

# Hypocretin/Orexin Selectively Increases Dopamine Efflux within the Prefrontal Cortex: Involvement of the Ventral Tegmental Area

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Hypocretins (HCRTs) modulate a variety of behavioral and physiological processes, in part via interactions with multiple ascending modulatory systems. Further, HCRT efferents from the lateral hypothalamus innervate midbrain dopamine (DA) nuclei, and DA cell bodies express HCRT receptors. Combined, these observations suggest that HCRT may influence behavioral state and/or state-dependent processes via modulation of DA neurotransmission. The current studies used *in vivo* microdialysis in the unanesthetized rat to first characterize the effect of intracerebroventricular infusion of HCRT-1 (0.07, 0.7 nmol) on extracellular levels of DA within the prefrontal cortex (PFC) and nucleus accumbens (Acc). Electroencephalographic/electromyographic measures of sleep–wake state were collected along with select behavioral measures (eg locomotor activity, grooming). HCRT-1 dose-dependently increased PFC dialysate DA levels, and these increases were closely correlated with increases in time spent awake. In contrast, Acc DA levels were unaffected. Additional studies examined whether HCRT-1 acts directly within the ventral tegmental area (VTA) to selectively increase PFC DA efflux and modulate behavioral state. Unilateral infusion of HCRT-1 (0.1, 1.0 nmol) within the VTA increased PFC, but not Acc, DA levels. Importantly, intra-VTA infusion of HCRT-1 increased the time spent awake and grooming. Moreover, HCRT-induced increases in both time spent awake and time spent grooming were significantly correlated with post-infusion PFC DA levels. The current observations predict a prominent modulatory influence of HCRT on PFC-dependent cognitive and affective processes that results, in part, from actions within the VTA. Additionally, these observations suggest that the activation of VTA DA neurons contributes to the behavioral state-modulatory actions of HCRT.

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## INTRODUCTION

The hypocretin (HCRT) (orexin) peptide family consists of two peptides (HCRT-1, HCRT-2) synthesized within the perifornical region of the lateral hypothalamus (de Lecea *et al*, 1998; Sakurai *et al*, 1998). These peptides have been implicated in the regulation of behavioral state and state-dependent processes. For example, central administration of HCRT-1 exerts potent wake-promoting actions (Piper *et al*, 2000; España *et al*, 2001). Moreover, dysregulated HCRT neurotransmission is associated with the sleep/arousal disorder narcolepsy (for reviews, see Hungs and Mignot, 2001; Scammell, 2001). In addition, central HCRT administration increases indices of both stressful and appetitive states, suggesting that HCRT may also participate

in physiological and behavioral processes associated with high-arousal environmental conditions (Sakurai *et al*, 1998; Ida *et al*, 2000; Jaszberenyi *et al*, 2000; Samson *et al*, 2002; España *et al*, 2003). Combined, these observations suggest a pivotal role for the HCRT system in the regulation of behavioral state and state-dependent processes associated with alert, active waking.

Dopaminergic systems also modulate behavioral state and state-dependent processes, particularly under high-arousal appetitive and aversive conditions (Trulson, 1985; Smith *et al*, 1992; Arnsten *et al*, 1994; Isaac and Berridge, 2003). Acting within the prefrontal cortex (PFC) and nucleus accumbens (Acc), dopamine (DA) exerts prominent modulatory actions on a variety of higher-level cognitive and affective processes (Ikemoto and Panksepp, 1999; Smith-Roe and Kelley, 2000; Baldwin *et al*, 2002). Thus, it is of interest that HCRT fibers are found in close association with DA neurons located within the ventral tegmental area (VTA) and substantia nigra (Peyron *et al*, 1998; Fadel and Deutch, 2002; Baldo *et al*, 2003). Further, VTA and substantia nigra neurons express HCRT1 and HCRT2 receptor mRNA (Marcus *et al*, 2001). Finally, *in vitro*,

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HCRT-1 activates VTA (though not substantia nigra) DA neurons (Uramura *et al*, 2001; Korotkova *et al*, 2002, 2003). Combined, these observations suggest that HCRT modulates DA neurotransmission and that this action may contribute to HCRT-induced alterations in behavioral state and state-dependent processes.

A critical question unaddressed in the previous studies is the extent to which HCRT impacts DA neurotransmission in the intact animal. Dopaminergic neurons within the VTA project to a variety of terminal fields, including the PFC, Acc and striatum. Importantly, these various terminal fields display differential sensitivity to environmental and pharmacological manipulations. In particular, DA terminals within the PFC display the greatest sensitivity to both appetitive and aversive (eg stress) conditions (Thierry *et al*, 1976; Dunn, 1988; Roth *et al*, 1988; Cenci *et al*, 1992). Under similar conditions, Acc DA terminals typically display reduced, and striatal terminals minimal, reactivity (Dunn, 1988; Abercrombie *et al*, 1989; Cenci *et al*, 1992). Each of these regions support a variety of distinct cognitive and affective processes. Thus, it is essential to delineate the actions of this peptide on DA neurotransmission across distinct forebrain terminal fields.

Toward this goal, the current studies first sought to characterize the degree to which intracerebroventricular (ICV)-administered HCRT-1 activates DA neurotransmission across various terminal fields utilizing *in vivo* microdialysis in the unanesthetized rat. Electroencephalographic (EEG) and electromyographic (EMG) measures of sleep-wake state as well as behavioral measures of locomotion and grooming were collected to preliminarily assess the degree to which HCRT-induced activation of DA neurotransmission might contribute to the behavioral actions of this peptide. In these studies, ICV HCRT-1 dose-dependently increased extracellular DA levels within the PFC. Interestingly, Acc DA levels were unaffected by HCRT-1 administration. The VTA is a major source of DA fibers projecting to both PFC and Acc. Additional studies were conducted to better determine the degree to which the selective increase in DA efflux within the PFC involves actions of HCRT-1 directly within the VTA. In these studies, HCRT-1 was infused directly into VTA and DA efflux measured within the PFC and Acc.

## MATERIALS AND METHODS

### Animals and Surgery

Male Sprague-Dawley rats weighing 260–280 g (Charles River, Wilmington, MA) were housed in groups of 2–3 on a 13/11-h light/dark cycle (lights on at 0600 h) and provided *ad lib* access to food and water for 1–3 weeks following arrival. At 1–2 days prior to testing, a microdialysis probe (see below) was lowered unilaterally either into PFC (+3.2 A/P;  $\pm$ 1.0 M/L; –5.0 D/V at an angle of 4° from vertical) or into Acc (+1.7 A/P;  $\pm$ 1.4 M/L; –7.9 D/V) under halothane anesthesia. All coordinates are referenced to Bregma with level skull. Probes were inserted at a rate of approximately 500  $\mu$ m/min to minimize tissue damage and ensure adequate levels of DA within the cortical dialysate. In animals with PFC microdialysis probes, a bipolar EEG electrode was implanted into the contralateral frontal cortex

(+3.0 A/P;  $\pm$ 1.5 M/L) and a bipolar EMG electrode was implanted into the dorsal neck muscle (see below). A ground screw was implanted over the cerebellum. The free ends of the EEG, EMG and ground wires were threaded into a plastic connector. A guide cannula (Plastics One, Roanoke, VA) was implanted unilaterally, aimed at either the lateral ventricle contralateral to the dialysis probe (–1.0 A/P;  $\pm$ 1.35 M/L; –1.4 D/V) or the VTA (–5.4 A/P;  $\pm$ 1.6 M/L; –3.8 D/V at an angle of 5° from vertical) ipsilateral to the probe. For ICV studies, a 26-gauge cannula and 33-gauge infusion needle were used. For intra-VTA studies, a 23-gauge cannula and 30-gauge infusion needle were used, due to a tendency for needle clogging at higher peptide concentrations. Probe placement was counterbalanced across the left and right hemispheres. The connector, cannula, and dialysis probe were cemented into position using acrylic cement (Plastics One). A stainless steel wire stylet was inserted and attached to the cannula using a threaded plastic connector (Plastics One). Following probe implantation, animals were transferred to the testing chamber (see below) and artificial extracellular fluid (AECF) was perfused through the dialysis probe overnight at a flow rate of 1.5  $\mu$ l/min. All surgical and experimental procedures were performed in accordance with University of Wisconsin Institutional Animal Care and Use Committee guidelines, and the National Institutes of Health (1996) *Guide for the Care and Use of Laboratory Animals*.

### Experimental Procedures

Following probe implant, animals were placed in a Plexiglas testing chamber (32  $\times$  32  $\times$  40 cm<sup>3</sup>) overnight. Each Plexiglas testing chamber was housed in a wooden, sound-attenuating outer chamber containing a 15 W light bulb, a speaker through which white noise (60 dB) was played, and a 12 V fan running at reduced speed. A 10-cm hole in the top panel of the outer chamber permitted entry of EEG cables. A second hole in the front panel of the outer chamber permitted videotaping of the animal.

On the morning of testing, between 0900 and 1000 h, an infusion needle (Plastics One) was connected to a PE20 tubing threaded through a stainless steel spring. For ICV studies, animals received a unilateral 2  $\mu$ l infusion of AECF, 0.07 nmol HCRT-1 or 0.7 nmol HCRT-1 (Orexin-A; Penninsula Laboratories, Merseyside, UK; dissolved in AECF). For the intra-VTA experiment, animals received a unilateral 250 nl infusion of AECF, 0.1 nmol HCRT-1 or 1.0 nmol HCRT-1 (Orexin-A; Bachem, King of Prussia, PA; dissolved in AECF). In all cases, an air bubble (approximately 150 nl) was used to separate infusate from water in the PE20 tubing. The needle was then inserted into the cannula and secured with a plastic threaded sleeve, the needle tip extending 2.5 mm (ICV) or 4 mm (intra-VTA) beyond the end of the cannula. Infusions were made using a microprocessor-controlled pump (Harvard Apparatus, South Natick, MA) at a rate of 1  $\mu$ l/min (ICV) or 150 nl/min (intra-VTA). At least four 16-min dialysis samples were collected prior to infusion. Infusions were typically made following at least two consecutive samples in which the majority of the time was spent asleep. Collection of the first post-infusion sample began just prior to the infusion. Sample collection continued for at least eight samples following infusion or

until DA levels had returned to baseline, generally between 1500 and 1600. For purposes of statistical analyses, DA values were calculated as a percent of baseline. The baseline value was determined by averaging three pre-infusion samples from epochs in which the animal spent the majority of time asleep.

### Microdialysis and High-Pressured Liquid Chromatography (HPLC) Analysis of DA Concentrations

DA was measured in dialysate samples using HPLC with electrochemical detection, as described previously (Berridge and Stalnaker, 2002). Briefly, AECF (147 mM NaCl, 1.3 mM CaCl<sub>2</sub>, 0.9 mM MgCl<sub>2</sub>, 2.5 mM KCl; pH 7.4) was delivered at a rate of 1.5 µl/min through PE10 tubing to a length of Spectra/Por hollow fiber (Spectrum Labs, Rancho Dominguez, CA; MW cutoff 13 000; o.d. 250 µm) or modified cellulose membrane (Gambro, Lakewood, CO; MW cutoff 6000; o.d. 250 µm). The dialysis membrane was sealed and attached to the PE10 tubing with 5-min epoxy. The length of functional dialysis membrane in contact with tissue was 4 mm for PFC and 2 mm for Acc. A 75 cm length of fused silica (150 µm o.d., 75 µm i.d.) provided outflow to a sample collection vial outside the sound-attenuating chamber. *In vitro* recovery tests were performed prior to implantation; only probes with recovery > 10% were used.

Dialysis samples were collected at intervals of approximately 16 min prior to and following vehicle or HCRT administration. Samples of 20 µl were injected onto an HPLC-EC system consisting of an ESA Model 582 pump set at 0.65 ml/min, a Velosep C18 100 × 3.2 mm cartridge, and an ESA 5100A Coulochem II detector with two electrodes in series: -0.025 V, +0.220 V. The mobile phase consisted of 200 mM sodium phosphate (pH 3.0–5.2), 0.1 mM EDTA, 0.3 mM sodium octyl-sulfate, and 5% v/v methanol. The quantitation limit for DA was approximately 0.2 pg (approx. 3 × noise). The mean amount of DA recovered per 20 µl baseline sample (low-arousal) was 1.04 ± 0.05 pg (*n* = 35) within the PFC and 9.52 ± 1.58 pg (*n* = 11) within the Acc (see Figure 1 for sample PFC chromatogram).

### EEG/EMG and Behavior Analyses

In the subset of animals with PFC probes (both ICV- and intra-VTA-treated animals), a bipolar surface-to-depth EEG electrode was inserted into the contralateral frontal cortex, as described previously (Berridge and Foote, 1996). EMG was recorded using two 4 cm lengths of flexible, insulated wire (Cooner Wire, Chatsworth, CA) threaded into the dorsal neck muscle and positioned such that an approximately 3 mm portion of exposed wire was in direct contact with tissue. EEG and EMG signals were amplified and filtered (1.0–100.0 Hz, EEG; 1.0–30.0 Hz, EMG) and recorded on a chart recorder and VCR recording tape.

EEG and EMG were scored for the following behavioral state categories: (1) slow-wave sleep (high-voltage EEG, low-voltage EMG); (2) REM sleep (low-voltage EEG combined with EMG activity approximately 50% lower amplitude than that observed in slow-wave sleep, with occasional short-duration, large-amplitude deflections due to muscle twitches); (3) quiet waking (low-voltage EEG with EMG

activity of an average amplitude twice that observed in slow-wave sleep); (4) active waking (low-voltage EEG, sustained high-voltage EMG of approximately twice that observed in quiet waking, with frequent movement deflections). To be scored as a distinct epoch, the appropriate cortical EEG and EMG activity patterns needed to persist for a minimum of 15 s. Time spent in each state was scored and totaled for each 16-min dialysis sample collection period by an observer blind to experimental conditions.

Behavioral analyses were also performed on the subset of animals with PFC probes. In these analyses, the frequency of quadrant entries and rears as well as time spent grooming were scored from a videotape, using a computer-based event recorder (Noldus Information, Wageningen, Netherlands). Behavioral analyses were conducted for the 30-min epoch immediately preceding infusion and the first three 30-min post-infusion epochs by an observer blind to experimental conditions.

### Histological Analyses and Data Selection

After testing, animals were deeply anesthetized with halothane and transcardially perfused with 60 ml of 4% formaldehyde. In the ICV study, animals received 2 µl ICV of 0.5% Chicago Blue dye approximately 5 min prior to perfusion. Placement of microdialysis probes were histologically verified in 50-µm-thick coronal sections stained with Neutral Red dye. Infusion needle placement was verified by histological examination and, in the ICV study, by observing dye distribution. Data were included only when (1) histological analyses verified accurate placement of needles and microdialysis probes, and (2) DA concentrations during sleeping baseline epochs were stable ( $\pm 10\%$ ).

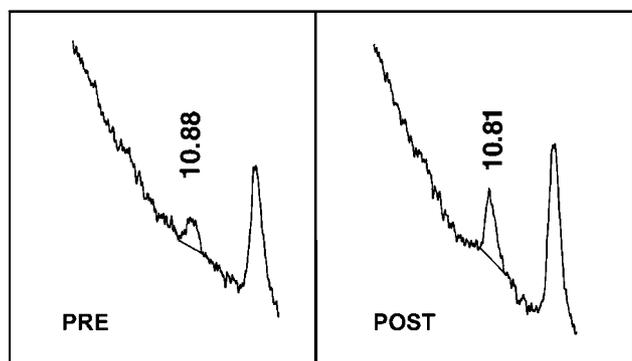
### Statistical Analyses

PFC DA (percent baseline) and EEG/EMG data were analyzed using a split-design two-way ANOVA for treatment (between-subjects, three levels) and time (within-subjects, 10 levels). Where between-subjects omnibus tests proved significant, pairwise *post hoc* group comparisons for each time point (sample number) were made using Fischer's LSD test. For Acc DA levels (percent baseline), animals only received the highest dose of HCRT-1. For these cases, DA data were analyzed using a one-way ANOVA, with time as a within-subjects variable (10 levels). Additional analyses for both dialysis and behavioral data utilized planned within-group matched-pair *t*-tests between the last baseline sample and each post-infusion sample. Best-fit regressions between post-infusion levels of waking or behaviors and PFC DA levels were also performed.

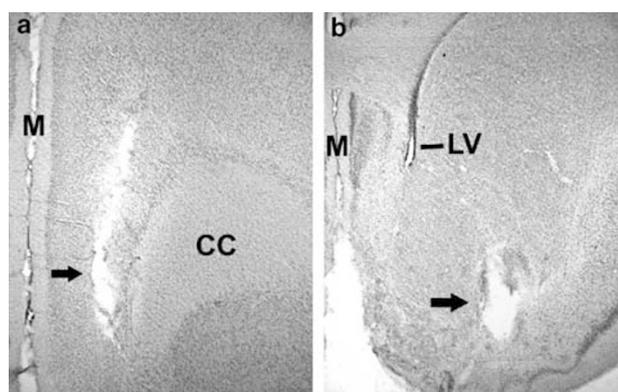
## RESULTS

### Experiment 1: ICV HCRT-1

*Effects of ICV HCRT-1 administration on PFC and Acc DA levels.* To characterize the effects of HCRT-1 on extracellular levels of DA within the PFC, dialysis samples were collected from the medial PFC (Figures 1 and 2a) prior to and after ICV infusions of either vehicle (*n* = 6) or HCRT-1 (0.07 nmol, *n* = 6; 0.7 nmol, *n* = 6). These doses



**Figure 1** Representative chromatogram showing PFC DA levels prior to (PRE) and after (POST) ICV HCRT-1 infusion (0.7 nmol). Numbers denote retention times for the DA peak. It is apparent that HCRT-1 increased the height of the DA peak.

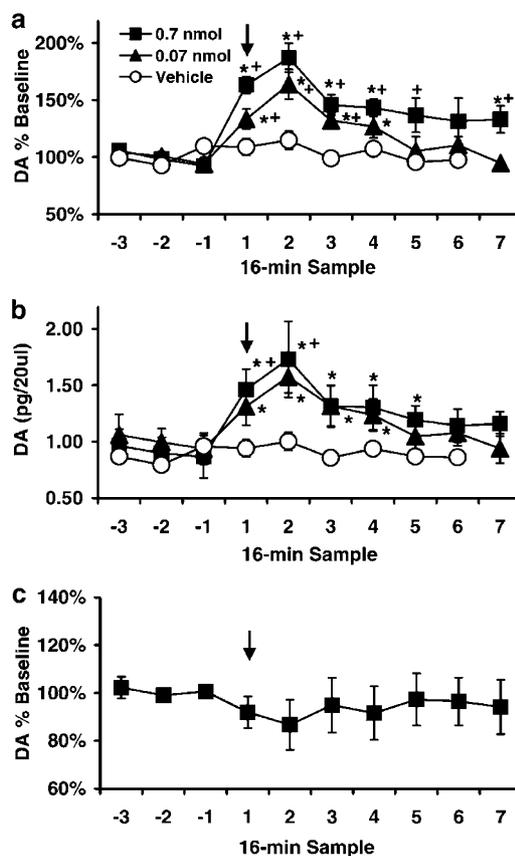


**Figure 2** Photomicrographs depicting placement of dialysis probes within PFC (a) and Acc (b). For PFC, probes were placed within the medial subdivision of this region. For Acc, probes were placed, in general, in the central portion corresponding to the core subdivision. Coronal sections (50  $\mu$ m) were stained with Neutral Red. Arrow indicates probe track. M = midline; CC = corpus colosum; AC = anterior commissure; LV = lateral ventricle.

were chosen based on previous studies demonstrating the dose-dependent wake-promoting actions of ICV HCRT-1 across this dose range (España *et al*, 2001). HCRT-1 dose-dependently increased DA levels in PFC dialysate samples (treatment  $F_{2,15} = 11.90$ ,  $P < 0.001$ ; time  $F_{9,135} = 16.53$ ,  $P < 0.001$ ; treatment  $\times$  time interaction  $F_{18,135} = 3.07$ ,  $P < 0.001$ ). Maximal increases of 64 and 87% above baseline were observed within the second post-infusion sample following low- and high-dose HCRT-1, respectively (Figure 3a and b). For both doses, PFC DA efflux remained elevated for approximately 60 min after infusion. Vehicle infusions did not alter PFC DA levels.

Additional studies examined the effects of the highest dose of ICV-administered HCRT-1 on extracellular DA levels in Acc (0.7 nmol,  $n = 6$ ; Figure 2b). In contrast to that observed within the PFC, there was no significant effect of this dose of HCRT-1 on Acc DA levels ( $F_{9,36} = 0.40$ ,  $P = 0.93$ ; Figure 3c).

**Relationship between ICV HCRT-1-induced alterations in behavior and PFC DA levels.** Similar to previous observations (España *et al*, 2001), ICV-administered HCRT-1 dose-



**Figure 3** Effects of ICV-administered HCRT-1 on PFC and Acc DA levels. Panels a and b depict the effects of ICV-administered vehicle, 0.07 nmol HCRT-1, and 0.7 nmol HCRT on DA efflux within PFC. Panel a displays mean ( $\pm$  SEM) PFC DA levels as a percentage of baseline. Panel b displays mean ( $\pm$  SEM) PFC DA levels in pg/20  $\mu$ l. Panel c displays mean  $\pm$  SEM Acc DA levels expressed as a percentage of baseline following 0.7 nmol HCRT-1. In all panels, data are shown for pre- (negative numbers) and post-infusion (positive numbers) 16-min samples. The start of infusion was aligned with the beginning of post-infusion Sample 1 (arrow). Within PFC, maximal increases in DA concentrations occurred during post-infusion Sample 2. In contrast, HCRT-1 had no significant effect on Acc DA levels.  $^+P < 0.05$  compared to vehicle;  $*P < 0.05$  compared to sample immediately preceding infusion (Sample -1).

dependently increased EEG/EMG measures of time spent awake (treatment  $F_{2,13} = 13.13$ ,  $P < 0.01$ ; time  $F_{9,117} = 9.76$ ,  $P < 0.001$ ; treatment  $\times$  time  $F_{18,117} = 4.16$ ,  $P < 0.001$ ). Two animals were excluded from these analyses due to inadequate EEG/EMG recordings. As previously described (España *et al*, 2001), the highest dose of HCRT-1 (0.7 nmol,  $n = 6$ ) significantly increased the time spent awake for approximately 120 min, while the lower dose (0.07 nmol,  $n = 5$ ) elicited a shorter-duration increase in waking (data not shown). Vehicle infusion ( $n = 5$ ) did not alter the time spent awake. Additionally, ICV-administered HCRT-1 (0.7 nmol,  $n = 5$ ; 0.07 nmol,  $n = 6$ ; vehicle,  $n = 6$ ) elicited moderate increases in locomotor activity, as measured by both quadrant entries (treatment  $F_{2,14} = 2.41$ ,  $P = 0.13$ ; time  $F_{3,42} = 10.87$ ,  $P < 0.001$ ; treatment  $\times$  time  $F_{6,42} = 3.00$ ,  $P < 0.05$ ) and rears (treatment  $F_{2,14} = 14.60$ ,  $P < 0.001$ ; time  $F_{3,42} = 10.37$ ,  $P < 0.001$ ; treatment  $\times$  time  $F_{6,42} = 7.98$ ,  $P < 0.001$ ), similar to that described previously (España

et al, 2001). Quadrant entries and rears were significantly increased over vehicle for the low dose during the first 30 min post-infusion epoch (Table 1). HCRT-1 also significantly increased the time spent grooming (treatment  $F_{2,14} = 0.83$ ,  $P = 0.46$ ; time  $F_{3,42} = 4.65$ ,  $P < 0.01$ ; treatment  $\times$  time interaction  $F_{6,42} = 0.80$ ,  $P = 0.57$ ). Within the high-dose group, grooming increased significantly over vehicle during the third post-infusion epoch (Table 1).

Correlation analyses were conducted to better assess the degree to which HCRT-1-induced increases in DA release are associated with HCRT-1-induced alterations in sleep-wake state and behavior. As shown in Figure 4a, there was a close temporal association between HCRT-1-induced increases in PFC DA levels and time spent awake. Consistent with this, best-fit regression analyses on data pooled across all groups and all post-infusion epochs (seven 16-min samples for waking; three 30-min samples for behavior) indicated a strong positive correlation between post-infusion PFC DA levels and time spent awake ( $r = 0.61$ ,  $r^2 = 0.37$ ,  $P < 0.001$ ; Figure 4b). Positive correlations were also observed when data from each group were analyzed separately. Less robust correlations were observed between PFC DA levels and quadrant entries ( $r = 0.46$ ,  $r^2 = 0.21$ ,

$P < 0.01$ ), rears ( $r = 0.45$ ,  $r^2 = 0.20$ ,  $P < 0.05$ ), and grooming ( $r = 0.30$ ,  $r^2 = 0.09$ ,  $P < 0.05$ ).

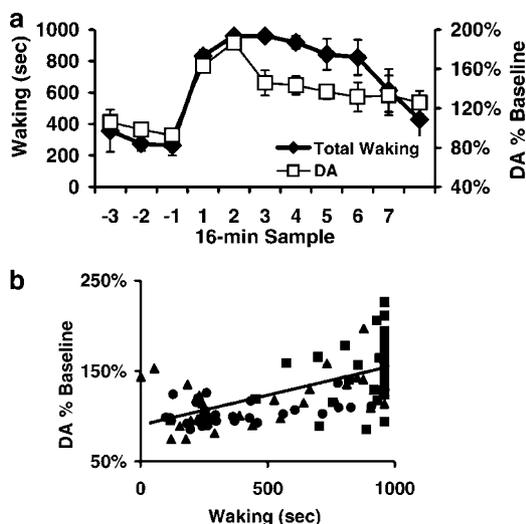
### Experiment 2: Intra-VTA HCRT-1

*Effects of intra-VTA HCRT-1 administration on PFC and Acc DA levels.* To assess the degree to which HCRT acts within the VTA to elicit a selective activation of DA terminals within the PFC, we examined the effects of intra-VTA infusion of HCRT-1 on PFC DA levels. Animals received intra-VTA infusions (see Figures 5 and 6) of vehicle ( $n = 6$ ) or HCRT-1 (0.1 nmol,  $n = 5$ ; 1.0 nmol,  $n = 6$ ). HCRT-1 dose-dependently increased DA levels in PFC dialysate samples (treatment  $F_{2,14} = 3.13$ ,  $P = 0.08$ , time  $F_{9,126} = 8.19$ ,  $P < 0.001$ , treatment  $\times$  time interaction  $F_{18,126} = 1.60$ ,  $P = 0.07$ ). Maximal increases of 35% above baseline were observed within the second post-infusion sample following low-dose HCRT-1, and 48% in the third sample following high-dose HCRT-1 (Figure 7a). PFC DA levels returned to baseline within approximately five samples following both doses of HCRT-1. Vehicle infusions did not significantly alter PFC DA levels, although there was a trend toward a modest increase in the second

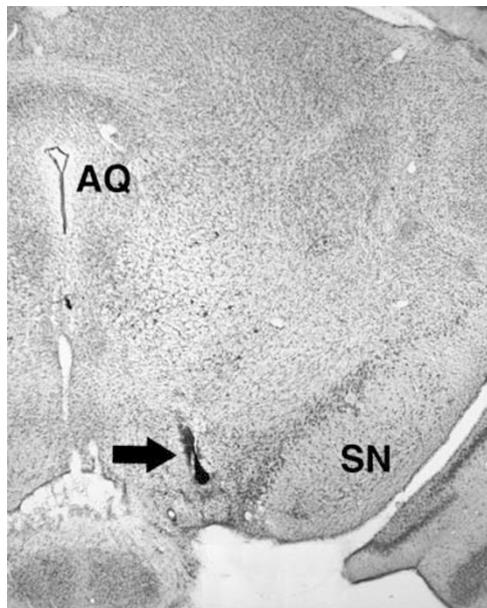
**Table 1** Behavioral Effects of ICV and Intra-VTA HCRT-1

Route		ICV											
		0.7 nmol				0.07 nmol				Vehicle			
Dose													
Testing epoch		-1	1	2	3	-1	1	2	3	-1	1	2	3
Quadrant entries	Mean	1.20	10.80*	5.00	1.60	3.67	13.17* <sup>+</sup>	3.33	0.00	1.67	2.50	0.83	2.50
	SEM	0.97	1.36	2.86	0.87	2.33	4.21	0.88	0.00	1.12	1.71	0.83	1.36
Rears	Mean	0.00	0.60	0.40	0.80 <sup>+</sup>	0.50	4.33* <sup>+</sup>	0.33	0.00	0.00	0.33	0.17	0.00
	SEM	0.00	0.24	0.24	0.49	0.34	1.02	0.21	0.00	0.00	0.33	0.17	0.00
Grooming	Mean	2.55	141.59	155.66*	85.40* <sup>+</sup>	14.33	93.17	104.82	1.12	45.61	113.82	49.64	21.40
	SEM	1.59	59.20	43.21	27.74	12.61	35.37	45.70	0.94	30.04	88.13	43.29	9.53
Route		Intra-VTA											
		1.0 nmol				0.1 nmol				Vehicle			
Dose													
Testing epoch		-1	1	2	3	-1	1	2	3	-1	1	2	3
Quadrant entries	Mean	1.17	5.50	3.17	0.50	3.60	3.40	7.60	1.20	3.00	1.00	1.33	0.00
	SEM	0.98	2.39	1.30	0.50	1.69	1.60	5.63	0.97	1.63	0.82	0.84	0.00
Rears	Mean	0.33	3.17	1.00	0.33	0.00	1.60*	2.00	0.00	0.00	0.00	0.17	0.00
	SEM	0.33	2.20	0.63	0.33	0.00	0.51	2.00	0.00	0.00	0.00	0.17	0.00
Grooming	Mean	167.83	284.19	445.27	225.52 <sup>+</sup>	96.30	179.23	234.23	62.98	139.22	82.30	214.68	23.51
	SEM	125.54	94.22	87.85	73.69	49.34	91.17	152.74	29.50	119.86	34.76	105.24	17.72

Shown are mean (and SEM) frequency of quadrant entries and rears, as well as time spent grooming (sec) across 30-min pre- (-1) and post (1-3) testing epochs. ICV-administered HCRT-1 significantly increased the frequency of quadrant entries and rears and time spent grooming. Intra-VTA-administered HCRT-1 significantly increased rears and grooming. <sup>+</sup> $P < 0.05$  compared to vehicle; \* $P < 0.05$  compared to baseline (Epoch -1).

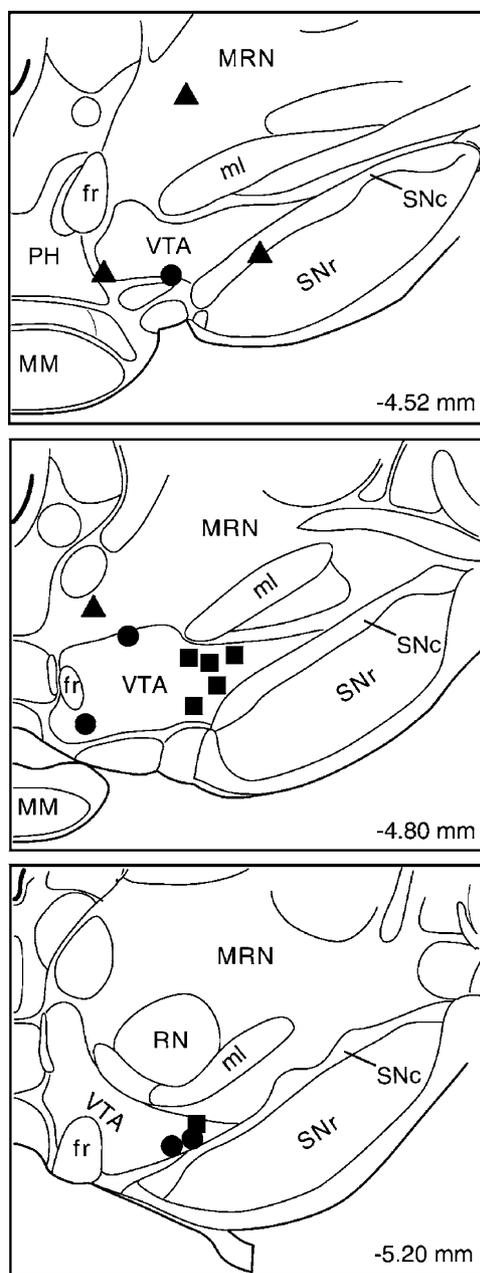


**Figure 4** Relationship between ICV-administered HCRT-1-induced increases in PFC DA and waking. (a) Displayed are mean ( $\pm$ SEM) DA levels (percent baseline) and time spent awake (sec). It is evident that there is a relatively close temporal relationship between HCRT-1-induced (0.7 nmol) increases in DA levels and waking. (b) Scatterplot showing the best fit line between waking and DA levels over all seven post-infusion samples. Different shapes represent treatment groups: squares (0.7 nmol); triangles (0.07 nmol); circles (vehicle). Regression analyses indicated a strong positive correlation between PFC DA levels and time spent awake after ICV infusion of HCRT-1 (best-fit regression  $r=0.61$ ,  $r^2=0.37$ ). During initial post-infusion samples, HCRT-1 elicited sustained waking throughout the sample epoch.



**Figure 5** Photomicrograph depicting infusion needle placement within VTA. Coronal sections (50  $\mu$ m) were stained with Neutral Red. Arrow indicates the most ventral extent of the infusion needle; AQ = cerebral aqueduct, SN = substantia nigra.

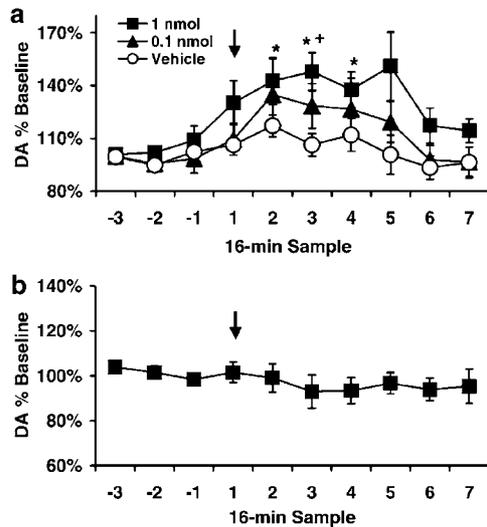
post-infusion sample. Although a full mapping study was not carried out, infusions placed dorsal to VTA, within the extreme medial region of VTA or within the medial substantia nigra, had no significant effect on PFC DA levels



**Figure 6** Diagram depicting infusion needle placements within VTA. The placements of needles in animals included in the PFC DA analyses are indicated by squares (1.0 nmol dose) and circles (0.1 nmol dose). Triangles indicate infusions of 1.0 nmol HCRT-1 located outside the main body of VTA that did not alter PFC DA levels (modified from Swanson, 1992).

(Figure 6). The mean ( $\pm$ SEM) post-infusion PFC DA levels in terms of percentage baseline for the four ineffective placements were  $93 \pm 11\%$  in Sample 1;  $103 \pm 16\%$  in Sample 2;  $114 \pm 9\%$  in Sample 3; and  $104 \pm 13\%$  in Sample 4.

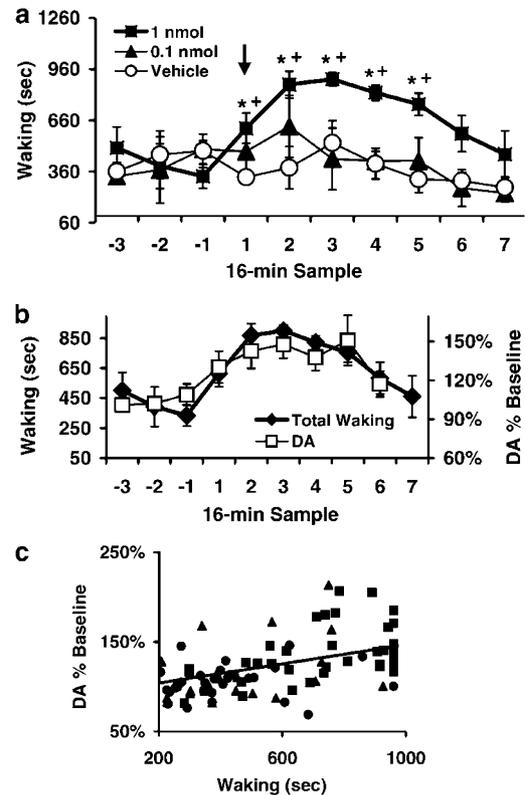
Similar to that observed with ICV infusions, intra-VTA infusion of the highest dose of HCRT-1 (1.0 nmol,  $n=5$ ) had no significant effect on Acc DA levels (time  $F_{9,36} = 0.65$ ,  $P=0.75$ ; Figure 7b). In all of these cases, the placement of the infusion needles within the VTA was comparable to needle placements that resulted in an increase in PFC DA levels.



**Figure 7** Effects of intra-VTA-administered vehicle, 0.1 nmol and 1.0 nmol HCRT-1 on PFC DA levels, and effects of 1.0 nmol HCRT-1 on Acc DA levels. DA levels are displayed as a percentage of baseline (mean  $\pm$  SEM) for 16-min samples before (negative numbers) and following infusion (positive numbers). The start of the intra-VTA infusion was aligned with the beginning of post-infusion Sample 1 (arrow). Panel a depicts PFC DA levels. HCRT-1 increased DA levels within PFC, with maximum concentrations observed during post-infusion Samples 2–5. Panel b depicts Acc DA levels. HCRT-1 had no significant effect on Acc DA levels.  $^+P < 0.05$  compared to vehicle;  $^*P < 0.05$  compared to sample immediately preceding infusion (Sample -1).

**Effects of intra-VTA HCRT-1 on sleep-wake state and behavior.** The degree to which unilateral intra-VTA infusion of HCRT-1 affected the sleep-wake state as well as select behaviors was also assessed. One animal was excluded from these analyses due to inadequate EEG/EMG recordings. HCRT-1 infused directly into VTA significantly increased the time spent awake, as determined from combined EEG/EMG measures (treatment  $F_{2,13} = 15.03$ ,  $P < 0.001$ ; time  $F_{9,117} = 3.08$ ,  $P < 0.01$ ; treatment  $\times$  time  $F_{18,117} = 1.65$ ,  $P = 0.06$ ). The highest dose of HCRT-1 (1.0 nmol,  $n = 6$ ) significantly increased the time spent awake for approximately 60 min, with near maximal waking during samples 2–3 (Figure 8a). Neither low-dose HCRT-1 (0.1 nmol,  $n = 4$ ) nor vehicle ( $n = 6$ ) infusion significantly altered the time spent awake.

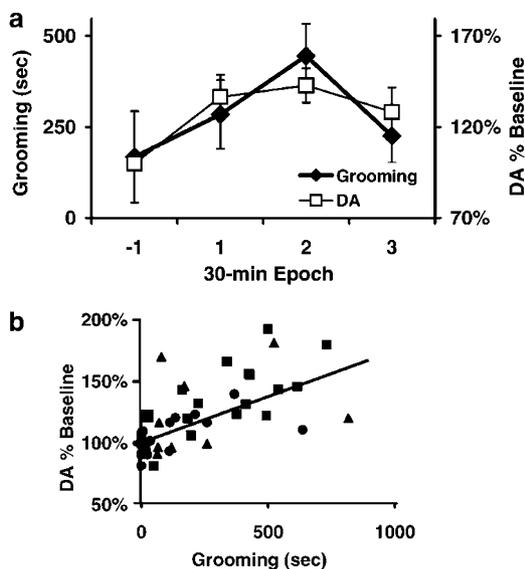
Intra-VTA HCRT-1 infusion (1.0 nmol,  $n = 6$ ; 0.1 nmol,  $n = 5$ ; vehicle,  $n = 6$ ) did not significantly alter locomotor activity as measured by quadrant entries (treatment  $F_{2,14} = 3.23$ ,  $P = 0.07$ ; time  $F_{3,42} = 1.61$ ,  $P = 0.20$ ; treatment  $\times$  time  $F_{6,42} = 0.85$ ,  $P = 0.54$ ; Table 1) or rears (treatment  $F_{2,14} = 2.23$ ,  $P = 0.14$ ; time  $F_{3,42} = 1.95$ ,  $P = 0.14$ ; treatment  $\times$  time  $F_{6,42} = 0.82$ ,  $P = 0.56$ ; Table 1). Nonetheless, planned comparisons within the low-dose group revealed a significant increase in rears during the first post-infusion sample relative to baseline, similar to that seen in the ICV study ( $t_4 = 3.14$ ,  $P < 0.05$ ). Finally, intra-VTA HCRT-1 significantly increased time spent grooming (treatment  $F_{2,14} = 4.17$ ,  $P < 0.05$ ; time  $F_{3,42} = 2.56$ ,  $P = 0.07$ ; treatment  $\times$  time  $F_{6,42} = 0.32$ ,  $P = 0.92$ ). Significantly more time was spent grooming in the high dose than the vehicle group in the third post-infusion epoch (Table 1). Interest-



**Figure 8** Effects of intra-VTA-administered vehicle, 0.1 nmol and 1.0 nmol HCRT-1 on time spent awake and relationship between intra-VTA HCRT-1-induced increases in PFC DA and waking. (a) Time spent awake based on EEG/EMG measures across 16-min periods. HCRT-1 elicited dose-dependent increases in time spent awake. The high dose of HCRT-1 induced near-maximal waking for several epochs, followed by a gradual return to baseline. (b) Displayed are mean ( $\pm$  SEM) DA levels (percent baseline) and time spent awake (sec). It is evident that there is a close temporal relationship between HCRT-1-induced (1.0 nmol intra-VTA) increases in DA levels and waking. (c) Scatterplot showing the best fit line between waking and DA levels over all seven post-infusion samples. Different shapes represent treatment groups: squares (1.0 nmol); triangles (0.1 nmol); circles (vehicle). Regression analyses indicated a strong positive correlation between PFC DA levels and time spent awake after intra-VTA infusion of HCRT-1 (best-fit regression  $r = 0.56$ ,  $r^2 = 0.31$ ).  $^+P < 0.05$  compared to vehicle;  $^*P < 0.05$  compared to epoch immediately preceding infusion (Epoch -1).

ingly, the increase in grooming observed following intra-VTA infusion was significantly greater than that observed following ICV infusion of high-dose HCRT-1 (treatment  $F_{1,9} = 8.11$ ,  $P < 0.05$ ; time  $F_{3,27} = 2.90$ ,  $P = 0.05$ ; treatment  $\times$  time  $F_{3,27} = 0.44$ ,  $P = 0.73$ ), particularly during the second post-infusion epoch ( $F_{1,9} = 7.67$ ,  $P < 0.05$ ; data not shown).

**Relationship between intra-VTA HCRT-1-induced alterations in behavior and PFC DA levels.** Correlation analyses were conducted to better assess the degree to which the intra-VTA HCRT-1-induced increase in PFC DA efflux might contribute to HCRT-1-induced alterations in behavioral state and specific behaviors. As shown in Figure 8b, there was a close temporal association between HCRT-1-induced increases in PFC DA levels and time spent awake. Additionally, best-fit regression analyses on data pooled across all groups and post-infusion epochs (seven 16-min



**Figure 9** Relationship between intra-VTA HCRT-1-induced increases in PFC DA and grooming. (a) Displayed are mean ( $\pm$ SEM) DA levels (percent baseline) and time spent grooming (sec). It is evident that there is a relatively close temporal relationship between HCRT-1-induced (1.0 nmol intra-VTA) increases in DA levels and grooming. (b) Scatterplot showing the best-fit line between grooming and DA levels across the first 90-min post-infusion. Different shapes represent treatment groups: squares (1.0 nmol); triangles (0.1 nmol); circles (vehicle). Regression analyses indicated a strong positive correlation between PFC DA levels and time spent grooming after intra-VTA infusion of HCRT-1 (best-fit regression  $r=0.65$ ,  $r^2=0.43$ ).

samples for waking; three 30-min samples for behavior) indicated a strong positive correlation between post-infusion PFC DA levels and time spent awake ( $r=0.56$ ,  $r^2=0.31$ ,  $P<0.001$ ; Figure 8c). The time course of the DA response also largely corresponds with the time course of HCRT-1-induced grooming (Figure 9a). Furthermore, there was a strong positive correlation between post-infusion DA levels in PFC and time spent grooming ( $r=0.65$ ,  $r^2=0.43$ ,  $P<0.001$ ; Figure 9b). Positive correlations were also observed when data from each group were analyzed separately. Less robust correlations were observed with quadrant entries ( $r=0.37$ ,  $r^2=0.14$ ,  $P<0.01$ ) and rears ( $r=0.38$ ,  $r^2=0.14$ ,  $P<0.01$ ).

## DISCUSSION

Available evidence indicates that the HCRTs act within multiple systems associated with the modulation of behavioral state. These include basal forebrain (España *et al*, 2001; Thakkar *et al*, 2001) and brainstem structures such as the locus coeruleus (Hagan *et al*, 1999; Horvath *et al*, 1999; Bourgin *et al*, 2000), the dorsal raphe (Liu *et al*, 2002; Kohlmeier *et al*, 2004), the lateral dorsal tegmental nucleus (Burllet *et al*, 2002; Kohlmeier *et al*, 2004) and the tuberomammillary nucleus (Eriksson *et al*, 2001). DA systems have also been implicated in the modulation of behavioral state and state-dependent processes, particularly under high arousal appetitive and aversive conditions (Trulson, 1985; Smith *et al*, 1992; Arnsten *et al*, 1994; Isaac and Berridge, 2003). Thus, it is of interest that VTA and

substantia nigra neurons express HCRT receptor mRNA (Marcus *et al*, 2001) and that HCRT activates VTA (though not substantia nigra) DA neurons *in vitro* (Uramura *et al*, 2001; Korotkova *et al*, 2002, 2003).

To examine initially the modulatory actions of HCRT on DA neurotransmission *in vivo*, the current studies utilized microdialysis measures of DA release within the PFC and Acc in the unanesthetized rat. These studies demonstrate a robust and dose-dependent increase in DA efflux within the PFC, but not within Acc, following ICV-administered HCRT-1. HCRT fibers are located in close proximity to DA cell bodies in the VTA, a major source of PFC- and Acc-projecting DA neurons (Fadel and Deutch, 2002; Baldo *et al*, 2003). To test the hypothesis that HCRT acts directly within the VTA to increase DA neurotransmission, we examined the effects of intra-VTA infusion of HCRT-1 on DA efflux within the PFC and Acc. Intra-VTA administration of HCRT-1 increased DA levels within the PFC and not Acc, a pattern similar to that observed with ICV administration.

## Methodological Considerations

Microdialysis probes were lowered slowly over a 5–10-min period 1 or 2 days prior to testing. This procedure was based on previous observations indicating that slow implantation results in substantially higher extracellular levels of norepinephrine and DA within the PFC relative to rapid insertion via an indwelling cannula. The magnitude of stressor-induced activation of DA efflux within the PFC and Acc estimated with these procedures is comparable to that observed with both postmortem measures and dialysis probe implantation 7 days following surgical implantation of a guide cannula (Abercrombie *et al*, 1989; Berridge *et al*, 1999a; Feenstra *et al*, 2000). Moreover, the magnitude of HCRT-induced waking observed following only 1–2 days recovery from surgery in the current studies was comparable to previous observations in animals given a recovery period of at least 7 days (España *et al*, 2001). Therefore, the stress of surgery 1–2 days prior to testing does not appear to compromise the reactivity of DA terminals to arousal-increasing environments or the wake-promoting actions of HCRT.

Although we did not observe an increase in Acc DA levels after either ICV or intra-VTA HCRT infusion, it has been well established by our laboratory and others that such an increase can occur under similar experimental conditions (Westerink *et al*, 1996; Ikemoto *et al*, 1997; Berridge *et al*, 1999a, 2003).

## Circuitry Underlying Selective Increase in DA Efflux by HCRT

The current observations demonstrate the selective increase by HCRT-1 of DA efflux within the PFC relative to Acc. These two regions are of particular interest because previous work demonstrates that DA terminals within the PFC and Acc display greater responsivity to salient stimuli relative to other cortical (eg cingulate cortex) and subcortical (eg striatum, hypothalamus) DA terminal fields (Dunn, 1988; Roth *et al*, 1988; Feenstra *et al*, 2000). Thus, given the apparent lack of sensitivity of Acc DA terminals to HCRT-1, it seems unlikely that HCRT-1 activates DA

terminals within these other areas. An exception to the general rule of reduced responsivity of DA terminals outside of PFC and Acc is the central nucleus of the amygdala (Kilts and Anderson, 1987; Inglis and Moghaddam, 1999). The extent to which HCRT influences rates of DA neurotransmission within the amygdala remains to be determined.

The selective increase in PFC DA efflux following intra-VTA infusion of HCRT-1 is somewhat surprising, given that VTA (and substantia nigra) DA neurons project to both PFC and Acc. This could result from a number of mechanisms. First, differential actions of autoreceptors (Chiodo and Bunney, 1983) and/or DA transporters (Sesack *et al*, 1998) across terminal fields may contribute to enhanced DA efflux within the PFC. Additionally, anatomical and physiological evidence indicates that PFC-projecting DA neurons differ from Acc-projecting DA neurons in receptor expression and distribution within the VTA (Swanson, 1982; Deutch *et al*, 1991; Roeper *et al*, 2003; Garzon and Pickel, 2004; Margolis *et al*, 2004). Thus, it is possible that this subpopulation of DA neurons display a greater sensitivity to salient environmental stimuli and/or HCRT-1 than other, non-PFC-projecting, DA neurons. Finally, ICV infusion of HCRT may activate LH HCRT neurons via neighboring glutamatergic neurons (Li *et al*, 2002), which project both to VTA and PFC, but only sparsely to Acc (Fadel and Deutch, 2002).

In the case of intra-VTA infusion of HCRT-1, comparable or larger doses were required to elicit an increase in PFC DA efflux similar to that observed with ICV infusion. This could reflect a number of possible mechanisms. First, it may be related to the relatively low density of HCRT synapses onto DA neurons within the VTA (Balcita-Pedicino and Sesack, 2003). Second, ICV infusions will impact multiple sources of PFC-projecting DA efferents not targeted by unilateral intra-VTA infusions (eg, A8, A9, contralateral VTA; Swanson, 1982; Deutch *et al*, 1991). However, it is important to note that, consistent with the results of our limited mapping study, HCRT-1 does not excite substantia nigra DA neurons *in vitro* (Korotkova *et al*, 2002). Third, PFC-projecting DA neurons are located primarily within the caudal portion of the VTA (Swanson, 1982; Deutch *et al*, 1991; Roeper *et al*, 2003; Garzon and Pickel, 2004). Given that this neuronal population was not specifically targeted in this study and given relatively small infusion volumes, higher doses may have been required to permit consistent diffusion of HCRT-1 to this region of the VTA. Additionally, ICV-administered HCRT-1 may also act via a multisynaptic pathway and/or directly within the PFC to modulate rates of DA neurotransmission. Finally, ICV HCRT-induced activation of the locus coeruleus (Hagan *et al*, 1999) may result in efflux of DA from noradrenergic terminals within the PFC (Devoto *et al*, 2003). Regardless of whether the VTA is the sole site of action, the current observations nonetheless demonstrate that HCRT acts directly within the VTA to increase rates of DA release within the PFC. Further studies are needed to fully delineate the circuitry underlying HCRT-dependent activation of PFC DA neurotransmission.

### HCRT-DA Interactions: Implications for Behavioral State

Intra-VTA infusion of HCRT-1 increased the time spent awake, providing the first demonstration that HCRT-1 acts

within the VTA to modulate behavioral state. It is not clear from these observations alone whether this involves HCRT-induced activation of DA neurotransmission. However, there was a strong positive relationship between HCRT-1-induced increases in waking and rates of PFC DA release following both ICV and intra-VTA infusions. Combined, these observations suggest that the wake-promoting actions of HCRT likely involve the activation of DA neurons located within the VTA. Future studies will need to determine the degree to which HCRT acts on DA neurons or other neurons within the VTA to promote waking.

Importantly, HCRT-induced increases in PFC DA efflux do not appear to result simply from the wake-promoting actions of this peptide. The waking state is associated with an approximately 25% increase in PFC DA dialysate levels over sleeping baseline (Berridge and Stalnaker, 2002), much less than the observed PFC DA response to ICV or intra-VTA HCRT infusion. The observed HCRT-induced increases in PFC DA efflux are comparable to those observed under high-arousal conditions (see below).

In the current studies, the dose of intra-VTA HCRT-1 required to promote waking was somewhat higher than that observed for basal forebrain infusions (España *et al*, 2001). However, it is important to note that the current studies utilized unilateral infusions, whereas these previous studies infused HCRT-1 bilaterally. Similarly, when infused into the basal forebrain, amphetamine displays substantially greater potency when infused bilaterally (Berridge *et al*, 1999b). Thus, it is predicted that the dose-response curve for the wake-promoting actions of intra-VTA HCRT-1 would be shifted to the left with bilateral infusions.

### HCRT-DA Interactions in Aversive and Appetitive Conditions

Accumulating evidence suggests a role for HCRT neurotransmission in high-arousal processes associated with stress and/or appetitive states. For example, the feeding-promoting actions of HCRT may reflect a potentially broader role of HCRT in appetitive processes (Willie *et al*, 2001). Additionally, ICV HCRT-1 elicits a variety of stress-like physiological and behavioral responses, including activation of the hypothalamo-pituitary-adrenal axis (Jaszberenyi *et al*, 2000; Kuru *et al*, 2000), increased *c-fos* expression (España *et al*, 2003), locomotor activation, grooming, and the chewing of inedible material (Nakamura *et al*, 2000; España *et al*, 2001). Intra-VTA infusions of HCRT-1 also provoked certain stress-like behaviors, including grooming. The increase in time spent grooming was closely associated with HCRT-induced increases in PFC DA efflux. Moreover, ICV HCRT-induced grooming is attenuated by pretreatment with DA receptor antagonists (Nakamura *et al*, 2000). Combined, these observations suggest that HCRT participates in regulating behavior in stress, including the induction of a high-arousal state, via actions upon VTA DA neurons.

The current studies demonstrate that HCRT elicits an activation of PFC DA systems comparable to that observed in both aversive and appetitive high-arousal states (Abercrombie *et al*, 1989; Cenci *et al*, 1992; Gresch *et al*, 1994; Di Chiara *et al*, 1999; Feenstra *et al*, 2000), and that this involves, at least in part, actions within the VTA. These

observations suggest that HCRT contributes to the relatively selective activation of PFC DA projections under appetitive and/or aversive conditions. It should be noted that, often, stress-related increases in PFC DA release are accompanied by smaller, though substantial, increases in Acc DA release (Abercrombie *et al*, 1989; Cenci *et al*, 1992; Gresch *et al*, 1994; Di Chiara *et al*, 1999; Feenstra *et al*, 2000). Thus, the effect of HCRT-1 on DA neurotransmission is not entirely similar to that observed in stress. Nonetheless, the selective activation of DA projections to PFC relative to Acc in stress has been consistently observed (Roth *et al*, 1988). As such, the degree to which HCRT-1-induced activation of PFC DA projections mimics that observed in stress and/or appetitive states remains unclear.

### Potential Role of HCRT in the Modulation of PFC-Dependent Cognitive and Affective Processes

PFC DA neurotransmission plays a pivotal role in a variety of higher-order cognitive and affective processes. These include learning under both appetitive and aversive conditions (Sokolowski and Salamone, 1994; Wilkinson *et al*, 1998; Bassareo and Di Chiara, 1997; Baldwin *et al*, 2002), working memory (Arnsten *et al*, 1994; Williams and Goldman-Rakic, 1995; Murphy *et al*, 1996; Phillips *et al*, 2004), and attention (Robbins, 2000). Additional evidence indicates a prominent role of the PFC and PFC DA in a variety of affective processes (for example, see Jentsch *et al*, 2000; Davidson, 2003). Given this, it is of interest that HCRT-1 produces a robust and apparently selective increase in PFC DA efflux. These observations suggest that HCRT likely impacts PFC-dependent cognitive and affective processes via alterations in rates of DA neurotransmission within the PFC. Clearly, the degree to which HCRT modulates PFC-dependent affect and cognition, and may contribute to affective and/or cognitive dysfunction associated with a variety of psychiatric/behavioral disorders, is an important question for future studies.

### Conclusions

The present studies demonstrated the selective increase in PFC DA efflux by HCRT-1 relative to Acc. This activation involved, at least in part, direct actions of HCRT within the VTA. Moreover, intra-VTA infusions of HCRT-1 produced an increase in time spent awake that was closely associated with HCRT-1-induced increases in PFC DA efflux. Combined, these observations suggest a prominent role of the VTA and DA neurotransmission in the modulatory actions of HCRT on behavioral state and PFC-dependent affective and cognitive processes.

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