. For both orexin and ppDyn mRNA levels in the LH, two-way ANOVA showed a significant effect of cocaine conditioning [F(1,16)=7.27, P<0.05]. For the orexin mRNA, there was a significant decrease [planned comparison, F(1,16)=5.13, P<0.05) (Fig. 1B)]. For the ppDyn mRNA, there was a tendency toward a decrease, without reaching statistical significance [planned comparison, F(1,16)=3.85, P=0.07]. In the MH, however, there was no significant effect on orexin mRNA level (Fig. 1B).

Since ppDyn is co-expressed with orexin in the LH (Chou et al., 2001), the relationship between ppDyn and orexin mRNA levels was examined in this region. No significant correlation was found in the animals after either saline or cocaine treatment (data not shown).

Experiment 2: Effects of cocaine administration in CPP pattern regimen without conditioning on mRNA levels of orexin and ppDyn in the LH of SD rats

As shown in Table 1, there was no significant effect on either orexin or ppDyn mRNA levels in the LH of SD rats after cocaine administration in a pattern identical to CPP regimen.

Experiment 3: Effect of acute (1-day), chronic (14-day) steady-dose "binge" pattern cocaine administration and acute (1-day) withdrawal from chronic cocaine on mRNA levels of orexin and ppDyn in the LH of F344 rats

As shown in Fig. 2A and 2B, there was no significant effect on either orexin or ppDyn mRNA levels after acute (1 day), chronic (14 days) steady-dose "binge" cocaine or acute (1-day) withdrawal.

Experiment 4: Effect of chronic (14-day) escalating-dose "binge" pattern cocaine administration, its acute (1-day) withdrawal with naloxone, and chronic (14-day) withdrawal on mRNA levels of orexin and ppDyn in the LH of F344 and SD rats

Orexin and ppDyn mRNA level in the LH of F344 rats. There was a significant decrease in the LH orexin mRNA level after 14 days of escalating-dose cocaine administration (t=5.84, df=11, P<0.05) (Fig. 3A). For 1-day acute withdrawal, two-way ANOVA showed a significant main effect of acute withdrawal on the orexin mRNA level [F(1,19)=8.75, P<0.01) (Fig. 3A). However, there was no significant effect of either naloxone or the acute withdrawal×naloxone interaction. Furthermore, the orexin mRNA level was unaltered by chronic (14 days) cocaine withdrawal.

As shown in Fig. 3B, ppDyn mRNA level showed a similar pattern of variation to that of orexin. There was no

Table 1. Effects of cocaine (10 mg/kg) in CPP pattern regimen without conditioning on orexin and ppDyn mRNA levels (pg/μg total RNA) in the lateral hypothalamus of SD rats (experiment 2)

	Saline control (n=7)	Cocaine in CPP regimen (n=8)
Orexin	1.18±0.07	1.17±0.11
ppDyn	0.96±0.09	1.05±0.09

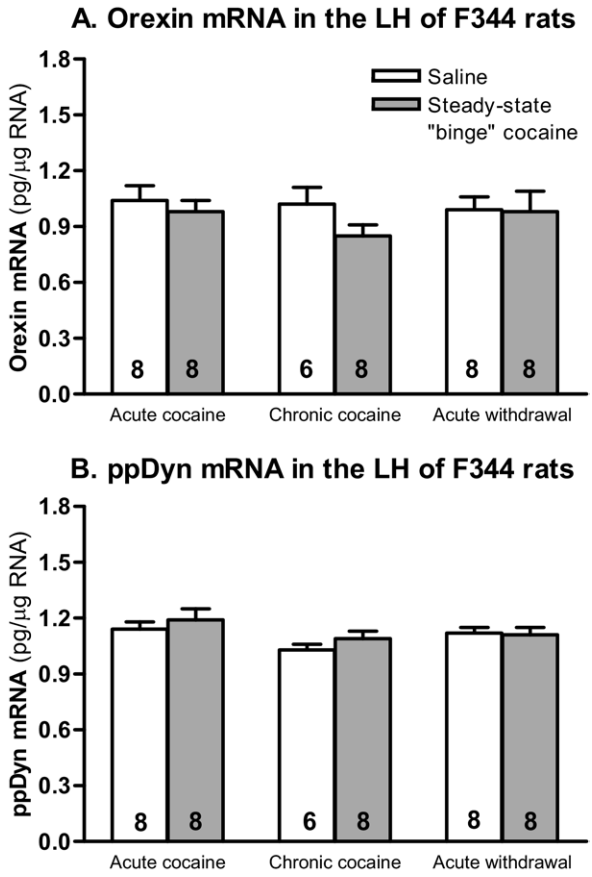

Fig. 2. Effects of acute (1-day), chronic (14-day) steady-dose "binge" pattern cocaine administration (45 mg/kg/day) and its acute (1-day) withdrawal on mRNA levels (pg/μg total RNA) of orexin (A) and ppDyn (B) in the LH of F344 rats (experiment 3). Data shown in graphs are treatment group mean±S.E.M. n=6–8.

significant effect on the LH ppDyn mRNA level after 14 days of escalating-dose cocaine administration. For 1-day acute withdrawal, two-way ANOVA showed a significant main effect of acute withdrawal on the ppDyn mRNA level [$F(1,21)=5.32$, $P<0.05$] (Fig. 3B). However, there was no significant effect of either naloxone or the acute withdrawal×naloxone interaction. Finally, the ppDyn mRNA level was unaltered by chronic (14 days) cocaine withdrawal.

The cocaine-treated animals displayed a significant positive correlation between orexin and ppDyn mRNA levels after 14-day chronic withdrawal ($r^2=0.92$, $n=6$, $P<0.005$) and a marginally significant positive correlation after 1-day acute withdrawal ($r^2=0.66$, $n=6$, $P=0.05$). No significant correlation between the orexin and ppDyn mRNA levels was found in the animals after saline, naloxone or acute cocaine withdrawal with naloxone (data not shown).

Orexin and ppDyn mRNA levels in the LH of SD rats. There was a significant decrease in the LH orexin mRNA level after 14 days of escalating-dose cocaine administration ($t=5.54$, $df=10$, $P<0.05$) (Fig. 4A). One-day acute withdrawal, however, had no significant effect on the orexin mRNA level. For the LH ppDyn mRNA level, there was a slight, but not statistically significant, decrease after 14 days of escalating-dose cocaine administration (Fig. 4B). One-day acute withdrawal had no significant effect on the ppDyn mRNA level.

No significant correlation between the orexin and ppDyn mRNA levels was found in the SD rats after saline, chronic cocaine or acute cocaine withdrawal (data not shown).

DISCUSSION

The first objective of these experiments was to investigate the effects of cocaine place conditioning on orexin mRNA level in two hypothalamic orexin subpopulations of SD rats: the LH and PFA-DMH regions. Orexin mRNA level modulated by expression of cocaine place preference was primarily found in the LH, whereas that in PFA-DMH was not affected, suggesting that LH orexin gene expression is involved in cocaine-conditioned behaviors in a region-specific manner. This observation is consistent with recent studies, showing that the cues previously conditioned with either cocaine or morphine reward (as measured by CPP)

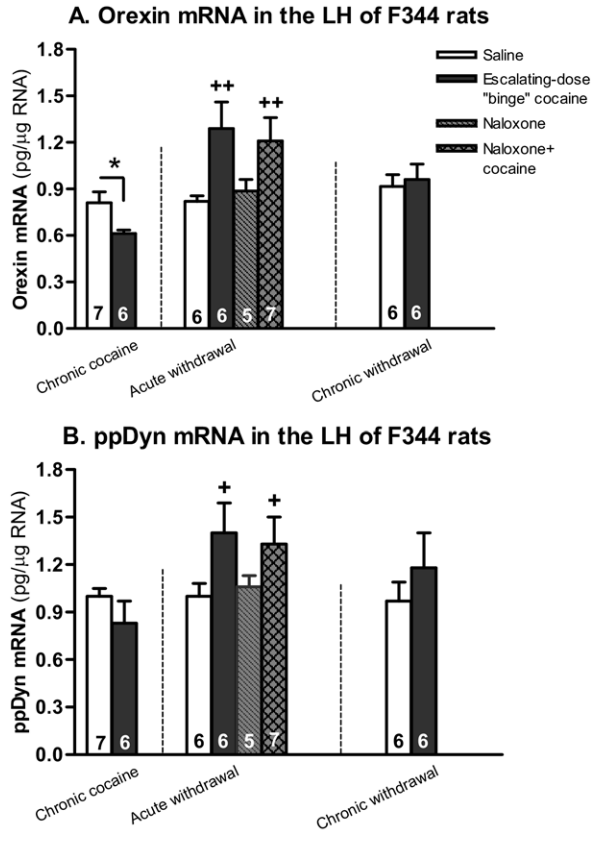

Fig. 3. Effects of chronic (14-day) escalating-dose "binge" pattern cocaine administration (45 up to 90 mg/kg/day), its acute (1-day) withdrawal with opioid receptor antagonist naloxone (1 mg/kg) and chronic (14-day) withdrawal on mRNA levels (pg/μg total RNA) of orexin (A) and ppDyn (B) in the LH of F344 rats (experiment 4). Data shown in graphs are treatment group mean±S.E.M. Significant differences are indicated: * $P<0.05$, Chronic cocaine vs. Saline control; + $P<0.05$ or ++ $P<0.01$, two-way ANOVA with a significant main effect for acute cocaine withdrawal. n=5–7.

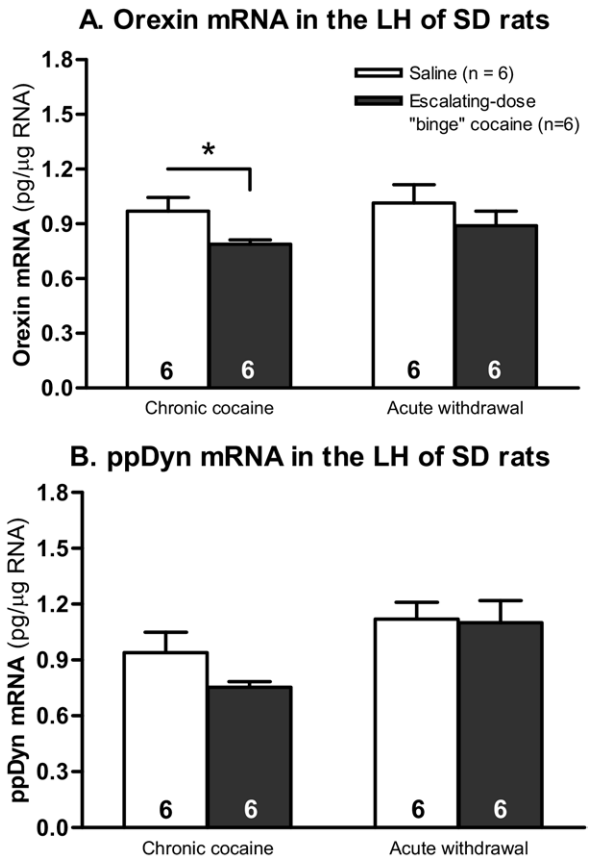

Fig. 4. Effects of chronic (14-day) escalating-dose "binge" pattern cocaine administration (45 up to 90 mg/kg/day) and its acute (1-day) withdrawal on mRNA levels (pg/μg total RNA) of orexin (A) and ppDyn (B) in the LH of SD rats (experiment 4). Data shown in graphs are treatment group mean±S.E.M. Significant difference is indicated: * $P<0.05$, Chronic cocaine vs. Saline control. $n=6$.

activated Fos-like immunoreactivity specifically within the LH orexin neurons of SD rats, whereas Fos expression in PFA-DMH orexin neurons was unaltered in response to these same cues (Harris et al., 2005, 2007). Together, these results suggest that the orexin gene in LH neurons, as different from PFA-DMH subpopulations, responds to drug-induced reward processes.

Decreased brain reward function (as reflected by the increases in ICSS thresholds) after cocaine administration is considered central to cocaine addiction (Koob and Le Moal, 2001). Interestingly, central infusion of orexin A (or Hcrt-1) has been recently found to elevate ICSS thresholds in the LH, indicating a decrease in excitability of brain reward systems (Boutrel et al., 2005). In fact, a recent study by Leri et al. (2006a) using Pavlovian-to-instrumental transfer procedure has shown that cocaine seeking behavior is associated with decreased LH orexin mRNA level in SD rats. Here, using the CPP model, we found a decrease in the LH orexin mRNA level shortly after the expression of cocaine place conditioning in SD rats. When SD rats received cocaine administration in a pattern identical to CPP regimen, but without cocaine-associated conditioning, the orexin mRNA level was unaltered in the LH. Together, the results indicate that decreased orexin gene expression (and therefore possibly decreased biosynthesis and release) may contribute to the expression of cocaine conditioning-related reward processes.

The second objective of these experiments was to investigate the effects of chronic cocaine exposure and its withdrawal on the orexin gene expression in the LH. First, we tested the steady-dose "binge" cocaine treatment in F334 rats. This moderate dose paradigm (45 mg/kg/day) was chosen for two reasons: (1) stress responsive corticotropin-releasing factor (CRF) is a potent neuropeptide that elevates ICSS thresholds in the LH (Macey et al., 2000); and (2) chronic cocaine at this moderate dose decreases CRF gene expression and function in the hypothalamus (Zhou et al., 1996). In the present study, however, LH orexin mRNA level was unaltered after chronic steady-dose cocaine treatment for 14 days.

Therefore, we examined the effects of the escalating-dose "binge" cocaine paradigm in both F344 and SD rats, in which the initial dose (45 mg/kg/day) was doubled after 10 days of dose escalation to 90 mg/kg/day that was maintained throughout the last 4 days. In contrast to steady-dose treatment, chronic escalating-dose cocaine led to a significant decrease in LH orexin mRNA level in both strains. After 1 day of acute withdrawal, the LH orexin mRNA level returned to the control in SD rats. Different from SD rats, however, the F344 rats showed a "rebound" increase in acute withdrawal. This increase was not found at the same time point after acute withdrawal from chronic steady-dose cocaine treatment in F344 rats. Furthermore, the orexin mRNA increase was insensitive to opioid receptor blockade, since we observed no effect of naloxone on this change. The increase suggests an enhanced biosynthesis in the orexin gene; though it cannot be determined from assays of mRNA levels alone which steps (gene transcription, processing, and/or degradation of mRNA) are affected. Although the stimulatory factors influencing elevation of orexin mRNA level are not yet fully elucidated, it is possible that an increased release is responsible for the increase in mRNA to compensate for acute withdrawal-induced peptide depletion. Together, our data demonstrate that acute withdrawal from chronic administration of high (but not moderate) dose cocaine increased orexin mRNA level in the LH of F344 rats, as a rebound after a decrease by chronic cocaine exposure. This "rebound" increase was strain-specific, since we observed no change in the SD rats, which only showed a decrease after chronic escalating-dose cocaine. In support of this interpretation, it has been found that when compared with SD and Lewis strains, F344 rats have a hyperresponsivity to a variety of stressors (for review, Kosten and Ambrosio, 2002). Therefore, it is possible that the large amount of cocaine administered led to more profound dysregulation of stress pathways in F344 rats that contribute to this enhanced orexin gene expression during acute withdrawal (for review, Kreek and Koob, 1998).

Central infusion of dynorphin A (1–17) prevents the development of cocaine-induced CPP (Zhang et al., 2004), and kappa opioid receptor (KOP-r) agonists elevate the

ICSS threshold in the LH (Todtenkopf et al., 2004). The ppDyn gene is co-expressed in most LH orexin neurons (Chou et al., 2001) and dynorphin A (1–13) has regulatory effects on the orexin system (Li and van den Pol, 2006). Chronic i.c.v. infusion of cocaine results in a significant decrease in ppDyn mRNA level in the rat hypothalamus (Romualdi et al., 1996). In the present study, we found a decrease (although this effect did not reach statistical significance) in LH ppDyn mRNA level, after the expression of cocaine place conditioning in SD rats. In contrast, an increase in LH ppDyn mRNA level was observed in acute withdrawal from chronic escalating-dose cocaine in F344 rats, similar to the LH orexin mRNA changes. Our results are in agreement with the established hypothesis that increased expression of dynorphin system in the nucleus accumbens that opposes reward processes (anhedonia) contributes to the decrease in the rewarding impact of cocaine conditioning (as measured by CPP) (Carlezon et al., 1998; Nestler and Carlezon, 2006). In the LH, it seems likely that alteration of the ppDyn gene expression in acute withdrawal is cocaine specific, since our recent study on morphine withdrawal showed LH orexin mRNA increase without LH ppDyn mRNA change in F344 rats (Zhou et al., 2006). Together, activation of both ppDyn and orexin gene expressions in the LH of F344 rats is likely involved in the negative affective state during cocaine withdrawal, which is found to be important in the maintenance of cocaine self-administration behaviors (for reviews, Koob et al., 2004; Koob and Kreek, 2007).

The gradual increase in cocaine intake which results from long access to cocaine self-administration is analogous to the loss of control over drug-taking found in human addicts (Ahmed and Koob, 1998; Ahmed et al., 2002; Kenny et al., 2003; Paterson and Markou, 2003; Mantsch et al., 2004; Vanderschuren and Everitt, 2004; Ferrario et al., 2005). In contrast to short access, with long access to cocaine self-administration, cocaine intake gradually escalates over days to high doses, with a parallel progressive elevation of ICSS thresholds (Ahmed et al., 2002). Specifically, this progressive elevation in basal ICSS thresholds observed in acute withdrawal (about 20 h after daily self-administration session) is correlated with escalation in cocaine intake (Ahmed et al., 2002), which is not found in short access to cocaine self-administration. In the present study, acute (24 h) withdrawal from high-dose cocaine showed an increase in basal LH orexin and ppDyn mRNA levels in F344 rats, while acute withdrawal (24 h) from moderate-dose cocaine did not show such changes. Our results suggest that the activation of orexin and ppDyn genes in the LH neurons leading to a new set point of basal gene expression levels is involved in brain reward dysfunction during acute withdrawal from chronic exposure to escalated high doses of cocaine. It has been found that there is a greater stress response in acute cocaine withdrawal from involuntary rather than voluntary cocaine administration (Mutschler and Miczek, 1998). Therefore, it should be kept in mind that in our study, the LH orexin and ppDyn mRNA levels were measured in rats that had received cocaine administered by the experimenters.

CONCLUSION

In summary, the data presented here showed that: (1) expression of cocaine place conditioning was associated with a region-specific decrease in LH orexin gene expression in SD rats; (2) in contrast to moderate steady-dose cocaine administration, chronic high escalating-dose cocaine resulted in a decreased LH orexin gene expression in both SD and F344 strains; (3) of interest, a rebound increase in LH orexin gene expression was observed in acute withdrawal from chronic escalating-dose cocaine in F344 rats only, which was not sensitive to opioid receptor antagonist naloxone. In accordance with this finding, we have recently reported an increase in LH orexin mRNA levels during acute withdrawal from chronic escalating-dose morphine in F344 rats (Zhou et al., 2006); and (4) in contrast to acute opiate withdrawal, acute withdrawal from chronic escalating-dose cocaine increased LH ppDyn gene expression in F344 rats, which was unaltered by opioid receptor blockade. Because both orexins and dynorphins are neuropeptides well known to negatively regulate brain reward functions, our results suggest that the LH orexin–dynorphin system is a critical component of neural circuitry underlying the negative affective consequences of drug withdrawal from chronic escalating-dose cocaine.

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