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Circadian rhythm disruption by a novel running wheel: Roles of exercise and arousal in blockade of the luteinizing hormone surge

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Abstract

Exposure of proestrous Syrian hamsters to a new room, cage, and novel running wheel blocks the luteinizing hormone (LH) surge until the next day in ~75% of hamsters (Legan et al, 2010) [1]. The studies described here tested the hypotheses that 1) exercise and/or 2) orexinergic neurotransmission mediate novel wheel blockade of the LH surge and circadian phase advances. Female hamsters were exposed to a 14L:10D photoperiod and activity rhythms were monitored with infra-red detectors. In Expt. 1, to test the effect of exercise, hamsters received jugular cannulae and on the next day, proestrus (Day 1), shortly before zeitgeber time 5 (ZT 5, 7 hours before lights-off) the hamsters were transported to the laboratory. After obtaining a blood sample at ZT 5, the hamsters were transferred to a new cage with a novel wheel that was either freely rotating (unlocked), or locked until ZT 9, and exposed to constant darkness (DD). Blood samples were collected hourly for 2 days from ZT 5–11 under red light for determination of plasma LH levels by radioimmunoassay. Running rhythms were monitored continuously for the next 10–14 days. The locked wheels were as effective as unlocked wheels in blocking LH surges (no Day 1 LH surge in 6/9 versus 8/8 hamsters, $P > 0.05$) and phase advances in the activity rhythms did not differ between the groups ($P = 0.28$), suggesting that intense exercise is not essential for novel wheel blockade and phase advance of the proestrous LH surge. Expt. 2 tested whether orexin neurotransmission is essential for these effects. Hamsters were treated the same as in Expt. 1 except they were injected (i.p.) at ZT 4.5 and 5 with either the orexin 1 receptor antagonist SB334867 (15 mg/kg per injection) or vehicle (25% DMSO in 2-hydroxypropyl-beta-cyclodextrin (HCD)). SB-334867 inhibited novel wheel blockade of the LH surge (surges blocked in 2/6

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SB334867-injected animals versus 16/18 vehicle-injected animals, $P < 0.02$) and also inhibited wheel running and circadian phase shifts, indicating that activation of orexin 1 receptors is necessary for these effects. Expt. 3 tested the hypothesis that novel wheel exposure activates orexin neurons. Proestrous hamsters were transferred at ZT 5 to a nearby room within the animal facility and were exposed to a new cage with a locked or unlocked novel wheel or left in their home cages. At ZT 8, the hamsters were anesthetized, blood was withdrawn, they were perfused with fixative and brains were removed for immunohistochemical localization of Fos, GnRH, and orexin. Exposure to a wheel, whether locked or unlocked, suppressed circulating LH concentrations at ZT 8, decreased the proportion of Fos-activated GnRH neurons, and increased Fos-immunoreactive orexin cells. Unlocked wheels had greater effects than locked wheels on all three endpoints. Thus in a familiar environment, exercise potentiated the effect of the novel wheel on Fos expression because a locked wheel was not a sufficient stimulus to block the LH surge. In conclusion, these studies indicate that novel wheel exposure activates orexin neurons and that blockade of orexin 1 receptors prevents novel wheel blockade of the LH surge. These findings are consistent with a role for both exercise and arousal in mediating novel wheel blockade of the LH surge.

Keywords

circadian rhythms; luteinizing hormone; orexin; hypocretin; novelty; phase shifts

1. Introduction

Circadian (~24 h) rhythms are essential for survival because they coordinate and synchronize the cycles of internal physiological processes, such as hormone secretion, with external environmental cycles (e.g., the daily light:dark cycle) and with each other. A variety of stimuli, both photic and nonphotic, influence circadian rhythms. Whether these timing signals (zeitgebers) improve or interfere with the synchrony of rhythms depends on the time of presentation, nature, and duration of the signals. Shift workers, who routinely undergo abrupt transitions to a new time schedule and experience an acute demand to be alert and active during a previous rest phase, often exhibit attenuation or desynchrony of circadian rhythms [2–4]. Chronic disruption of circadian rhythms has deleterious health effects, including increased risk of metabolic and cardiovascular disease [5, 6]. Among women, shift work is also associated with increased incidence of menstrual cycle irregularities, miscarriages, pre-term births, and breast cancer [7, 8]. Although women constitute a significant proportion of the workforce, the majority of experimental studies of circadian rhythms in rodents have focused on males.

In order to elucidate the mechanisms underlying circadian rhythm disruption by shift work in females, we investigated the effects of rapid transitions to activity and wakefulness in female hamsters on the circadian locomotor activity rhythm and the preovulatory LH surge. In hamsters and other rodents, the LH surge not only depends on an antecedent increase in circulating estradiol concentrations, but is also regulated by the circadian timing system, as demonstrated by findings that the LH surge persists under constant conditions [9], can be entrained by the light (L):dark (D) cycle (photoperiod) [10], and depends on the master

circadian pacemaker in the suprachiasmatic nucleus (SCN) [11, 12]. Circadian regulation ensures that the LH surge occurs consistently at the same time of day, i.e., in Syrian hamsters maintained in a 14 hour light (L):10 hour dark (D) photoperiod, the LH surge occurs once every 4 days on proestrous afternoon and peaks at zeitgeber time (ZT) 9 (i.e., 3 hours before lights-off, defined as ZT 12) [10, 13, 14]. In addition, in rats and hamsters the LH surge is associated with increased activation of the hypothalamic gonadotropin releasing hormone (GnRH) neurons, which is directly responsible for triggering the LH surge [15, 16].

Presentation of an arousing stimulus, a novel running wheel, at ZT 5 on proestrus blocks the expected LH surge in the majority (~75%) of hamsters and delays it until the next day, when it occurs ~2 hours earlier [1]. This effect depends on the novelty of the running wheel, because it is not observed in hamsters with prior running wheel experience that are transferred to a new cage with a new wheel after cannulation and identical transport from the animal facility to the laboratory [1]. The mechanisms underlying novel wheel blockade of the LH surge are unknown. One possible mechanism whereby novel wheel exposure blocks the LH surge is physical exercise, because previous findings suggested that the novel wheel-induced delay of the LH surge or the estrous cycle and mating behavior correlates well with the intensity of wheel running [1, 17]. Alternatively, it is possible that novel wheel blockade of the LH surge does not depend on intense physical exercise, but may result from an increased level of arousal induced by a novel stimulus. As noted above, mere transportation to a new room followed by transfer to a clean cage containing a clean wheel is not a novel enough stimulus to block the LH surge in hamsters previously housed with wheels [1], therefore the arousal must exceed a minimum threshold.

Arousal is associated with stimulation of orexin (hypocretin) neurons, located in the lateral hypothalamus, which participate in regulation of a variety of behaviors and physiological functions, including wakefulness, alertness, feeding, and secretion of pituitary hormones, e.g., LH [18–23]. Fos expression and firing activity of orexin neurons are greater during wakefulness than sleep [24, 25]. Furthermore, the firing rate of orexin neurons is higher during exploratory behavior than during quiet wakefulness [25]. In rats and hamsters, orexin neurons not only innervate the noradrenergic locus coeruleus, whose cortical projections promote wakefulness and attention [26], but also project broadly throughout the brain [27, 28]. Orexin projections innervate several hypothalamic regions, e.g., the medial preoptic area, the ventromedial nucleus (VMH), dorsomedial nucleus (DMH), and arcuate nucleus (ARC), as well as the GnRH neurons, which are located primarily in the medial septum/diagonal band and directly control LH release [19, 27–29]. Indeed, a large majority of rat hypothalamic GnRH neurons (~85%) are contacted by orexin-immunoreactive fibers and exhibit orexin-1 receptor-immunoreactivity [30], suggesting that orexin regulates GnRH neurons and thereby modulates LH release.

Orexin has complex effects on both the tonic (i.e., basal), and surge modes of LH secretion, which depend on the dose and brain region [31–34]. Thus, suppression of orexinergic neurotransmission by administration of either an orexin-1 receptor antagonist in the rostral preoptic area [35] or anti-orexin A antiserum into the lateral ventricle [36] can suppress peak LH levels or abolish LH surges in steroid-treated ovariectomized rats. These findings

suggest that orexin neurotransmission is required for LH surges. In contrast however, microinjection of a large dose of orexin A (1 µg/0.5 µl) in the medial preoptic area or arcuate/median eminence, suppresses peak post-injection LH levels during the expected time of the LH surge in steroid-primed ovariectomized rats, suggesting that increased levels of orexin A either delay or block LH surges [35]. Therefore, if novel wheel exposure during the critical period activates orexin neurons in proestrous hamsters, the resultant increase in orexinergic neurotransmission might block the preovulatory LH surge.

Based on these observations, we tested the hypotheses that 1) exercise and/or 2) arousal-induced increases in orexin neurotransmission mediate novel wheel-induced blockade of the LH surge. The roles of exercise and orexin neurotransmission on novel wheel induction of a phase shift in the activity rhythm and on timing of the LH surge were also investigated.

2. Materials and methods

2.1 Animals

Adult female Syrian hamsters (Harlan, HsdHan:AURA, 3–4 months old) were housed individually in polypropylene cages (10.25 × 8.25 × 18.75 inches) within light-tight, ventilated compartments containing soiled bedding from male hamsters. Temperature was maintained at 21° C. Food and water were available ad libitum. Estrous cyclicity was monitored by daily examination of vaginal discharge [37] until 2–3 consecutive 4-day cycles were observed, after which animals were examined every 4th day on expected estrus. Only hamsters exhibiting at least three consecutive estrous cycles were used. The hamsters were exposed to a 14L:10D photoperiod (lights on at 0300 h) or to constant darkness, as indicated in each experiment described below. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and are consistent with the Institute of Laboratory Animal Resources Guide for Care and Use of Laboratory Animals, 8th edition.

2.2 Experiment 1. Effect of wheel immobilization on novel wheel blockade of the LH surge and circadian phase advances

Activity rhythms were monitored continuously using infra-red motion detectors interfaced with a ClockLab data collection system (Coulbourn Instruments, Whitehall, PA). After baseline activity was recorded for at least seven days, hamsters were anesthetized with isoflurane and fitted with a jugular cannula on the day before proestrus (diestrus 2). On proestrus (Day 1) shortly before zeitgeber time (ZT) 5, hamsters were transported from the animal facility to the laboratory. A blood sample was obtained and each hamster was placed into a new cage containing a novel running wheel (13.5 inches in diameter) and exposed to constant darkness (DD). Half the animals were exposed to a wheel that rotated freely; the other half received a wheel that was locked from ZT 5 to ZT 9 after which it rotated freely for the remainder of the experiment. Blood samples (300 µl) were obtained on Day 1 at the onset of DD and hourly thereafter in dim red light for the next 6 hours, and on Day 2 (250 µl) at the same clock times. The blood samples were immediately centrifuged and the plasmas were separated and stored at –20 ° C. To prevent lowering of hematocrit from repeated blood withdrawal, the red blood cells from each sample were resuspended in an

equal volume of sterile heparinized saline (50 IU/ml), and re-injected via the cannula after collecting the next blood sample. During the two days of blood sampling, the number of wheel revolutions was monitored by an event counter and recorded at each sampling time. After the last sample, animals were briefly anesthetized with isoflurane to interiorize their cannulae before being returned to the animal facility. To determine phase shifts, wheel running activity in DD was monitored in the animal facility for the next ~10–14 days. Actograms depicting daily activity rhythms were generated and circadian phase shifts were calculated by the linear regression method with ClockLab software as described previously [38]. Day and time of occurrence of LH surges as indicated by plasma LH concentrations were determined by RIA as described previously [38].

2.3 Experiment 2. Effect of antagonism of orexin 1 receptors on novel wheel blockade of the LH surge and circadian phase advances

The experimental protocol was the same as that for Experiment 1 except that before obtaining the first blood sample at ZT 5, either the orexin 1 receptor antagonist SB334867 or vehicle (25% DMSO in 2-hydroxypropyl-beta-cyclodextrin) was injected i.p. (100 µl/130 g body weight). Animals were randomly assigned to one of three treatment groups: 1) home cage, vehicle, 2) novel wheel, vehicle, or 3) novel wheel, SB334867 (two 15 mg/kg doses at ZT 4.5 and 5).

SB334867 (Tocris Bioscience or NIMH Chemical Synthesis and Drug Supply Program) was selected based on a previous report that SB334867 at a dose of 30 mg/kg i.p. inhibited arousal (i.e., by preventing stimulus-induced increases in locomotor activity, heart rate, and blood pressure) in rats exposed to a panic-inducing stimulus [39]. Because SB334867 is insoluble in aqueous solutions, a 30mg/kg dose was first dissolved in DMSO, as reported in previous studies [39]. However, because we found that injection of 100% DMSO i.p. interferes with novel wheel blockade of the LH surge (described in Results), two other diluents were also tested that contained 2-hydroxypropyl-β-cyclodextrin (HCD, 45% in H₂O) in attempts to decrease the proportion of DMSO yet still solubilize the antagonist: 1) 10% DMSO, 10% HCD, and 80% water and 2) 25% DMSO and 75% HCD. In addition, to enhance the antagonists's solubility, the dose was decreased to 15 mg/kg, which was injected twice. All injection solutions were prepared by first dissolving SB334867 completely in the appropriate volume of DMSO; then while vortexing, HCD either alone or followed by water was added by slow drip.

2.4 Experiment 3. Effect of novel wheel exposure on Fos expression

At ZT 5 on the day of expected proestrus, hamsters were transported in their home cages to a nearby room in the animal facility and randomly assigned to one of three treatments: 1) remain in home cage without a wheel, 2) transfer to a new cage with a freely moving (unlocked) wheel, or 3) transfer to a clean cage with an immobilized (locked) wheel. The unlocked wheels were interfaced with Clocklab, which detected steady running in all animals in this group. All three groups were exposed to DD for 3 hours. At ZT 8, animals were exposed to dim red light and were anesthetized with xylazine (20 mg/kg, i.p.) and ketamine (125 mg/kg, i.p.). While under deep anesthesia, a blood sample was obtained by cardiac puncture, after which the animals were perfused intracardially with phosphate-

buffered saline (PBS) followed by buffered 4% paraformaldehyde. The brains were removed, post-fixed for three hours in buffered 4% paraformaldehyde at room temperature, and infiltrated with sucrose. Serial coronal sections (30 μ m) were cut on a cryostat and stored in sets of 1 in 6 in cryoprotectant [40] at -20° C until processed for immunohistochemistry. The blood samples were allowed to clot at room temperature (for ~30 minutes) and then were centrifuged at $2,500 \times g$ for 20 minutes. Serum samples were aspirated and stored at -20° C for determination of LH concentrations by RIA. Immunohistochemistry for Fos was conducted as described below. Fos expression was examined in GnRH and orexin neurons, as well as in two regions which receive orexin innervation and project to the GnRH neurons (e.g., the ARC and VMH), and in two brain regions regulating circadian phase (i.e., the SCN and the intergeniculate leaflet [IGL] of the thalamus). Fos expression in the SCN was quantified in the SCN as a whole and in its two main subregions, the ventrolateral and dorsomedial areas).

2.5 LH Radioimmunoassay

Circulating LH concentrations were determined in 1–60 μ l aliquots by means of an RIA described previously [38], and are reported in terms of nanograms RP-3 per milliliter (NHPP, NIDDK). All samples were analyzed in 4 assays for which the sensitivity (100% – 2 SD of maximum binding) averaged 0.11 ng/tube, and the inter-assay coefficient of variation was 8.5%. In Expts. 1 and 2, an LH surge was defined as an increase in plasma LH levels at least 2 standard deviations above basal levels (at ZT 5) in at least 2 consecutive samples. In Expt. 3, the same criteria were applied for the single sample obtained from each animal, the basal LH concentrations being determined from the mean LH concentrations in the hamsters receiving freely moving wheels.

2.6 Immunohistochemistry

Three sets of a 1 in 6 series of brain sections (30 microns thick) from each brain were used, one set for Fos alone, one set for colocalization of Fos and GnRH, and another set for Fos and orexin. Within each set the sections were 180 microns apart and spanned a rostral to caudal range within each region corresponding to the following figures in A Stereotaxic Atlas of the Golden Hamster Brain [41]: GnRH neurons (Figs. 14–18), orexin neurons (Figs. 26–32), ARC (Figs. 28–34), VMH (Figs. 28–32), SCN (Figs. 23–25), IGL (Figs. 26–34). The orexin neurons were localized in the lateral hypothalamus, with greatest abundance in the retrochiasmatic area and lesser abundance at the level of the arcuate nucleus and median eminence, closely resembling the distribution of these neurons in male hamsters [28].

The sections were cleared of cryoprotectant with two 10 minute washes in 50 mM PBS, pH 7.3. Endogenous peroxidase activity was blocked or inhibited by incubation for 30 minutes in 0.5% hydrogen peroxide in PBS, followed by three 10 minute washes in PBS. Sections were then subjected to 15 minutes of antigen retrieval in 10 mM citrate buffer, pH 8.5, at 80° C and cooled to room temperature. Following two final PBS washes for 10 minutes each, the sections were pre-treated in blocking buffer (2% horse serum/0.2% triton X-100/PBS) for one hour. Before incubation with primary antibody, tissues were exposed to 0.1 mg/ml avidin (Vector Labs, Burlingame, CA) for 15 minutes, rinsed in PBS, incubated in 0.5 mg/ml biotin (Vector Labs) for 15 minutes, and again rinsed with PBS. The tissue

sections were incubated in the primary antibody, goat-anti c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5000 in blocking buffer), for one hour at room temperature and then for ~40 hours at 4° C. To remove the antibody, the tissues were washed with PBS at room temperature, five times for 10 minutes each. Sections were then incubated with biotinylated donkey-anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA; 1:600 in blocking buffer) for one hour at room temperature. Sections were again washed in PBS and then incubated in streptavidin/biotin/peroxidase solution (ABC Vectastain Elite; Vector Labs) for one hour at room temperature. After three 5 minute washes with PBS followed by three 5 minute washes with 0.11 M sodium acetate (NaAc, pH 6.5.), sections were stained for 20 minutes with a solution of 2.5% nickel (II) sulfate hexahydrate (Alfa Aesar, Ward Hill, MA), 0.01% diaminobenzadine tetrahydrochloride (DAB; Sigma), and 4 µl/100 ml 30% H₂O₂ in NaAc (NiDAB). Dual labeling of cells was accomplished by first washing the sections once with NaAc for 10 minutes and five times with PBS. Next, the above sequence of incubations and washes was repeated, with the following exceptions: in one set of sections the primary antibody was goat anti-orexin A (Santa Cruz Biotechnology, 1:10,000 in blocking buffer) and the second set was incubated with rabbit anti-GnRH (LR-5, Dr. Robert Benoit, McGill University, 1:120,000 in blocking buffer); the biotinylated secondary antibody was either donkey-anti-goat or anti-rabbit gamma globulin (Jackson ImmunoResearch), respectively. Before chromogen development for 20 minutes at room temperature, sections were washed three times in 50 mM Tris, pH 7.4; the chromogen was 0.01% DAB (Sigma) prepared in Tris buffer with 4 µl/100 ml 30% H₂O₂. Sections were washed three times after DAB incubation with Tris buffer. The sections were mounted on Superfrost slides (VWR) in Tris buffer and allowed to air dry overnight. Slides were then subjected to dehydration with a graded alcohol series followed by three changes of xylene, and cover slipped with DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA).

Micrographs of the sections (~7–9/brain region) were analyzed bilaterally by an individual blind to the treatments to determine the numbers of orexin-immunoreactive cells and GnRH-immunoreactive cells, and the number and percentage of these cells that were also Fos-immunoreactive. Potential differences in Fos expression in distinct regional populations of the SCN were also analyzed. The numbers of Fos-immunoreactive nuclei in the SCN and IGL were determined because exposure of male hamsters to a novel wheel and darkness has previously been shown to decrease c-Fos immunoreactivity in the SCN and increase it in the IGL [42]. Fos immunoreactivity was assessed in the ARC and VMH because these regions exhibit orexin immunoreactive fibers and send afferents to GnRH neurons [27, 29].

2.7 Data Analyses

The proportion of animals whose LH surge was delayed was compared between groups by a Chi Square test or Fisher's exact test. The mean peaks of LH surges, i.e., the maximum levels attained, and the times at which the peak levels occurred were compared between groups by Student's t-test, Kruskal-Wallis ANOVA on ranks or Mann-Whitney Rank Sum test. The correlations between phase shifts in wheel running activity with intensity of running or phase shifts in LH surges were determined by Spearman rank correlation test. Comparisons of the mean phase shifts among groups, the mean LH levels, and the

immunohistochemical data were analyzed by one-way ANOVA. When main effects of the ANOVA were significant, post hoc analyses were performed by the Holm-Sidak multiple comparison procedure (LH levels) or Student-Newman-Keuls tests (ICC data). The significance level was $P < 0.05$.

3. Results

3.1 Experiment 1. Effect of exposure to an immobilized novel wheel on blockade of the LH surge and circadian phase advance

LH surge—Presentation of a novel wheel at ZT 5 on proestrus followed by release into DD caused a one-day blockade of the LH surge regardless of whether the wheel was locked during the first 4 hours or was freely moving (surge delayed in 6/9 versus 8/8 hamsters, respectively, $P = 0.20$) (Fig. 1). The novel wheel-induced blockade of the LH surge was associated with an alteration in the timing, but not the concentration, of peak LH levels. Thus, when the LH surge was blocked until Day 2, it was phase advanced approximately two hours such that the peak occurred at ZT 7, rather than at ZT 9 ($P < 0.02$, Fig. 1). This phase advance in timing of delayed LH surges occurred regardless of whether the wheel was locked or not. There was no difference in the peak level of LH surges between days, although the peak tended to be higher on Day 2 (Fig. 1, $P = 0.17$).

Phase shifts—Introduction of locked or unlocked novel wheels at ZT 5 on proestrus caused phase shifts in the wheel-running rhythm that were highly variable within each group and were not different among the groups (Fig. 2). The phase advance in the wheel running rhythm caused by novel wheel and DD exposure at ZT 5 was independent of whether the wheel was locked or freely moving, or whether the LH surge was delayed or not (Mean $\pm$ SE phase shifts: Unlocked, 2.2 ± 0.4 h; Locked Day 1 surge, 1.3 ± 0.8 h; Locked Day 2 surge, 1.4 ± 0.3 h, $P = 0.28$, Fig. 2). Further, correlation analyses indicated that the phase shifts in wheel running rhythms were not related to intensity of running ($P > 0.05$) or to phase advances in the LH surge ($P > 0.05$).

3.2 Experiment 2. Effect of antagonism of orexin 1 receptors on novel wheel blockade of the LH surge and circadian phase advances

Solubilization of SB334867—The orexin receptor antagonist, SB334867, is readily soluble only in DMSO; however when 100% DMSO was injected, it interfered with the novel wheel blockade of the LH surge, which was blocked in only 5/13 (38%) of hamsters, as compared to 8/8 in Experiment 1. Also, injection of 100% DMSO appeared to cause discomfort, because it caused the animals to curl to the side for < 1 minute. Therefore, two additional formulations of SB334867 were attempted with either 10% or 25% DMSO in HCD to increase its solubility. Neither of these formulations caused discomfort when injected; however, SB334867 was very difficult to dissolve in these formulations, and only completely dissolved in 6 of 10 attempts with 25% DMSO in HCD. In the remaining attempts with both formulations, a milky suspension resulted, in which it was later reported that SB334867 is not in solution [43]; therefore, the results from animals that received these suspensions were excluded from data analyses. SB334867 had to be dissolved just prior to each injection, therefore whenever it could not be dissolved, those hamsters whose daily

activity rhythms had been monitored and who had already been cannulated and transported to the laboratory were re-assigned to the vehicle-injected, novel wheel-exposed group, resulting in a larger final sample size in this treatment group.

LH Surge—There was an overall effect of treatment on blockade of the LH surge on Day 1 ($P<0.002$; Fig. 3). Vehicle injections had no adverse effect on posture or novel wheel blockade of the LH surge (Day 1 surge blocked in 16/18 hamsters, Fig. 3, middle panel). Injection of SB334867 (15 mg/kg at ZT 4.5 and ZT 5) prevented novel wheel-induced blockade of LH surges in most hamsters (Fig. 3, bottom versus middle panel). Thus, the proportion of blocked LH surges on Day 1 in SB334867-treated, wheel-exposed hamsters was different from that in vehicle-treated, novel wheel-exposed hamsters (2/6 vs 16/18, $P<0.02$), but no different from that in vehicle-injected, home cage control hamsters (1/6, $P=1.000$) (Fig. 3). Neither peak LH levels nor the times of peak LH concentrations were different among groups, although the peaks of Day 2 LH surges tended to occur earlier (Fig. 3).

Phase shifts—Phase shifts in locomotor activity are reported from all except two hamsters (one each in the novel wheel, vehicle group and the novel wheel, SB334867 group), whose activity was very sparse and erratic. Overall, the phase shifts were significantly affected by treatment ($P<0.01$; Fig. 4). Novel wheel-exposed, SB334867-injected hamsters had smaller phase shifts than those exposed to a novel wheel and injected with vehicle ($P<0.05$; Fig. 4); there were no other differences due to the high variability in the home cage, vehicle-injected and novel wheel-exposed, SB334867-treated groups.

Activity counts—The amount of wheel running between ZT 6 and ZT 9 varied significantly with treatment ($P<0.005$) and there was a significant interaction between treatment and time ($P<0.05$) (Fig. 5). The average number of wheel revolutions per hour exhibited by the novel wheel, SB334867-injected group was significantly less than that exhibited by the novel wheel, vehicle-injected group at ZT 7, 8, and 9 ($P<0.005$).

3.3 Experiment 3. Effect of novel wheel exposure on Fos expression

LH levels—There was an overall effect of treatment on circulating LH levels ($P<0.02$) such that LH levels at ZT 8 in the group exposed to the unlocked (free) wheel were lower than those in the no wheel group ($P<0.02$, Fig. 6A). All of the hamsters in both the home cage control group and the locked wheel group exhibited LH levels at ZT 8 consistent with the occurrence of an LH surge that day, but only one of the hamsters in the free wheel group exhibited an LH level that might have been indicative of a small or delayed LH surge. Thus, in contrast to the results from Experiment 1, presentation of a locked wheel and exposure to DD at ZT 5 did not delay the LH surge until the next day, although it may have been suppressed and/or delayed it on the same day. In this regard, it should be noted that in this study, in contrast to Experiment 1, the animals were not cannulated on the previous day or transported out of the animal facility to the laboratory.

Fos immunoreactivity—The proportion of activated GnRH neurons, i.e., those expressing Fos, paralleled circulating LH levels, in agreement with previous reports of

increased Fos expression in GnRH neurons during the rising phase of the LH surge in rats [15]. The percentage of dual-labeled GnRH neurons was lowest in the hamsters whose LH surge was blocked, namely those receiving an unlocked wheel (Fig. 6B). Thus, exposure to a new cage and novel wheel blocks both the proestrous increase in Fos expression in GnRH neurons ($P < 0.001$) and the increase in LH levels at ZT 8 on proestrus; Fig. 6A&B). In this regard, although either a locked or an unlocked wheel decreased the percentage of GnRH-immunoreactive (-ir) neurons that coexpressed Fos, the unlocked wheel had the greater effect. The number of GnRH-ir neurons was not significantly affected by treatment (mean $\pm$ SEM: no wheel, 69 ± 7.9 ; locked wheel, 64 ± 9.5 ; free wheel, 75 ± 13 ; $P > 0.05$).

Interestingly, the proportion of Fos-immunoreactive GnRH neurons was inversely correlated with Fos expression in the orexin neurons in the lateral hypothalamus (Fig. 6C&D).

Exposure to a new cage and novel wheel increased the percentage of orexin-ir neurons that expressed Fos above that in home cage controls, regardless of whether the wheel was locked or not ($P < 0.001$; Fig. 6C & Fig. 7). Once again, exposure to a freely moving wheel had the greatest effect on Fos activation. Fos expression in orexin-ir neurons was highest in hamsters that had basal LH concentrations (i.e., those with the freely turning wheel) and was greater than that in hamsters exhibiting elevated circulating LH concentrations (home cage controls and locked wheel-exposed) (Fig. 6C). Novel wheel exposure did not affect the number of orexin-ir neurons (no wheel, 170 ± 15 ; locked wheel, 170 ± 10 ; free wheel, 150 ± 14 ; $P > 0.05$). In contrast to its activation of the orexin neurons, novel wheel exposure did not significantly alter Fos expression in the other regions examined, i.e., the SCN and IGL, or in the VMH or ARC (Table 1). Also, Fos expression in the SCN was not affected by either treatment ($P = 0.485$) or subregion (dorsomedial or ventrolateral, $P = 0.982$). In the VMH, a trend towards stimulation by novel wheel was observed ($P = 0.093$). Furthermore, when the data were grouped by surge-like LH levels vs basal LH levels, the VMH was the only region among those examined in which the number of Fos-ir neurons differed between hamsters with surge-like LH concentrations (64.0 ± 9.6) and those with basal LH levels (110 ± 22.0) ($P < 0.05$), although a trend towards decreased Fos expression in hamsters with surge-like LH was observed in the IGL (high LH, 12.8 ± 1.8 ; basal LH, 18.3 ± 1.7 ; $P = 0.08$).

4. Discussion

These studies explored two potential mechanisms, exercise and arousal-induced activation of orexin neurotransmission, by which exposure to a novel running wheel on proestrous afternoon could block the LH surge, as well as advance the circadian time of its peak. In order to determine whether exercise is an essential part of this mechanism, Experiment 1 compared the effects of exposure to a freely moving versus a locked wheel on the LH surge. The results of this experiment clearly demonstrate that after transportation from the animal facility to the laboratory, even a locked novel wheel presented at the beginning of the critical period is a sufficient stimulus to cause a one-day delay and a circadian phase advance of the LH surge in most hamsters, as well as a phase advance in the locomotor activity rhythm. Thus, under these conditions, intense exercise is not required for blockade and phase advance of the LH surge.

In contrast, in Experiment 3, all of the animals exposed to a locked novel wheel had elevated levels of LH at ZT 8, indicating that the LH surge occurred on Day 1, i.e., was not blocked. The critical difference between these experiments is the transport of animals to different locations for the novel wheel stimulus. In Experiment 3, the animals were moved to similar light-tight compartments in a neighboring room in the animal facility, whereas in Experiment 1, the animals were transferred to a room outside of the animal facility, requiring transport in an elevator and into another wing of the building. Thus the hamsters in Experiment 1 experienced more sensory stimuli such as motion and vibration in the elevator, and odors and noises that they had also experienced on the previous day, when they were cannulated, and likely received a more robust arousal signal. The locked novel wheel was sufficient to prevent the LH surge only when associated with the additional sensory cues in Experiment 1. When the environment did not include these strong sensory stimuli, as in Experiment 3, only the unlocked wheel allowing exercise was sufficient to prevent the LH surge. Taken together, these findings suggest that high levels of arousal in the absence of intense exercise in a novel environment can block the LH surge on the afternoon of proestrus, and that intense running, induced by presentation of an unlocked wheel in an otherwise familiar environment, sustains robust arousal.

In a familiar environment, exposure to either a locked or unlocked wheel increased Fos expression in orexin-immunoreactive neurons as compared to home cage (no wheel) controls, and this effect was approximately 30% greater in hamsters exposed to the freely turning wheels. Thus arousal associated with exercise leads to greater activation of orexin neurons than a novel wheel alone, at least under the conditions tested here (i.e., new cage and new room within the animal facility). Furthermore, the percentage of orexin-immunoreactive neurons expressing Fos was higher in hamsters with basal rather than elevated LH levels, indicating that increased activation of orexin neurons is associated with blockade of the LH surge.

Novel wheel exposure is also associated with decreased Fos expression in GnRH neurons, indicating that decreased activation of GnRH neurons is likely part of the mechanism by which this arousing stimulus blocks the LH surge. Interestingly, lower levels of both circulating LH and the proportion of Fos-activated GnRH neurons were observed in the animals exposed to a locked wheel versus an unlocked wheel. Because the data were only obtained at a single time point during the expected time of the LH surge, it is not possible to distinguish whether the intermediate LH levels associated with the locked wheel represent a decrease in amplitude or a delay in the phase of the LH surge, or both. The finding that the proportion of GnRH neurons expressing Fos paralleled plasma LH levels during the expected time of the proestrous LH surge in hamsters agrees with previous reports that Fos expression in GnRH neurons only increases during an LH surge in rats [15, 16].

In the current study, the percentage of Fos-positive GnRH-ir neurons was inversely correlated with the percentage of Fos-positive orexin-ir neurons, lending strong support to the hypothesis that increased activation of orexin neurons is associated with novel wheel-induced blockade of the LH surge. Because orexin neurons communicate with the GnRH neurons by several pathways, including projections to the medial preoptic area and the arcuate nuclei [29], and administration of orexin A in either of these regions inhibits rat LH

surges [35], a role for orexin neurons in novel wheel inhibition of the LH surge in hamsters is quite plausible. Although previous studies have indicated that experimental manipulation of orexin neurotransmission can either stimulate or suppress LH levels during the time of an expected LH surge in rodents, neither release of orexin nor activation of orexin neurons at the time of the LH surge have yet been reported. Experiment 2 directly tested whether orexinergic neurotransmission is necessary for novel wheel blockade of the LH surge. Administration of the orexin-1 receptor antagonist, SB334867, prevented novel wheel blockade of the LH surge and inhibited wheel running indicating that orexinergic neurotransmission is required for novel wheel blockade of the LH surge and robust wheel running.

The finding that presentation of a novel running wheel to female hamsters induces Fos expression in orexin neurons is consistent with similar findings in male hamsters and mice [20, 44] and extends these findings by revealing that the effect is potentiated by running, at least in females. This potentiation by running suggests that the opportunity to run enhances the level of arousal induced by the novel wheel. In contrast, in male hamsters, equivalent levels of Fos expression in orexin neurons were observed following either running in a novel wheel or sleep deprivation induced by gentle handling in the absence of exercise [44]. Furthermore, in the same study there were no differences in the induction of Fos expression in orexin neurons between animals exposed to arousing stimuli that reset the circadian pacemaker (e.g., novel wheel exposure or gentle handling) and those that did not (e.g., stress-loaded physical restraint), leading to the conclusion that activation of orexin neurons alone is not sufficient to induce a phase shift [44]. In keeping with this finding, the present studies did not reveal an effect of novel wheel exposure on Fos expression in the SCN, the site of the master circadian pacemaker, or in the IGL, a structure which conveys nonphotic information to the SCN.

Both locked and freely moving wheels induced equivalent phase advances in the circadian locomotor rhythm, in spite of a large difference in Fos expression in orexin neurons; thus the magnitude of the phase shift was not associated with the degree of activation of the orexin neurons. However, administration of SB334867 decreased circadian phase shifts in the female hamsters in Experiment 2, suggesting that orexin neurotransmission participates in the induction of large phase shifts. It was surprising that large phase shifts were exhibited by home-caged hamsters as well as novel wheel-exposed hamsters. It is possible that the vehicle injections potentiated the phase shifts in the home-caged hamsters.

In the IGL, Fos expression was not greater in novel wheel-exposed female hamsters than in home cage-housed female hamsters, in contrast to previous results from male hamsters [42, 45]. As well as the sex difference, a possible reason for this difference in results may be the difference in lighting conditions between the studies. In the present study, the female hamsters were transferred into darkness at the time of novel wheel presentation, whereas the male hamsters in previous studies had been exposed to constant darkness for at least 40 hours before novel wheel presentation. Although exposure to dark pulses during the daytime induces phase shifts [46], whether this treatment induces Fos expression in the IGL, similar to other nonphotic stimuli, has not previously been reported to the best of our knowledge. Our results from female hamsters suggest that acute exposure to darkness alone induces Fos

expression in the IGL to the same levels as acute exposure to darkness and a novel wheel. In addition, this level of expression (~13 immunopositive cells) is equivalent to that reported previously in male hamsters exposed to novel wheels in constant darkness, which was greater than the expression (~3 immunopositive cells) in male home cage controls [42].

The foregoing results show that running is not required for a novel wheel-induced phase advance in female hamsters, similar to previous findings in male hamsters. Although activity-inducing stimuli (e.g., cage changes, triazolam injections, and exercise) induce phase shifts in male hamsters [47–50], exercise is not always associated with large phase shifts [51] and sleep deprivation by gentle handling induces phase shifts without exercise [52, 53]. The findings herein show that nonphotic phase shifts in females also do not depend on intense exercise, consistent with our previous finding that phenobarbital injection induces phase shifts while acutely suppressing activity [38]. It is likely that presentation of a locked wheel in a new cage elicited investigatory behavior and movement. Therefore, the present results are consistent with the finding in male hamsters that phase shifts require some minimal level of locomotion for their occurrence [52]. In this regard, the sensory signals associated with transporting animals to the location of the novel wheel exposure, described above, may have contributed to the phase shifting stimulus in the present study.

Our results demonstrate that novel wheel exposure activates orexin neurons and that orexinergic neurotransmission through orexin 1 receptors is necessary for novel wheel blockade of the LH surge. Because running increases Fos expression in orexin neurons while antagonism of orexin 1 receptors decreases running, exercise and activation of orexin neurons appear to be positively coupled, such that each can potentiate the other. These findings are consistent with the concept that a heightened state of arousal, induced by novel stimuli and exercise, is a condition that interferes with the preovulatory LH surge. To our knowledge, the effect of acute arousal on the LH surge has not been previously reported in any species.

The present findings indicate that a 2-fold increase in the number of activated, Fos-positive, orexin neurons near the time of the expected peak of the LH surge is strongly associated with novel wheel blockade of the surge, and thus suggest that high levels of orexin release interfere with LH secretion. In support of this concept, antagonism of orexinergic neurotransmission inhibited novel wheel blockade of the LH surge. These results extend previous findings that a variety of arousal-inducing stimuli, e.g., wheel running, food restriction, stress, and drugs of abuse, activate orexin neurons [54–56]. Furthermore, these findings suggest that increased orexin neurotransmission may interfere with the secretion of LH and/or perhaps other hormones in human females and may contribute to the increased risk of menstrual cycle irregularities and infertility in women engaged in shift work.

In conclusion, the current findings are consistent with the hypothesis that arousal-associated novel wheel exposure induces activation of orexin neurons, leading to blockade of the LH surge. Although this sequence of events does not absolutely depend on running, running potentiates novel wheel activation of orexin neurons as well as novel wheel inhibition of GnRH neurons.

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Highlights

Novel wheel blockade of the LH surge does not absolutely require exercise.

Novel wheel blockade of the LH surge is associated with activation of orexin neurons.

Injection of SB334867 (ORX1 antagonist) inhibits novel wheel blockade of LH surges.

SB334867 attenuates novel wheel-induced phase shifts in activity rhythms.

Fig. 1.

Plasma LH levels in hamsters transferred to a new cage with a novel wheel at ZT 5 on proestrus. Profiles of plasma LH concentrations (mean $\pm$ SE) are illustrated on expected proestrus (Day 1) and estrus (Day 2) from ZT 5–11. The shaded area indicates exposure to DD. Closed circles and solid lines depict LH levels in animals whose LH surge was blocked on proestrus and occurred on estrus. Open circles and broken lines depict LH concentrations in hamsters whose LH surge was not blocked. The ZT time at which peak LH levels occurred is indicated. Novel wheel-induced blockade of the proestrous LH surge occurred in

8/8 hamsters receiving an unlocked wheel and in 6/9 hamsters presented with a wheel that was locked from ZT 5–9 and is thus not dependent on exercise.

Fig. 2. Effects of novel wheel exposure at ZT 5 on proestrus on phase shifts in activity rhythms. Open circles depict the phase shifts in individual animals; bars represent mean + S.E.M. and numbers within bars indicate sample size. Phase advances did not differ among hamsters exposed to locked or unlocked wheels, regardless of whether the LH surge occurred on Day 1 (D1) or 2 (D2) ($P=0.28$).

Fig. 3. Plasma LH levels (mean $\pm$ SE) in home caged hamsters injected with vehicle (top panel) or novel wheel-exposed hamsters treated with either vehicle (middle panel) or the orexin-1 receptor antagonist, SB334867 (bottom panel). The shaded area indicates exposure to DD.

Fig. 4. Effect of SB334867 or vehicle (VEH) on novel wheel-induced phase shifts. Home cage (HC) controls did not receive a novel wheel (NW). Bars represent the mean + S.E.M. for each group. Numbers in parentheses indicate the group sizes. * $P < 0.05$.

Fig. 5.

Acute effect of SB334867 or vehicle on wheel running activity depicted as the mean $\pm$ S.E.M. of wheel revolutions per hour. The shaded area represents exposure to constant darkness (DD). * $P < 0.05$ compared to Vehicle group.

Fig. 6.

Effect of novel wheel exposure on LH levels and Fos expression in orexin-ir and GnRH-ir neurons at ZT 8. Bars represent mean + S.E.M. (N=4/group); those with the same letter are significantly different from each other. **A.** Novel wheel exposure significantly reduced circulating LH levels ($P<0.05$). a: $P<0.05$. **B.** The percentage of GnRH-ir neurons coexpressing Fos-ir was decreased by novel wheel exposure; the locked wheel had an intermediate effect. b: $P<0.01$; c,d: $P<0.05$. **C.** The percentage of orexin-ir neurons coexpressing Fos-ir was increased by novel wheel presentation, with the locked wheel

inducing an intermediate effect. e: $P < 0.001$; f: $P < 0.01$; g: $P < 0.05$. **D.** The percentage of GnRH-ir neurons expressing Fos-ir was inversely correlated with the percentage of orexins-ir neurons expressing Fos-ir ($P < 0.001$).

Fig. 7.

Photomicrographs depicting Fos expression (black stain) in orexin-immunoreactive cells in the lateral hypothalamus (brown stain, panels A, B and C), and in GnRH-immunoreactive neurons (brown stain, panels D, E and F). No wheel: **A** and **D**; Locked wheel: **B** and **E**; Unlocked wheel: **C** and **F**. Bar in panel **A** represents 50 microns. Insets are at a 2-fold higher magnification.

Table 1

Lack of effect of novel wheel exposure on Fos immunoreactivity in selected brain regions.

Brain region	No wheel	Locked wheel	Free wheel	P value
SCN	177.3 $\pm$ 45.5	218.7 $\pm$ 49.0	127.7 $\pm$ 28.3	0.420
IGL	12.0 $\pm$ 2.1	13.5 $\pm$ 3.3	13.0 $\pm$ 1.7	0.220
ARC	39.3 $\pm$ 6.6	58.9 $\pm$ 11.9	76.1 $\pm$ 11.9	0.201
VMH	28.5 $\pm$ 5.8	34.8 $\pm$ 7.6	56.7 $\pm$ 10.8	0.093

Values represent the mean $\pm$ S.E.M. of the average number of Fos-ir cells per section per region; N=3–4 animals/group and 7–9 sections/region/animal.