**RESEARCH ARTICLE | Physical Activity and Inactivity**

Orexin signaling in rostral lateral hypothalamus and nucleus accumbens shell in the control of spontaneous physical activity in high- and low-activity rats

Claudio Perez-Leighton,1,5 Morgan R. Little,2,3 Martha Grace,2 Charles Billington,2,3,4 and Catherine M. Kotz2,3,6

1Center for Integrative Medicine and Innovative Science, Facultad de Medicina, Universidad Andres Bello, Santiago, Region Metropolitana, Chile; 2Geriatric Research Education and Clinical Center, Minneapolis Veterans Affairs Health Care System, Minneapolis, Minnesota; 3Minnesota Obesity Center, University of Minnesota, Minnesota; 4Department of Medicine, University of Minnesota, Minneapolis, St. Paul, Minnesota; 5Department of Food Science and Nutrition, University of Minnesota, Minneapolis, St. Paul, Minnesota; and 6Department of Integrative Biology and Physiology, University of Minnesota, Minneapolis, Minnesota

Submitted 2 August 2016; accepted in final form 21 December 2016

Orexin signaling in rostral lateral hypothalamus and nucleus accumbens shell in the control of spontaneous physical activity in high- and low-activity rats. Am J Physiol Regul Integr Comp Physiol 312: R338–R346, 2017. First published December 30, 2016; doi:10.1152/ajpregu.00339.2016.—Spontaneous physical activity (SPA) describes activity outside of formal exercise and shows large interindividual variability. The hypothalamic orexin/hypocretin peptides are key regulators of SPA. Orexins drive SPA within multiple brain sites, including rostral lateral hypothalamus (LH) and nucleus accumbens shell (NAcSh). Rats with high basal SPA (high activity, HA) show higher orexin mRNA expression and SPA after injection of orexin-A in rostral LH compared with low-activity (LA) rats. Here, we explored the contribution of orexin signaling in rostral LH and NAcSh to the HA/LA phenotype. We found that HA rats have higher sensitivity to SPA after injection of orexin-A in rostral LH, but not in NAcSh. HA and LA rats showed similar levels of orexin receptor expression in rostral LH, and activation of orexin-producing neurons after orexin-A injection in rostral LH. Also, in HA and LA rats, the coinjection of orexin-A in rostral LH and NAcSh failed to further increase SPA beyond the effects of orexin-A in rostral LH. Pretreatment with muscimol, a GABA A receptor agonist, in NAcSh potentiated SPA produced by orexin-A injection in rostral LH in HA but not in LA rats. Our results suggest that a feedback loop from orexin-responsive neurons in rostral LH to orexin neurons and a the NAcSh–orexin neuron–rostral LH circuit regulate SPA. Overall, our data suggest that differences in orexin sensitivity in rostral LH and its modulation by GABA afferents from NAcSh contribute to individual SPA differences.

orexin; physical activity; spontaneous physical activity; lateral hypothalamus; accumbens

**SPONTANEOUS PHYSICAL ACTIVITY (SPA) describes low-intensity, nonstructured physical activity (6). In humans, SPA is best exemplified by standing, fidgeting, and ambulating (15, 38), while in rodents it is measured by quantifying ambulatory and rearing activity in a home cage or specialty cage after acclimation, to avoid novelty or stress-induced activity (39). A neuronal network involving multiple brain sites and neuropeptidergic signaling regulates SPA (11), among which the orexin/hypocretin peptides play a key role.

Orexin-A and orexin-B are synthesized and released by neurons located in the lateral hypothalamic (LH), which project to multiple brain sites (1, 29). The orexin peptides act through two G protein-coupled receptors (orexin receptor 1, OX1R and orexin receptor 2, OX2R). Orexin-A has a higher affinity for OX1R, while orexin-B has an equal affinity for both receptors (30, 32). The orexin peptides modulate behaviors such as SPA, reward, food intake, and the sleep/wake cycle, with orexin-A showing more robust effects in all of these behaviors (41). By promoting SPA and energy expenditure by their action at multiple brain sites (8, 9, 12, 17, 26, 35), the activity of orexin neurons and peptides is sufficient to reduce susceptibility to diet-induced obesity (5, 7, 22, 27).

SPA is highly variable among individual rodents and humans (15, 16, 31), and rodent data suggest this variability is associated with orexin function. Rats selectively bred for resistance to diet-induced obesity (obesity-resistant, OR rats) have higher SPA and orexin mRNA expression compared with rats selectively bred for susceptibility to obesity [obesity-prone (OP) rats] (14, 36–38). When rats are classified on the basis of natural variations in SPA, rats with high SPA [high-activity (HA) rats] show higher basal energy expenditure and expression of orexin mRNA compared with rats with low SPA [low-activity (LA) rats] (26). These two rat models support the hypothesis that variations in orexin function underlie individual differences in SPA.

The rostral LH is a brain region located anterior to orexin-producing neurons (12), where injection of orexin-A robustly increases SPA and energy expenditure compared with administration in other brain sites (12, 27, 38). Repeated injections of orexin-A in rostral LH prevent obesity caused by a high-fat diet in rats (27). As previously reported, HA and OR rats have greater SPA increases after orexin-A injection in rostral LH compared with LA or OP rats, respectively (26, 38). These data suggest that rostral LH is a relevant brain site for individual differences in SPA and orexin function. Currently, the downstream effectors of orexin-responsive neurons located in rostral LH are not known.
The orexin peptides also promote SPA in the nucleus accumbens shell (NAcSh) (40). There are monosynaptic projections from orexin neurons to NAcSh (2), and orexin neuronal activation increases dopamine content in NAcSh, both by direct projections and indirectly through activation of the ventral tegmental area (24, 43). In addition to orexin projections to NAcSh, the medium spiny neurons (MSN) in NAcSh project to LH (23). Bilateral injection of the GABA_A agonist muscimol in NAcSh inhibits the MSN and modulates the activity of the orexin neurons, as evidenced by increased c-Fos expression in orexin neurons (2, 48). These data suggest a bidirectional pathway between orexin neurons and NACSh, but the importance of this pathway in regulation of SPA has not been studied.

Despite evidence suggesting the orexin peptides can modulate SPA through different brain sites, the interaction between different brain sites involved in the SPA regulatory network has not been studied. Studies in rats suggest the possibility of a functional connection between rostral LH and NACSh involving orexin peptides. Injection of orexin-A in the rostral LH increases c-Fos in the NACSh (20), efferent projections from NACSh overlap with the rostral LH (42), and manipulation of MSN in NACSh activates orexin-producing neurons (2, 48), which could include those projecting to rostral LH. However, whether there are interactions between orexin signaling in rostral LH and NACSh that are relevant for SPA control has not been determined.

The goal of this study was to understand the differences in orexin regulation of SPA between HA and LA rats, focusing on the action of orexin-A as a model for the orexin peptides, as this peptide has more robust behavioral effects compared with those driven by orexin-B (32). We aimed to determine first, whether orexin action in NACSh contributes to the HA/LA phenotype; second, whether the downstream effectors of orexin function in the rostral LH, and third, what the relevance of interaction is between orexin function in rostral LH and NACSh for control of SPA and the HA/LA phenotype.

METHODS

Animals

Sprague-Dawley rats (Charles River, Kingston, NY; Taconic, Cambridge, City, IN) were used in these experiments. Animals were maintained at 22–25°C and fed with free access to standard chow (Teklad, Envigo, UK) and water throughout all of the experiments. Upon arrival, rats were housed in clear plastic, solid-bottom cages with corn-cob bedding and acclimated to the housing facility for 1 wk before any study procedures began. The studies were approved and carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the Minneapolis Veterans Affairs Health Care System and the University of Minnesota.

Surgical Procedures

Rats were anesthetized with either a mixture of ketamine (50 mg/kg) and xylazine (15 mg/kg) via intraperitoneal injection, inhaled isoflurane (5% for induction and 1% for maintenance), or pentobarbital sodium (50 mg/kg ip) and implanted with a 26-gauge stainless-steel cannula (Plastics One, Roanoke, VA) directed at the rostral LH and/or the NACSh in the right brain hemisphere. Stereotaxic coordinates for rostral LH (−2.2 mm posterior, 1.9 mm lateral to bregma, and 7.3 mm below the skull surface) and NACSh (1.75 mm posterior, 1.9 mm lateral to bregma, and 6.4 mm below the skull surface) were obtained from the Paxinos and Watson brain atlas (25). For rats with cannulas in both rostral LH and NACSh, cannulas were implanted ipsilateral to each other. Surgical procedures are described in detail elsewhere (26, 39). Postsurgical analgesia (meloxicam, 2 mg/kg sc) was administered on the day of surgery and once daily for two consecutive days after surgery.

Drugs

Muscimol (Sigma-Aldrich, St. Louis, MO) and orexin-A (American Peptide, Sunnyvale, CA) were dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA). Drugs were aliquoted for single use and stored at −20°C until use. Equal amounts of RNA were used to measure expression levels of OX1R, OX2R, and GAPDH with the LightCycler RNA Master SYBR Green I kit (Roche) in a LightCycler 2 thermocycler (Roche). Primer sequences were described previously (38). Efficiency of each PCR reaction was determined using the dilution method (30). Expression levels of OX1R and OX2R were normalized against GAPDH using an efficiency-corrected formula (30).

SPA Measurements

SPA was measured in a 17 × 17 inch square acrylic cage surrounded by three sets of 16-beam infrared activity sensors (Med Associates, St. Albans, VT). Two sets of arrays were in the x-y plane, and the third set was elevated 3 inches above the x-y plane. Movement was recorded by beam breaks with 100-ms resolution. SPA was defined as time spent ambulating (moving in the x-y plane and rearing); this did not include time in stereotypic movement (movement occurring within the 3.25 × 3.25-inch square around the center of mass of the animal).

For SPA measurements of over 24 h, rats were first acclimated to the SPA cage for at least 24 h before data recording began. Cages were serviced daily (cleaned of feces, urine, and recovery of chow spillage). On each day, data from the first hour after servicing the cages were eliminated to avoid potential confounding effects on the measures of SPA. For SPA measurements after intracranial injections, rats were acclimated to the SPA cages for at least 2 h before the injection, and SPA was recorded for up to 3 h after injection.

Injections

In all injections, 0.5 μl was injected over 30 s with a 33-gauge injector (Plastics One, Roanoke, VA) that extended 1.0 mm beyond the tip of the guide cannula. The injector was left in place for an additional 30 s. All injections were done within 3 h of the start of the light period. For all injection studies, we allowed 48 h of rest between injections, which has been shown to be sufficient to clear any behavioral effects of orexin-A injections in sleep and SPA (4, 10, 17, 19, 27).

Immunofluorescence

Rats were exsanguinated via intracardiac perfusion with 0.9% sterile saline followed by 0.1 M phosphate-buffered (PB) 4% paraformaldehyde solution. The brains were removed, postfixed

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00339.2016 • www.ajpregu.org

Downloaded from http://ajpregu.physiology.org/ by 10.220.33.1 on March 31, 2017
overnight in the same fixative used for perfusion, and subjected to a 2-day sucrose gradient (15% then 30% in PB). Brains were embedded in optimal cutting temperature compound (Tissue-Tek, Torrance, CA), frozen in a bath of 100% ethanol (Pharmco-Aaper, Brookfield, CT), and cooled with dry ice. Blocks were held at −20°C until cut into 40-μm sections on a CM 1850 cryostat (Leica, Buffalo Grove, IL), and were collected into 0.1 M PBS. Sections were rinsed with 0.01 M PBS, blocked with 5% normal horse serum (NHS; Vector, Burlingame, CA) for 2 h, probed with goat-anti-orexin (1:5,000 vol/vol; sc-8070; Santa Cruz Biotechnology, Dallas, TX) and rabbit-anti-c-Fos (1:1,500 vol/vol; sc-52; Santa Cruz Biotechnology) for 20 h, followed by Alexa-Fluor-488 conjugated donkey-anti-goat (1:1,000 vol/vol; 705-545-003; Jackson ImmunoResearch, West Grove, PA) and Cy3-conjugated donkey-anti-rabbit (1:1,000 vol/vol; 711-165-152; Jackson ImmunoResearch). All antibody cocktails were prepared with 3% NHS, 0.01 M PBS, and 0.3% Triton X-100 (Promega, Madison, WI); all incubations and washes were performed free floating, at room temperature (RT), and in dark conditions from the application of secondary antibody onward. Prolong Gold with DAPI (Molecular Probes, Waltham, MA) mounting medium was used for coverslipping. Slides were allowed to cure for 48 h at RT before storage at 4°C.

Unbiased Stereology

The number of c-Fos-positive, orexin-positive, and double-labeled cell populations were quantified within the hemisphere ipsilateral to the cannulation site. Unbiased stereological analysis was performed using the optical fractionator probe within the Stereo Investigator 11.1.2 software (MBF Bioscience, Williston, VT). Every third section was imaged using an Axios Imager M2 fluorescence microscope (Zeiss, Oberkochen, Germany). The rostral boundaries of the hypothalamus were defined as −2.0 to −2.56 mm from bregma; the caudal boundaries were −2.57 to −3.2 mm from bregma (25). The medial, lateral, dorsal, and ventral boundaries (47) were used to outline contours at ×5 magnification. Image stacks were collected at the intersection points of a 200 × 200-μm grid, at ×63 magnification (oil). The counting frame dimensions were 80 × 80 μm, with dissector height of 18 μm and step height of 2 μm. The average coefficient of error (CE, m = 1) ratio for all rats imaged (n = 12) was 0.34.

Classification of HA and LA Phenotype

In all of the studies, HA and LA rats were selected on the basis of the 24-h SPA determined from the second day of recording after 24-h acclimation to the SPA cages (26). The criteria for classifying HA/LA rats was based on the 30th and 70th percentile within each cohort tested, rather than the absolute SPA levels reported previously (26).

Study Protocols

Study 1: robustness of HA/LA phenotype. To determine the robustness of the HA/LA phenotype, in one cohort of rats (n = 32), SPA was measured for 4 consecutive days. The stability of the HA/LA phenotype was analyzed by comparing the difference in daily SPA between HA and LA rats throughout the 4 days of recording.

Study 2: dose-response curves for orexin-A in rostral LH and NAcSh. HA and LA rats with either a single cannula aiming at rostral LH or with ipsilateral cannulas aiming at rostral LH and NAcSh were used in this study. All rats were acclimated to the intracranial injection procedure for 3 consecutive days with a single daily aCSF injection. Next, all rats were injected in each rostral LH or NAcSh with orexin-A (0, 125, 250, and 500 pmol) in a Latin-square design, with at least 48 h between injections.

Study 3: expression of orexin receptors in rostral LH and effects of orexin-A injection in rostral LH on c-Fos expression in orexin neurons in caudal LH. Expression of OX1R and OX2R was measured in rostral LH samples from HA (n = 18) and LA rats (n = 9) that were singly housed without any experimental manipulations except SPA measurement for 48 h. A separate group of HA and LA rats with cannulas aiming at rostral LH were acclimated to the injection procedure for 3 consecutive days by receiving a single daily aCSF injection in rostral LH. On the 4th day, rats were injected with either orexin-A (200 pmol, n = 3/group for HA and LA rats) or aCSF (n = 3/group for HA and LA rats) in rostral LH and euthanized 90 min after injection with excess of ketamine-xylazine, and then prepared for immunofluorescent analysis of orexin and c-Fos in caudal LH.

Study 4: effect of coinjection of orexin-A in rostral LH and NacSh on SPA. In this study, HA and LA rats with ipsilateral cannulas aiming at rostral LH and NAcSh were used. All rats were acclimated to a simultaneous injection in rostral LH and NAcSh for three consecutive days with a single daily aCSF injection in each brain site. Next, all rats were injected with the following combinations of aCSF or orexin-A (200 pmol) 1) aCSF in rostral LH and NAcSh, 2) orexin-A in rostral LH and aCSF in NAcSh, 3) orexin-A in rostral LH and NAcSh, and 4) orexin-A in rostral LH and in NAcSh. Treatments were administered following a Latin-square design with at least 48 h between injections.

Study 5: effects of pretreatment with muscimol in NAcSh on SPA induced by orexin-A injection in rostral LH. HA and LA rats with ipsilateral cannulas aiming at rostral LH and NAcSh were used in this study. All rats were acclimated to the injection procedure for 3 consecutive days by receiving a daily aCSF injection in NAcSh followed 15 min later by an aCSF injection in rostral LH. Next, rats were injected in the following combinations of either muscimol (500 pmol) or aCSF in NAcSh and 15 min later with either orexin-A (200 pmol) or aCSF in rostral LH: 1) muscimol in NAcSh and orexin-A in rostral LH, 2) aCSF in NAcSh and orexin-A in rostral LH, 3) muscimol in NAcSh and aCSF in rostral LH, and 4) aCSF in NAcSh and rostral LH. Treatments were administered following a Latin-square design with at least 48 h between injections.

Statistical Analysis

All statistical analyses were performed and graphs were created using the software R 3.1.2 with extensive use of the packages plyr, ggplot2, and reshape2 (44 – 46). For injection studies, animals with incomplete data or misplaced cannula, as verified by histological methods (27), were excluded from data analysis. Figure 1 shows cannula placement for all animals included in these studies. The final number of rats used for analysis in each experiment is indicated in the legends of the respective figures.

In all experiments in which SPA was the end point, we analyzed the cumulative SPA for the first hour postinjection after eliminating the first 5-min postinjection, which are confounded by an injection effect (27). Results from experiments involving a repeated-measure design were analyzed by ANOVA with rat (study 1) or injections (studies 4 and 5) as the repeated measure. Comparison of slopes for the linear regression between SPA and orexin-A between rats injected with orexin-A in rostral LH and NAcSh (study 2) was done with a nonpaired t-test. All nonrepeated experimental designs were analyzed with ANOVA. Pairwise comparisons after two-way repeated-measures ANOVA were done by paired t-tests corrected for multiple comparisons (Hochberg correction) and by Tukey HSD after nonrepeated-measures ANOVA. The percentage of orexin neurons expressing c-Fos estimated from stereology cell counts was analyzed with ANCOVA using total number of orexin neurons as a covariate. For all analyses, normality and heterocedasticity were verified by inspection of diagnostic
residual plots. Statistical significance was established at $P \leq 0.05$. All data are presented as means ± SE.

**RESULTS**

Studies 1–2: Robustness of the HA/LA Phenotype and Effects of Orexin-A in Rostral LH and NAcSh on SPA

There were significant differences in SPA measured over 24 h across cohorts of rats used in the studies presented here ($F_{1,237} = 18.06$, $P < 0.01$; Table 1). Therefore, we classified rats as HA or LA on the basis of whether their SPA was above the 70% (HA) or below the 30% (LA) of SPA measured over 24 h within each cohort (26, 39), instead of using the absolute value of SPA as cut-offs across cohorts (26). In one cohort of rats, we evaluated the robustness of the HA/LA phenotype over four consecutive days (Fig. 2). There were significant effects of the HA/LA phenotype ($F_{1,17} = 54.39$, $P < 0.01$), day ($F_{3,51} = 7.39$, $P < 0.01$), and the interaction between day and HA/LA phenotype ($F_{3,51} = 3.51$, $P = 0.02$). Pairwise analysis comparing SPA between HA and LA rats across days confirmed a significant difference in SPA between phenotypes on each day of recording (Fig. 2). These data show the robustness of the HA/LA phenotype independent of the variability in absolute SPA levels across cohorts.

<table>
<thead>
<tr>
<th>Cohort, mo, yr*</th>
<th>24 h SPA, min†</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 2012</td>
<td>96.20 ± 4.34 ($n = 32$)*</td>
</tr>
<tr>
<td>October 2012</td>
<td>88.51 ± 4.28 ($n = 32$)*</td>
</tr>
<tr>
<td>March 2013</td>
<td>102.52 ± 3.84 ($n = 32$)*</td>
</tr>
<tr>
<td>January 2014</td>
<td>136.05 ± 7.02 ($n = 48$)*</td>
</tr>
<tr>
<td>September 2014</td>
<td>71.75 ± 6.46 ($n = 31$)*</td>
</tr>
<tr>
<td>March 2015</td>
<td>107.63 ± 7.42 ($n = 32$)*</td>
</tr>
<tr>
<td>August 2015</td>
<td>140.53 ± 8.09 ($n = 32$)*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. *Indicates month and year cohort was purchased, and 24-h spontaneous activity (SPA) was measured. †Superscript lowercase letters indicate significant difference between cohorts for pairwise comparisons.

Fig. 1. Cannula placement. Placement of cannulas in nucleus accumbens (bregma 1.20 to 2.28) and rostral lateral hypothalamus (bregma −1.56 to −2.56) for all of the animals used in the experiments.

Fig. 2. Robustness of the high-activity/low-activity (HA/LA) model. Twenty-four hours of spontaneous physical activity (SPA) in male Sprague-Dawley rats ($n = 32$) were measured over four consecutive days. The graph shows the average 24-h SPA on each day for all rats. Rats were classified as HA ($n = 10$) or LA ($n = 9$) on the basis of being within the upper and lower 30th percentile of 24 h SPA, respectively, on the second day of recording. *Significant differences in SPA between HA and LA rats compared on each day corrected for multiple comparisons, $P < 0.05$. 

Table 1. Variation in 24-h SPA levels across cohorts

Fig. 2. Robustness of the high-activity/low-activity (HA/LA) model. Twenty-four hours of spontaneous physical activity (SPA) in male Sprague-Dawley rats ($n = 32$) were measured over four consecutive days. The graph shows the average 24-h SPA on each day for all rats. Rats were classified as HA ($n = 10$) or LA ($n = 9$) on the basis of being within the upper and lower 30th percentile of 24 h SPA, respectively, on the second day of recording. *Significant differences in SPA between HA and LA rats compared on each day corrected for multiple comparisons, $P < 0.05$. 

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00339.2016 • www.ajpregu.org
Figs. 3A and 3B: Differences in SPA caused by orexin-A injection in rostral LH and its interaction with the HA/LA phenotype. (A) Significantly higher SPA in HA rats compared with LA rats at the 500 pmol dose (P < 0.05) and the line over the bars indicates a significant difference between HA and LA rats at a given orexin-A dose. B: line over the bars indicates a significant difference between doses for HA rats.

magnitude of SPA and the stability of the HA/LA phenotype over time.

In their original description, HA rats showed higher sensitivity to SPA caused by injection of orexin-A in rostral LH compared with LA rats (26). To validate the selection criteria for HA and LA rats, based on percentiles, we examined differences in SPA caused by orexin-A injection in rostral LH between HA/LA rats (Fig. 3A). Orexin-A in rostral LH significantly increased SPA (F3,81 = 24.14, P < 0.001) with significant differences between HA and LA rats (F1,27 = 6.04, P = 0.02), but without a significant interaction between orexin-A dose and HA/LA phenotype (F3,81 = 0.97, P = 0.41). The lack of interaction suggested the higher response for HA rats is not localized to any particular dose of orexin-A, but rather present in all doses tested. This was confirmed by comparing the slopes obtained from the linear regression between SPA and orexin-A dose, which showed significant differences between HA and LA rats (t40 = 3.29, P = 0.001). Pairwise analyses showed significantly higher SPA in HA compared with LA rats at the 125- and 500-pmol orexin-A doses, with the difference at 250 pmol close to statistical significance (Fig. 3A). These data confirm that relative to LA rats, HA rats have higher sensitivity to SPA induction by orexin-A injections in rostral LH.

As orexin-A can increase SPA after injection in NAcSh (40), we tested whether there are differences in orexin-A sensitivity in NAcSh between HA and LA rats. Orexin-A in NAcSh significantly increased SPA (F3,39 = 8.5, P < 0.001) without significant effects of the HA/LA phenotype (F1,13 = 0.02, P = 0.91) or the interaction between orexin-A dose and HA/LA phenotype (F3,39 = 0.47, P = 0.73). Pairwise analyses showed no significant differences between HA and LA rats at any dose of orexin-A tested (Fig. 3B). These data show that HA rats are more sensitive than LA rats to SPA effects of orexin-A after injection in rostral LH, but not NAcSh.

Study 3: Expression of Orexin Receptors in Rostral LH and Effects of Orexin-A Injection in Rostral LH on c-Fos Expression in Orexin Neurons in Caudal LH

The mechanism behind higher sensitivity to orexin-A in rostral LH among HA rats is not known. Analysis of OX1R and OX2R expression in rostral LH by real-time PCR revealed no significant differences between HA and LA rats (Fig. 4A). Injection of orexin-A in rostral LH increased c-Fos expression within the caudal hypothalamus (20), which led us to hypothesize this injection would increase activation of orexin neurons more in HA compared with LA rats. Analysis of total c-Fos-positive neurons in the caudal LH showed a significant effect of orexin-A dose (F1,8 = 7.52, P = 0.02), without significant effects of the HA/LA phenotype (P = 0.62) or the interaction between orexin-A dose and the HA/LA phenotype (P = 0.46). Analysis of the percentage of orexin neurons expressing c-Fos using number of orexin neurons as a covariate (Fig. 4B) showed a significant increase after orexin-A injection in rostral LH (F1,8 = 14.95, P = 0.004) without significant effects of the covariate (P = 0.27), HA/LA phenotype (P = 0.99), or the interaction between orexin-A and the HA/LA phenotype (P = 0.27). Together, these data suggest equal expression of orexin receptors in rostral LH and equal activation of orexin-producing neurons after orexin-A injection in rostral LH in HA and LA rats.

Studies 4–5: Interaction Between Orexin-A Signaling in Rostral LH and NAcSh and Effect of Pretreatment with Muscimol in NAcSh on Orexin-A Effects in Rostral LH

To understand how orexin-A signaling at rostral LH interacts with other brain sites, we examined the effect of co-injecting orexin-A in rostral LH and NAcSh on SPA compared with injecting orexin-A injection in each brain site. There was a significant main effect of treatment...
GABAA receptors in NAcSh increases c-Fos expression with orexin-A injection in either brain site (Fig. 5). These results were classified as HA or LA based on the 30th and 70th percentile within each cohort tested, rather than the absolute SPA levels reported previously (26). This method of classifying HA and LA rats produced the higher SPA caused by orexin-A injection in rostral LH in HA compared with LA rats (26), which further supports the conclusion that differences in orexin function contribute to natural differences in SPA, irrespective of absolute SPA levels.

Orexin-A increases SPA in multiple brain sites, including rostral LH and NAcSh (40). While our data confirm the differential responsiveness of HA and LA rats to SPA induced by orexin-A injection in rostral LH, we did not observe such differences after orexin-A injection in NAcSh. This lack of difference mirrors the similar response of HA and LA rats in SPA after injection of orexin-A into the substantia nigra pars compacta (26). Together, these data suggest that although orexin-A can modulate SPA within multiple brain sites (i.e., rostral LH, NAcSh and substantia nigra pars compacta), only some of these brain sites are differentially responsive between HA and LA rats.

DISCUSSION

The current studies extend the characterization of the HA/LA rat model (26) by focusing on orexin-A action in the NAcSh and rostral LH and the interaction between these brain sites regarding individual differences in SPA. Our data confirm the robustness of the HA/LA rat phenotype. When first characterized, the HA and LA rats were classified on the basis of absolute SPA levels measured over 24 h, such that rats with SPA of more than 120 min were considered HA, and rats with SPA equal or less than 90 min were considered LA (26). The studies presented here were conducted over the course of three years in rats from several cohorts with significant variability in the distribution and range of SPA between cohorts (Table 1). Therefore, rats were classified as HA or LA based on the 30th and 70th percentile within each cohort tested, rather than the absolute SPA levels reported previously (26). This method of classifying HA and LA rats reproduced the higher SPA caused by orexin-A injection in rostral LH in HA compared with LA rats (26), which further supports the conclusion that differences in orexin function contribute to natural differences in SPA, irrespective of absolute SPA levels.

Injection of muscimol in NAcSh and subsequent activation of GABAA receptors in NAcSh increases c-Fos expression in orexin neurons (2, 48). This suggests that muscimol injection in NAcSh could potentiate SPA caused by orexin-A action in rostral LH by activating orexin-producing neurons that project to rostral LH. To test this hypothesis, HA and LA rats were injected with muscimol (500 pmol), and 15 min later, they were injected with orexin-A (200 pmol) in rostral LH (Fig. 6). There were significant effects of treatment ($F_{3,63} = 26.68, P = 0.001$), the HA/LA phenotype ($F_{1,21} = 4.23, P = 0.05$), and the interaction between treatment and the HA/LA phenotype ($F_{3,63} = 4.13, P = 0.001$). Pairwise analysis indicated that orexin-A alone significantly increased SPA in HA and LA rats, without significant differences between phenotypes. Muscimol potentiated the response to orexin-A injection only in HA rats (Fig. 6). Together, these data suggest that activation of GABAA receptors in NAcSh potentiates orexin-A-induced SPA in rostral LH only in HA rats.

**Fig. 5. SPA after coinjection of orexin-A in rostral LH and NAcSh in HA/LA rats.** HA and LA rats were injected with orexin-A (0, 200 pmol) in NAcSh and rostral LH (HA, n = 5; LA, n = 10). *Significant differences from vehicle (0 pmol injection of orexin-A in rostral LH and NAcSh), $P < 0.05$. A line across histogram bars indicates significant pairwise differences. #Significant difference between treatment and the HA/LA phenotype ($P < 0.01$) but no significant effect of the HA/LA phenotype ($F_{1,13} = 0.78, P = 0.39$) or the interaction between treatment and the HA/LA phenotype ($F_{3,39} = 2.40, P = 0.08$). Pairwise analysis showed that injection of orexin-A (200 pmol) in rostral LH increased SPA in both HA and LA rats, with a higher response in HA rats compared with LA rats (Fig. 5). However, injection of orexin-A alone in NAcSh failed to increase SPA in HA and LA rats, and coinjection of orexin-A in rostral LH and NAcSh did not produce an additive effect on SPA compared with orexin-A injection in either brain site (Fig. 5). These data suggest that orexin signaling in NAcSh and rostral LH does not converge to regulate SPA.

**Fig. 6. Effect of pretreatment with muscimol in NAcSh on SPA resulting from orexin-A injection in rostral LH.** HA (n = 8) and LA rats (n = 18) were injected with muscimol (0, 500 pmol) in NAcSh and 15 min later with orexin-A in rostral LH (0, 200 pmol). *Significant difference from baseline (aCSF injection in NAcSh and rostral LH) for HA or LA rats. A line across the bars indicates a significant difference in response between HA and LA rats at a given orexin-A dose. #Significant difference ($P < 0.05$) in response between HA and LA rats at a given treatment.
relevant for the contribution of orexin peptides to individual variability in SPA.

The HA/LA model shares similarities with rats bred for differential susceptibility to diet-induced obesity, the OP, and OR rats, including the differential susceptibility to diet-induced obesity and SPA response after injection of orexin-A in rostral LH (14, 36–38). However, unlike the OP/OR rats (38), we did not find higher expression of orexin receptors mRNA in rostral LH in HA rats compared with LA rats (Fig. 4A). Although this suggests that mechanisms downstream of orexin-responsive neurons in rostral LH mediate the difference in responsiveness between HA and LA rats, it remains necessary to confirm whether there are differences in protein expression level or signaling pathway for orexin receptors in rostral LH that could contribute to the observed differences between HA and LA rats.

As injection of orexin-A in rostral LH increased c-Fos expression in the caudal lateral hypothalamus (20), we hypothesized that there would be higher neuronal activation in orexin neurons in HA rats compared with LA rats. Our data did not support this hypothesis, as orexin-A injection in rostral LH increased c-Fos expression equally in orexin- and nonorexin-producing neurons in HA and LA rats (Fig. 4B). However, robust activation of orexin-producing neurons after injection of orexin-A in rostral LH could explain that the highest sensitivity to SPA induced by orexin-A injection is found in rostral LH compared with other brain sites (28). Whether this proposed feedback loop is exclusive to orexin-responsive neurons in rostral LH and orexin-induced SPA needs to be determined.

Injection of muscimol in NACSh potentiates SPA caused by orexin-A injection in rostral LH in HA rats (Fig. 6). This effect is consistent with data showing that injection of muscimol in NACSh increases c-Fos expression in caudal LH (2, 48). Medium spiny neurons in NACSh are the primary source of efferent projections from this brain site and have monosynaptic projections to orexin neurons in the LH (33). We hypothesized that the orexin neurons activated after muscimol injection in NACSh are the same as those that project to rostral LH, but additional tracing studies are necessary to verify this hypothesis. Coinjection of orexin-A in rostral LH and NACSh failed to further increase SPA above that observed with injection into a single brain site (rostral LH or NACSh) in HA and LA rats, while pretreatment with muscimol potentiated SPA in HA rats (Figs. 5 and 6). Together, with the lack of difference between HA and LA in activation of orexin neurons after orexin-A injection in rostral LH, these data suggest that both differences in orexin sensitivity in rostral LH and modulation of this response by GABA afferents from NACSh to PHD contribute to SPA differences between HA and LA rats.

The studies of coinjection of orexin-A in rostral LH and NACSh or preinjection of muscimol in NACSh (Figs. 5 and 6) suggest that orexin circuits regulating SPA have a lower threshold for activation in HA compared with the threshold in LA rats. In addition to differences in orexin receptor expression or signaling pathways, there are several mechanisms that could explain higher sensitivity to orexin-A in HA rats. HA rats have elevated orexin mRNA expression compared with LA rats (26), which could lead to higher synaptic release of orexin-A. Also, it is possible that orexin-producing neurons have a lower threshold for activation in HA compared with LA rats, thereby contributing to potentiation of responses in HA rats after preinjection with muscimol (Fig. 6). Finally, orexin neurons also express other neuropeptides and neurotransmitters, which are known to interact with orexin to regulate its function, including dynorphin and glutamate (3, 21, 34); thus, it is conceivable that orexin neurons in HA rats have higher glutamate release compared with orexin neurons in LA rats. Future studies of electrophysiological analysis of the intrinsic properties of orexin-producing neurons and their synaptic connectivity between HA and LA rats will greatly enhance our understanding of the differences between HA and LA rats in their SPA responses.

**Perspectives and Significance**

The orexin peptides are key regulators of SPA, yet the neuronal mechanisms regulating SPA at an individual level remain largely unknown. Our results demonstrate robustness of the HA/LA phenotype and identify two functional circuits involved in regulation of SPA: a feedback from orexin-responsive neurons in rostral LH to orexin-producing neurons in caudal LH, and inhibitory projections from NACSh to LH that modulate rostral LH orexin-A-induced SPA (Fig. 7). The first circuit (rostral LH → orexin neurons in caudal LH) could be a positive feedback loop to potentiate SPA in HA and LA rats, while the second circuit (NACSh → orexin neurons → rostral LH) could provide negative feedback to modulate orexin responses (2) and appears to be active in the HA rats only. Our data confirm that HA rats appear to have lower threshold for activation of orexin responses compared with LA rats. In conclusion, these data suggest that differences in orexin sensitivity in rostral LH and modulation of this response by GABA afferents from NACSh contribute to SPA differences between HA and LA rats. Overall, our data support a central role for orexin function variation as a key regulator of individual physical activity differences.

**GRANTS**

This work was supported by National Institutes of Health Grant ROI DK-100281-01A1 (to C. M. Kotz and C. J. Billington) and Grant CONICYT FONDECYT Regular 1150274 (to C. Perez-Leighton).

**DISCLOSURES**

The authors declare no conflicts of interest, financial or otherwise.

**AUTHOR CONTRIBUTIONS**

C.P-L., C.B., C.K. conceived and designed the research; C.P-L., M.G, M.R.L performed the experiments. C.P-L., M.R.L. analyzed data and prepared figures; C.P-L. drafted the manuscript. All authors edited, revised and approved the final version of the manuscript.

**REFERENCES**


2. Baldo BA, Gual-Bonilla L, Sijapati K, Daniel RA, Landry CF, Kelley AE. Activation of a subpopulation of orexin/hypocretin-containing hypothalamic neurons by GABAergic receptor-mediated inhibition of the nucleus accumbens shell, but not by exposure to a novel


