Behavioral/Systems/Cognitive

A Role for Hypocretin (Orexin) in Male Sexual Behavior

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The role of hypocretin (orexin; hcrt/orx) neurons in regulation of arousal is well established. Recently, hcrt/orx has been implicated in food reward and drug-seeking behavior. We report here that in male rats, Fos immunoreactivity (ir) in hcrt/orx neurons increases markedly during copulation, whereas castration produces decreases in hcrt/orx neuron cell counts and protein levels in a time course consistent with postcastration impairments in copulatory behavior. This effect was reversed by estradiol replacement. Immunolabeling for androgen (AR) and estrogen (ERα) receptors revealed no colocalization of hcrt/orx with AR and few hcrt/orx neurons expressing ERα, suggesting that hormonal regulation of hcrt/orx expression is via afferents from neurons containing those receptors. We also demonstrate that systemic administration of the orexin-1 receptor antagonist SB 334867 [*N*-(2-methyl-6-benzoxazolyl)-*N*″-1,5-naphthyridin-4-yl urea] impairs copulatory behavior. One locus for the prosexual effects of hcrt/orx may be the ventral tegmental area (VTA). We show here that hcrt-1/orx-A produces dose-dependent increases in firing rate and population activity of VTA dopamine (DA) neurons *in vivo*. Activation of hcrt/orx during copulation, and in turn, excitation of VTA DA neurons by hcrt/orx, may contribute to the robust increases in nucleus accumbens DA previously observed during male sexual behavior. Subsequent triple immunolabeling in anterior VTA showed that Fos-ir in tyrosine hydroxylase-positive neurons apposed to hcrt/orx fibers increases during copulation. Together, these data support the view that hcrt/orx peptides may act in a steroid-sensitive manner to facilitate the energized pursuit of natural rewards like sex via activation of the mesolimbic DA system.

Key words: **hypocretin/orexin; dopamine; ventral tegmental area; sexual behavior; male rats; electrophysiology**

Introduction

Since its discovery in the 1990s (de Lecea et al., 1998; Sakurai et al., 1998), the hypocretin (orexin; hcrt/orx) system has been viewed mainly in the context of arousal and ingestive behavior (Sutcliffe and de Lecea, 2002; Siegel, 2004). Mutant mice with impaired hcrt/orx signaling display uneven patterns of arousal marked by frequent transitions between sleep and wakefulness (Mochizuki et al., 2004). The hcrt/orx peptides are believed to facilitate uninterrupted arousal by robust projections to wake-active aminergic cell groups in the brainstem (e.g., locus ceruleus, raphe) (Peyron et al., 1998; Nambu et al., 1999; Saper et al., 2005). In addition to their projections to sites that regulate vigilance and arousal, the hcrt/orx neurons also project to the origin of the mesolimbic dopamine (DA) pathway in the ventral tegmental area (VTA) (Fadel and Deutch, 2002). This pattern of connectivity has prompted a number of research groups to evaluate the role of hcrt/orx in reward-related reinforcement (DiLeone et al., 2003; Harris et al., 2005; Harris and Aston-Jones, 2006).

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Studies in animals moving *ad libitum* show that hcrt/orx neurons increase their firing rate during periods of energized, exploratory behavior (Mileykovskiy et al., 2005) and that enhanced locomotor output after intracerebroventricular hcrt/orx infusion is attenuated by DA receptor blockade (Nakamura et al., 2000). Other studies have demonstrated increased DA cell activity *in vitro* by hcrt/orx (Korotkova et al., 2003), as well as augmented nucleus accumbens (NAc) DA release *in vivo* after application of hcrt/orx to the VTA (Narita et al., 2006). Recently, behavioral studies have found that activation of the hcrt/orx system may be integral to motivation for drugs of abuse (Boutrel et al., 2005; Harris et al., 2005; Borgland et al., 2006), as well as natural food rewards whose reinforcement strength is determined homeostatically by hypothalamic structures (Hoebel, 1969; Rolls et al., 1980; Fulton et al., 2000; Thorpe et al., 2005).

Just as exogenous hcrt/orx can enhance feeding and food seeking (Kotz, 2006), intrahypothalamic infusion of hcrt/orx facilitates male rat sexual behavior (Gulia et al., 2003). In light of classic studies in which feeding or male copulatory behavior was elicited by electrical stimulation of sites in the lateral hypothalamic area (LHA) that are now known to contain hcrt/orx neurons (Vaughan and Fisher, 1962; Caggiula and Hoebel, 1966; Swanson et al., 2005), we sought evidence for the activation of hcrt/orx neurons during copulation and for impairment of the behavior by orexin-1 receptor (OX_1) blockade. We also evaluated the neuroendocrine regulation of the hcrt/orx system by measur-

ing the effects of castration and hormone replacement on central hcrt/orx content and by double immunolabeling for hcrt/orx and steroid hormone receptors. Finally, we assessed the extent to which the mesolimbic DA system could be activated by hcrt/orx *in vivo* and whether DA neurons in the VTA that receive hcrt/orx inputs show increased Fos immunoreactivity (ir) during copulation.

Materials and Methods

Fos immunohistochemistry, behavior, and cell counts. Adult (~325 g), sexually experienced male Long–Evans rats (Harlan, Indianapolis, IN) were kept and used in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*, and all procedures were approved by the universities' Institutional Animal Care and Use Committees. In the week before testing, all animals were allowed to have four daily 1 h sexual experience sessions with an ovariectomized female brought to estrus by estradiol benzoate (10 μg, s.c.) and progesterone 48 h later (400 μg, s.c.; given 4 h before testing; Sigma, St. Louis, MO). Behavioral testing in all experiments was performed 2 h into the animals' usual nocturnal period in their home cage under light from a single 40 W red incandescent bulb. For copulation experiments, one group ($n = 6$) of males was allowed to copulate in their home cage with an estrous female to a single ejaculation, after which the female was removed. All animals mounted almost immediately and intromitted in <4 min. The mean ejaculation latency for this group was 632.6 ± 169.64 s (mean ± SEM; $n = 6$). A control group ($n = 6$) was used to control for activity level. These animals were visually monitored to verify that they were awake and active for a 15 min period, which corresponded to the duration of the experimental group's copulation testing period. Cage lids were opened and closed at the beginning and end of this 15 min period, but otherwise, animals were left undisturbed. Control animals that were quiescent during this period were not used. Animals were judged to be quiescent if they appeared to rest on their flank or in a head-down posture (adapted from Espana et al., 2003). Sixty minutes after the start of copulation sessions or control observations, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg) and perfused with 0.1 M PBS, pH 7.4, and 4% paraformaldehyde in PBS. Brains were cryoprotected in 20% sucrose–PBS, and every other 40 μm cryostat section (30 per animal) from the vicinity of the hypothalamic hcrt/orx cell population was immunolabeled for prepro-hcrt/orx (1:100; Millipore, Billerica, MA) and Fos (1:10,000; EMD Biosciences, San Diego, CA) with 3′,3′-diaminobenzidine (DAB) and nickel-intensified DAB, respectively, according to immunohistochemistry procedures described previously (Espana et al., 2003; Sato et al., 2005). Cell counts were performed under high magnification using an Olympus (Tokyo, Japan) microscope and camera on a single section at a consistent rostrocaudal level (see Fig. 1*B*,*C*) (2.45 mm posterior to bregma) (Swanson, 2004). In each hemisphere, cells that fell within two 470 × 630 μm counting fields were tagged using digital image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD), by an examiner blind to experimental conditions. In both hemispheres, lateral counting fields used the fornices as their medial boundary. Medial counting fields used the fornices as their lateral boundary. The numbers of Fos-only, hcrt/orx-only, and double-labeled cells with both hcrt/orx and Fos-ir were recorded.

Castration experiments and immunoblotting. Adult male (~325 g at the beginning of experiments) Long–Evans rats were anesthetized (75 mg/kg ketamine HCl and 10 mg/kg xylazine HCl, i.p.) and castrated. Animals were allowed 7, 14, or 28 d of survival time after surgery before they were anesthetized and perfused and their brains were immunolabeled for prepro-hcrt/orx as above (all groups, $n = 5$). These animals were compared with a sham-surgery group that received a scrotal incision under identical anesthesia ($n = 5$). Shams were also killed at 28 d. Cell counts were performed as above at the same coronal plane under lower magnification.

In a separate experiment, 28 d castrates ($n = 5$) and sham-treated controls ($n = 5$) were deeply anesthetized with sodium pentobarbital (100 mg/kg), and their brains were removed and rapidly frozen in 2-methylbutane chilled by dry ice. Frozen hypothalami were then blocked by two coronal cuts, one through the medial preoptic area (mPOA) just rostral to the decussation of the anterior commissure, and the other through the posterior hypothalamic nucleus just caudal to the division of the third ventricle into its hypothalamic and mammillary recesses. Blocks were then trimmed with two sagittal cuts at the medial end of either cerebral peduncle and by a horizontal cut at the dorsal end of the third ventricle's hypothalamic recess. Tissue blocks were then homogenized with sonication, protein was extracted into modified radioimmunoprecipitation buffer, pH 8.0, with protease inhibitors (Roche Complete Mini protease mixture; Roche Diagnostics, Indianapolis, IN), and extracts were aliquoted after centrifugation. After determining total protein concentration for extracts from each subject, 40 μg of protein from each animal was loaded into separate wells of a 15% SDS-PAGE gel. Electrophoretic separation, transfer to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), and immunolabeling was performed according to the method of Laemmli (Cleveland et al., 1977), using 1:1000 of the previously noted anti-prepro-hcrt/orx antibody, and later 1:2000 anti-β-actin (Sigma) and 1:5000 goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in 5% powdered milk in Tris-buffered saline. Protein bands were visualized by enhanced chemiluminescence kit (ECL; GE Healthcare, Piscataway, NJ) and Kodak (Rochester, NY) BioMax films. Films were exposed for 40 s and digitally scanned, and optical densities were measured using publicly available ImageJ software. For each animal, optical density values for hcrt/orx as a percentage of β-actin loading control were determined and subjected to statistical analysis.

In a third experiment, male rats as described above were given sham surgeries ($n = 3$) or castrated and injected every second day for 28 d with dihydrotestosterone (500 μg, s.c.; $n = 4$), estradiol benzoate (20 μg, s.c.; $n = 3$), or oil vehicle (0.1 μl; $n = 3$). Both hormones were purchased from Sigma. Hormone doses were chosen for their ability in previous studies to maintain copulation in castrates (Putnam et al., 2005). On the 28th day after surgeries, animals were killed, and their hypothalami were blocked for measurement of prepro-hcrt/orx content by Western immunoblot as described above.

Steroid hormone receptor and hcrt/orx double-label immunohistochemistry. Brain sections from the LHA of intact, sexually naive adult male (~350 g) Long–Evans rats were immunolabeled for androgen receptor (AR) (1:750; $n = 6$; Santa Cruz Biotechnology) or estrogen receptor (ERα) (1:2500; $n = 9$; Santa Cruz Biotechnology) using nickel-intensified DAB. Subsequently, AR-labeled sections were double labeled for prepro-hcrt/orx (as above), and ERα-labeled sections were double stained for prepro-hcrt/orx ($n = 5$). After mounting, sections that closely matched one of four coronal layers of the LHA in the atlas of Swanson (2004) were hand drawn under the microscope using a camera lucida attachment, and double- and single-labeled cells in each section were tagged. Drawings were digitally scanned and superimposed on atlas levels using Adobe (San Jose, CA) Illustrator, and each population of cells was mapped onto atlas illustrations (Swanson, 2004). In the case of ERα-plus-hcrt/orx-labeled sections, numbers of double- and single-labeled hcrt/orx neurons were counted.

Pharmacology and copulatory behavior. Adult male (~350 g at the start of experiments) Long–Evans rats ($n = 9$) were given four 1 h sexual experience sessions with a sexually receptive female during the week before behavioral testing. Animals that failed to ejaculate at least once during the first 30 min of the final test were excluded from the experiment. Experience sessions and behavioral testing were performed under 40 W red incandescent light 2 h into the animal's nocturnal period. Tests of copulatory behavior were performed in the male's home cage and were 30 min in duration. Distinct features of male copulatory behavior (i.e., mounts, intromissions, and ejaculations) were scored by an observer blind to experimental treatments using custom computer software that recorded frequency and latency data for each behavioral event. Thirty minutes before behavioral testing, animals were injected with either the OX_1 antagonist *N*-(2-methyl-6-benzoxazolyl)-*N*″-1,5-naphthyridin-4-yl urea (SB 334867) (20 mg/kg, i.p.) or DMSO vehicle (0.5 ml/kg). The experiment followed a simple counterbalanced within-subjects design such that five animals received drug injections on the first day's testing and the remaining four received vehicle. After a 48 h drug washout pe-

Table 1. Increased Fos-ir during copulation in hcrt/orx neurons of male rats

Treatment	Total hcrt/orx	Double label (Fos plus hcrt/orx)	Total Fos	Total Fos in hcrt/orx (%)	Hcrt/orx double labeled (%)
Noncopulation	269.3 ± 25.5	31.8 ± 5.8	54.3 ± 11.3	64.4 ± 7.5	12.0 ± 2.1
Copulation	284.8 ± 20.2	114.3 ± 12.4***	146.5 ± 6.9***	77.6 ± 6.8	40.0 ± 2.9**

Values are given as mean ± SEM. ** $p < 0.01$; *** $p < 0.001$.

riod, animals treated with drug on day one were given vehicle and vice versa.

Electrophysiology. Adult male Sprague Dawley rats (~350 g) were anesthetized with chloral hydrate (400 mg/kg, i.p. for induction; 100 mg · $kg^{-1} \cdot h^{-1}$ thereafter for maintenance) and mounted on a stereotaxic instrument. The skull and dura over the brainstem region containing the VTA were removed. Each animal first received an infusion of artificial CSF (aCSF) [0.5 μl/5 min; from lambda, anteroposterior, +2.6 or +3.4 mm; mediolateral, ±0.8 mm; dorsoventral, −7.0 mm; flat skull according to a stereotaxic atlas (Paxinos and Watson, 1998)], with a 32 ga Hamilton syringe (0.5 μl/5 min) on one side of the VTA. Then, 10 min later, the cells-per-track sampling procedure was performed on the ipsilateral side of the VTA. Animals next received an infusion of hcrt-1/orx-A (0.014 nmol, $n = 4$; 1.4 nmol, $n = 5$; or 140 nmol, $n = 6$ dissolved in aCSF; American Peptide, Sunnyvale, CA) into the contralateral VTA before the cells-per-track procedure was repeated on that side. Injections were counterbalanced by hemisphere and by anterior or posterior injection site. Extracellular single-unit recordings were performed with single-barrel glass micropipettes (1.5 mm outer diameter before pulling; World Precision Instruments, Sarasota, FL) filled with 2 M NaCl and back-broken. Electrode impedance ranged from 2 to 4 MΩ at 135 Hz. DA neurons were identified by their positive–negative extracellular action potentials, which often have a prominent initial segment/somatodendritic (IS/SD) break, wide action potential duration, slow firing rate, and irregular single spike or burst firing pattern (Grace and Bunney, 1983). To perform the cells-per-track experiment, the recording electrode was passed through a stereotaxically defined block in the VTA (2.8–3.4 mm anterior to lambda; 0.6–1.0 mm lateral to midline; 6.5–8.5 mm below the brain surface) systematically six times. Each identified DA neuron was recorded for 2–5 min on-line using the Chart data acquisition system (AD Instruments, Mountain View, CA). The average number of spontaneously active DA neurons encountered per electrode track from each animal (cells per track) was the index for VTA DA neuron population activity. The mean firing rate of DA neurons was determined from all DA neurons sampled from all animals within each group.

To test for depolarization inactivation in the 140 nmol hcrt/orx group, apomorphine HCl (20 μg/kg, i.p.; $n = 4$; Sigma) was administered immediately after the completion of post-hcrt-1/orx-A sampling. Ten minutes after apomorphine injection, six additional electrode tracks were sampled in the side of VTA that previously received hcrt-1/orx-A.

Behavior, triple label immunohistochemistry, and cell counts. Adult (~300 g) male Long–Evans rats were given four 1 h sexual experience sessions with a receptive female as above. Before (1 h) anesthesia, perfusion, and preparation of tissue for immunolabeling, animals ($n = 6$) were allowed to copulate to a single ejaculation. As above, animals mounted and intromitted almost immediately, and mean ejaculation latency for this group was 588.50 ± 85.03 s (mean ± SEM). The experimental group was compared with sexually experienced controls that were not given access to estrous females for copulation ($n = 6$). As above, testing occurred 2 h into the animal's usual nocturnal period under red incandescent light. After the animals were killed, fixated, and cold microtome sectioned (as above), four sections from each animal representing four rostrocaudal levels of VTA were selected for immunohistochemistry. For the number of subjects (n) used for cell counts at each level, see Table 4. Sections were labeled for Fos (1:7500; Santa Cruz Biotechnology) with DAB as above. Sections were then labeled for prepro-hcrt/orx (1:500) and tyrosine hydroxylase (TH; 1:2000; Millipore) with cyanine-conjugated fluorescent secondary antibodies (1:200; Cy3 and Cy2, respectively; Jackson ImmunoResearch, West Grove, PA). Cell counts were performed under high (40×) magnification on a Leica (Nussloch, Germany) DM 4000B microscope using Stereo Investigator software (MBF Bioscience, Williston, VT) by an experimenter blind to treatment conditions. Stereo Investigator software was used to define a counting field that included all DA cells within each level of VTA. At a single focal length in each of the four rostrocaudal levels of VTA, five cell types were tagged: TH-positive neurons, TH-positive neurons showing Fos-ir, TH-positive neurons showing direct (onto somatic plasmalemma) appositions by hcrt/orx fibers, TH-positive neurons showing both Fos-ir and hcrt/orx appositions, and finally, solo Fos-positive nuclei not in TH neurons.

Data analysis. Group means for cell counts in the Fos experiment and optical density measures of Western blots from the first such experiment were compared by independent-samples t test. Matched-samples t testing was performed on means from the OX_1 antagonist behavioral experiment. Cell counts in the castration, triple-labeling experiments, and mean firing rate and cells-per-track measures were subjected to ANOVA.

Results

Copulation increases Fos immunoreactivity in hcrt/orx neurons

The percentage of hcrt/orx-ir cells that showed Fos-ir differed significantly between groups, with copulating animals showing increased Fos-ir in hcrt/orx cells ($t_{(10)} = 7.71$; $p < 0.01$) (Table 1, Fig. 1). Mean numbers of double-labeled hcrt/orx also differed significantly between groups ($t_{(10)} = 6.03$; $p < 0.001$). Remarkably, in copulating animals, no difference in number of Fos-positive hcrt/orx neurons was detected between the medial and lateral counting fields (data not shown) (Harris and Aston-Jones, 2006). The total mean number of Fos-ir nuclei (Fos-only and double-labeled hcrt/orx neurons) was significantly greater in copulating animals than in noncopulating controls ($t_{(10)} = 16.97$; $p < 0.001$) (Table 1).

Castration decreases hcrt/orx immunoreactivity and protein in hypothalamus

When compared with sham-treated controls, counts of hcrt/orx-ir neurons showed a significant decrease in cell number 28 d after castration (Fig. 2*A*) ($F_{(3,16)} = 7.60$; $p < 0.005$). This represents a 31.8% decrease in the number of cells in the observed population. *Post hoc* (Tukey) testing revealed no significant differences among counts of sham-treated rats or 7 or 14 d castrates. Subsequent Western immunoblot analysis of hypothalamic prepro-hcrt/orx found a significant decrease in mean optical density of hcrt/orx bands from 28 d castrates when compared with sham-treated controls (Fig. 2*B*) ($t_{(8)} = 2.99$; $p < 0.05$.).

Estradiol restores hypothalamic hcrt/orx protein levels

At 28 d after castration or sham surgery, prepro-hcrt/orx levels in oil-treated animals were significantly lower than those for sham- and estradiol (E_2)-treated groups ($F_{(3,9)} = 8.47$; $p < 0.005$) (Fig. 2*C*). One-way ANOVA with *post hoc* (Tukey) tests found that prepro-hcrt/orx levels measured in sham- and E_2-treated animals did not differ from each other and that the E_2 group did not differ from DHT-treated animals.

ERα is coextensive with hcrt/orx and is coexpressed in an anatomically distinct population of hcrt/orx neurons

Nuclear AR was ventrally removed from the main hcrt/orx neuron population, spreading mediolaterally from the arcuate nu-

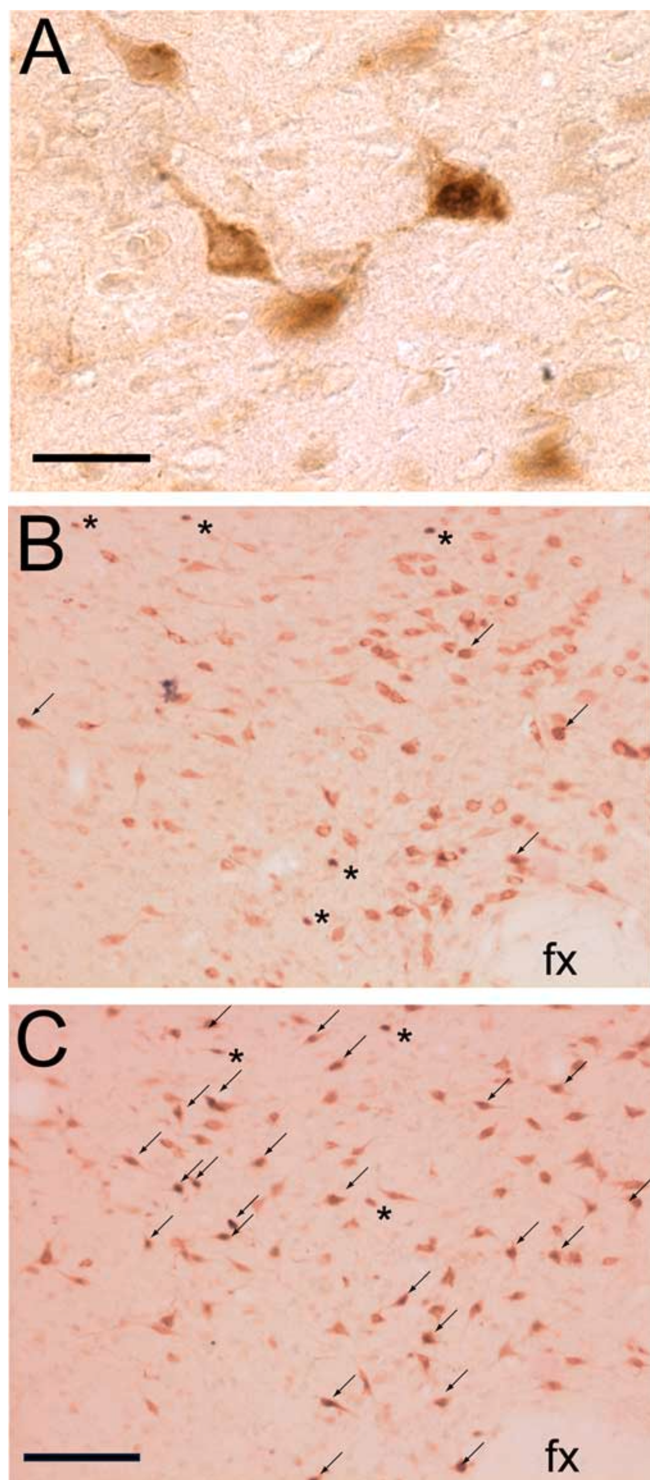

Figure 1. Increased Fos immunoreactivity in hcrt/orx neurons during copulation. ***A***, Hcrt/orx neuron (on right) showing Fos-ir. Scale bar, 25 μm. ***B***, Representative micrograph from right counting field in left hemisphere of noncopulating control male. Scale bar (in ***A***), 200 μm. ***C***, Micrograph from copulating male. Scale bar, 200 μm. Arrows indicate hcrt/orx neurons showing Fos-ir; asterisks indicate Fos-ir nuclei in non-hcrt/orx cells; fx, fornix.

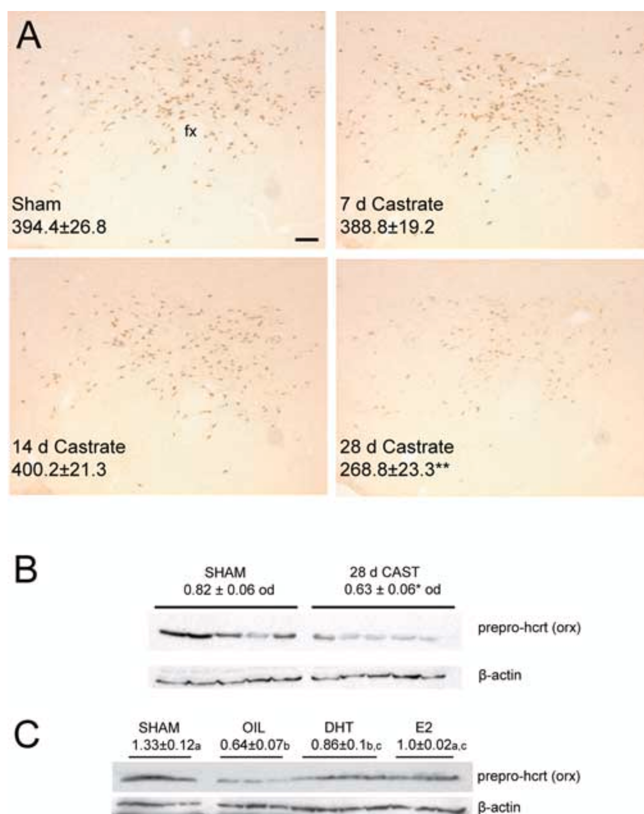

Figure 2. Castration decreases hcrt/orx-ir in male rat hypothalamus. ***A***, Representative micrographs of hcrt/orx-labeled neurons in one hemisphere show significant decreases in cell number by 28 d after castration. Inset values are mean cell counts for both hemispheres ± SEM; $^{**}p < 0.005$. Scale bar, 200 μm; fx, fornix. ***B***, Western immunoblots show significant decreases in hypothalamic prepro-hcrt/orx of 28 d castrates. Each band represents the signal from one animal in either group. Values are mean ± SEM optical density (od) units for hcrt/orx relative to β-actin; $^{*}p < 0.05$. ***C***, Immunoblots for prepro-hcrt/orx in 28 d castrates show E_2 to maintain hypothalamic hcrt/orx content equivalent to that of shams when compared with oil-treated controls. Groups with the same lowercase letter are not significantly different ($p < 0.05$).

cleus to the optic tract. In no cases were nuclear ARs and hcrt/orx found in the same neuron (data not shown). Although ERα showed a similar pattern of distribution in the ventral extent of the hypothalamus, ERα labeling was more extensive in the ventromedial nucleus. More dorsally, ERα labeling was found in tapered bands of cells that extended medially from the internal capsule beneath the zona incerta to the dorsomedial hypothalamus (DMH). Label for ERα was seldom found in hcrt/orx neurons (<1% of the population surveyed). When ERα was seen to colocalize with hcrt/orx, it was typically in neurons within or just rostral to the DMH (Fig. 3). Thus, although very few hcrt/orx neurons in the hypothalamus express ERα, a high proportion (~65%) of those located in or near the DMH do (Fig. 3).

Hcrt/orx receptor blockade impairs copulation

Compared with vehicle, pretreatment with the OX_1 antagonist SB 334867 increased mean latencies to intromit and decreased mean ejaculation frequency (Table 2). Paired-samples *t* tests revealed a significant effect of SB 334867 on intromission latencies ($t_{(8)} = 3.31$; $p < 0.05$) and ejaculation frequency ($t_{(8)} = 2.40$; $p < 0.05$). Drug treatment also appeared to produce nonsignificant increases in mean latencies to mount and ejaculate and decreases in mean number of intromissions (Table 2).

Hcrt-1/orx-A increases VTA DA neuron firing rate and population activity and induces depolarization inactivation

The lowest dose of hcrt-1/orx-A (0.014 nmol) increased mean VTA DA neuron firing rate compared with vehicle-treated controls (Fig. 4*B*,*C*) (pairwise comparison; $F_{(1,18)} = 13.83$, $p < 0.01$; after a mixed 2 × 3 ANOVA, $F_{(1,10)} = 13.67$, $p < 0.005$), but no

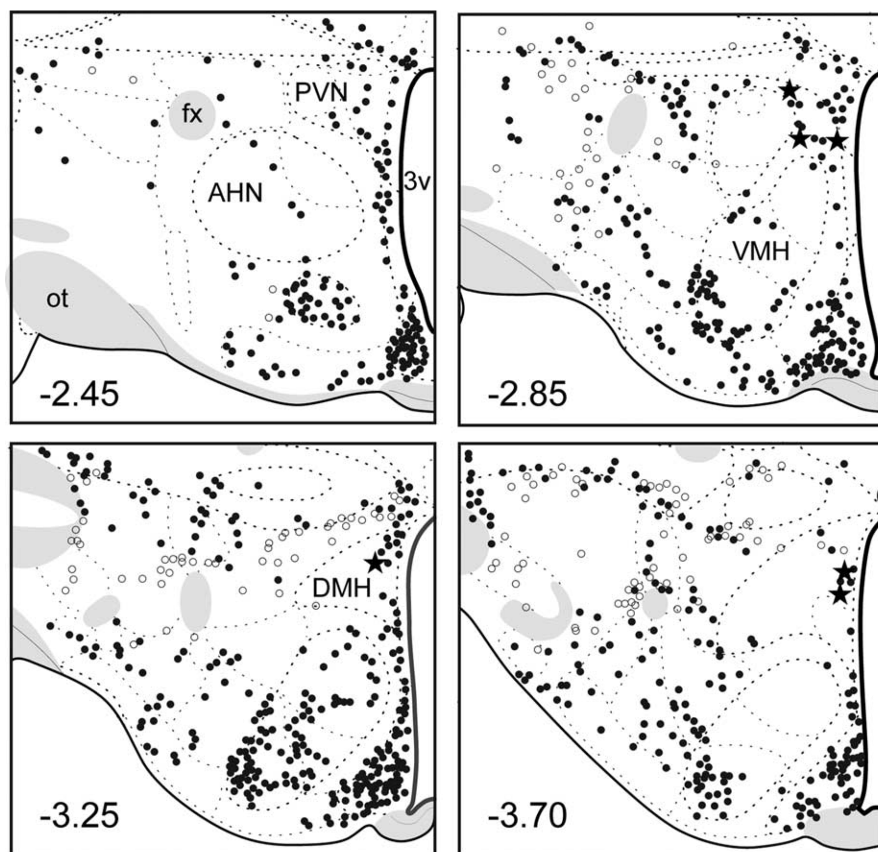

Figure 3. ERα is coextensive with hcrt/orx. ERα nuclei are represented by closed circles, hcrt/orx neurons are represented by open circles, and double-labeled ERα plus hcrt/orx cells are represented by stars. Numbers are in millimeters caudal to bregma. AHN, Anterior hypothalamic nucleus; fx, fornix; ot, optic tract; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; 3v, third ventricle.

other dose of hcrt-1/orx-A had this effect. This dose appears responsible for a significant main effect of hcrt/orx on firing rate detected in the 2 × 3 ANOVA. A *post hoc* (Tukey) test revealed no significant difference in rate between aCSF-treated control groups across dose. The 1.4 nmol dose of hcrt-1/orx-A significantly increased VTA DA neuron population activity (number of spontaneously active neurons detected in each electrode track (Fig. 4*D*) (pairwise comparison, $F_{(1,24)} = 6.42$, $p < 0.05$; after a significant interaction in a 2 × 3 mixed ANOVA, $F_{(2,10)} = 16.71$, $p < .001$). The highest dose of hcrt-1/orx-A tested (140 nmol) significantly decreased the population activity of DA neurons (cells per track) (Fig. 4*D*) (one-way repeated measures ANOVA on 140 nmol dose, $F_{(2,6)} = 12.20$; $p < 0.01$). This decrease was reversed by systemic apomorphine injection (Tukey's *post hoc* test).

TH-positive VTA neurons with hcrt/orx appositions show increases in Fos-ir after copulation

Two-way ANOVA on mean number of Fos-ir nuclei in non-TH positive neurons showed significant effects of treatment ($F_{(1,38)} = 38.88$; $p < 0.001$) and anatomical level ($F_{(7,38)} = 12.59$; $p < 0.001$), as well as a significant interaction between these two factors ($F_{(7,38)} = 7.45$; $p < 0.001$), suggesting that Fos induced during copulation appears preferentially in the anterior counting levels. This was confirmed by subsequent one-way ANOVA and *post hoc* (Tukey) tests, which reveal the two anteriormost levels ("rostral" and "middle 1") to have significantly greater numbers of Fos-positive nuclei ($F_{(3,24)} = 9.48$; $p < 0.001$). The same was not true for noncopulating controls, in which basal Fos-ir did not differ by level (Table 3). The percentage of TH-labeled neurons with hcrt/orx appositions did not differ by experimental treatment; however, this measure did show a marked rostrocaudal gradient, with the most rostral level having a significantly higher percentage of these neurons in both groups (Table 4) ($F_{(3,38)} = 133.57$; $p < 0.001$). This finding is consistent with the higher density of hcrt/orx fibers that we observed in the anterior VTA. The percentage of TH-labeled neurons showing Fos-ir (but not having hcrt/orx appositions) differed neither by treatment or by rostrocaudal level. The percentage of TH neurons showing both Fos and hcrt/orx appositions showed a significant effect of treatment ($F_{(1,38)} = 8.62$; $p < 0.01$), level ($F_{(3,38)} = 4.53$; $p < 0.01$), and their interaction ($F_{(3,38)} = 4.53$; $p < 0.01$). One-way ANOVA on means from the experimental group suggests that copulation-induced Fos-ir in TH neurons with hcrt/orx appositions occurs most prominently within cells located in the rostral VTA ($F_{(3,24)} = 4.85$; $p < 0.05$).

Discussion

The increased Fos-ir in hcrt/orx neurons in the LHA after copulation suggests that activation of these cells accompanies male reproductive behavior (Morgan and Curran, 1991). These data are consistent with earlier 2-deoxyglucose studies reporting increased metabolic activity in LHA after exposure to estrous female odors (Orsini et al., 1985). This effect may reflect enhanced hcrt/orx transmission in hcrt/orx neuron terminal areas like the mPOA, in which hcrt/orx has been shown to facilitate male copulatory behavior (Gulia et al., 2003). Sex-related Fos induction in hcrt/orx neurons is consistent with the notion that the hcrt/orx neurons are sensitive to natural reinforcers. A recent study demonstrated that Fos-ir increased in hcrt/orx neurons of rats conditioned to expect a food reward and that this increase in Fos-ir correlated with the animals' preference score in the conditioned place preference paradigm (Harris et al., 2005). The activation of hcrt/orx neurons may be a reward-related phenomenon, because the above study showed a lack of robust increases in Fos-ir hcrt/orx cells in animals exposed to a novel object stimulus. These authors also report percentages of hcrt/orx neurons expressing basal (15%), novelty-induced (18%), and food-conditioned (50%) Fos-ir that are compatible with those we report here (12% basal vs 40% copulation-induced). These observations suggest that hcrt/orx neurons are activated by natural rewards such as food and sex.

The estrogenic regulation of hcrt/orx described here provides additional evidence for possible hypocretinergic control of male sexual behavior. It is notable that the time course of hcrt/orx loss reported here is compatible with classic behavioral data showing that male sexual behavior takes weeks to decline after castration, and furthermore, that it is E_2 rather than DHT that is required for

Table 2. OX_1 antagonist SB 334867 impairs male copulatory behavior

Treatment	Mount latency (sec)	Intromission latency (s)	Ejaculation latency (s)	Mount frequency	Intromission frequency	Ejaculation frequency	PEI (s)
Vehicle	22.4 ± 7.7	39.0 ± 9.2	585.7 ± 80.7	13.7 ± 1.5	16.9 ± 1.5	2.1 ± 0.2	485.0 ± 37.3
SB 334867	48.2 ± 8.8	107.7 ± 20.4**	804.5 ± 103.9	13.7 ± 1.7	12.4 ± 1.7	1.3 ± 0.2*	548.0 ± 44.5

Values are given as mean ± SEM. *$p < 0.05$; **$p < 0.01$. PEI, postejaculatory interval.

reinstatement of behavior (Hull et al., 2006). Without further experiment, we can only speculate on the mechanisms underlying the continued presence of hcrt/orx in the absence of E_2. The simplest explanation may be that the lag time to decreased hcrt/orx levels mirrors the latency to a readily quantifiable depletion in vesicular stores of the transmitter. As discussed below, the absence of ERs in hcrt/orx neurons suggests regulation of hcrt/orx expression by inputs from neurons containing those receptors. Because neuropeptide synthesis (Enyeart et al., 1987), motility (Shakiryanova et al., 2005), and release are activity-dependent phenomena (Fulop et al., 2005), any postcastration decreases in hcrt/orx neuronal excitability (Smith et al., 2002) may negatively affect these processes, slowing the kinetics of peptide release such that reduced levels of synthesis might not be apparent for some time. By whatever mechanism, it seems likely that action of the steroid is the first element of a complex cascade responsible for maintaining basal levels of the peptide.

Just as hcrt/orx neurons seem to regulate food intake in response to humoral factors related to energy balance (Olszewski et al., 2003; Burdakov et al., 2006), hcrt/orx neurons also appear to be sensitive to the hormonal milieu and may facilitate reproductive behavior in a similar manner. Data presented here suggest that basal hcrt/orx expression is maintained by E_2. In gonadally intact animals expressing the full complement of hcrt/orx, this transmitter would presumably facilitate processing in structures important to male sexual behavior and reward. The hcrt/orx neurons enjoy substantial reciprocal connections with areas like the mPOA, bed nucleus of the stria terminalis (BNST), and VTA (Peyron et al., 1998; Sakurai et al., 2005), which are known to be important for expression of male sexual behavior (for review, see Hull et al., 2006). Decreases in hcrt/orx after castration would be expected to diminish an important source of excitatory input to these structures, thereby impairing behavior.

The manner in which ER activation maintains basal hcrt/orx expression awaits additional study; however, it is likely to be

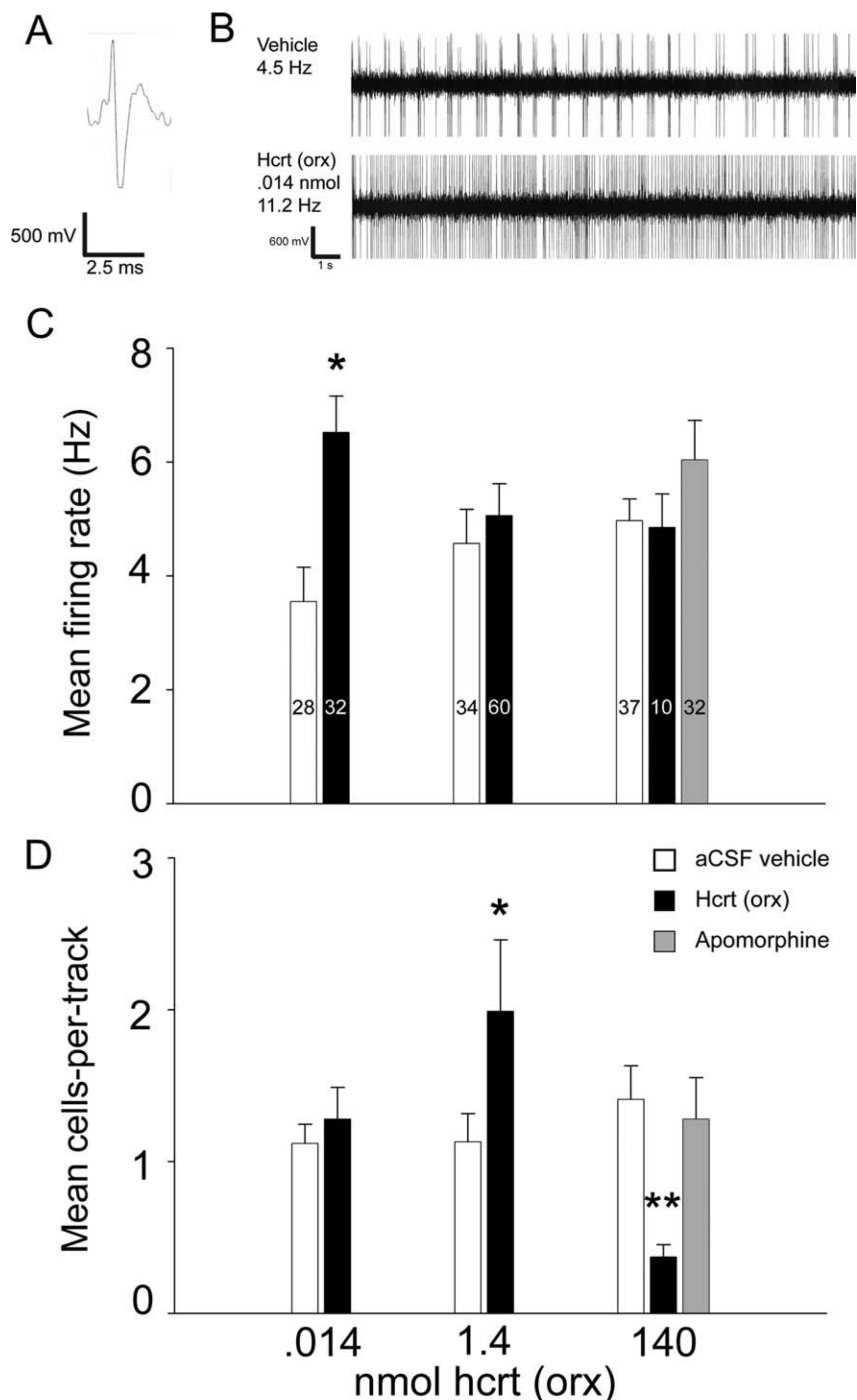

Figure 4. Hcrt/orx regulates VTA DA neuronal activity *in vivo*. ***A***, Waveform of a typical VTA DA neuron showing characteristic wide action potential and an IS/SD break. ***B***, Top, Firing rate and pattern of the same vehicle-treated neuron showing typical bursting activity; bottom, a fast-firing neuron observed after local infusion of 0.014 nmol of hcrt 1/orx A ($n = 4$). ***C***, ***D***, Dose relationship of locally infused hcrt-1/orx-A on the firing rate and population activity of VTA DA neurons. Hcrt-1/orx-A (1.4 nmol; $n = 5$) increases population activity. The decreased population activity after 140 nmol of hcrt 1/orx A ($n = 4$) was reversed by systemic apomorphine (20 μg/kg). The number inside each bar shows the number of neurons recorded. Shown are means ± SEM. *$p < 0.05$; **$p < 0.01$.

Table 3. Number of Fos-ir nuclei in non-TH-positive cells of VTA at four rostrocaudal levels

Treatment	Rostral	Middle 1	Middle 2	Caudal
Noncopulation	2.8 ± 1.3^a	0.4 ± 0.2^a	2.6 ± 1.3^a	0.6 ± 0.4^a
Copulation	21.2 ± 2.5^b	18.5 ± 4.0^b	6.3 ± 3.5^c	2.0 ± 0.8^c

Means ± SEM with the same lowercase letter do not differ significantly ($p < 0.05$).

Table 4. Percentage of TH neurons showing hcrt/orx appositions, Fos, or both

Level of VTA Treatment	TH-hcrt/orx	TH-Fos (nonapposed)	TH-Fos-hcrt/orx
Rostral			
Noncopulation ($n = 6$)	47.6 ± 6.8^a	1.3 ± 0.8^a	0^a
Copulation ($n = 6$)	53.7 ± 8.7^a	0.9 ± 0.9^a	9.5 ± 3.9^b
Middle 1			
Noncopulation ($n = 5$)	12.5 ± 2.1^b	0^a	0^a
Copulation ($n = 6$)	10.3 ± 1.2^b	0.8 ± 0.3^a	$2.2 \pm 0.7^{b,c}$
Middle 2			
Noncopulation ($n = 6$)	6.2 ± 0.9^b	0^a	$0^{a,c}$
Copulation ($n = 6$)	6.0 ± 0.9^b	0.8 ± 0.4^a	$0.5 \pm 0.3^{a,c}$
Caudal			
Noncopulation ($n = 5$)	5.2 ± 0.6^b	0^a	$0^{a,c}$
Copulation ($n = 6$)	3.5 ± 0.4^b	0.1 ± 0.1^a	$0^{a,c}$

In each counting column, means ± SEM with the same lowercase letter do not differ significantly ($p < 0.05$).

driven by afferents from ERα-expressing brain areas that project to LHA (Simerly et al., 1990; Yoshida et al., 2006), particularly those structures found to have some excitatory projections [e.g., BNST and mPOA (Georges and Aston-Jones, 2002; Henny and Jones, 2006)]. We report no colocalization of AR with hcrt/orx, and, although a few hcrt/orx neurons were ERα-immunopositive, these cells are not numerous enough to explain the marked effects of castration, nor are they in register with those seen to decrease their hcrt/orx content after castration (Figs. 2*A*, 3). We also report that ERα-ir nuclei and hcrt/orx neurons are often juxtaposed, raising the possibility of local regulation of hcrt/orx neuronal and gene expression activity by neighboring ERα-containing cells. The importance of excitatory local circuit activity of this type has been described in the hcrt/orx system (Li et al., 2002). However, until the requisite anatomical experiments show excitatory synapses made by ERα-containing cells onto hcrt/orx neurons, hormone-dependent, afferent-driven expression of hcrt/orx cannot be assumed. *Hcrt* expression fluctuates diurnally (Taheri et al., 2000), during pregnancy (Kanenishi et al., 2004), and in response to various dietary manipulations (Cai et al., 1999; Griffond et al., 1999). The molecular mechanisms that regulate the dynamics of *hcrt* expression have not been characterized, thus tracing a path from nucleus to membrane, and naming candidate signaling molecules that may affect hcrt/orx expression is difficult at this time.

The notion that hcrt/orx signaling is involved in reinforcing behaviors like sex is also supported by data showing impairments in sexual behavior after treatment with the OX_1 antagonist SB 334867 (Table 2). At a dose similar to those used to block stress-induced reinstatement of cocaine self-administration (Boutrel et al., 2005), SB 334867 significantly increased intromission latency and decreased numbers of ejaculations, suggesting that blockade of hcrt/orx transmission may affect the incentive properties of estrous females.

DA is an important neurotransmitter for reward, incentive motivation, and adaptive behavior (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Wise, 2004). We observed a potent dose-dependent excitatory effect of hcrt/orx on VTA DA neuron activity. This finding supports the role of hcrt/orx in reinforcement and suggests that the mesolimbic DA system is a locus outside the mPOA in which hcrt/orx projections may act to enhance male sexual behavior. At the lowest dose tested (0.014 nmol), hcrt/orx increased firing rate without affecting population activity (cells/track) (Fig. 4*C*). At the intermediate dose (1.4 nmol), the population activity of DA neurons was increased, indicating that previously quiescent, hyperpolarized neurons were activated (Fig. 4*D*). At the highest dose (140 nmol), the population activity of VTA DA neurons was decreased. Interestingly, the hcrt/orx-induced reduction in VTA DA neuron population activity was reversed by acute administration of the DA agonist apomorphine (Fig. 4*D*). In normal animals, apomorphine hyperpolarizes DA neurons by activating autoreceptors and reduces their firing rate and population activity. However, after chronic antipsychotic treatment or repeated treatment with drugs of abuse, apomorphine can reverse drug-induced decreases in population activity (Grace et al., 1997; Shen and Choong, 2006). Apomorphine is thought to reverse depolarization inactivation by repolarizing overexcited cells enough to resume firing. Together, these data suggest that hcrt/orx exerts a dose-dependent excitatory effect on VTA DA neurons, consistent with previous *in vitro* work (Korotkova et al., 2003).

The importance of descending pathways from the LHA to VTA was explored in a number of experiments (Bielajew and Shizgal, 1986; Shizgal, 1989; You et al., 2001). These data suggest that increases in NAc DA during hypothalamically mediated motivated behaviors like copulation (Pfaus et al., 1990; Wenkstern et al., 1993) or feeding (Hernandez and Hoebel, 1988; Rada et al., 2005) may rely on these descending projections. That such projections contain hcrt/orx is supported by experiments in which NAc DA efflux is seen to increase after intra-VTA injection of hcrt/orx (Narita et al., 2006). Within the hypothalamus, serotonin [5-hydroxytryptamine (5-HT)] can potently hyperpolarize hcrt/orx neurons in the LHA (Li et al., 2002). Selective serotonin reuptake inhibitors or 5-HT itself, reverse dialyzed into the LHA near the main population of hcrt/orx-expressing cells, reduces basal and female-elicited NAc DA release and impairs copulation (Lorrain et al., 1997, 1999). In light of data presented above showing activation of hcrt/orx neurons during copulation and of VTA DA neurons by hcrt/orx, we argue that descending hcrt/orx projections to the VTA could mediate sex-related NAc DA release. Furthermore, inhibition of these projections by intra-LHA 5-HT may explain the inhibitory effect of 5-HT on NAc DA release and sexual behavior.

An anatomical substrate for hcrt/orx-DA interactions is apparent in our finding that TH-positive VTA neurons are innervated by the hcrt/orx system and show increased Fos-ir with exposure to rewarding stimuli like copulation (Fig. 5, Table 4). This effect showed both behavioral relevance and spatial specificity, as Fos induction in hcrt/orx-apposed TH neurons was detected only in the anterior VTA of copulating animals. It is perhaps unremarkable that this pattern of activation appears in this portion of VTA, as DA cells there lie just caudal to the main population of hcrt/orx neurons, whose descending fibers traverse this area before disbursing to more distal targets in the brainstem (Peyron et al., 1998).

These data suggest a novel pathway by which gonadal steroids may affect a hypothalamic input to DA systems implicated in motivated behavior (Fig. 6). They also add sexual behavior to the growing list of behaviors that are regulated by hcrt/orx, including arousal, ingestive behavior, and drug seeking.

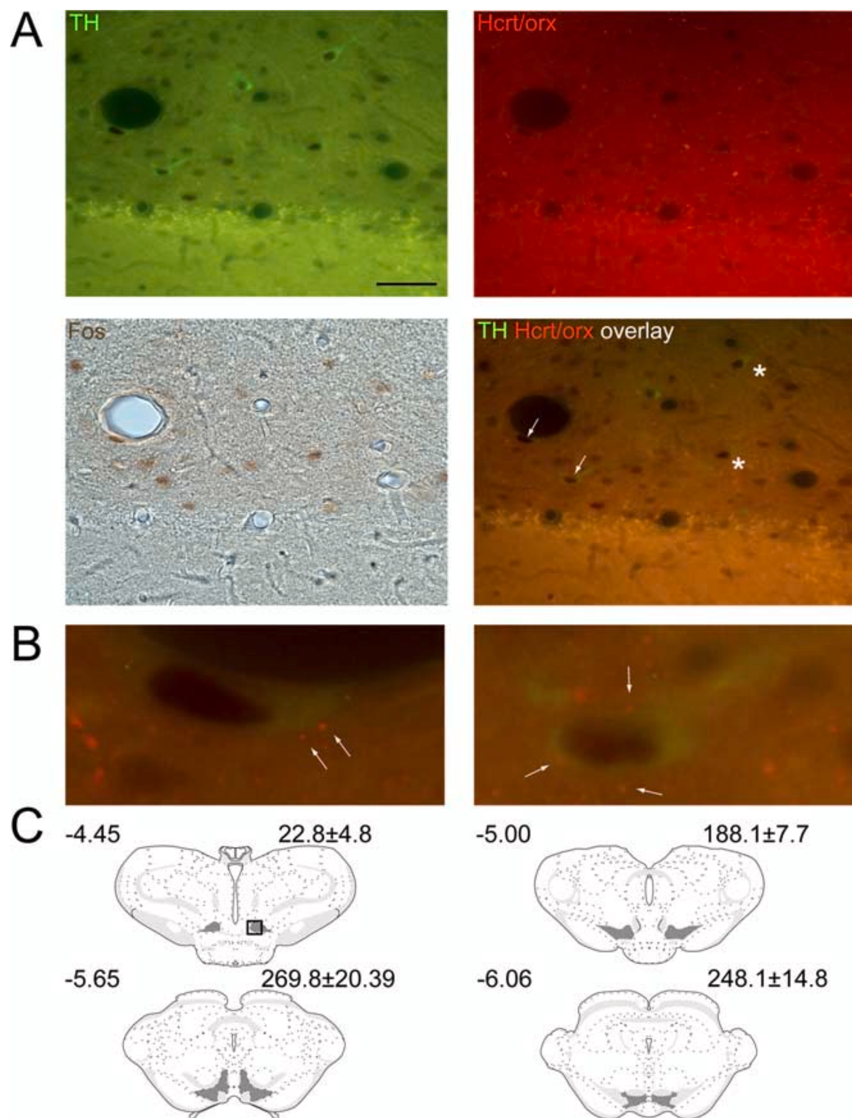

Figure 5. Copulation induces Fos-ir in hcrt/orx-apposed VTA DA neurons. ***A***, Micrographs showing TH, hcrt/orx, and Fos labeling in VTA. Arrows denote Fos-positive TH neurons with hcrt/orx appositions; asterisks mark double-labeled neurons without appositions. Scale bar, 45 μm. ***B***, Detail of Fos-positive TH neurons with arrows indicating sites of hcrt/orx boutons in apposition. ***C***, Coronal sections showing rostrocaudal levels of VTA used in counting (dark shaded). Numbers at the top left of each section are in millimeters from bregma. Numbers at the top right are mean $\pm$ SEM estimates of cell density at that level. The box indicates the area of micrographs in ***A***.

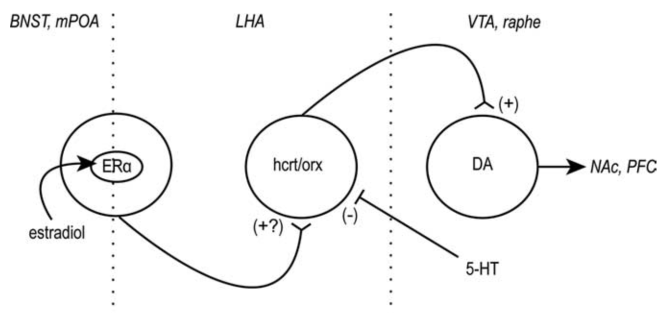

Figure 6. Model for regulation of hcrt/orx by gonadal steroids and VTA DA by hcrt/orx. Estradiol, synthesized from gonadal testosterone by aromatase, acts on ERα-containing neurons in BNST, mPOA, and LHA. These structures project to hypothalamic hcrt/orx neurons. Excitatory projections from these structures may influence hcrt/orx neuronal and gene expression activity in a steroid-sensitive manner. Hcrt/orx projections to VTA enhance midbrain DA neuronal activity during male sexual behavior. This effect may be blocked by intra-LHA infusions of 5-HT that inhibit hcrt/orx activity, impairing sexual behavior and NAc DA release.

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