Role of the locus coeruleus in enhanced orexin A-induced spontaneous physical activity in obesity-resistant rats

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Teske JA, Perez-Leighton CE, Billington CJ, Kotz CM. Role of the locus coeruleus in enhanced orexin A-induced spontaneous physical activity in obesity-resistant rats. Am J Physiol Regul Integr Comp Physiol 305: R1337–R1345, 2013. First published October 2, 2013; doi:10.1152/ajpregu.00229.2013.—Orexin/hypocretin terminals innervate noradrenergic locus coeruleus (LC) neurons that project to the prefrontal cortex, which may influence spontaneous physical activity (SPA) and energy balance. Obesity-resistant (OR) rats have higher orexin receptors (OXR) mRNA in the LC and other brain regions, as well as lower adiposity compared with obese rats. These findings led us to hypothesize that orexin activity in the LC is relevant for the OR phenotype. We compared OR rats to Sprague-Dawley rats. We predicted that: 1) brain OXR expression pattern is sufficient to differentiate OR from non-bred Sprague-Dawley rats; 2) nonresting energy expenditure (NREE) and orexin A (OXA)-stimulated SPA after injection in LC would be greater in OR rats; and 3) the effect of OXA on SPA would be greater than its effect on feeding. OXR mRNA from 11 brain sites and the SPA and feeding responses to OXA in the LC were determined. Body composition, basal SPA, and EE were determined. Principal component analysis of the OXR expression pattern differentiates OR and Sprague-Dawley rats and suggests the OXR mRNA in the LC is important in defining the OR phenotype. Compared with Sprague-Dawley rats, OR rats had greater SPA and NREE and lower resting EE and adiposity. SPA responsiveness to OXA in the LC was greater in OR rats compared with Sprague-Dawley rats. OXA in the LC did not stimulate feeding in OR or Sprague-Dawley rats. These data suggest that the LC is a prominent site modulating OXA-stimulated SPA, which promotes lower adiposity and higher nonresting EE.

diet-induced obesity; brain

THE HYPOTHALAMIC NEUROPEPTIDE orexin A (10, 42) is crucial for body weight regulation (14). In rodents, orexin A in various brain sites stimulates feeding in a circadian-dependent manner (50), spontaneous physical activity (SPA), and energy expenditure (13, 20, 42). That orexin A can increase energy intake as well as energy expenditure makes it difficult to reconcile its role in body weight regulation. Evidence suggests the impact of orexin A on SPA is more relevant to energy balance than its effects on feeding. First, orexin A therapy elicits weight loss (33) and leaner body composition (36). Also, mice lacking orexin show obesity caused by reduced physical activity despite hypophagia (14). Second, SPA stimulated by orexin A persists longer than the feeding response. The dose required for the SPA response is lower than that for feeding effects, and the positive effects of orexin A on SPA are distributed among multiple brain sites with different functions (20). While the effect of orexin A on feeding also appears to be distributed in the sense that multiple brain sites contribute to the feeding response to orexin A, we have observed lack of orexin A feeding response in sites that elicited a SPA response. Importantly, obesity-resistant (OR) rats are more responsive to the SPA-promoting effect of orexin A relative to obese rats (32, 49). Together, these data suggest orexin A-mediated SPA contributes to energy balance, although a better understanding of the central network conferring enhanced SPA is needed.

Noradrenergic neurons in the locus coeruleus (LC) modulate arousal (4, 7) and feeding behavior (56) and are subject to orexin modulation. The LC receives dense orexin innervation and contains both orexin receptors (12, 37, 44), and orexin A increases activity of LC neurons (3, 5, 13, 15, 54). In a reciprocal manner, central orexin A increases arousal (13), muscle tone (17), and wakefulness (1), while silencing orexin neurons promotes sleep (53). Interestingly, orexin A infusion into the LC increases SPA without altering feeding (20). These data suggest a prominent role for the LC in mediating the behavioral effects of orexin A.

We sought to characterize if orexin modulation of LC was relevant for obesity resistance and also to uncover additional brain sites conferring protection from obesity. We focused on the LC based on functional and neuroanatomical evidence for the LC in modulating orexin signaling and SPA. Additional rationale was the finding that orexin receptor one and two mRNA in the LC was elevated in OR rats (25). We used a selectively bred rodent model of obesity resistance, the OR rats, whereby phenotypic traits are expressed on a low-fat diet (39). We compared OR rats to Sprague-Dawley rats instead of the selectively bred obesity-prone rats, as we have found that Sprague-Dawley and obesity-prone rats are similar behaviorally and phenotypically and respond to orexin similarly (48, 49). We hypothesized that 1) analysis of orexin receptors from multiple brain sites would differentiate OR and Sprague-Dawley rats and identify orexin receptors in the LC as significant to the OR phenotype; 2) compared with Sprague-Dawley rats, OR rats would have greater SPA, energy expenditure, SPA following orexin A infusion into the LC, and lower adiposity; and 3) orexin A-induced hyperphagia would be similar between

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groups. The current results are novel and show that the brain orexin receptor pattern, nonresting energy expenditure, and SPA response to orexin A in the LC distinguishes OR rats from Sprague-Dawley rats, identifies the LC as an important site modulating orexin A-enhanced SPA, and further suggests that the LC is an important brain site mediating high SPA and an OR phenotype.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley and selectively bred OR rats (Charles River, Kingston, NY) were housed individually in wire-bottom cages with resting platforms and chewing substrate (Nylabone, natural flavor; BioServ, Frenchtown, NJ), with a 12:12-h light-dark photocycle (lights on at 0600) in a temperature-controlled room (21–22°C). Rodent chow (Harlan Teklad 8604) and water were allowed ad libitum. Studies were approved by the Institutional Animal Care and Use Committee at the Minneapolis Veterans Affairs Health Care System and the University of Minnesota. Two sets of rats were used for the studies. One set of rats was used for the principal component analysis \((n = 9–10/\text{group})\), and a second set of rats \((n = 8–10/\text{group})\) was used for the behavioral studies.

Surgery

Animals were anesthetized with ketamine (50 mg/kg) and xylazine (15 mg/kg). A 26-gauge stainless steel cannula (Plastics One, Roanoke, VA) was directed towards the LC. The basic methods have been described previously (21, 22, 49). Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson (35) and are as follows: −9.7 mm posterior, 1.3 mm lateral to bregma, and 6.4 mm below the skull surface. For all cannulations, the incisor bar was set at 3.3 mm below the ear bars. Animals recovered from surgery for at least 7 days before experimental trials began.

Drugs

Orexin A (American Peptides, Sunnyvale, CA) was dissolved in artificial cerebrospinal fluid, which was used as the vehicle control for the injection studies. All drugs were stored at 4°C for <48 h.

Injections

A volume of 0.5 \(\mu\)l was injected evenly over 30 s with a 33-gauge injector (Plastics One, Roanoke, VA) that extended 1.0 mm beyond the tip of the guide cannula (21). The injector was left in place an additional 10 s to ensure extrusion from the tip and to minimize distribution of the drug upwards on the cannula tract. Injections were performed between 0900 and 1100. At least 48 h elapsed between treatments. Repeated injections do not cause tissue damage as measured by lack of extensive gliosis around the injection site under \(\times 100\) microscopy after 50 injections (38). Additionally, we observed no diminution of behavioral responses following repeated injections, suggesting that tissue and cellular integrity is not lost using this method. Because of technical error, we were unable to directly verify cannula placement with histology. Based on diffusion coefficients of the injection volume delivered, injected OXA should remain confined in the LC (28). Nonetheless, we performed a biological verification based on the magnitude of SPA stimulated by orexin A after injection into the LC in our past study (20). Rats were included in the data analysis if their SPA response to orexin A was 56% greater than their SPA response to the vehicle injection. Based on this biological verification, 14 of 16 rats or 88% of animals had correctly placed cannulae.

SPA Measurement

SPA was measured by infrared activity sensors placed around an acrylic cage (17.0 × 17.0; Med Associates, St. Albans, VT) as described previously (49). Briefly, ambulation was detected by two infrared arrays in the x- and y-axes and vertical movement was detected by a third elevated x-array and thus movement was simultaneously detected in all three axes. The amount of time spent ambulating (locomotor activity) and in the vertical position (rearing or standing) was calculated from the beam-break data. Spontaneous physical activity was defined as the sum of time spent ambulating and vertical or “time spent moving.” Before the start of the studies, animals were acclimated to SPA chambers for 140 min on three separate occasions.

Indirect Calorimetry

Energy expenditure was determined at 6 mo of age in an eight-chamber (inner dimensions: 10 W × 12 L × 8 H) open-circuit indirect calorimeter customized to permit simultaneous and continuous measurement of \(O_2\) consumption and \(CO_2\) production from each chamber (Columbus Instruments, Columbus, OH) (55). Gas sensors were calibrated before each test with a primary gas standard. Chamber air-flow was maintained at 3.1 l/min, and experiments were performed at 22°C. Rodents were acclimated to the chambers for 3 days before the 24-h test, and food and water were available ad libitum during acclimation and testing. The volume of \(O_2\) and \(CO_2\) was measured at 30-s intervals, and reference measurements from room air were determined at 15-min intervals over the 24-h testing period. The respiratory exchange ratio was defined as the mean of the respiratory exchange ratio values taken over the 24-h period. Energy expenditure (heat) was calculated as described previously (55). Total energy expenditure was calculated at the sum of heat measurements during the 24-h period. Resting energy expenditure was defined as the lowest metabolic rate during the light cycle extrapolated over the 24-h period. Nonresting energy expenditure was defined as the difference between total and resting energy expenditure. The percentage of energy expenditure due to rest was defined as the ratio of resting energy expenditure to total energy expenditure multiplied by 100. Nonresting energy expenditure was defined as the ratio of nonresting energy expenditure to total energy expenditure multiplied by 100.

Body Composition Measurement

Total fat and fat-free mass were measured using a quantitative magnetic resonance body composition analyzer (EchoMRI-900, Houston, TX) (29, 47). Animals were weighed and placed in a small cylindrical Plexiglas chamber. The chamber was inserted into the body composition analyzer, and the measurement was taken over a 1- to 2-min period. The percent values representing body fat and fat-free mass were calculated from the ratio of absolute fat and fat-free mass, respectively, and body weight.

Specific Experimental Designs

Study 1: principal component analysis. The following brain sites were dissected with a micropunch technique (19) from 3-mo-old OR and Sprague-Dawley rats: arcuate nucleus, caudal lateral hypothalamus, dorsal raphe, hypothalamic paraventricular nucleus, LC, rostral lateral hypothalamus, substantia nigra pars compacta, substantia innominata magnocellular basal nucleus, tuberomammillary nucleus, ventrolateral preoptic area, and ventral tegmental area. Brain sites were immediately frozen in liquid nitrogen and stored at −80°C. To avoid potential effects of recent food intake on gene expression, food was removed from the cages between 0700 and 0800. At this time, food intake is at a minimum in rodents and is similar between OR and Sprague-Dawley rats (48). Animals were euthanized between 1100 and 1200. Relative gene expression for the orexin one and two receptor and the housekeeping gene glyceraldehyde 3-phosphate de-
hydrogenase was measured by one-step real time RT-PCR. Data are expressed as a ratio of target (orexin receptor one or two) to the housekeeping gene as described previously (31, 49).

Study 2: development and characterization of the phenotype. Spontaneous physical activity was measured at 3 and 6 mo of age, and energy expenditure was measured at 6 mo of age in OR and Sprague-Dawley rats. Body weight and body composition were determined weekly from 3 to 6 mo of age, and 24-h food intake was determined at 3, 4.5, 5, and 6 mo of age.

Study 3: effect of orexin A in the LC on SPA. Orexin A (62.5, 125, 250, and 500 pmol/0.5 μl) or vehicle was infused into the LC in a repeated-meaures latin-square counterbalanced design. Doses of orexin A were determined based on previous work (20, 49). Continuous SPA was measured for 140 min postinjection. Since handling involved in the injection procedure increases SPA for up to 20 min postinjection independent of treatment, the first 20 min of data postinjection were not included in the data analysis (21). Thus the data analysis was performed on SPA measured in the 20- to 140-min postinjection period.

Study 4: effect of OXA in the LC on food intake. Orexin A (62.5, 125, 250, and 500 pmol/0.5 μl) or vehicle was infused into the LC in a repeated-measures latin-square counterbalanced design. Preweighted food hoppers and food spillage were measured 1, 2, and 24 h after injections to determine food intake corrected for spillage.

Statistical Analyses

Study 1. The $2^{-ΔΔCT}$ method was used to calculate relative gene expression (23). Orexin receptor gene expression was analyzed with a principal component analysis (36), using the free available R statistical software (46). The goal of this analysis is to determine whether the expression of orexin receptors across multiple brain sites is sufficient to classify an animal as an OR or Sprague-Dawley rat. Although there are other analytical methods to reveal differences between two groups (i.e., a multiple measures ANOVA with group as dependent variable), principal component analysis is a statistical tool used to understand the underlying structure of multivariate datasets and is widely used in multivariate analysis of gene expression (40) to understand how multiple variables (i.e., the expression of the orexin receptor across multiple sites) contribute to the overall variability in a dataset. Briefly, a principal component analysis will generate two or more vectors (the principal component vectors) that describe the major sources of variability in a given dataset (40). For our dataset, each dimension is the expression of either the orexin one receptor or the orexin two receptor in each 1 of the 11 brain regions tested. Each resulting principal component vector contains scores (a particular linear combination of the original dimensions of the dataset, i.e., the expression of each orexin receptor in each brain site) for each one of the animals in the original dataset. The principal component vectors are organized such that each successive vector describes a particular percent of the original variability of the dataset in a decreasing order. Therefore, a principal component analysis describes the variability between OR and Sprague-Dawley rats using orexin receptor expression data from all brain sites simultaneously. This analysis allows one to determine if the combined information from the orexin receptors across brain sites tested is sufficient to discriminate OR from Sprague-Dawley rats. To account for incomplete data, we used an implementation of the svdImpute algorithm (43, 51). To analyze the results of the principal component analysis, we plotted the principal component scores for the first two principal component analysis vectors in (Fig. 1A) and performed a cluster analysis using the fuzzy K-means clustering algorithm (8) to verify whether the clusters corresponded to the OR and Sprague-Dawley rats.

To identify the contribution of each orexin receptor to the differences between OR and Sprague-Dawley rats, we used the loadings of the principal component analysis (Fig. 1B). The loadings are the correlation between each one of the original dimensions of the dataset relative to each of the principal component vectors. The original dimensions of the dataset are the expression of either the orexin one receptor or the orexin two receptor in each brain site. Thus Fig. 1B shows how much the expression of each orexin receptor in each brain site contributes to principal component one or principal component two.

Study 2. Differences between OR and Sprague-Dawley rats were analyzed by t-test for the following end points: total energy expenditure, percentage of resting and nonresting energy expenditure, respiratory exchange ratio, SPA, body weight, composition data and food intake (Prism 5.0b; GraphPad Software, San Diego, CA). An alpha

Fig. 1. Study 1: expression of orexin receptors across multiple brain sites distinguishes obesity-resistant (OR) from Sprague-Dawley (SD) rats. A: scatter plot of OR and SD rats based on the first two principal component vectors. Each point represents a single animal; it is known a priori whether the point represents data from an OR or SD rat. Inspection of this plot shows that, based on orexin receptor expression alone, OR rats can be distinguished from SD rats. This plot suggests that the variability expressed by the first principal component described better the differences between OR and SD rats. The ellipsoids for each cluster were calculated to be the minimal area to include all the points within each cluster using the center the two-dimensional mean of each cluster. B: loadings from principal component one and the second principal component. All (brain site x orexin receptor subtype) combinations are plotted against the loadings for principal component one and principal component analysis two. ARC, arcuate nucleus; cLH, caudal lateral hypothalamus; DR, dorsal raphe; PVN, hypothalamic paraventricular nucleus; LC, locus coeruleus; rLH, rostral lateral hypothalamus; SN, substantia nigra pars compacta; SIMBN, substantia innominata magnocellular basal nucleus; TMN, tuberomammillary nucleus; VLPO, ventrolateral preoptic area; VTA, ventral tegmental area.
level of 0.05 was used for all statistical tests. Data are expressed as means ± SE.

Study 3 and 4. The results were the same with or without excluding two rats after biological verification of cannula placement. Orexin A-induced SPA and food intake data were analyzed by two-factor repeated-measures ANOVA (Prism 5.0b; GraphPad Software, San Diego, CA). When significant main effects were observed, the data were separated by group (OR and Sprague-Dawley) and then analyzed by repeated-measures ANOVA followed by post hoc t-tests to compare individual treatment means within each group. Therefore, “group” and “treatment-orexin A” were the between-subjects factors, while SPA (e.g., time spent moving 0–1 h, 1–2 h, and 0–2 h) and food intake (0–1 h, 1–2 h, 0–2 h, 2–24 h, and 0–24 h) were the dependent variables. An alpha level of 0.05 was used for all statistical tests. Data are expressed as means ± SE.

RESULTS

Study 1: Brain Orexin Receptor mRNA Differentiates OR and Sprague-Dawley Rats

We used principal component analysis to first ask whether orexin receptor mRNA was sufficient to differentiate OR from Sprague-Dawley rats (n = 9–10/group, see MATERIALS AND METHODS for rationale). A principal component analysis reduces the dimensionality of a dataset by creating linear combinations (scores) of each of the original dimensions (i.e., expression of each orexin receptor in each brain site tested) for each of the individuals sampled (OR and Sprague-Dawley rats). Figure 1A shows a scatterplot of the scores for the first two principal component vectors, which describe 40% of the total variability in the original dataset. In this scatterplot (Fig. 1A), each point represents a single animal, and we know a priori whether the sample is an OR or Sprague-Dawley rat. Inspection of this plot (Fig. 1A) reveals two clusters that correspond to the OR and Sprague-Dawley rats, which was confirmed with cluster analysis (see MATERIALS AND METHODS). A feature of the principal component analysis is that each principal component vector describes a portion of the total variability observed in the original dataset. Figure 1A shows that the variability described by the first principal component vector drives the separation between OR and Sprague-Dawley rats. Hence, orexin one and two receptor gene expression from 11 brain sites is sufficient to distinguish OR from Sprague-Dawley rats (Fig. 1A). Next we determined the contribution of each brain region x orexin receptor mRNA combination to the difference between OR and Sprague-Dawley rats. To accomplish this, we plotted the loadings of the principal component analysis (Fig. 1B) for principal component analysis one and principal component two. The loadings are the correlation between each one of the original dimensions of the dataset relative to each of the principal component vectors. As principal component one is the vector that better separates OR and Sprague-Dawley rats, we were interested in brain sites with large loadings for principal component one and low loadings for principal component two. Figure 1B shows that expression of orexin one and two receptors in several brain sites have larger loadings for principal component one. However, in this dataset only the LC and caudal lateral hypothalamus have higher loadings for principal component one and lower loadings for principal component two for both OXR. This indicates that expression of both orexin receptors in the LC and caudal lateral hypothalamus equally explain the phenotypic difference between OR and Sprague-Dawley rats.

Study 2: Adiposity Differences Develop During Maturation and Track With Greater SPA and Nonresting Energy Expenditure in OR Rats

OR (n = 10) rats showed a leaner phenotype compared with Sprague-Dawley rats (n = 10). OR rats had significantly lower body weight, fat and fat-free mass compared with Sprague-Dawley rats (P < 0.0001 for each weekly measurement, Fig. 2,

Fig. 2. Study 2: body weight (A), percent body fat (B), fat-free mass (C), time ambulatory (D), time vertical (E), and time ambulatory + vertical (F) in OR and SD rats. *P < 0.05 compared with age-matched SD rats; n = 22 10/group. Please note different y-axes. Data represent means ± SE.
Adiposity, expressed as percent body fat, began to diverge between OR and Sprague-Dawley rats at 5 mo of age, with OR rats showing significantly less adiposity \((P < 0.05)\) for each weekly measurement, Fig. 2B. Percent fat-free mass was similar between OR and Sprague-Dawley rats with one exception at 3.25 mo of age (Fig. 2C), where Sprague-Dawley rats had significantly greater fat-free mass at 3.25 mo of age compared with OR rats \((t = 3.71, \text{degrees of freedom } 18, P < 0.0016)\).

OR rats spent more time ambulating at 3 and 6 mo of age and spent more time vertical at 6 mo of age only (Fig. 2, D and E). This resulted in OR rats spending significantly more time moving at 6 mo of age \((P = 0.0162)\) but not at three mo of age \((P = 0.26)\). During the 24-h calorimetry test, OR rats consumed significantly fewer calories than Sprague-Dawley rats \((\text{OR: } 76.4 \pm 1.7 \text{ g and Sprague-Dawley: } 92.9 \pm 2.5 \text{ g; } P < 0.0001)\).

Total energy expenditure was not significantly different between OR and Sprague-Dawley rats measured over 24 h, dark and light periods (Fig. 3, A–C). These data, together with the lower body weight and greater 24 h SPA in OR compared with Sprague-Dawley rats (Fig. 2, A and F) suggest that OR rats have higher energy expenditure despite reduced body weight and lean mass. Indeed, OR rats have higher energy expenditure per gram of lean mass compared with Sprague-Dawley rats \((\text{OR: } 0.178 \pm 0.007 \text{ and Sprague-Dawley: } 0.131 \pm 0.005, P < 0.0001)\). Recently, it has been suggested that analysis of covariance (ANCOVA) techniques can be used to control for influences of body weight in energy expenditure \((2, 6, 11, 52)\). However, in this case an ANCOVA is not appropriate, as the covariates (body weight or lean mass) are confounded between OR and Sprague-Dawley rats, which violates the ANCOVA assumptions (see DISCUSSION).

The OR rats have higher SPA, lower body weight, and yet the same daily energy expenditure as Sprague-Dawley rats. This suggests that OR rats expend more energy moving during the dark period despite their lower lean mass. To confirm this, we calculated resting and nonresting energy expenditure in OR and Sprague-Dawley rats (Fig. 3D). The percentages of resting and nonresting energy expenditure were significantly different between groups (Fig. 4D). OR rats expended a significantly lower percentage of calories during rest and a greater percentage during nonrest \((P = 0.0301, \text{Fig. 3E})\). When expressed as absolute calories during the 24-h period, OR rats expended fewer calories during rest \((\text{Fig. 3B, } P < 0.0001)\), but the value difference in nonresting energy expenditure between groups did not reach statistical significance \((\text{Fig. 3C, } P = 0.1079)\). The respiratory exchange ratio was significantly lower
in OR rats relative to Sprague-Dawley rats (OR: 0.77 ± 0.009 and Sprague-Dawley: 0.81 4 ± 0.004; t = 3.72, degrees of freedom = 18, \( P = 0.0016 \)). Together, these data show that OR rats, despite lower body weight, have the same energy expenditure as Sprague-Dawley rats, which appears to be mediated by their increased energy expenditure associated to physical activity.

**Study 3: OR Rats Have a Greater SPA Response to Orexin A Infusion Into the LC**

Two-factor repeated measures ANOVA indicated a main effect of group and treatment on all indices of SPA 1 and 2 h postinjection (\( P < 0.05 \) for all effects). Figure 4 shows that OR rats moved significantly more than Sprague-Dawley rats in response to the three highest doses of orexin A (Fig. 3C, \( P < 0.01 \) for all comparisons) and that orexin A infusion into the LC significantly increased SPA 1 h postinjection in both OR (\( P = 0.001 \)) and Sprague-Dawley rats (\( P = 0.0225 \)). In OR rats, the two highest doses of orexin A significantly increased SPA (\( P < 0.05 \) for both doses) and this effect was dose dependent, as total movement stimulated by the 500-pmol dose was significantly greater than movement stimulated by 62.5 pmol OXA (\( P < 0.05 \)). In contrast, in Sprague-Dawley rats only the highest dose of orexin A significantly increased SPA relative to vehicle (\( P < 0.05 \)). During the 1- to 2-h postinjection interval, there was no main effect of group and treatment on SPA indicated by the two-factor repeated measures ANOVA (group: \( P = 0.0548 \) and treatment: \( P = 0.56 \), data not shown). The enhanced response to orexin A in OR rats persisted 2 h postinjection (Fig. 4F); albeit the response during the 0- to 2-h postinjection period was largely due to the effect of OXA in the 0- to 1-h period since there was no effect of OXA in the 1- to 2-h period. The two highest doses of orexin A significantly increased SPA more robustly in OR rats relative to Sprague-Dawley rats (250 pmol: \( P = 0.0034 \) and 500 pmol: \( P = 0.0158 \)). Orexin A infusion into the LC had a significant effect on SPA in both OR (\( P = 0.0109 \)) and Sprague-Dawley rats (\( P = 0.0389 \)). In OR rats the 250-pmol dose of orexin A significantly increased SPA in OR rats relative to vehicle and in Sprague-Dawley rats the 500-pmol dose of orexin A significantly increased SPA relative to vehicle (\( P < 0.05 \) for both comparisons).

The enhanced SPA response to orexin A in OR rats relative to Sprague-Dawley rats was primarily due to greater time spent in ambulatory movement in OR rats relative to vertical movement (Fig. 4, A and B). Two hours postinjection, orexin A significantly increased ambulation but not vertical movement in OR rats. In contrast, orexin A stimulated vertical movement but not ambulation in Sprague-Dawley rats.

**Study 4: Orexin A-Induced Feeding is Similar in OR and Sprague-Dawley Rats**

Orexin A infusion into the LC did not stimulate food intake 1, 2, or 24 h postinjection in either OR or Sprague-Dawley rats (Fig. 5). There was also no effect of orexin A in the LC on food intake during the 1- to 2-h or 2- to 24-h intervals, and a two-factor repeated-measures ANOVA indicated no main effect of group, treatment or interaction for the 1-, 2-, and 24-h intervals postinjection.

**DISCUSSION**

In the current study, orexin A infused into the LC stimulated SPA but not feeding. OR rats had less fat and resting energy expenditure but similar total energy expenditure and greater nonresting energy expenditure compared with Sprague-Dawley rats. The brain orexin receptor expression pattern and differential SPA sensitivity to orexin A in the LC distinguished OR from Sprague-Dawley rats. Together, these data suggest that the brain orexin receptor enhanced SPA by orexin A in the LC and greater nonresting energy expenditure contributes to lower adiposity in OR rats. While previous work showed that brain orexin receptor mRNA differentiates rats with dissimilar severity for diet-induced obesity and differential SPA levels (36) and that OR rats have greater orexin receptor mRNA in several brain areas (25), a novelty of the current study is that we suggest that differences in brain orexin receptor expression patterns translate into distinct behavioral (e.g., enhanced the response to orexin A in the LC-mediated SPA and greater nonresting energy expenditure) phenotypic profiles driven by orexin signaling through the LC that contribute to lower adiposity in OR rats.

Principal component analysis reduces variability within a multidimensional dataset to two or more vectors that consecutively describe major sources of variability (40). We expected OR rats could be differentiated from Sprague-Dawley rats based on the brain orexin receptor expression pattern given previous phenotypic characterization of OR rats (18, 20, 32, 47, 49). As orexin receptor expression is widely distributed, we reasoned that the differences in orexin receptor expression would be distributed across multiple brain sites. Cluster analysis confirmed a separation of the data from OR and Sprague-

![Figure 5](https://example.com/image.png)

**Fig. 5.** Study 4: orexin A in the locus coeruleus does not stimulate feeding in OR rats and SD rats. Data represent means ± SE; \( n = 5–6 \)/group. Please note different y-axes (A–C).
Dawley rats into two clusters. Based on this, principal component analysis of orexin receptor mRNA levels provides a neuroanatomical basis for previously observed basal differences in antiobesity behaviors such as high SPA.

To identify additional brain sites that contribute to the variability between OR and Sprague-Dawley rats, the loadings for the principal component vectors were plotted. The LC mRNA data were highly correlated to the first principal component vector, which indicates that orexin one and two receptor mRNA in LC accounts for significant phenotypic variability between OR and Sprague-Dawley rats. Expression of both orexin receptor expression in the hypothalamic paraventricular nucleus and caudal lateral hypothalamus also contributes to the distinguishing clusters of data from OR and Sprague-Dawley rats. Together this suggests that enhanced LC orexin signaling functions within a network conferring behaviors that promote phenotypic traits in OR rats. Whether elevated mRNA parallels peptide levels remains to be determined, but OR rats do have greater orexin one receptor peptide levels in the rostral lateral hypothalamus (18).

We had previously shown that orexin A in the LC robustly stimulated SPA but had no effect on feeding in Sprague-Dawley rats (20) and elevated orexin receptor expression in the LC of OR rats (25). Hence, we expected a positive SPA response and null feeding response to orexin A in the LC and expected OR rats to be more responsive. That OR rats had greater orexin A-induced SPA compared with Sprague-Dawley rats despite no effect on feeding indicates that OR rats are not more responsive to orexin A in general. Instead, the lack of feeding response to orexin A in the LC in OR rats undoes the site specificity of the feeding and SPA response to orexin A. This finding is consistent with the null feeding response to OXA in the tuberomammillary nucleus and substantia nigra (18) among Sprague-Dawley rats but contrasts to the orexin A-induced hyperphagia shown in OR rats (32, 49) after injection in rostral lateral hypothalamus. Orexin stimulates release of norepinephrine in the LC, histamine in the tuberomammillary nucleus, and dopamine in the substantia nigra monoamines that inhibit feeding (9, 41, 45). This fits with the current data indicating that orexin A action in the LC more profoundly influences energy expenditure over energy intake by stimulating SPA. In addition to a heightened SPA response to OXA, OR rats have elevated orexin receptor mRNA in the LC, a prominent sleep regulatory brain site, and better sleep quality (25). Therefore, enhanced signaling through orexin receptors in the LC may also function to maintain appropriate sleep/wake status, which promotes a lean phenotype. Thus, like other brain sites in OR rats, orexin receptor expression in the LC (25, 49) appears to contribute to functional differences in behavior.

We found lower resting energy expenditure and greater nonresting energy expenditure leading to similar total energy expenditure but lower adiposity and weight in OR rats. At 3 and 6 mo of age, OR rats moved more and consumed fewer calories for their metabolic mass. Interestingly, body fat percentage was not different between groups from 3–5 mo of age but was significantly lower in OR rats from 5–6 mo of age. This suggests that elevated SPA and associated energy expenditure at this young age may contribute to the lean phenotype by dampening fat accretion over time.

Several strategies for analyzing energy expenditure data in rodents of varying body mass or composition have been described (2, 6, 11, 52). While some of the literature favors using ANCOVA (2, 6, 11, 52), we used an alternative to ANCOVA since the covariates (body weight or fat-free mass) were confounded within the OR and Sprague-Dawley phenotypes. Analysis of covariance is invalid for correcting or controlling for preexisting rat group differences on a potential covariate (body weight and fat-free mass) (26). This problem is particularly acute in cases where animals cannot be randomly assigned to groups. Our analysis shows that there are no differences in overall energy expenditure between OR and Sprague-Dawley rats despite lower total body weight and greater SPA in OR rats. Our data indicate that OR rats have higher energy expenditure during the dark period when rats are most active and lower energy expenditure during rest periods (Fig. 3). OR rats have lower body weight and fat-free mass and higher SPA, while Sprague-Dawley rats show the opposite pattern. These data match with the analysis showing higher nonresting or activity energy expenditure in OR rats (probably mediated by higher SPA) and higher resting energy expenditure in Sprague-Dawley rats (probably mediated by higher body mass). Therefore, these data suggest that differences in total energy expenditure are not accounted for by differences in fat-free mass but are instead due to differences in SPA-associated nonresting energy expenditure.

OR rats had significantly greater nonresting energy expenditure when data were expressed as a percentage of total energy expenditure, and the same trend, although it did not reach statistical significance, was observed when analyzing absolute kilocalories. The failure to reach statistical significance for kilocalories of nonresting energy expenditure is likely due to variability in SPA and nonresting energy expenditure. Greater nonresting energy expenditure in lean rats parallels high nonresting energy expenditure in rodents that had never been obese (24), and lean humans exhibiting elevated activity-related energy expenditure and time spent in physical activity (16). Together, these studies suggest that nonresting energy expenditure contributes to obesity prevention in OR rats. The age of 3–6 mo in a rat coincides with ~18–30 yr of age in humans, which is the life stage where physical activity levels declines, food choice worsens, and obesity rates increase (27). The potential to attenuate fat accretion with elevated SPA-associated nonresting energy expenditure during this critical time frame is important to obesity prevention efforts.

The current findings suggest that nonresting energy expenditure as a percentage of total energy expenditure is greater in OR rats than in Sprague-Dawley rats. Similar total energy expenditure between these groups with such different body composition and activity profiles suggests that additional factors contribute to total energy expenditure and/or that difficulties inherent to estimating energy expenditure from indirect calorimetry chambers preclude an exact accounting of true energy expenditure. Some of these challenges include sensitivity of indirect calorimetry systems, such that small changes in energy expenditure below the detection limits remain uncaptured. Heat may be lost due to the high turnover rate of chamber air during reference air measurements, and as indirect calorimetry is typically conducted below thermoneutral for rodents (34), as in this experiment, this makes detecting changes in energy expenditure difficult. Potential group differences in the thermic effect of feeding were not accounted for in the assumptions used to calculate total energy expenditure, and separating
energy expenditure into resting energy expenditure and nonresting energy expenditure also has inherent difficulties (2). The current estimation methods are consistent with others (24, 30), but further work is needed to get a more complete indication of resting energy expenditure and nonresting energy expenditure in this rodent model. We acknowledge that lack of histology is a limitation of the study. Finally, while OR rats are part of the Sprague-Dawley rat line, our study would be strengthened with a demonstration that OXA activates the LC in OR rats, although OXA in the LC activates LC neurons in Sprague-Dawley rats as indicated by c-fos (5).

Perspectives and Significance

These data show that higher nonresting energy expenditure in OR rats parallels enhanced SPA and lower adiposity. The data also suggest that brain orexin signaling specifically in LC is a prominent mechanism underlying phenotypic variation in behavior and adiposity between OR and obese rats. These data imply that elevated orexin signaling in LC promotes behaviors conferring negative energy balance and reducing adiposity gain. The present findings have strong implications for obesity therapies targeting the orexin system and behavioral programs to increase SPA in humans.

REFERENCES


