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OREXIN A MEDIATION OF TIME SPENT MOVING IN RATS: NEURAL MECHANISMS

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Abstract—The brain regulates energy balance and spontaneous physical activity, including both small- and large-motor activities. Neural mediators of spontaneous physical activity are currently undefined, although the amount of time spent in sedentary positions versus standing and ambulating may be important in the energetics of human obesity. Orexin A, a neuropeptide produced in caudal hypothalamic areas and projecting throughout the neuraxis, enhances arousal and spontaneous physical activity. To test the hypothesis that orexin A affects the amount of time spent moving, we injected orexin A (0–1000 pmol) into three orexin projection sites in male Sprague–Dawley rats: hypothalamic paraventricular nucleus, rostral lateral hypothalamic area and substantia nigra pars compacta, and measured spontaneous physical activity. Orexin A affects local GABA release and we co-injected orexin A with a GABA agonist, muscimol, in each brain site. Dopamine signaling is important to substantia nigra function and so we also co-injected a dopamine 1 receptor antagonist (SCH 23390) in the substantia nigra pars compacta. In all brain sites orexin A significantly increased time spent vertical and ambulating. Muscimol significantly and dose-dependently inhibited orexin A effects on time spent moving only when administered to the rostral lateral hypothalamic area. In the substantia nigra pars compacta, SCH 23390 completely blocked orexin A–induced ambulation. These data indicate that orexin A influences time spent moving, in three brain sites utilizing separate signaling mechanisms. That orexin A modulation of spontaneous physical activity occurs in brain areas with multiple roles indicates generalization across brain site, and may reflect a fundamental mechanism for enhancing activity levels. This potential for conferring physical activity stimulation may be useful for inducing shifts in time spent moving, which has important implications for obesity.

Key words: orexin, hypocretin, hypothalamic paraventricular nucleus, lateral hypothalamus, substantia nigra, locomotor activity.

The distribution of time devoted to various activities, including standing and ambulating, sitting, and lying, may be important in non-exercise activity thermogenesis (NEAT) and energy balance. Obese subjects spend approximately 164 more minutes per day sitting compared with non-obese, sedentary counterparts. Importantly, individual daily amount of time spent moving vs. sitting does not change with shifts in body weight, regardless of body weight phenotype (obese or lean) (Levine et al., 2005). This suggests that the amount of time spent moving may be biologically driven and important in body weight regulation and human obesity (Zurlo et al., 1992; Ravussin and Gautier, 2002; Ravussin, 2005). However, the mechanism by which this occurs is unknown.

The brain regulates physical activity and energy balance and many neurotransmitters modulate spontaneous physical activities, including small- and large-motor activities, which can profoundly influence the activity thermogenesis component of energy expenditure. However, neural mediators of spontaneous physical activity are unknown. Recent evidence from our laboratories suggests that orexin A, a neuropeptide produced exclusively in hypothalamic areas (de Lecea et al., 1998; Sakurai et al., 1998) and projecting throughout the neuraxis (Peyron et al., 1998) profoundly enhances arousal and small and large motor activities, activities that contribute substantially to energy expenditure. Orexin A is a 33 amino acid peptide with two disulfide bonds, which along with orexin B, a 28 amino acid linear peptide, is proteolytically cleaved from a common 130 amino acid precursor protein, prepro-orexin (de Lecea et al., 1998; Sakurai et al., 1998). Orexins are synthesized in the lateral (LHa), dorso-medial, posterior and perifornical areas of the hypothalamus, and orexin-containing fibers project to several areas implicated in arousal and activity (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998). The orexin receptors, OX1R and OX2R, are G-coupled protein receptors and substrate binding phosphorylates protein kinase which in turn increases intracellular calcium (Sakurai et al., 1998). The receptors bind to both orexin A and orexin B; however,

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Abbreviations: aCSF, artificial cerebrospinal fluid; NEAT, non-exercise activity thermogenesis; OX1R, orexin 1 receptor; OX2R, orexin 2 receptor; PVN, hypothalamic paraventricular nucleus; rLHa, rostral lateral hypothalamic area; substantia $nigra_{pc}$, substantia nigra pars compacta.

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orexin A has a 10-fold greater affinity for OXR1 compared with OXR2 (Sakurai et al., 1998).

We have previously demonstrated that orexin A injected into hypothalamic orexin projection sites (hypothalamic paraventricular nucleus, PVN, and rostral lateral hypothalamic area, rLHa) enhances spontaneous running wheel activity (Kotz et al., 2002), spontaneous physical activity, and NEAT (Kotz et al., 2002; Kiwaki et al., 2004; Novak et al., 2006; Teske et al., 2006). Whether orexin A modulates daily allocation of time devoted to ambulating versus time spent resting, has not been specifically tested. This could be an important mechanistic question for understanding the regulation of human NEAT and obesity.

Previous studies indicated that orexin A affects local GABA release (van den Pol et al., 1998); therefore, our secondary hypothesis was that orexin modulation of posture allocation is dependent upon GABA signaling. We also sought to determine whether orexin A in the substantia nigra pars compacta (substantia nigra$_{pc}$) is important to time spent moving, and since the substantia nigra relies on dopamine signaling, we co-injected a dopamine 1 receptor antagonist to determine whether blockade of dopamine 1 receptors would influence orexin A effects on time spent moving.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (3–5 mo, Harlan, Madison, WI, USA) weighing 325–450 g were housed individually in cages with a 12-h light/dark (since birth) photo-cycle (lights on at 07:00 h) in a room at 21–22 °C. Teklad Laboratory Chow (Teklad, Madison, WI, USA) and water were allowed *ad libitum*, except where noted. All efforts were made to minimize animal suffering and reduce the number of animals used in these studies, and all experiments received local Institutional Animal Care and Use Committee approval, and were carried out in accordance with the National Institutes of Health Guide for the Care and use of Laboratory Animals revised 1996.

Cannulation and verification of placement

Rats were anesthetized with Nembutal (40 mg/kg) and fitted with a 28 gauge stainless steel guide cannula placed just above the area of interest. Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson (1998) and are as follows: rLHA: −2.0 mm lateral, −2.1 mm posterior to bregma, 7.3 mm below the skull surface; PVN: −1.9 mm lateral, −0.5 mm from bregma, 7.3 mm below the skull surface; substantia nigra$_{pc}$: −2.4 mm lateral, −5.3 mm from bregma, −7.6. The injector extended 1 mm beyond the end of the guide cannula. For all cannulations, the incisor bar was set at 3.3 mm below the ear bars. At least 10 days elapsed following surgery before experimental trials. After the experiments brains were dissected out and stored in a 10% formaldehyde solution for placement verification by histology. A cannula was deemed incorrect 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. For all brain sites, there were two to four rats deemed to have incorrect cannula placement, and the data from these animals were excluded from the final analysis. The number of rats listed in the specific experimental protocols represents the number of rats in the final analysis (all cannulae correctly placed). The location of correct injection sites is illustrated in Fig. 1.

Peptides

Orexin A (American Peptides, Sunnyvale, CA, USA), muscimol (Sigma, St. Louis, MO, USA) and SCH 23390 (Sigma, St. Louis, MO, USA) were dissolved in artificial cerebrospinal fluid (aCSF), aliquoted and kept at 4 °C until needed. All injections given to the control animals contained the vehicle corresponding to that used for the treated animals.

Injections

Injection cannulae (33 gauge; Plastics One, Inc., Roanoke, VA, USA) were fabricated such that when inserted to their maximum depth, they protrude 1.0 mm beyond the tip of the guide cannulae. Volume of 0.5 μl was injected slowly over 30 s, with injector left in place an additional 15 s to ensure extrusion from the tip, and to minimize distribution of drug upwards on the cannula tract. After injection, this cannula was withdrawn, the stylet replaced, and the rat returned to the activity chamber. All injections were unilateral. The total number of injections for each animal was less than 12. Injection sites were examined by light microscopy for tissue damage in the present studies and none was found.

Oxygen consumption, carbon dioxide production and time spent moving (experiment 1a)

Oxygen consumption and carbon dioxide production were measured by using a customized, high-precision, single-chamber indirect calorimeter (Columbus Instruments, Columbus, OH, USA) as we have reported previously. Thermogenesis was calculated from oxygen consumption and carbon dioxide production. Calibration of the calorimeter was performed at the beginning of each measurement day. The animal was placed inside the cylindrical calorimeter chamber (acrylic; diameter 30 cm, height 20 cm, volume 15 l) without food or water for the 80 min measurement period. The chamber lid was attached and sealed, and room air was pumped at atmospheric pressure through the chamber at 3.4–3.7 l/min. Data on oxygen consumption and carbon dioxide production were then collected every minute and stored on a PC. Each data-point was identified by a time-stamp. Ambulation was measured simultaneously with the oxygen consumption and carbon dioxide production measurements. Measurements were performed using customized, high-precision racks of collimated infrared activity sensors (Columbus Instruments) placed around the acrylic chamber. There were 45 collimated beams of infrared light crossing the 30-cm-diameter cage, allowing the detection of 1 inch of movement in three orthogonal axes. Photosensors registered an activity unit each time a beam was interrupted. In this fashion, activity was simultaneously detected in all three axes: forward-and-backward, side-to-side, and up-and-down. Data for ambulation were summed for every minute and stored on the PC with use of the time stamp for identification. Data were thereby derived simultaneously for oxygen consumption and ambulation, for each animal, minute-by-minute over the 80 min measurement period.

Measures of time spent moving (experiments 1b, 2a–b, and 3a–c)

Amount of time spent moving (time spent in ambulation and vertical movement) was measured for 80 min following injection using the data from the beam break measurements taken as indicated above. Activity was assessed in 43.2×43.2 cm open field activity chambers equipped with 45 collimated beams of infrared light crossing the chamber (MED Associates, East Fairfield, VT, USA). Data collection and processing were performed using “Open Field Activity Software” (MED Associates). Data for ambulation (i.e. “new” beam breaks, accounted for by ambulation

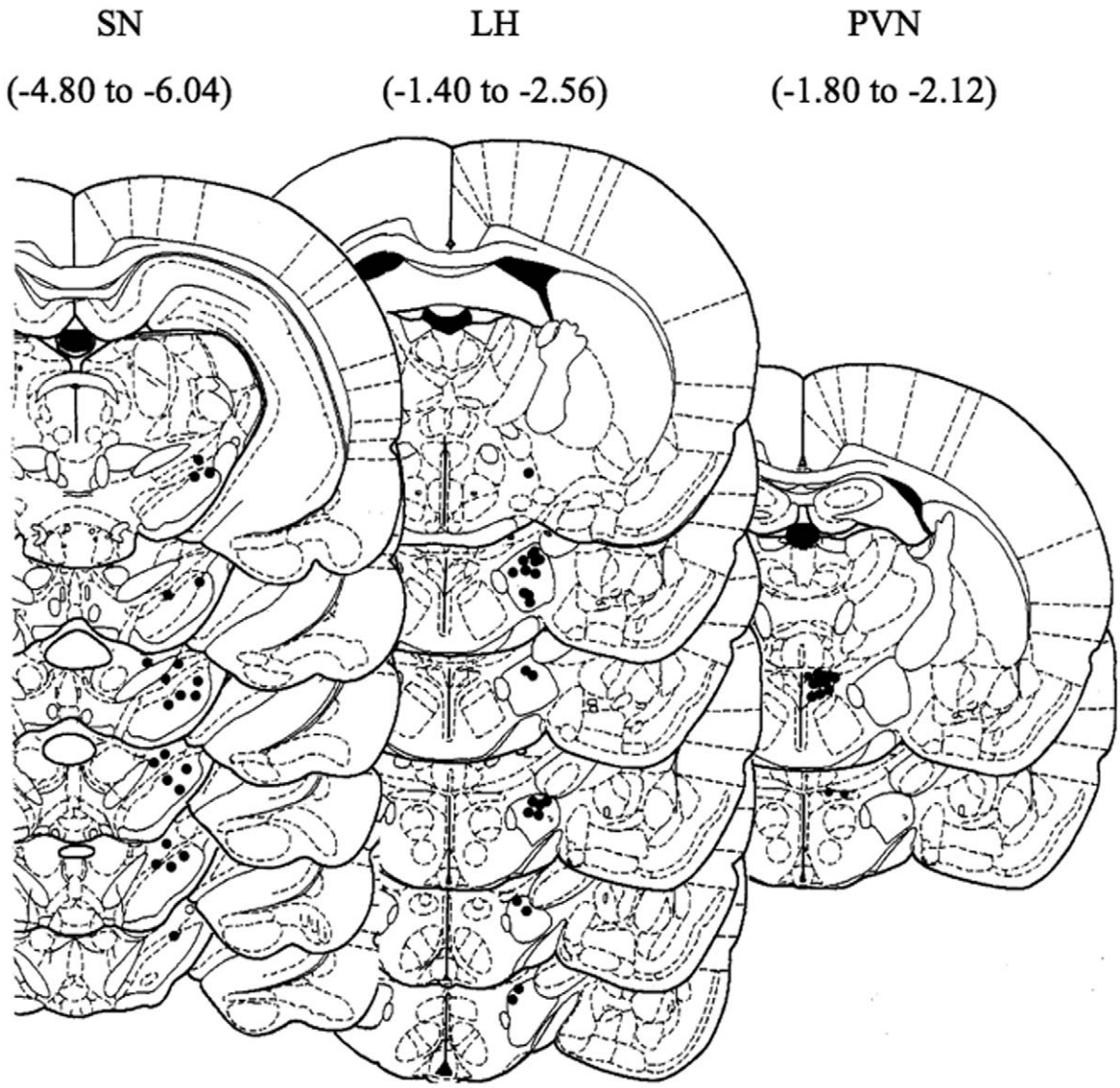

Fig. 1. Location of correctly placed injections within each brain area under study. LH=lateral hypothalamus, SN=substantia nigra.

or standing versus sedentary activity such as grooming) were summed for every second and stored on the PC with use of the time stamp for identification. "Outside the box" vertical beam break counts reflect either standing (lower and upper beams are broken) or jumping (lower beam not broken, upper beam broken). Outside the box horizontal beam breaks represent ambulation. "Inside the box" vertical counts reflect stereotypic movement, such as grooming. We report time spent moving, which is the sum of time spent in ambulatory and vertical movement.

Specific experimental designs

There were at least 48 h between treatments. Doses of drugs were chosen based on our previous work (Sweet et al., 1999; Kotz et al., 2002; Kiwaki et al., 2004). Our previous work also indicates that food availability results in minor and insignificant differences in horizontal and vertical outside the box measurements (Kotz et al., 2002; Kiwaki et al., 2004; Novak et al., 2006; Teske et al., 2006) but to avoid activity associated with food consumption, food was made unavailable during the 80-min measurement period.

Experiment 1: effect of orexin A in PVN

Dose response: time spent moving and thermogenesis. Orexin A (0, 0.1, 0.25, 0.5 and 1 nmol) was injected intra-PVN (N=5). Horizontal and vertical counts, and thermogenesis were measured every min for 80 min. Injections occurred in the light cycle (07:00 h–19:00 h). These data were derived from measurements taken in one of our previously published studies (Kiwaki et al., 2004). These data were collected in a chamber that recorded activity units per minute vs. per second in the subsequent experiments (Expts. 1b, 2 and 3); thus the absolute values for time spent moving are higher (approximately four-fold) than that for experiments 1b, 2 and 3, because each minute that contained at least one activity count was included as one full minute of movement.

Effect of muscimol. In a separate set of PVN-cannulated animals (N=8), muscimol (20 ng) was injected 15 min prior to injection of orexin A (0.5 nmol). Horizontal and vertical counts were determined every second for 80 min. Injections were performed between 10:00 h and 12:30 h.

Experiment 2: effect of orexin A in rLHa

Dose response: time spent moving. Orexin A (0, 0.1, 0.5 and 1 nmol) was injected intra-rLHa ($N=10$). Horizontal and vertical counts were determined every second for 80 min. Injections were performed between 10:00 h and 12:30 h.

Effect of muscimol. In a separate set of rLHa-cannulated animals ($N=6–9$), muscimol (4, 12 and 20 ng) was injected 15 min prior to injection of orexin A (0.5 nmol). Horizontal and vertical counts were determined every second for 80 min. Injections were performed between 10:00 h and 12:30 h.

Experiment 3: effect of orexin A in substantia nigra$_{pc}$

Dose response. Orexin A (0, 0.05, 0.1, 0.25 and 0.5 nmol) was injected intra–substantia nigra$_{pc}$ ($N=12$). Horizontal and vertical counts were determined every second for 80 min. Injections were performed between 10:00 h and 12:30 h.

Effect of muscimol. In a separate set of substantia nigra$_{pc}$–cannulated animals ($N=6–7$) muscimol (4, 12 and 20 ng) was injected 15 min prior to injection of orexin A (0.5 nmol). Horizontal and vertical counts were determined every second for 80 min. Injections were performed between 10:00 h and 12:30 h.

Effect of SCH 23390. In the same set of rats used in study 3b ($N=6–8$), SCH 23390 (dopamine 1 receptor antagonist, 30 pmol) was pre-injected 15 min prior to injection of 0.5 nmol orexin A. Horizontal and vertical counts were determined every second for 80 min. Injections were performed between 10:00 h and 12:30 h.

Statistical analyses

Data were analyzed by repeated measures ANOVA, with treatment as the independent variable (orexin A±SCH 23390, or muscimol) and total time spent moving (standing+ambulatory) as the dependent variable. In the cases where some data were lost due to computer software issues (experiments 2b, 3b and 3c), the rest of those rats' data was included in the data-set and a non-repeated measures one factor ANOVA was used. In all analyses data were normally distributed and paired *t*-tests were used to determine differences between individual groups. For all analyses, data from the first 20 min after injection were discarded as the increased activity in this time-period represents the behavioral response to the injection procedure rather than a drug effect (Kotz et al., 2002).

RESULTS

Study 1a ($N=5$)

Repeated measures ANOVA indicated that orexin A in the PVN significantly elevated time spent moving ($F_{4,16}=38.881$, $P<0.0001$, Fig. 2a) and energy expenditure ($F_{4,16}=10.713$, $P=0.0002$, Fig. 2b). Paired *t*-tests indicated that the 0.25, 0.5 and 1 nmol dose orexin A significantly elevated time spent moving over control levels ($P=0.0375$, $P=0.0035$ and $P=0.0002$, respectively); all doses of orexin A significantly elevated energy expenditure (0.1 nmol: $P=0.003$; 0.25 nmol: $P=0.02$; 0.5 nmol: $P=0.0061$; 1 nmol: $P=0.0045$).

Study 1b ($N=8$)

There was a main effect of treatment on time spent moving ($F_{3,21}=8.319$, $P=0.0008$, data not shown). Paired *t*-tests indicated that orexin A (0.5 nmol) significantly increased time spent moving ($P=0.0003$), but that muscimol (20 ng) did not significantly block this effect ($P=0.1202$, data not shown).

Study 2a ($N=10$)

Repeated measures ANOVA indicated that orexin A in the rLHa significantly elevated time spent moving ($F_{3,27}=6.113$, $P=0.0026$, Fig. 3a). Paired *t*-tests indicated that 0.5 nmol and1 nmol orexin A significantly elevated time spent moving ($P=0.0007$ and $P=0.0032$, respectively; Fig. 3a).

Study 2b ($N=6–9$)

There was a main effect of treatment on time spent moving ($F_{5,37}=2.748$, $P=0.0329$, Fig. 3b) and muscimol at the 12 and 20 ng doses significantly blocked the effect of orexin A (0.5 nmol) on time spent moving ($P=0.0344$ and $P=0.0229$, respectively; Fig. 3b).

Study 3a ($N=11$)

Repeated measures ANOVA indicated that orexin A in the substantia nigra$_{pc}$ significantly elevated time spent moving ($F_{5,50}=4.047$, $P=0.007$, Fig. 4a). Paired *t*-tests indicated that the 0.25 and 0.5 nmol doses of orexin A significantly elevated time spent moving ($P=0.0106$ and $P=0.004$, respectively; Fig. 4a).

Study 3b ($N=6–7$)

Muscimol did not block orexin A-induced time spent moving ($P>0.05$, data not shown).

Study 3c ($N=6–8$)

There was a main effect of treatment on time spent moving ($F_{2,19}=11.406$, $P=0.0005$, Fig. 4b) and post hoc *t*-tests indicate that SCH 23390 (30 nmol) completely inhibited orexin A induced increases in time spent moving ($P=0.0007$, Fig. 4b).

DISCUSSION

Individual variation in daily amount of time spent moving vs. resting may be important in NEAT and energy expenditure in obesity. However, the biological controls mediating these activities are unexplained. We report that in rats, the amount of time spent moving can be modulated centrally by orexin A in multiple brain sites. Orexin A injected in the PVN, rLHa and the substantia nigra$_{pc}$ induced significant dose-dependent increases in the amount of time animals spent moving (Figs. 2–4) and in NEAT (Fig. 2b). Thus orexin A is a putative mediator of time spent moving and NEAT.

Orexin producing neurons reside in the caudal aspect of the lateral, perifornical, posterior and dorsomedial hypothalamus, and project diffusely throughout the CNS in a pattern that matches the wide orexin receptor distribution (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998). Orexin neurons project to the PVN, rLHa and sub-

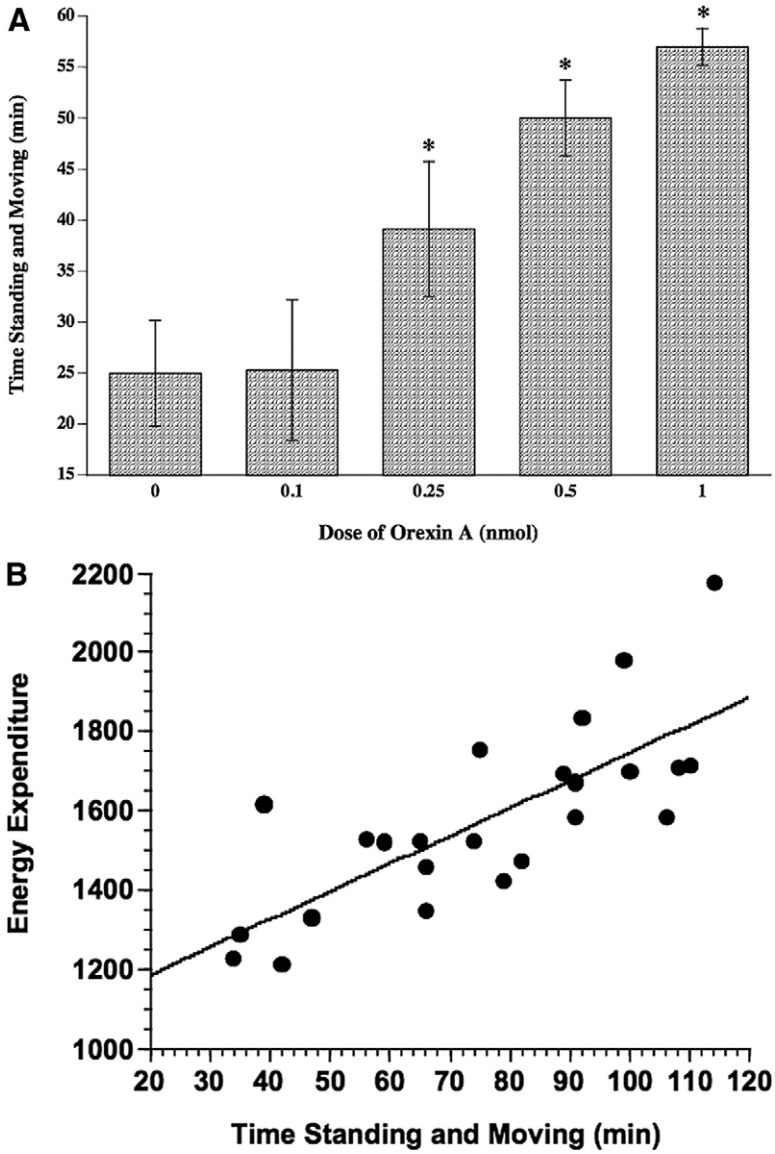

Fig. 2. Effect of orexin A (0–1 nmol) in hypothalamic PVN on (a) time spent moving and (b) energy expenditure (oxygen consumption, ml/kg body weight/h). * $P<0.05$; $N=5$.

stantia nigra$_{pc}$, and both OX1R and OX2R have been identified within each of these regions (Trivedi et al., 1998; Marcus et al., 2001; Backberg et al., 2002). As would be predicted based upon the diverse functional, neurochemical and receptor populations with each of the sites tested in the current studies, the mechanisms by which orexin induces shifts in postural allocation are distinct within each region. In the rLHa, orexin A shifts in postural allocation are dependent upon inhibition of GABA inhibitory signaling (Fig. 3b), as co-administration of the GABA agonist muscimol completely reversed the effects of orexin A on time spent moving. In the PVN, GABA inhibitory signals do not appear to be important to orexin signaling, as muscimol had no effect on orexin A shifts in time spent moving within this region. It has been previously shown that orexin A in substantia nigra increases the firing rate of GABAergic neurons, which increases arousal (Timmerman and Westerink, 1997; Balon et al., 2002). Thus, one might expect that injection of muscimol, a GABA agonist, into the substantia nigra would increase time spent moving. However, we observed no effect of muscimol on time spent moving after injection into the substantia nigra, and it is possible that the level of GABAergic signaling induced by orexin A was already at maximum and thus co-administration of a GABA agonist had no further effect. In contrast, dopamine signaling in the substantia nigra appears to be critically important for substantia nigra orexin A–induced shifts in time spent moving, as blockade of dopamine 1 receptors in this region completely abolished substantia nigra orexin A stimulated movement (Fig. 4b). In the PVN, the only site in which we tested the thermogenic consequences of our treatments, orexin A–induced shifts in time spent moving

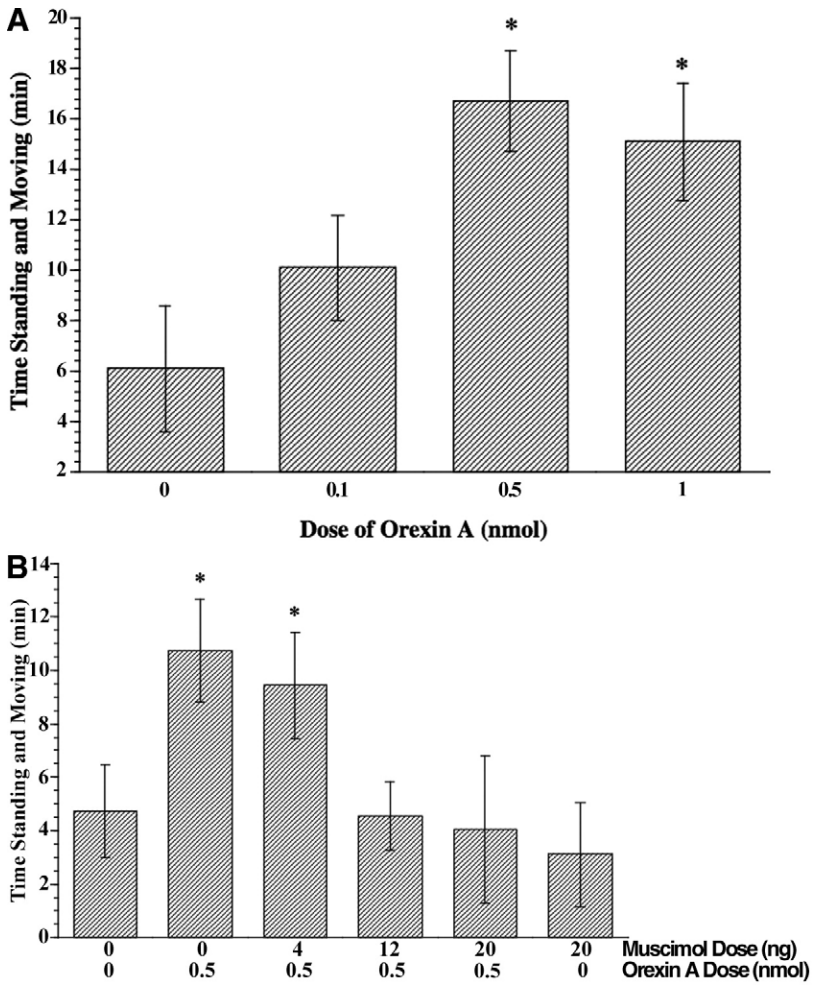

Fig. 3. (a) Effect of orexin A (0–1 nmol) in rLHa on time spent moving, (N=10); (b) effect of rLHA-injected muscimol (4–20 ng) on rLHa-injected orexin A (0.5 nmol) induced shifts in time spent moving (N=6–9). * P<0.05 as compared with control (aCSF) group.

were clearly linked to changes in energy expenditure (Fig. 2b), as expected from our previous study of orexin A in the PVN (Kiwaki et al., 2004). Our previous work indicates that the resulting thermogenesis is dose-dependently linked to activity rather than changes in resting energy expenditure (Kiwaki et al., 2004), which has important implications for energy balance.

We are not asserting that the sites we tested are the only ones supported in orexin-postural allocation mechanisms. Several additional brain regions regulate arousal and physical activity, including the cortical ascending arousal pathway, which involves the histaminergic tuberomammillary nucleus, the noradrenergic locus coeruleus, serotonergic dorsal raphe and the acetylcholinergic prepuduncle pontine nucleus (Willie et al., 2001). The ventral tegmental area is a dopaminergic site important to motor arousal, and the raphe pallidus nuclei are important to shivering thermogenesis. Thus there are likely other, as of yet untested sites that may prove to be important for orexin A modulation of postural allocation.

Lack of orexin function is linked to narcolepsy and orexin clearly stimulates wakefulness (Mignot, 2004). Therefore, orexin A stimulation of spontaneous physical activity and time spent moving could be thought of as a by-product of orexin effects on wakefulness. While impossible to completely dissociate the waking and spontaneous physical activity effects of orexin A, we have three arguments for supporting an additional role for orexin A in time spent moving: first, the condition of wakefulness is a binary state: one is either awake or asleep. Alertness, and active waking (being awake and moving around) are analog states: continuous and variable. Orexin is not implicated in the switch between sleep and wake, but rather in maintaining wakefulness (being awake for longer periods of time) (Mignot, 2004) and the acute and dose-response nature of orexin effects on physical activity suggests that orexin is also important to regulation of such activity. Second, several lines of evidence implicate orexin in regulation of motor activity per se (Wu et al., 2002; Peever et al., 2003; Akiyama et al., 2004; Mieda et al., 2004), which give support to the idea that orexin A stimulates the motor activity associated with spontaneous physical activity. Finally, we have previously shown that orexin A stimulates activity when animals are already awake (Kotz et al., 2002;

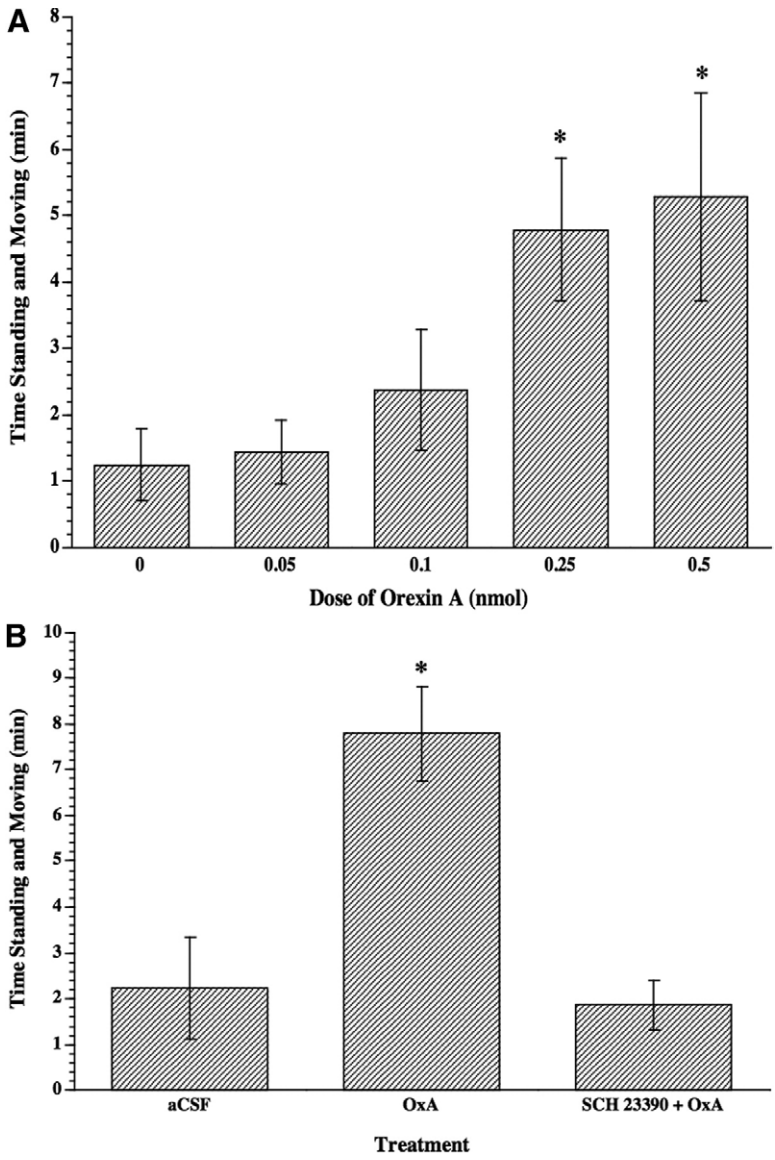

Fig. 4. (a) Effect of orexin A (0–0.5 nmol) in substantia nigra$_{pc}$ on time spent moving, (N=12); (b) effect of substantia nigra$_{pc}$–injected SCH 23390 (30 pmol) on substantia nigra$_{pc}$–injected orexin A (0.5 nmol) induced shifts in time spent moving (N=6–8). * P<0.05 as compared with control (aCSF) group. OxA=orexin A.

Kiwaki et al., 2004) and that there is no circadian rhythm to orexin A effectiveness to stimulate activity (Kiwaki et al., 2004), such that orexin enhances spontaneous physical activity at all times of the light/dark (non-active/active) cycle.

This study has limitations that we recognize. First, we did not examine the effect of prolonged orexin A infusions on time spent moving and body weight. Second, we did not examine the interface of orexin A and obesity. Third, there were differences in baseline activity between studies, which most likely reflect slight differences in the age of the rats at time of testing, as basal locomotor activity in rodents decreases with age (Tou and Wade, 2002). All studies however, were of paired design so that baseline activity was always compared with post-injection activity for that rat. Despite the limitations of these studies, we feel that we robustly examined our primary hypothesis that orexin A impacts time spent moving. Our primary conclusion that orexin A impacts postural allocation in a dose-dependent fashion in the PVN, rLHA and substantia nigra$_{pc}$ is supported by the data we present.

Recent data suggest that time spent standing and ambulating may be important to human obesity (Levine, 2002; Levine et al., 2005). The mechanism by which this effect is mediated is unknown. Based on previous studies demonstrating that orexin A mediates physical activity and NEAT as a whole, we conjectured that orexin A could mediate time spent moving. By injecting orexin A into principal hypothalamic and hindbrain nuclei, to which

orexin neurons project, we found that indeed orexin A mediated time spent moving in rats in a dose-dependent manner. We do not maintain that orexin A is the sole mediator of time spent moving in obesity, but rather assert that orexin A demonstrates the principle that central mediation of time spent moving occurs and that orexin is one such mediator of this effect. The role of orexin A in the central mediation of time spent moving and obesity is deserving of scrutiny.

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