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Elevated hypothalamic orexin signaling, sensitivity to orexin A and spontaneous physical activity in obesity resistant rats.

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Running Head: Enhanced SPA response to OxA in obesity resistant rats

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ABSTRACT

Selectively-bred obesity resistant (DR, diet resistant) rats weigh less than obesity prone (DIO, diet-induced obese) rats despite comparable daily caloric intake, suggesting phenotypic energy expenditure differences. Human data suggest that obesity is maintained by reduced ambulatory or spontaneous physical activity (SPA). The neuropeptide orexin A robustly stimulates SPA. We hypothesized that DR rats have greater: 1) basal SPA, 2) orexin A-induced SPA and 3) preproorexin, OX1R and OX2R mRNA, compared to DIO rats. A group of age-matched out-bred Sprague-Dawley rats were used as additional controls for the behavioral studies. DIO, DR and Sprague-Dawley rats with dorsal-rostral lateral hypothalamic (rLHa) cannulae were injected with orexin A (0, 31.25, 62.5, 125, 250, and 500 pmol/0.5 µl). SPA and food intake were measured for 2 h after injection. Preproorexin and orexin 1 and 2 receptor (OX1R and OX2R) mRNA in the rLHa and whole hypothalamus were measured by real-time RT-PCR. Orexin A significantly stimulated feeding in all rats. Orexin A-induced SPA was significantly greater in DR and Sprague-Dawley rats than in DIO rats. Two month old DR rats had significantly greater rLHa OX1R and OX2R mRNA than DIO rats but comparable preproorexin levels. Eight-month old DR rats had elevated OX1R and OX2R mRNA compared to DIO rats, although this increase was significant for OX2R only at this age. Thus, DR rats show elevated basal and orexin A-induced SPA associated with increased OX1R and OX2R gene expression, suggesting that differences in orexin A signaling through OX1R and OX2R may mediate DIO and DR phenotypes.

Keywords: hypocretin, lateral hypothalamus, locomotor activity, diet induced obesity.

INTRODUCTION

Out-bred male Sprague-Dawley rats fed a high-energy diet display divergent body weight gain patterns. Approximately half of the rats become obese (DIO rats) while the other half remain lean (DR rats). DIO and DR rats can be prospectively identified prior to exposure to a high-energy diet based upon sympathetic activation (32) and monoaminergic function (13, 24, 26), suggesting that differences in the propensity towards weight gain are due to differences in CNS function. To better understand the mechanism underlying the propensity or resistance to weight gain observed in the out-bred DIO and DR rats, Sprague-Dawley rats fed a high-fat diet were selectively bred for their weight gain. This selective breeding scheme produced two substrains of Sprague-Dawley rats that displayed divergent body weight gain patterns despite comparable caloric intake following chow feeding (29, 47), suggesting that differences in energy expenditure primarily underlie phenotypic differences in body weight gain patterns. It has been demonstrated that lean individuals spent more time standing and ambulating than obese individuals, and this amount of time remained fixed independent of body weight despite overfeeding lean or underfeeding mildly obese individuals (33). Similarly, differences in SPA between lean and obese out-bred Sprague-Dawley rats were maintained independent of changes in body weight after removal from the high energy diet (27). Together these studies suggest that neural mechanism(s) modulate energy expenditure to either protect against or increase propensity towards excessive weight gain.

Orexin A, a neuropeptide involved in feeding and arousal, is synthesized in discrete neurons within the lateral, dorso-medial, posterior and perifornical areas of the hypothalamus (7, 45, 48). Orexin neurons have widespread projections throughout the neural axis with dense projections to areas implicated in the regulation of feeding and arousal (45, 49). Central

administration of orexin A stimulates food-seeking and eating behavior in rodents (6, 17, 49) and robustly stimulates SPA (22) and non-exercise activity thermogenesis (heat produced by SPA) (19). Orexin neurons project to the dopaminergic neurons in the substantia nigra that innervate the striatum and are a critical component of motor activity, supporting the idea of orexin regulation of SPA. Mice lacking orexin are less active and weigh significantly more than wild-types despite reduced feeding behavior (12), suggesting that changes in SPA may have a more profound influence on body weight than changes in energy intake.

Functional distinction of the orexin receptors has not yet been established, mostly due to lack of availability of a specific OX2R antagonist. Some data suggest that OX1R may play a larger role in feeding regulation whereas OX2R may be more important to arousal and locomotor activity. Administration of SB334867A (OX1R antagonist) systemically or into the nucleus accumbens decreases orexin A-induced feeding (14, 56). Orexin A, which selectively binds OX1R, consistently stimulates feeding or drinking, and OX2R has equal affinity for orexin A and B (8, 15, 17, 23, 49). Furthermore, orexin A injected the hypothalamic paraventricular nucleus, which is densely innervated by orexin neurons and where OX2R is highly expressed, produces a robust locomotor activity response but a small or absent feeding response (8, 19, 36, 52). However, a few studies show that orexin effects on locomotor activity are reduced by SB334867A indicating that some orexin-induced locomotor effects may be mediated via OX1R (18, 19).

The neural mechanism(s) that modulate SPA in selectively-bred DIO and DR rats are unknown. Therefore, in the current study components of SPA, preproorexin, OX1R, and OX2R mRNA were determined in young selectively-bred DIO and DR rats. A group of Sprague-Dawley rats was included to allow comparison to results from DIO and DR rats, as out-bred

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Sprague-Dawley rats are predominately used as "normal" controls and could help determine which group (DIO or DR) is anomalous. We addressed three hypotheses: DR rats have elevated 1) basal SPA, 2) orexin A-induced SPA, and 3) orexin tone as measured by preproorexin, OX1R and OX2R mRNA, compared to DIO rats.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley and selectively-bred male DIO and DR rats (Charles River, Stoneridge, NY) were housed individually in cages with a 12-h light/12-h dark photo-cycle (lights on at 0700 h) in a temperature controlled room (21–22 ˚C). Rodent chow (Harlan Teklad 8604) and water were allowed ad libitum. All studies were approved by the local Institutional Animal Care and Use Committee at the Veterans Affairs Medical Center and the University of Minnesota. Six sets of animals were used. One set was used for study 1, one set for studies 2 and 3, one set for studies 4 and 5 and 3 sets for study 6.

Surgery: Animals were anesthetized with Ketamine (90 mg/kg) and Xylazine (15 mg/kg) and were fitted with a 26-gauge stainless steel cannula (Plastics One, Roanoke, VA) aimed at the rLHa. Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson (43) and are as follows: 2.2 mm posterior and 1.9 mm lateral to bregma, and 7.3 mm below the skull surface. For the animals used in studies 4 and 5 the coordinates were adjusted 0.1mm anterior per 25g over 300g body weight based on our previous experience. For all cannulations, the incisor bar was set at 3.3 mm below the ear bars. A dummy stylet was placed in the guide cannula that extended to the tip of the cannula after surgery and between injections. At least 7 days elapsed following surgery before experimental trials.

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Drugs: Orexin A (American Peptides, Sunnyvale, CA) was dissolved in artificial cerebrospinal fluid (aCSF) and stored at 4°C for less than 48 h. Orexin A from the same lot was used for studies 2 - 5.

Injections: A volume of 0.5 µl was injected slowly 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 an additional 10 s to ensure extrusion from the tip and to minimize distribution of the drug upwards on the cannula tract. After injection, the injector was withdrawn and the stylet replaced. The total number of injections for each animal was less than 15. In previous studies, lack of extensive tissue damage after 50 repeated injections as measured by gliosis around the injection site (46) and light microscopy at 100x was demonstrated.   In the current studies injection sites were examined by light microscopy for extensive tissue damage and none was found.

Verification of Cannula Placement: Brains from animals in studies 4 and 5 were dissected out and stored in a 10% formaldehyde solution for placement verification by histology. A cannula was deemed incorrectly placed if the actual injection site was farther than 0.25 mm away from the targeted site. This rationale is based on diffusion coefficients of injection volume delivered (40).   All cannulas were correctly placed, therefore, the number of rats listed in the figure legends represent the number of rats in the final analysis (all cannulas correctly placed). Due to continued experimental testing, we were unable to verify cannula placement for animals in studies 2 and 3, but based on the high percentage of correctly placed cannulae in study 4 and 5 (100%) and our previous experience with rLHa cannulations (21, 22, 38, 42, 52, 53, 57-59), we are confident that a high percentage (>95%) of the animals in studies 2 and 3 have correctly placed cannulas.

SPA measurement: SPA was measured using customized, high precision racks of infrared activity sensors (Med Associates, St. Albans, Vermont) placed around a square acrylic cage (17.0" x 17.0").   There were three 16-beam I/R arrays, two arrays in the x direction and one elevated x array above an x array.   Hence, ambulation was measured by arrays in the x and y-axes and vertical movement was measured by the second elevated x array.   Photosensors registered an activity unit each time a beam was interrupted.   Thus movement was simultaneously detected in all three axes. Data were collected by downloading to a PC every 50 ms during the measurement period.   From the SPA measurements we calculated time spent ambulating (locomotor activity) and vertical (rearing or standing) and we refer to this, the sum of time spent ambulating and vertical as "time spent moving".

Total RNA Extraction and Quantification: Total RNA was isolated from the rLHa (obtained using a brain micropunch procedure reported previously (20)) and whole hypothalamus according to the TRI Reagent protocol with minor modifications (4).   Briefly, tissue was homogenized with Trizol reagent (Gibco BRL, Paisley, UK) and chloroform.   After phase separation, the aqueous phase was removed and total RNA was precipitated with 70% ethanol and applied to a Qiagen column (Qiagen RNeasy micro kit, Qiagen, Valencia, CA).   The concentration and purity of the RNA were determined by the 260nm and 280nm readings on a spectrophotometer (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE).

One-Step Real Time RT-PCR: The primers for preproorexin, OX1R, OX2R and the housekeeping gene, ribosomal protein L32 (Rpl32), were created using MacVector 7.2 (Accerlys, San Diego, CA.) (table 1). One-step real time RT-PCR was performed using 100 ng of total RNA, and the reagents provided in the Roche RNA Amplification Kit SYBR Green I, and a Roche LightCycler (Roche Applied Science, Indianapolis, IN.).   RT-PCR was performed

as follows: reverse transcription for 30 min at 42°C, denaturation for 30 sec at 95°C, followed by 35 cycles of cDNA amplification consisting of a 15 sec denaturation at 95°C, primer annealing for 20 sec at 60˚C (preproorexin) or 59°C (OX1R, OX2R and Rpl32), and product elongation for 15 sec at 72°C. Data acquisition was taken at the end of each amplification cycle at a temperature slightly lower than the temperature required to melt the PCR product, 84˚C (OX1R), 82°C (OX2R) and 79°C (preproorexin and Rpl32). Amplification products from PCR were purified (QIAquick PCR Purification Kit, Valencia, CA), determined by electrophoresis in a 3% Nuseive gel, and then verified by capillary electrophoresis.

Specific experimental designs:

Study 1. Basal 24 h SPA in the presence of food at 1-2, 4 and 7 months of age: Male age-matched DIO, DR and Sprague-Dawley rats (n = 4-10/group); were acclimated for 48 h to chambers that measured SPA prior to a continuous 24 h measurement period. We found that time spent moving was not significantly different between the last 24 h of the 48 h acclimation period and the 24 h test measurement period (data not shown) and thus the acclimation period was reduced from 48 h to 24 h for measurements at 4 and 7 months of age. Continuous 24 h SPA was measured at 1-2, 4 and 7 mo of age in the same group of rats. Variation in group sizes was due to rats that spontaneously died of unknown causes. Rodents had ad libitum access to pelleted rodent chow and water throughout the acclimation and measurement periods. Chow was placed on the chamber floor and water was provided in two 25mL test tubes filled attached to a stopper and secured in the corners of the chamber 2-3 inches from the chamber floor, ensuring that time spent vertical was not confounded by activity due to eating or drinking. The test tubes were refilled every 24 h. Body weight was measured prior to the 24 h measurement period. Cumulative food intake was measured during the acclimation and measurement periods by

subtracting the remaining food pellets and spillage from the original amount of food given to the rats and are reported as mean 24 h caloric intake per 100g body weight. Data are reported as time spent moving in the light cycle, dark cycle, and 24 h measurement period.

Study 2. Effect of orexin A on feeding in 2-3 month old DIO, DR, and Sprague-Dawley rats: One week after surgery, orexin A (31.25, 62.5, 125, 250 pmol/0.5 μl) or vehicle (aCSF) was injected into the rLHa of DIO, DR, and Sprague-Dawley rats in a latin-square counter-balanced design. Rats were randomly assigned to treatment, whereby each animal received each treatment once and each treatment was represented on a given day (DIO, N = 9; Sprague-Dawley, n = 10; DR, n = 11). At least 48 h elapsed between treatments. Food intake was measured 1 and 2 h after the injection. Injections were given between 1200 and 1230 h.

Study 3. Effect of orexin A on SPA in 2-3 month old DIO, DR, and Sprague-Dawley rats: Animals were acclimated to SPA chambers for 140 min on three separate occasions prior to the start of the study. Orexin A (31.25, 62.5, 125, 250 pmol/0.5 μl) or vehicle (aCSF) was injected into the rLHa of the same rats used for study 2 in a randomly assigned latin-square counter-balanced design (DIO, N = 9; Sprague-Dawley, n = 10; DR, n = 11). At least 48 h elapsed between treatments. Continuous SPA was measured for 140 min post-injection. Injections were given between 1100 and 1330 h. The data were collected into cumulative 10-min time bins for the 140 min measurement period and data are reported as 0-1 h, 1-2 h, and 0-2 h time spent moving. To equally distribute potential variability related to aging and repeated injections, studies 2 and 3 were performed concurrently and treatment days for testing OXA-induced SPA and feeding were alternated. Body weight was measured on the first and last treatment day and was used to calculate mean body weight for studies 2 and 3.

Study 4. Effect of orexin A on feeding in 6 mo old DIO and DR rats: In a separate group of rLHa-cannulated DIO and DR rats the effect of orexin A on feeding was measured as described above in study 2 (DIO, n = 10; DR, n = 7). Briefly, orexin A (62.5, 125, 250, 500 pmol/0.5 µl) or vehicle (aCSF) was injected into the rLHa of DIO and DR rats and food intake was measured 1 and 2 h post injection. Injections were given between 1200 and 1230 h. Body weight was measured on the first and last treatment day and was used to calculate mean body weight for studies 4 and 5.

Study 5. Effect of orexin A on SPA in 6 mo old DIO and DR rats: Orexin A (62.5, 125, 250, 500 pmol/0.5 µl) or vehicle (aCSF) was injected into rLHa-cannulated DIO and DR rats from study 4 (DIO, n = 8; DR, n = 7). Following acclimation to SPA chambers continuous SPA post injection was measured as described above in study 3. Injections were given between 1100 and 1330 h.

Study 6. Preproorexin, OX1R, and OX2R mRNA in whole hypothalamus and rLHa: Separate groups of 1.5 month old DIO and DR rats were fed a micro- and macronutrient specified and adequate low fat diet (Research Diets D12489B, New Brunswick, NJ) ad libitum for 1-2 weeks (whole hypothalamus: n = 10/group, young rLHa: n = 9 (DIO) and 10 (DR). Food intake was measured daily and body weight was measured 3 times each week. At 2 months of age, animals were sacrificed, the rLHa or whole hypothalamus was dissected out, immediately frozen in liquid nitrogen, and stored at -80 °C until analysis. In a separate group of 8 month old chow-fed DIO and DR rats OX1R and OX2R mRNA was measured in the rLHa (DIO, n = 10; DR, n = 4). To avoid potential effects of recent food intake on gene expression, food was removed from the cages of all animals between 0700-0800 h, and animals were sacrificed

between 1100-1200 h. Relative preproorexin, OX1R, OX2R, and Rpl32 gene expression was measured by one-step real time RT-PCR.

Statistical analyses: Data were analyzed using Statview 5.0 (Cary, North Carolina) and SAS version 9.1 (Cary, North Carolina) and are expressed as mean ± SEM. An alpha level of .05 was used for all statistical tests. Only statistically significant results were reported in full, except where non-significant findings are highlighted to contrast with positive findings.

Study 1: To determine if the effect of age (1-2, 4, 7 months) on SPA was different across phenotypes (DIO, DR, and Sprague-Dawley), a hierarchical (mixed multilevel) linear model (51) was fitted using data obtained from the same rats at different ages. The model estimated the effect of age within each animal and assessed the effect of phenotype on response (time spent moving, time ambulatory, time vertical) to age, while simultaneously controlling for body weight as a covariate. Separate mixed-linear models were run for time spent moving, time ambulatory, and time vertical in DR vs. DIO rats. An identical set of models was used to compare DR vs. Sprague-Dawley and DIO vs. Sprague-Dawley rats. When significant main effects were observed the data were separated by age and analyzed by one-factor ANOVA followed by Fisher's least significant difference t-tests to compare individual treatment means for cumulative time spent moving in the light, dark, and 24 h measurement period where phenotype (DIO, DR, or Sprague-Dawley) was the independent variable and cumulative SPA (time spent moving, time ambulatory, time vertical) in the light, dark, and 24 h period were the dependent variables. Body weight on the day SPA was measured and cumulative caloric intake during the acclimation and test periods at each age were analyzed separately by one-factor ANOVA followed by Fisher's least significant difference t-tests to compare individual treatment means where phenotype (DIO, DR, or Sprague-Dawley) was the independent variable and body weight and caloric intake were

the dependent variables. Caloric intake data are reported as mean 24 h caloric intake per 100g body weight.

Studies 2, 3, 4, and 5: To determine if the effect of orexin A on SPA and feeding response was different across phenotypes (DIO, DR, and Sprague-Dawley), a hierarchical linear model (51) was fitted using data obtained from the same rats after multiple doses of orexin A. The model estimated the effect of orexin A within each animal and assessed of the effect of phenotype on response to orexin A, while simultaneously controlling for body weight and response to the vehicle injection as covariates. Separate mixed-linear models were run for 0-1, 1-2h and 0-2 h feeding and for 0-1, 1-2h and 0-2 h time spent moving in young DR vs. DIO rats under different repeated doses of orexin A. An identical set of models was used to compare DR vs. DIO at 6 months of age. Additional models were constructed to compare young DR vs. Sprague-Dawley and young DIO vs. Sprague-Dawley rats. The feeding data for studies 2 and 4 were also corrected for body weight by dividing food intake by 100g body weight, and this value (food intake/100g body weight) was analyzed as described above, but without using body weight as a covariate. These data are shown in figures 2 and 4 and the uncorrected feeding data are shown in table 4.

When significant phenotype main effects were observed, data were then separated by phenotype and analyzed with a mixed-linear model to estimate orexin A dose effects within each phenotype. When there was a significant main effect of dose, the data were then separated by dose and analyzed by one-factor ANOVA followed by Fisher's least significant difference t-tests to compare individual treatment means at each dose. The independent variable was phenotype (DIO, DR, or Sprague-Dawley) and the dependent variables included: corrected and non-corrected food intake (0-1 h, 1-2h and 0-2 h) and time spent moving (0-1 h, 1-2h and 0-2 h).

Since handling involved in the injection procedure results in increased SPA for up to 20 min post-injection independent of treatment, the first 20 min post-injection were not included in the data analysis for studies 3 and 5. Thus, the data analysis was performed on time spent moving measured in the 20 - 140 min post-injection interval. Results for the 0-1 h and 1-2 h post-injection intervals for studies 2, 3, 4, and 5 are shown in figures 2, 3, 4, and 5, respectively. Results from the cumulative 0-2 h post injection interval are reported but not shown.

Study 6: The $2^{-\Delta\Delta CT}$ method was used to calculate relative gene expression (35). Gene expression, body weight and caloric intake were analyzed by separate one-factor ANOVAs for each dependent variable with phenotype (DIO or DR) as the independent variable and body weight on the day of sacrifice, cumulative caloric intake, and the ratio of the relative preproorexin/Rpl32 mRNA, OX1R/Rpl32 mRNA, OX2R/Rpl32 mRNA as dependent variables. Caloric intake data are reported as mean 24 h caloric intake per 100g body weight.

RESULTS

Study 1. Basal SPA in DIO, DR, and Sprague-Dawley rats at 1-2, 4, and 7 months of age: Body weight was significantly different between phenotypes at all ages (1-2 mo: $F_{2,27}$ = 4.2, p = .0255; 4 mo: $F_{2,19}$ = 9.3, p = .0015; 7 mo: $F_{2,18}$ = 10.6, p = .0009, table 2) and caloric intake was significantly different between phenotypes at 1-2 months of age (1-2 mo: $F_{2,27}$ = 4.9, p = .0151, table 2). The mixed linear model indicated that DR rats spent significantly more time moving than DIO rats in the dark and 24 h period ($F_{1,18}$ = 11.7, p = .0031 and $F_{1,18}$ = 12.0, p = .0027, respectively, fig. 1) and Sprague-Dawley rats in all time periods (light: $F_{1,18}$ = 8.7, p = .0084; dark: $F_{1,18}$ = 12.1, p = .0026; 24 h: $F_{1,18}$ = 22.0, p = .0002, fig. 1. Time spent moving

was not significantly different between Sprague-Dawley and DIO rats in all time periods but there was a main effect of age in the dark ($F_{1,31} = 23.0$, $p < .0001$) and 24 h period ($F_{1,31} = 9.3$, $p = .0047$).

When time spent moving between phenotypes was compared at each age there was a main effect of phenotype in 1-2 and 4 mo old rats in all time periods (1-2 mo, light: $F_{2,27} = 5.7$, $p = .0085$; dark: $F_{2,27} = 9.1$, $p = .0010$; 24 h: $F_{2,27} = 10.9$, $p = .0003$; 4 mo, light: $F_{2,19} = 4.3$, $p = .0296$; dark: $F_{2,19} = 11.9$, $p = .0004$; 24 h: $F_{2,19} = 17.5$, $p < .0001$, fig 1). DR rats spent significantly more time moving than both DIO and Sprague-Dawley rats in all time periods (dark: $p = .0008$ and $p = .0014$, 24 h: $p = .0013$ and $p = .0002$; 4 mo, light: $p = .0206$ and $p = .0134$, dark: $p = .0005$ and $p = .0002$, 24 h: $p < .0001$ and $p < .0001$) with the exception of 1-2 mo DIO and DR rats in the light (1-2 mo, light: DR vs. Sprague-Dawley $p = .0032$). Time spent moving between 1-2 and 4 mo old DIO and Sprague-Dawley rats was not significantly different at all time periods with one exception (1-2 mo, light: $p = .0204$). At 7 mo of age time spent moving was significantly different between phenotypes over 24 h ($F_{2,18} = 3.5$, $p = .0500$, fig. 1). DR rats spent significantly more time moving than DIO and Sprague-Dawley rats ($p = .0333$ and $p = .0204$) and there was no significant difference between DIO and Sprague-Dawley rats at this time point. SPA components (time spent ambulatory and vertical) were separated to determine if each component contributed equally to time spent moving (table 3). Briefly, among 1-2 and 4 mo old rats time vertical contributed more than time ambulatory to time spent moving and in 7 month old rats time ambulatory contributed most to time spent moving (table 3). Finally, like time spent moving, in general DR rats spent more time ambulatory and vertical than both DIO and Sprague-Dawley rats and the individual components were similar between DIO and

Sprague-Dawley rats. In summary, DR rats spent significantly more time moving than DIO and Sprague-Dawley rats at each age.

Study 2. Orexin A-induced feeding in young DIO, DR, and Sprague-Dawley rats: Mean body weight for studies 2 and 3 was significantly different between phenotypes ($F_{2,27} = 20.4$, p < .0001, table 4). There was a main effect of phenotype on feeding (DIO vs. DR, 0-1h: $F_{1,16} = 5.0$, p = .0404 and 0-2h: $F_{1,16} = 6.5$, p = .0213; Sprague-Dawley vs. DR, 0-1h: $F_{1,17} = 5.0$, p = . 0387 and 0-2h: $F_{1,17} = 6.6$, p = .0196 fig. 2 and table 4). There was a main effect of dose on feeding (DIO vs. DR, 0-1h: $F_{1,58} = 5.0$, p = . 0292 and 0-2h: $F_{1,58} = 5.7$, p = .0198; Sprague-Dawley vs. DIO, 0-1 h: $F_{1,55} = 9.6$, p = . 0031; 0-2h: $F_{1,55} = 6.9$, p = . 0111; Sprague-Dawley vs. DR, 0-1h: $F_{1,61} = 11.3$, p = .0013 and 0-2h: $F_{1,61} = 12.3$, p = .0008, fig. 2 and table 4).

When the data were separated by dose orexin A-induced feeding was significantly different between phenotypes in response to the vehicle (0-1h: $F_{2,27} = 3.7$, p = .0374) and 62.5 pmol orexin A (0-1h: $F_{2,27} = 3.7$, p = .0372 and 0-2h: $F_{2,27} = 3.8$, p =.0332, table 4). In response to vehicle and 62.5 pmol orexin A, feeding was significantly different between DR and Sprague-Dawley rats (vehicle, 0-1h: p = .0154 and 62.5 pmol, 0-2h: p = .0103). DR rats ate significantly more than both DIO and Sprague-Dawley rats in response to 62.5 pmol orexin A 1 h post injection (p = .0434 and p = .0185, respectively) .

When the data were separated by phenotype to compare orexin A-induced feeding between doses of orexin A within each phenotype, there was a main effect of dose for all phenotypes (DIO: $F_{1,35} = 5.6$, p = .0239; DR: $F_{1,35} = 5.5$, p = .0231; Sprague-Dawley: $F_{1,35} = 11.2$, p = .0018, table 4). Two h post injection orexin A significantly stimulated feeding in DR and SD rats but failed to significantly stimulate feeding in DIO rats (DIO: $F_{1,35} = 3.2$, p = .0815;

DR: $F_{1,35} = 8.7$, p = .0051; and Sprague-Dawley: $F_{1,35} = 5.2$, p = .0280). When the data were corrected for body weight the statistical results remained generally unchanged (fig. 2). Hence, orexin A significantly stimulated feeding in both young DIO, DR, and Sprague-Dawley rats.

Study 3. Orexin A-induced SPA in young DIO, DR, and Sprague-Dawley rats: There was a main effect of dose on time spent moving (DR vs. DIO: 0-1h: $F_{1,58} = 7.6$, p = .0080, Sprague-Dawley vs. DIO: 0-1h: $F_{1,55} = 21.2$, p < .0001 and 0-2h: $F_{1,55} = 11.1$, p = .0015; Sprague-Dawley vs DR: 0-1h: $F_{1,61} = 19.2$, p < .0001 and 0-2h: $F_{1,61} = 13.2$, p = .0006, fig. 3). During the 1-2h interval there was a main effect of phenotype between DR and Sprague-Dawley rats ($F_{1,17} = 7.6$, p = .0134, fig. 3) and when the data were separated by phenotype the analysis indicated that orexin A stimulated time spent moving in Sprague-Dawley rats ($F_{1,39} = 7.8$, p = .0008). Two hour post injection there was a main effect of phenotype (Sprague-Dawley vs. DIO: $F_{1,15} = 14.2$, p = .0019 and Sprague-Dawley vs. DR: $F_{1,17} = 5.3$, p = .0347, fig. 3).

When the data were separated by dose orexin A-induced time spent moving was significantly different between the phenotypes in response to the vehicle and all doses of orexin A 1 and 2 h post injection (vehicle: $F_{2,27} = 8.0$, p = .0019 and $F_{2,27} = 10.1$, p =.0005; 31.25 pmol: $F_{2,27} = 5.0$, p = .0148 and $F_{2,27} = 9.0$, p =.0010; 62.5 pmol: $F_{2,27} = 7.2$, p = .0031 and $F_{2,27} = 7.9$, p =.0020; 125 pmol: $F_{2,27} = 5.3$, p = .0117 and $F_{2,27} = 9.1$, p =.0010; 250 pmol: $F_{2,27} = 6.4$, p = .0053 and $F_{2,27} = 5.6$, p =.0096). Multiple comparisons indicated that DR rats spent significantly more time moving than both DIO and Sprague-Dawley rats in response to vehicle (0-1h: p = .0436 and p = .0005 and 0-2h: p = .0072 and p = .0002, respectively) 31.25 pmol orexin A (0-2h: p = .0311 and p = .0002, respectively) and the two highest doses of orexin A (125 pmol, 0-1h: p = .0198 and p = .0056 and 0-2h: p = .0035 and p = .0005 respectively; 250 pmol, 0-1h: p = .0104

and p = .0027 and 0-2h: p = .0095 and p = .0072, respectively). DR rats spent significantly more

time moving than Sprague-Dawley rats in response to 31.25 pmol orexin A (0-1h: p = .0041) and

62.5 pmol orexin A (0-1h: p = .0008 and 0-2h: p = .0005). However, time spent moving between

DIO vs. Sprague-Dawley rats was not significantly different in response to vehicle and all doses

of orexin A 1 and 2 h post injection (p > .05). When the data were separated by phenotype, the

analysis indicated that orexin A significantly stimulated time spent moving in DR ($F_{1,43}$ = 6.0, p

= .0187) and Sprague-Dawley rats ($F_{1,39}$ = 42.36, p < .0001) but failed to stimulate time spent

moving in DIO rats 2 h post injection (p > .05). In summary, young DR rats spent significantly

more time moving in response to orexin A than DIO rats.

Study 4. Orexin A-induced feeding in 6 mo old DIO and DR rats: DR rats weighed significantly

less than DIO rats ($F_{1,10}$ = 19.9, p = .0012, table 2). There was no main effect of phenotype or

dose, or interaction between phenotype and dose on 0-1 h, 1-2 h, or 0-2 h food intake (p > .05,

table 4). When food intake was corrected for body weight there was a main effect of phenotype

(0-1h: $F_{1,14}$ = 5.4, p = .0361 and 0-2h: $F_{1,14}$ = 4.8, p = .0465, respectively, fig. 4). However,

when the data were separated by phenotype there was no effect of dose on orexin A-induced

feeding in DIO or DR rats (p > .05 for all comparisons). Hence, the effect of orexin A on

feeding in 6 mo old DIO and DR rats was similar.

Study 5. Orexin A-induced SPA in 6 mo old DIO and DR rats: There was a main effect of dose

and phenotype on time spent moving (DIO vs. DR, dose, 0-1h: $F_{1,43}$ = 9.4, p = .0037, 1-2h: $F_{1,43}$

= 5.0, p = .0302, and 0-2h: $F_{1,43}$ = 9.1, p = .0042; phenotype, 0-1h: $F_{1,11}$ = 7.8, p = .0175, fig.

5). When the data were separated by dose DR rats spent significantly more time moving than

DIO rats in response to 62.5 pmol orexin A and the two highest doses of orexin A (0-1h, 62.5 pmol: $F_{1,13}$ = 4.0, p = .0118; 250 pmol: $F_{1,13}$ = 11.7, p = .0045; 500 pmol: $F_{1,13}$ = 10.6, p = .0062 and 0-2h: 250 pmol: $F_{1,13}$ = 7.7, p =.0156, 500 pmol: $F_{1,13}$ = 7.3, p = .0182). During the 1-2h interval DR rats spent more time moving than DIO rats in response to the highest dose of orexin A, however, the difference failed to reach statistical significance ($F_{1,13}$ = 3.8, p = .0717). When the data were separated by phenotype, the analysis indicated that orexin A stimulated time spent moving in both DIO and DR rats 1 h post injection (DIO, $F_{1,27}$ = 8.4, p = .0073 and DR, $F_{1,31}$ = 21.8, p < .0001). However, multiple comparisons indicated that when compared to vehicle, only the highest dose of orexin A stimulated time spent moving in DIO (p = .0007) whereas all doses of orexin A stimulated time spent moving in DR rats 1 h post injection (62.5 pmol: p < .0001, 125 pmol: p = .0011, 250 pmol: p = .0007 and 500 pmol: p < .0001). Therefore, time spent moving after orexin A injection was significantly greater in 6 mo old DR rats as compared to age-matched DIO rats.

Study 6. Hypothalamic and rLHa-preproorexin, Ox1 and Ox2R mRNA: The preproorexin, OX1R, OX2R, and Rpl32 primer sets yielded amplification products that were detected as single products at 143, 257, 244, and 122 base pairs, respectively (fig. 6). In all studies DR rats weighed significantly less than DIO rats (table 2). Hypothalamic and rLHa preproorexin mRNA were not significantly different between young DIO and DR rats (p > .05, fig. 7). Although hypothalamic OX1R and OX2R mRNA were also not significantly different between young DIO and DR rats (p > .05), young DR rats had significantly greater rLHA OX1R and OX2R mRNA than young DIO rats (OX1R: $F_{1,17}$ = 20.5, p = .0003 and OX2R: $F_{1,17}$ = 21.3, p = .0003, fig. 7). Eight mo old DR rats had significantly greater OX2R mRNA than 8 mo old DIO rats ($F_{1,10}$ =

41.7, p < .0001, fig. 7). There was no phenotypic difference in rLHa OX1R mRNA in 8 mo old DIO rats. Hence, both young and 8 m old DR rats had elevated OX2R mRNA in the rLHa compared to age-matched DIO rats.

DISCUSSION

We measured basal SPA, orexin A-induced feeding and SPA, and hypothalamic and rLHa-preproorexin, OX1R, and OX2R mRNA in selectively-bred DIO and DR rats to determine whether differences in SPA, orexin A responsiveness and OXR gene expression differed between phenotypes. We also studied Sprague-Dawley rats as a standard with which to compare both phenotypes. The current data demonstrate for the first time that in the basal state, young selectively-bred DR rats spend more time moving than DIO and Sprague-Dawley rats (fig. 1). These data also show that time spent moving in response to rLHa orexin A (fig. 3 and 5) is greater in DR rats. Finally, levels of rLHa-OX1R and OX2R mRNA (fig. 7) are greater in DR rats. These data suggest that the robust SPA response to orexin A in DR rats may be due to enhanced orexin signaling through rLHa OX1R and OX2R. In addition, these data demonstrate that orexin A significantly enhances time spent moving in both DR and Sprague-Dawley rats, albeit at different absolute levels (fig. 3), whereas time spent moving in the basal state (fig. 1) is more similar between DIO and Sprague-Dawley rats. Together these data indicate that orexin A-induced effects on SPA and feeding are phenotype dependent and differences in orexin A signaling through rLHa OX1R and OX2R may mediate phenotypic traits of selectively-bred DIO and DR rats.

The effect of rLHa-orexin A on feeding observed in the present study is consistent with previous studies in out-bred Sprague-Dawley rats (8, 48, 52, 56, 57). Further, others and we

have previously demonstrated the stimulatory effect of orexin A on SPA (19, 22, 56) and energy expenditure (37, 44, 60) in out-bred Sprague-Dawley and selectively-bred DIO and DR rats (41). The greater potency of orexin A to stimulate SPA (vs. food intake) is also consistent with previous studies (22, 56). Although the effect of orexin A on feeding and SPA during the 1-2 h time interval in the present study was largely insignificant, figs. 4 and 5 clearly show that orexin A induced SPA in DR rats continued into the 2nd h post-injection, whereas feeding induced by orexin A was similar between phenotypes in this time interval. Thus the significant effect of orexin A on cumulative 0-2 h SPA is due to a persistent elevation in SPA throughout the 2 h measurement interval whereas the effect of orexin A on cumulative 2 h food intake is due to responses in the 0-1h time interval. Together these data indicate that the time course of orexin A-mediated feeding and SPA differ, suggesting that these two responses, SPA and feeding, may be mediated by different mechanisms. The divergent time-courses also support the idea that orexin A-induced feeding is not merely a consequence of enhanced arousal or SPA, but rather, a motivated component of orexin stimulation (55).

Differences in caloric intake between phenotypes were not always observed but DIO rats weighed more than DR rats consistently across studies. Body weight alone, however, does not itself explain the results, as the data were corrected for body weight using covariate analysis. Discrepancies in reported caloric intake between our results and others are likely due to differences in the number of generations of selective breeding, as the phenotype becomes apparent earlier with each additional generation of selectively-bred rats (29, 47). It has been suggested that selective breeding has produced two distinct groups of rats that readily express phenotypic differences prior to exposure to a high energy diet, which is required for expression of phenotypic traits in out-bred DIO and DR rats (29, 47). The current data also demonstrate that

selectively-bred DR rats spend more time moving across age-groups, suggesting that differences in SPA between DIO and DR rats across the lifespan likely functions to maintain phenotypic differences in body weight. Our results contrast with previous reports showing no differences in locomotor activity between chow-fed out-bred retrospectively identified DIO and DR rats (27) and selectively-bred DR rats (41). Inconsistencies are likely due to differences in breeding scheme (out-bred vs. selectively-bred), type of SPA measurement chambers, and duration of acclimation and measurement periods between studies. Exploratory behavior increases when rats are placed in a novel environment, and thus the use and length of acclimation periods affect results. Further, exploratory behavior differs between selectively-bred DIO and DR rats (1, 27, 31, 41).

A group of Sprague-Dawley rats was included to allow comparison to our studies of DIO and DR rats, and to determine whether one phenotype (DIO or DR) was more similar to Sprague-Dawley rats. Given that DIO and DR rats were selectively-bred from Sprague-Dawley rats, as a whole, random groups of Sprague-Dawley rats would be expected to contain both obesity resistant and prone rats. Thus we anticipated that mean endpoint values in Sprague-Dawley rats would lie between those observed in DIO and DR rats. Contrary to our predictions, DR rats appear to be different from both Sprague-Dawley and DIO rats. In the basal state, 24 h time spent moving (fig. 1), was most similar between DIO and Sprague-Dawley rats. In terms of orexin A responsivity, Sprague-Dawley rats showed a significant response to orexin A whereas in most cases DIO rats did not (figs. 2 and 3). However, the feeding and SPA response to orexin A in Sprague-Dawley rats was at a much lower level than that of DR rats. Overall, compared to DIO and Sprague-Dawley rats, DR rats have greater basal SPA and more robust feeding and SPA responses to orexin A injections.

The mechanism(s) underlying enhanced responsiveness to orexin A in DR rats is unknown. One possibility is increased receptor numbers, and to test this we measured brain OX1R and OX2R mRNA in young DR and (pre-obese) DIO rats, and 8 mo old DIO and DR rats. To quantify OX1R and OX2R mRNA levels that would be expected to bind to rLHa-injected orexin A, we assayed a discrete area of the LH (rLHa), which corresponds to our injection site. As shown in figure 7, rLHa-OX1R and OX2R gene expression was significantly higher in young DR rats as compared to DIO rats. Consistent with the young rats, OX2R mRNA was also elevated in 8 mo old DR rats, suggesting that the gene expression differences may be maintained through adulthood. OX1R mRNA levels were not significantly increased in the 8 mo animals. Although these data need to be confirmed at the protein level, the large and fairly consistent increase in rLHa OXR in DR rats (esp. OX2R) is suggestive of relevant differences in receptor levels. The finding that expression of both OXRs was increased in DR rats in parallel with increased basal and OXA-induced SPA and feeding may indicate that both receptors mediate these behaviors. Yet in the older DR rats, only enhanced expression of OX2R remained, as did the SPA response to OXA, suggesting that OX2R may mediate SPA. It is yet possible that other brain sites contain phenotype dependant differences in OXR mRNA, an idea that requires further testing. Whole hypothalamus levels of OXR did not differ between groups, suggesting that receptor levels do not differ throughout the hypothalamus, and that rLHa may be an important hypothalamic site for orexin A mediated effects. These gene expression data together with the behavioral measures suggest that elevated OXR in rLHa may mediate enhanced basal SPA and responsivity to rLHa-injected orexin A in DR rats.

We also measured preproorexin mRNA to determine whether orexin "tone" is different between phenotypes. For this assay whole hypothalamus was taken to capture the entire orexin

neuron population as orexin neurons are located in the dorso-medial, posterior, perifornical areas of the hypothalamus and the caudal aspect of the lateral hypothalamus (7, 45, 48). Hypothalamic preproorexin mRNA was similar between the phenotypes, suggesting that at the whole hypothalamus level, orexin tone does not differ between groups. It is possible that distinct subsets of orexin neurons with unique functional connectivity may contain differential levels of preproorexin mRNA, but this was not determined in the current study.

To determine whether enhanced response to orexin A in DR rats may be related to differences in body weight, we analyzed the data with and without body weight as a covariate and found that the results remained largely unchanged, suggesting that body weight differences do not account for the phenotypic differences in orexin A responsivity. Orexin A significantly stimulated time spent moving in young DR and Sprague-Dawley rats but failed to do so in young DIO rats 2 h post injection even when body weight was included as a covariate. In a similar manner, orexin A stimulated feeding in young DR and Sprague-Dawley rats but failed to stimulate feeding in DIO rats 2 h post injection. Although young DIO rats weight more than young DR rats, this difference is likely due to lean body mass (47). In older rats, adiposity increases and contributes more to the phenotypic difference in body weight (29). Whether adiposity itself contributes to orexin A responsivity is unclear, but the data show that the phenotypic gap in responsivity is larger at 6 mo of age, suggesting that adiposity may influence phenotypic differences. However, using feeding response corrected for body weight in the analysis of the feeding data abolished any potential differences in feeding response to orexin A at this age. Thus, if adiposity differences affect responsivity to orexin A it appears to be important only for the feeding response to orexin A.

Finally, differences in responsivity to orexin A between phenotypes may be related to previously described differences between prospectively identified or selectively-bred DIO and DR rats. For instance, DIO and DR rats differ in monoaminergic function (13, 25, 26), sensitivity to glucose (32), leptin (28, 30), and/or insulin (5). However, since orexin A-induced effects are sensitive to metabolic status (3, 34) and the circadian cycle (10, 54), and are mediated downstream by monoamines (2, 9, 11, 16, 39), these differences could also be explained by variation in orexin signaling between DIO and DR rats. Dopaminergic neurons in the substantia nigra that innervate the striatum are a critical component of locomotor activity and dopamine receptor antagonists block orexin A-induced locomotor activity when administered in the ventral tegmental area (39). This supports a role for dopamine neurons in orexin A-induced locomotor activity, and may mediate the enhanced SPA observed in DR rats. Irrespective of the mechanism(s) underlying the observed orexin A-mediated effects on SPA and feeding, the results presented here together with the mouse (12) and human data (50) showing that lack of orexin increases body weight gain, indicate that orexin A-mediated differences in the central regulation of energy balance exist between DIO and DR rats and suggest that orexin activity may confer protection against excess weight gain.

In conclusion, the data presented here demonstrate that basal SPA is increased in DR rats, and DR rats have enhanced orexin A-induced SPA, OX1R and OX2R mRNA. Augmented sensitivity to the SPA-promoting effects of orexin A in DR rats may be attributable to alterations in orexin signaling mediated by OX1R and OX2R in the rLHa, and may perpetuate the lean phenotype. Studies in humans demonstrate that the propensity to remain lean or obese is dependent upon differences in time spent moving, which we have shown is modulated by orexin A in rodents. Therefore, previous studies and the data presented here suggest that enhanced SPA

and elevated OX1R and OX2R signaling contributes to the lean phenotype of DR rats, and supports the idea that orexin signaling is involved in mediating SPA and the energy expenditure associated with such. These findings may have important implications for human obesity.

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FIGURE LEGENDS:

Figure 1 (study 1): Cumulative 24 h time spent moving in obesity prone (DIO), obesity resistant (DR), and Sprague-Dawley (SD) rats at 1-2, 4, and 7 months of age in the light (open bars), dark (shaded bars) and cumulative 24 h period. Light: $+ p < .05$ and $\ddagger p < .005$, dark: $\# p < .05$ and $\#\# p < .005$, and 24 h: $* p < .05$ and $** p < .005$. Data represent mean $\pm$ SEM. N $= 10$/group (1-2mo), 9 (4 and 7 mo DIO), 5 (4 mo DR), 4 (7 mo DR), 8 (4 and 7 mo SD).

Figure 2 (study 2): Effect of orexin A (OxA) in rostral lateral hypothalamus (rLHa) on feeding in young obesity prone (DIO), obesity resistant, (DR), and Sprague-Dawley (SD) rats. $* p < .05$ as compared to DIO rats at each dose; $\# p < .05$ as compared SD rats at each dose. Data represent mean $\pm$ SEM. N $= 9$ (DIO), 11 (DR), and 10 (SD). Please note different y-axes.

Figure 3 (study 3): Effect of orexin A (OxA) in rostral lateral hypothalamus (rLHa) on time spent moving in young obesity prone (DIO), obesity resistant (DR), and Sprague-Dawley (SD) rats. $* p < .05$ and $** p < .005$ as compared to DIO rats at each dose; $\# p < .05$ and $\#\# p < .005$ as compared to DR rats at each dose. Data represent mean $\pm$ SEM. N $= 9$ (DIO), 11 (DR), and 10 (SD). Please note different y-axes.

Figure 4 (study 4): Effect of orexin A (OxA) in rostral lateral hypothalamus (rLHa) on feeding in 6 mo. obesity prone (DIO) and obesity resistant (DR) rats. Data represent mean ± SEM. N = 10 (DIO) and 7 (DR). Please note different y-axes.

Figure 5 (study 5): Effect of orexin A (OxA) in rostral lateral hypothalamus (rLHa) on time spent moving in 6 mo. obesity prone (DIO) and obesity resistant (DR) rats. *p < .05 and **p < .005 as compared to DIO rats at each dose. Data represent mean ± SEM. N = 8 (DIO) and 7 (DR). Please note different y-axes.

Figure 6 (study 6): (A) Gel indicating that preproorexin (OX), orexin 1 receptor (OX1R), orexin 2 receptor (OX2R) and ribosomal protein large 32 (Rpl) primer sets yielded PCR amplification products that were detected as single products at 143, 257, 244 and 122 base pairs, respectively.

Figure 7 (study 6): Relative hypothalamic and rLHa-preproorexin/ribosomal protein large 32 (hypo OXA and rLHa OXA), orexin 1 receptor/ribosomal protein large 32 (hypo OX1R and rLHa OX1R), orexin 2 receptor/ribosomal protein large 32 (hypo OX2R and rLHa OX2R) gene expression in 2 and 8-month old obesity prone (DIO) and obesity resistant (DR). * p < .005 as compared to DIO rats; + p < .05 as compared to 8 mo old DIO rats. Data represent mean ± SEM. N = 4-10 (DIO and DR).
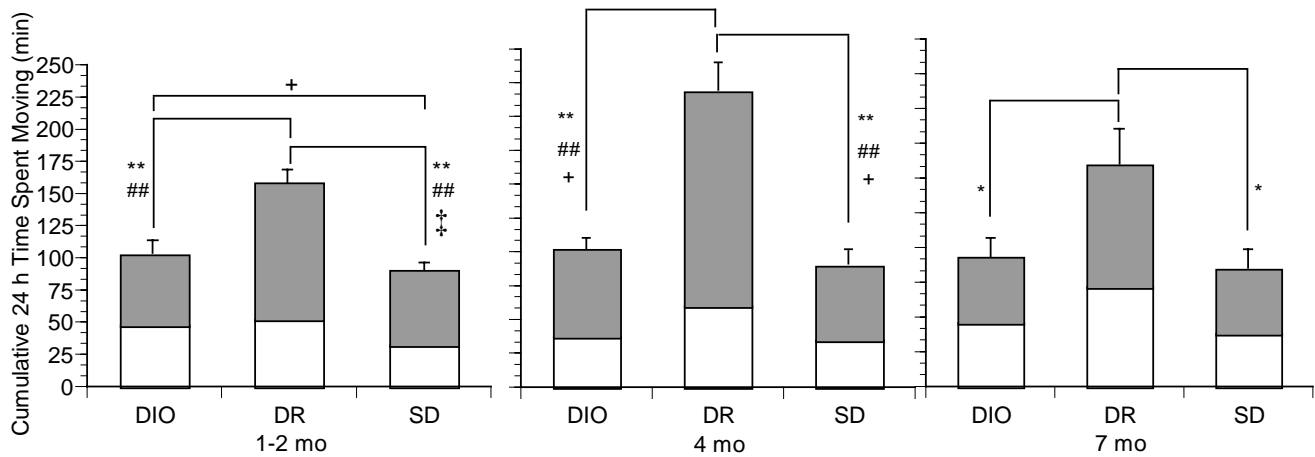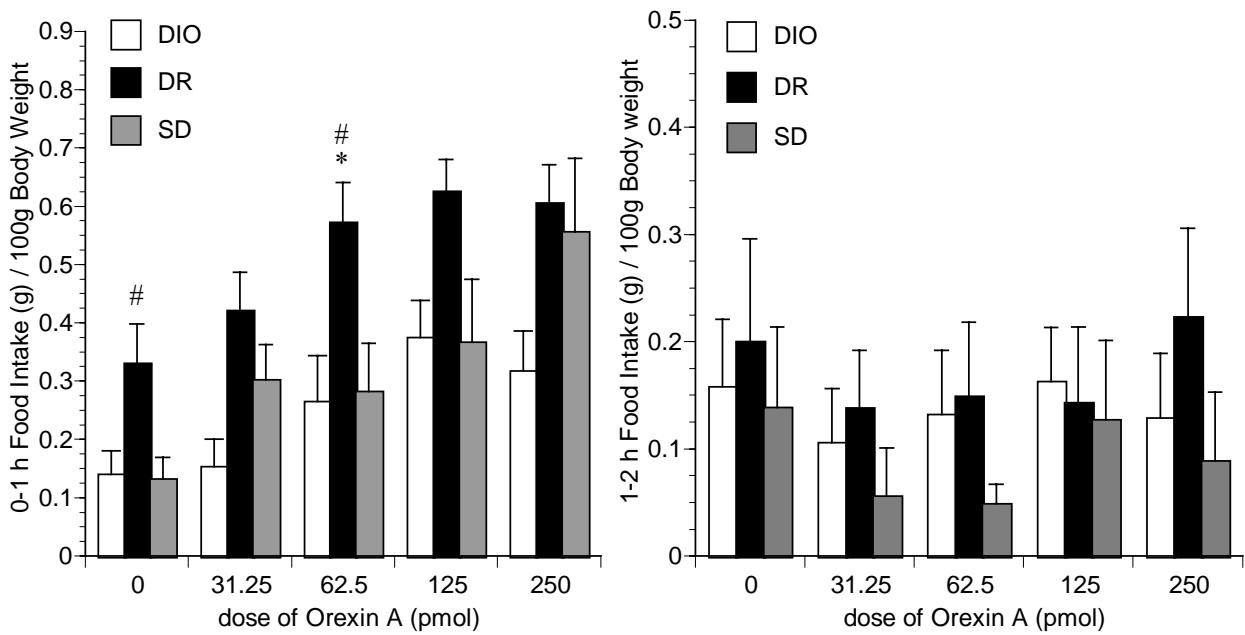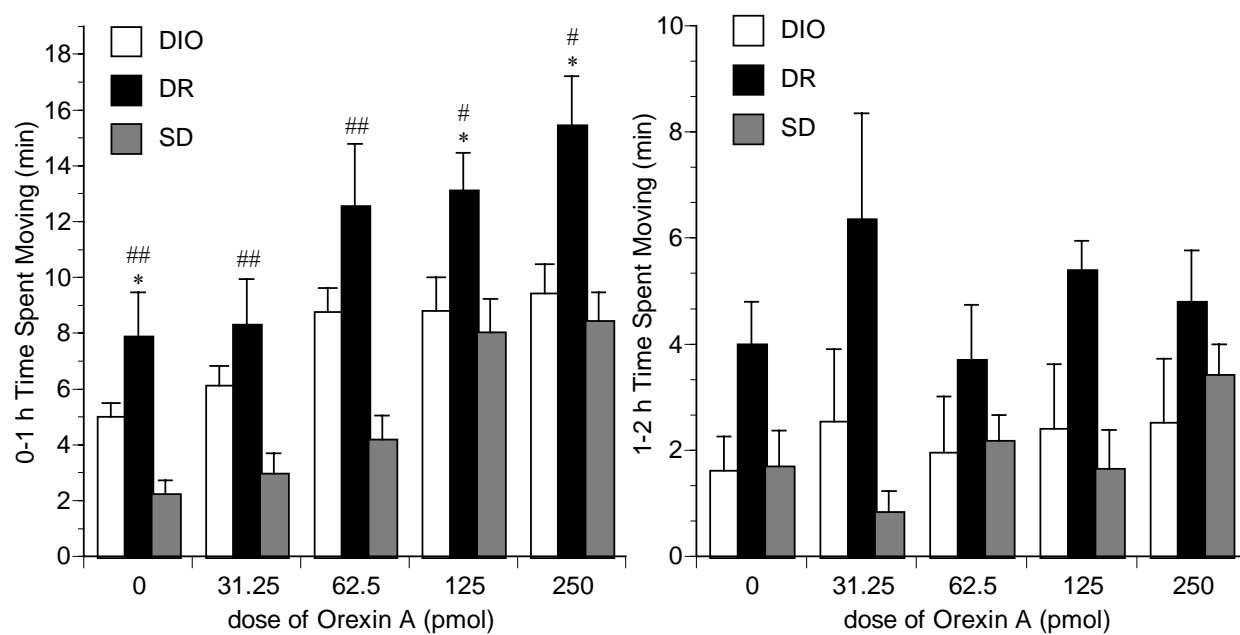
Figure 1.

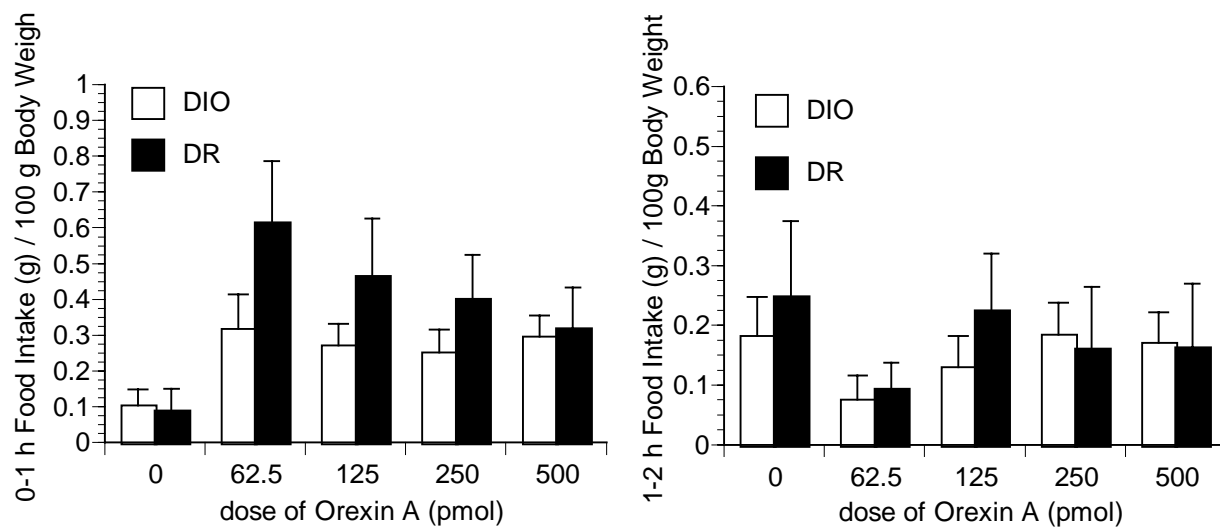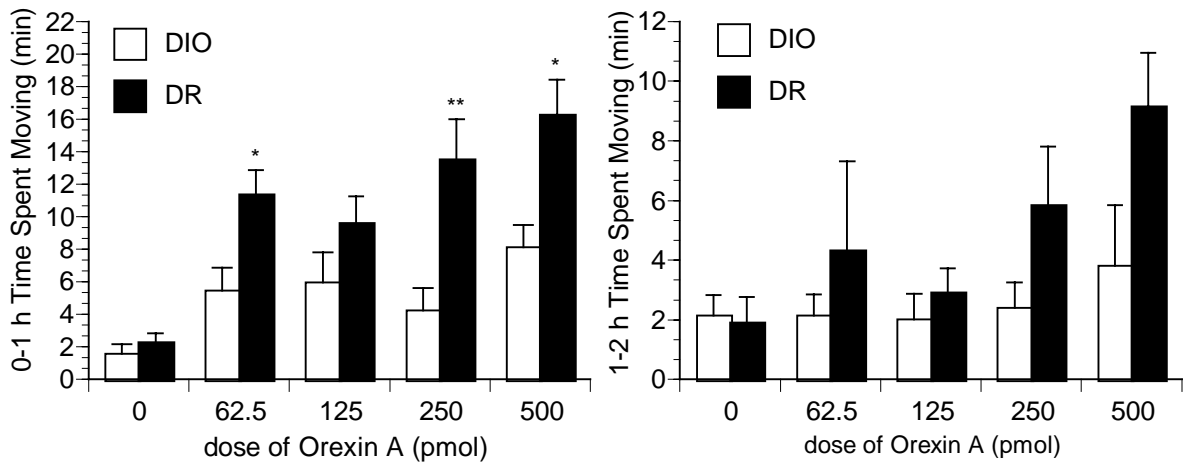Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.
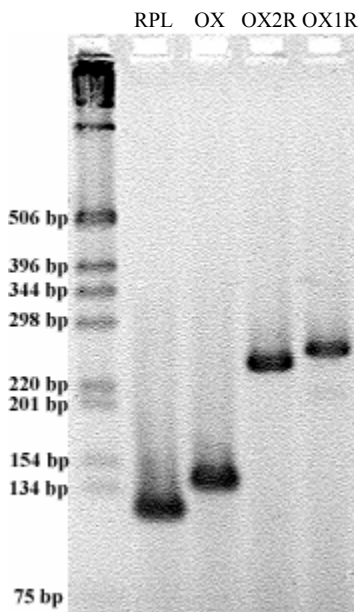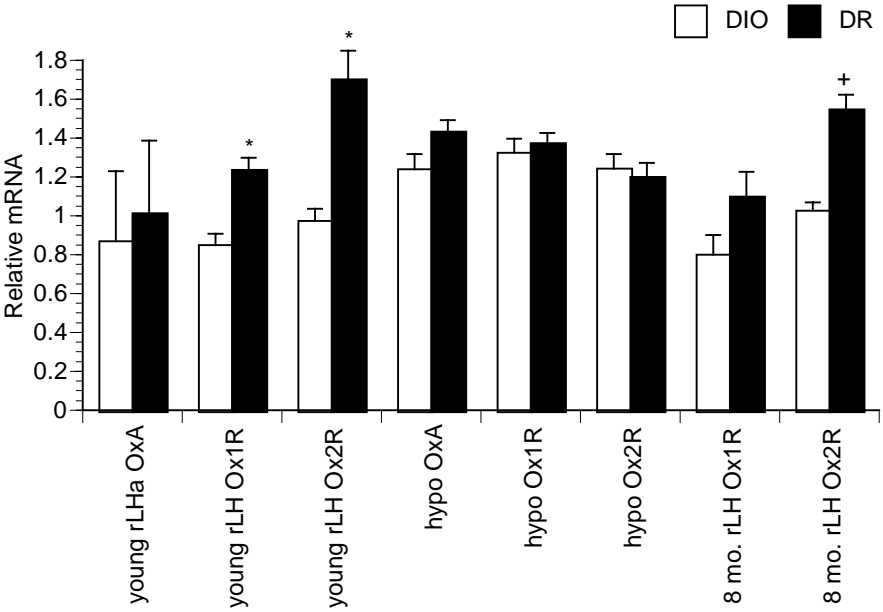
Figure 7.

Table 1

Primer sequences for preproorexin, orexin 1 receptor (OX1R), orexin 2 receptor (OX2R) and ribosomal protein L32 (RPl32)

Preproorexin (GenBank Accession, NM_013179)

   Forward 5' - CATCCTCACTCTGGGAAA G -3'

   Reverse 5' - AGGGATATGGCTCTAGCT C -3'

OX1R (GenBank Accession, NM_013064)

   Forward 5' - AGAGAGCAGAGAGCGTTGTAAACC -3'

   Reverse 5' - TTC ACA GGG ACA CAT TGG TGC -3'

OX2R (GenBank Accession, AF041246)

   Forward 5'-TGTTCAAGAGCACAGCCAAACG-3'

   Reverse 5'-GCCAATACCATAAGACACAGGGG-3'

Rpl32 (GenBank Accession, NM_013226)

   Forward 5'-CGGAAGTTTCTGGTCCACAATGTC-3'

   Reverse 5'-GCTCTTTCTACGATGGCTTTTCGG-3'

Table 2

Body weight and caloric intake for studies 1 to 6

	Body Weight (g) Mean ± SEM			Mean 24 h Caloric Intake/100 g Body weight (kcal/g) Mean ± SEM		
	DIO	DR	SD	DIO	DR	SD
Study 1						
1-2 mo[a]	236.6 ± 25.2	187.2 ± 13.1[+]	272.8 ± 22.5	19.6 ± 4.1	22.0 ± 2.6*[‡]	14.2 ± 2.7
4 mo[b]	512.6 ± 11.9	415.7 ± 6.5[§‡]	496.8 ± 19.7	14.2 ± 1.1	15.1 ±0.7	12.8 ± 1.3
7 mo[c]	582.2 ± 13.7	452.5 ±5.8[§‡]	580.5 ± 23.8	11.2 ± 0.8	14.1 ± 1.0	11.4 ± 0.6
Studies 2 and 3[d]	415.0 ± 9.8[‡]	331.8 ± 10.7[§]	343.5 ± 8.2	---	---	---
Studies 4[e] and 5[f]	591.7 ± 21.0	467.2 ± 18.4[§]	---	---	---	---
Study 6						
Young rLHa[g]	237.7 ± 8.2	208.7 ± 6.5*	---	35.9 ± 0.5	39.5 ± 0.7	---
Hypothalamus[h]	225.6 ± 5.4	186.3 ± 5.7[§]	---	43.4 ± 0.9	42.2 ± 0.3[§]	---
8mo rLHa[i]	595.5 ± 17.0	482.7 ± 10.2[§]	---	---	---	---

[a] n = 10/group, [b] n = 9 (DIO), 5 (DR), 8 (SD), [c] n = 9 (DIO), 4 (DR), 8 (SD), [d] n = 9 (DIO), 11 (DR), 10 (SD), [e] n = 10 (DIO) and 7 (DR), [f] n = 8 (DIO) and 7 (DR), [g] n = 9 (DIO), 10 (DR), 6 (SD), [h] n = 10/group, [i] n = 9 (DIO), 4 (DR). $* p < .05$ and $\S p < .005$ as compared to DIO rats. $+ p < .05$ and $\ddagger p < .005$ as compared to SD rats. Data represent mean ± SEM. ---: no data.

Table 3

Study 1: Basal time ambulatory and time vertical in the light, dark, and 24 h measurement period in DIO, DR, and SD rats at 1-2, 4, and 7 months of age.

	DIO	DR	SD
	time vertical / ambulatory	time vertical / ambulatory	time vertical / ambulatory
1-2 mo[a]			
Light *	$15.4 \pm 1.8\,^{\ddagger} / 32.0 \pm 3.8$	$22.4 \pm 2.2\,^{\S\ddagger} / 29.8 \pm 3.0$	$9.5 \pm 1.2 / 22.1 \pm 1.4$
Dark *#	$18.3 \pm 2.6 / 36.4 \pm 3.9$	$54.4 \pm 14.7\,^{\S\ddagger} / 51.3 \pm 6.1\,^{\S\ddagger}$	$19.8 \pm 3.3 / 37.9 \pm 2.2$
24 h *	$33.8 \pm 4.4 / 68.4 \pm 7.4$	$76.8 \pm 14.3\,^{\S\ddagger} / 81.2 \pm 7.9$	$29.3 \pm 4.3 / 60.4 \pm 3.0$
4 mo[b]			
Light *	$13.2 \pm 1.7 / 23.5 \pm 2.3$	$26.6 \pm 4.9\,^{\S\ddagger} / 32.9 \pm 5.0$	$12.1 \pm 4.2 / 22.2 \pm 2.8$
Dark *#	$22.0 \pm 2.5 / 42.2 \pm 1.5$	$54.4 \pm 9.1\,^{\S\ddagger} / 104.1 \pm 31.5\,^{\S\ddagger}$	$20.4 \pm 6.4 / 34.5 \pm 4.1$
24 h *#	$35.2 \pm 3.8 / 65.7 \pm 3.1$	$81.0 \pm 8.6\,^{\S\ddagger} / 137.0 \pm 28.7\,^{\S\ddagger}$	$32.5 \pm 10.5 / 56.7 \pm 6.3$
7 mo[c]			
Light #	$17.0 \pm 3.2 / 28.7 \pm 3.1$	$25.4 \pm 7.5 / 46.1 \pm 11.3\,^{\S}$	$17.1 \pm 5.0 / 20.8 \pm 2.8$
Dark #	$17.3 \pm 11.9 / 29.4 \pm 5.5$	$38.5 \pm 11.9 / 48.9 \pm 13.2$	$19.9 \pm 5.3 / 26.3 \pm 4.0$
24 h #	$34.4 \pm 5.3 / 58.1 \pm 7.9$	$63.9 \pm 17.3 / 95.0 \pm 22.5\,^{\S}$	$37.0 \pm 10.0 / 47.1 \pm 6.0$

[a] n = 10/group, [b] n = 9 (DIO), 5 (DR), 8 (SD), [c] n = 9 (DIO), 4 (DR), 8 (SD). * $p < .05$ for time vertical and # $p < .05$ for time ambulatory for ANOVA. § $p < .05$ as compared to DIO and ‡ $p < .05$ as compared to SD as determined by Fisher's PLSD test. Data represent mean $\pm$ SEM.

Table 4

Studies 2 and 4. Effect of orexin A on food intake in 2-3 mo and 6 mo old DIO, DR, and SD rats

	Young DIO	Young DR	Young SD	6 mo. DIO	6 mo. DR
0-1 h					
Vehicle*	0.6 ± 0.2	1.1 ± 0.3 §‡	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.3
31.25 pmol OxA*	0.7 ± .02	1.4 ± 0.3‡	1.0 ± 0.2	---	---
62.5 pmol OxA*	1.1 ± 0.3	1.9 ± 0.2 §‡	0.9 ± 0.3	1.8 ± 0.5	2.8 ± 0.8
125 pmol OxA*	1.5 ± 0.3	2.1 ± 0.2§‡	1.2 ± 0.4	1.5 ± 0.3	2.1 ± 0.7
250 pmol OxA	1.3 ± 0.3	2.0 ± 0.3	1.3 ± 0.4	1.4 ± 0.3	1.8 ± 0.6
500 pmol OxA	---	---	---	1.6 ± 0.3	1.4 ± 0.5
1-2 h					
Vehicle	0.6 ± 0.2	0.7 ± 0.3	0.4 ± 0.2	1.0 ± 0.4	1.1 ± 0.6
31.25 pmol OxA	0.4 ± 0.2	0.5 ± 0.2	0.2 ± 0.1	---	---
62.5 pmol OxA	0.6 ± 0.3	0.5 ± 0.2	0.1 ± 0.1	0.4 ± 0.2	0.4 ± 0.2
125 pmol OxA	0.7 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.7 ± 0.3	1.0 ± 0.5
250 pmol OxA	0.6 ± 0.2	0.8 ± 0.3	0.3 ± 0.2	2.0 ± 0.3	0.7 ± 0.4
500 pmol OxA	---	---	---	1.0 ± 0.3	0.7 ± 0.4

Young: n = 9 (DIO), 11 (DR), 10 (SD). 6 mo: n = 10 DIO and 7 DR. *p < .05 for ANOVA for young rats. §p < .05 as compared to DIO and ‡p < .05 as compared to SD for each age as determined by Fisher's PLSD test. Data represent mean ± SEM. ---: no data.