Daily Intraparaventricular Orexin-A Treatment Induces Weight Loss in Rats

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The neuropeptide orexin (hypocretin) increases energy expenditure partially through increasing spontaneous physical activity. The ability of exogenous orexin to alter body weight has never been established, however. We sought to determine whether orexin-A microinjected into the paraventricular nucleus of the hypothalamus (PVN) induced weight loss in rats. Chronic guide cannulae were implanted into rats, aimed at the PVN. Rats were given daily microinjections of orexin (0.5 nmol) or vehicle into the PVN for 6 days; food intake and body weight were measured daily. In a separate group of rats, we injected orexin-A and vehicle intra-PVN and measured daily activity levels. Daily orexin treatment induced weight loss: orexin-A-treated rats lost significantly more weight than their vehicle-injected counterparts without a significant difference in food intake. Rats were significantly more active after intra-PVN orexin compared to vehicle. These results support the concept that orexinergic agents have the potential to produce negative energy balance through increasing physical activity. This presents a promising, untapped potential resource for weight loss.

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INTRODUCTION

Obesity has become a serious burden on health-care systems and is inexorably linked with numerous diseases (1). The majority of investigations into the causes of and treatments for obesity, however, focus on energy intake. There is increasing acknowledgment of the importance of variations in energy expenditure to the propensity for obesity (2–4). Specifically, the energy expended in daily spontaneous activity, called nonexercise activity thermogenesis (NEAT), varies between individuals, is related to the tendency to gain weight or remain lean, and can have an significant impact on energy balance (3,4).

Several neural and endocrine factors influence energy balance by acting on the brain to alter physical activity levels (5). Orexins (hypocretins) are peptides known to increase food intake (6). Others and we have demonstrated that orexin microinjected site-specifically into brain regions, increases physical activity and the associated energy expenditure, and that this effect is dampened in obesity (7,8). These studies reveal the potential to tap into the orexin-mediated control of physical activity to alter energy balance and induce weight loss. Though it is established that orexin increases the energy expenditure of activity short-term (7,8), and that perturbation of the orexin system alters body weight, we do not know if orexin-induced energy expenditure can alter body weight over time. In this study, we administered daily microinjections into the paraventricular nucleus of the hypothalamus (PVN), a region known to contain orexin receptors (ORs), as well as mediate orexin-induced increases in physical activity and energy expenditure (5,8). We measured food intake and body weight daily to determine whether intra-PVN orexin-A, compared to vehicle, could induce weight loss in rats. Subsequently, we measured activity after orexin to determine whether a single dose of orexin could increase daily activity levels over baseline. Our aim was to determine whether orexin-induced NEAT could meaningfully affect energy balance and cause weight loss after a short-term treatment.

METHODS AND PROCEDURES

Twenty adult male Sprague–Dawley rats were used for the first study. Rats were individually housed in a 12:12 light:dark cycle (lights on at 0700 hours) in cages with wire mesh floors covered with absorbent paper, and given food (Laboratory Rodent Diet 5001; PMI Nutrition International, St. Louis, MO) and water *ad libitum*; ambient temperature was kept between 69 and 72 °F. Each rat received a chronically implanted guide cannula aimed at the PVN. This procedure has been previously described in detail (8). Briefly, rats were anesthetized with Nembutal (50 mg/kg, intraperitoneal) and placed in the stereotaxic apparatus. The following coordinates were used: anterior–posterior, −1.3 mm; medial–lateral, +0.5; dorsal–ventral, −6.3; and injection needle, 3 mm projection. After 5 weeks of recovery, rats were matched by body weight and assigned into groups. One day before the onset of injections (day −1), the body composition of the rats was measured using an EchoMRI-900 (Echo Medical Systems, Houston, TX), which measured total water as well as fat and lean mass, from which we could calculate fat-free mass (total mass minus fat mass; includes bone, skin, and fur).

On each injection day, in the first 2 h after lights on, the body weight of each rat was measured, and the remaining food and scattered food remnants were removed from the absorbent paper and weighed. Each rat received a single, unilateral intra-PVN microinjection of either orexin-A (0.5 nmol in 250 nl vehicle; American Peptide, Sunnyvale, CA) or vehicle (artificial cerebrospinal fluid; Harvard Apparatus, Holliston, MA) over 30 s. Each rat was given one microinjection per day for six consecutive days (days 1–6). On the 7th day (day 7), food and body weight were

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measured in the first hour after lights on, then body composition was again measured using the EchoMRI-900. Body weight was measured a final time 6 days after the final injection (day 12).

In a separate study, we measured daily activity levels in male rats ($N = 9$) after intra-PVN microinjections of orexin-A or vehicle. After acclimation for 1 day, rats were injected with either orexin-A (0.5 nmol) or vehicle (aCSF, 250 nl) between 0815 and 0835 hours. Activity was measured, as previously described (8), using infrared beam breaks to monitor horizontal, vertical, and ambulatory activity; data collection were stopped at 0740 hours the next day. Each rat received both orexin-A and vehicle injections in random order, separated by at least 48 h.

After the completion of the study, rats were given a terminal injection of 1 ml Nembutal and microinjected with 250 nl of India ink to allow easy identification of the injection site. The brains were removed and placed in 10% phosphate-buffered formalin with 30% sucrose. Brains were sectioned at 50 µm using a Reichert-Jung cryostat and mounted onto slides (Fisher Superfrost Plus; Fisher Scientific, Pittsburgh, PA); slides were dehydrated, delipidated, and stained using cresyl violet, and the injection sites were determined using a microscope and referencing the rat stereotaxic atlas (9). Rats with injection sites placed >300 µm from the border of the PVN were not included in the final analyses. The final group numbers for the daily injection study were 9 (orexin-A), 8 (vehicle), and 7 for the study measuring daily activity levels. To analyze the injection-induced damage, we measured the maximum width of the damaged area (to the nearest 25 µm) at the most ventral point of injection using a microscope with a calibrated reticle. The means were compared using an independent samples *t*-test.

Body weight was analyzed using a two-way mixed ANOVA, with day of treatment as the within-subjects independent variable and group (orexin-A- or vehicle-injected) as the between-subjects independent variable. Within-subjects *t*-tests, corrected for multiple comparisons (corrected α = uncorrected α/number of tests), were used to determine significance of pairwise comparisons of body weight between experimental days and baseline day. The same design was used to analyze the food intake and body composition data, which consisted of body lipid and protein (both in grams), and percent lipid and percent lean were calculated (arcsine-square root corrected). To examine feed efficiency before treatment for each rat, we calculated the total food intake for 2 days preceding the treatment onset (food intake measured on days −1 and 1); this was divided by the total body weight gained on the 2 days preceding the onset of the treatment (day 1 minus day −2). This was compared to the feed efficiency during the experimental treatment, which was the total food intake measured on days 2 through 7 divided by the body weight gained during the treatment (day 7 minus day 1). A two-way mixed ANOVA was used to analyze these data. Differences of $P < 0.05$ were considered significant.

RESULTS

We found a significant effect of orexin-A on body weight, with orexin causing more weight loss than vehicle (**Figure 1**). On the 5th and 6th day of injection, as well as the day after the final injection (day 7), the orexin-treated rats lost significant weight and the vehicle-injected rats did not. When rats were measured on day 12, the orexin-treated rats remained significantly lighter than they were the day prior to injection, whereas the vehicle-treated rats were significantly heavier than they were on day −1. The groups differed in body weight on days 7 and 12. Treatment significantly decreased body fat, but this was not specific to rats treated with orexin-A or vehicle (**Table 1**). Both groups showed a significant decrease in lean mass and fat-free mass, but the decreases were significantly larger in animals receiving orexin-A. No significant changes were seen from days −1 to 7 in the percent of body water in rats given orexin (from 91.5 ± 0.9 to 89.5 ± 0.4%) or vehicle (from 90.3 ± 0.5 to 90.4 ± 0.5%). Absolute daily food intake did not differ between groups (**Figure 2b**), and orexin-A had no significant effect on food intake corrected for body weight, compared to vehicle (**Figure 2a,b**). The analyses of food intake were complicated due to the changing body weights of the rats; the change in food intake over time was the only significant effect in the two-way mixed ANOVA. In both groups, food intake tended to

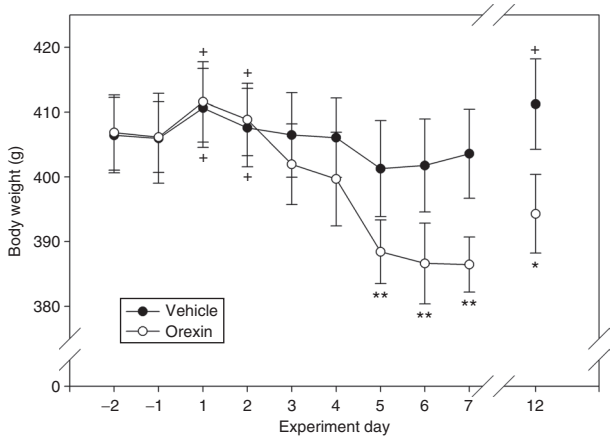

Figure 1 Intraparaventricular microinjections of orexin-A over 6 days resulted in weight loss in rats. Microinjections began on day 1 and continued through day 6. Rats were weighed again on day 12. +Compared to the day before the injections began (day −1), both groups showed significant weight gains on the first and second injections days. **The orexin-treated group (empty circles), however, showed significant decreases in body weight on days 5, 6, and 7 compared to day −1. *On day 12, 6 days after the final injection, the orexin-treated rats still weighed significantly less than they did on day −1, and also significantly less than the vehicle-injected rats (filled circles) on the same day, which gained weight compared to day −1.

Table 1 Fat and lean mass in orexin- and vehicle-treated rats (mean ± s.e.m.)

	Fat mass				Lean mass				Fat-free mass			
	Grams[a]		% of Body mass[a]		Grams[a,b]		% of Body mass[a]		Grams[a,b]		% of Body mass[a]	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Orexin-A	33.07 ± 2.07	30.83 ± 1.51	8.21 ± 0.48	7.98 ± 0.36	319.74 ± 5.16	302.29 ± 3.79	79.45 ± 0.87	78.23 ± 0.45	369.78 ± 7.50	355.57 ± 4.15	91.79 ± 0.48	92.02 ± 0.36
Vehicle	34.14 ± 1.96	30.20 ± 1.91	8.40 ± 0.40	7.49 ± 0.41	326.92 ± 6.08	320.50 ± 5.91	80.53 ± 0.38	79.41 ± 0.34	371.80 ± 6.35	373.36 ± 6.79	91.61 ± 0.40	92.51 ± 0.41

[a]Significant change, pre- vs. post-treatment, no difference between groups. [b]Significant interaction in the effect of treatment between groups.

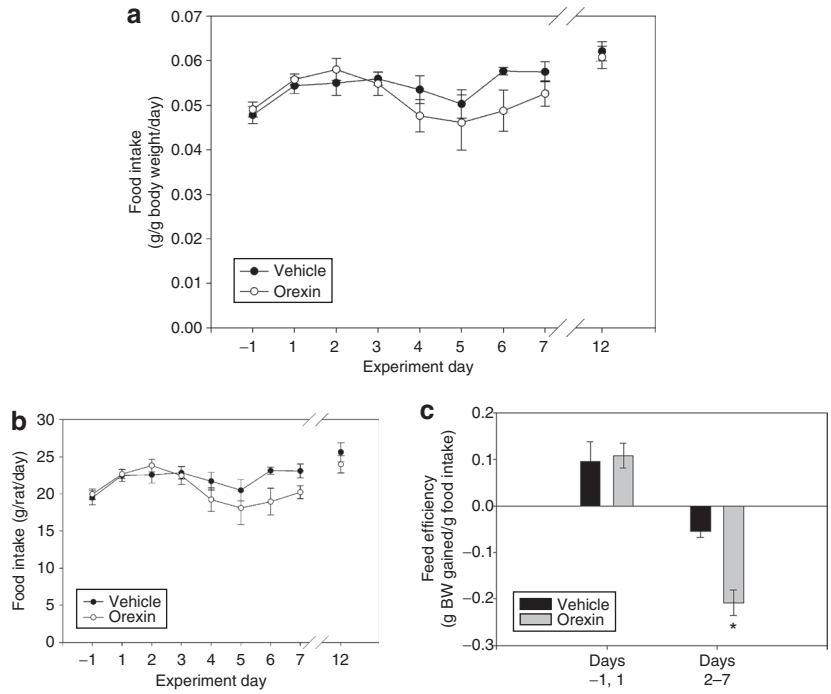

Figure 2 Food intake corrected for body weight (**a**) or absolute food intake (**b**) did not differ between rats treated with orexin-A or vehicle. (**c**) Feed efficiency (grams of body weight gained for each gram of food eaten) was calculated for each rat for the pretreatment period (measured on experiment days –1 and 1) and treatment period (measured on experiment days 2–7). *The rats microinjected with intraparaventricular orexin-A (gray bars) were significantly less feed efficient than their vehicle-treated counterparts.

increase as the injections began, decreased toward the end of the injections, then recovered in the days after injections were completed (**Figure 2b**). As shown in **Figure 2c**, when feed efficiency was analyzed, a significant interaction was found: the post-treatment feed efficiency was significantly lower in the orexin-treated rats compared to the vehicle-treated rats.

Figure 3 illustrates the neuroanatomical placements of daily injections of orexin-A and vehicle. Because damage to the ventral noradrenergic bundle near the PVN is likely to result in weight gain rather than weight loss (10), it is unlikely that microinjection-induced damage to the PVN region would bias our results. To make certain of this, however, we carefully examined the injection region of all rats included in the final analysis; examples are shown in **Figure 3**. The vehicle-injected rats had a damage of 118.8 ± 15.5 µm (mean ± s.e.m.), and the orexin-injected rats had a damage of 144.4 ± 19.4 µm (P = 0.33), thus damage is not a likely explanation for the weight loss seen in the orexin-injected rats. Finally, a single orexin-injected rat that had an injection site outside of the required distance from the PVN was not included in the analysis.

In the second study, rats were significantly more active after intra-PVN injections of orexin-A compared to vehicle. **Figure 4a** illustrates the effect of orexin-A on ambulatory activity; horizontal activity was also significantly increased after orexin-A compared to vehicle in the same rat (mean counts/min ± s.e.m. for vehicle: 21.38 ± 1.79; orexin-A: 25.95 ± 2.31); an hourly analysis of horizontal activity is illustrated in **Figure 4b**.

DISCUSSION

This study demonstrates that daily intra-PVN orexin-A decreases body weight over the course of several days. Furthermore, the orexin-treated rats remained significantly lighter than control rats 6 days after the orexin treatment had ended (**Figure 1**). (The one exception was an orexin-treated rat that gained weight (4.5 g) from days 1 to 7; this rat's injection site did not include the PVN.) Because there was no significant difference in body weight–corrected food intake in these rats (**Figure 1**), we can infer that the orexin treatment altered energy expenditure. In support of this, orexin-treated rats were significantly less feed efficient than vehicle-treated rats, demonstrating that relatively more energy was being expended rather than added to body mass (**Figure 2c**). Orexin-A also increased daily ambulatory activity over baseline levels (**Figure 4a**). Within-animal comparisons are critical for the detection of the effects of orexin-A on daily activity; this is not the case for orexin-induced short-term activity (7,8,11,12). These results, though preliminary, support the concept that daily exogenous orexin treatment can induce weight loss. We propose that the weight loss seen in the orexin-treated rats was, at least in part, a result of increased physical activity and the associated energy expenditure. It is well established that orexin acts in the brain to increase short-term physical activity and energy expenditure (7,8,11,12), but here we demonstrate that daily activity as a whole is also increased by a single orexin treatment. It remains possible that energy expenditure outside of activity-associated expenditure was also increased due to orexin-A (13,14), resulting in negative energy balance

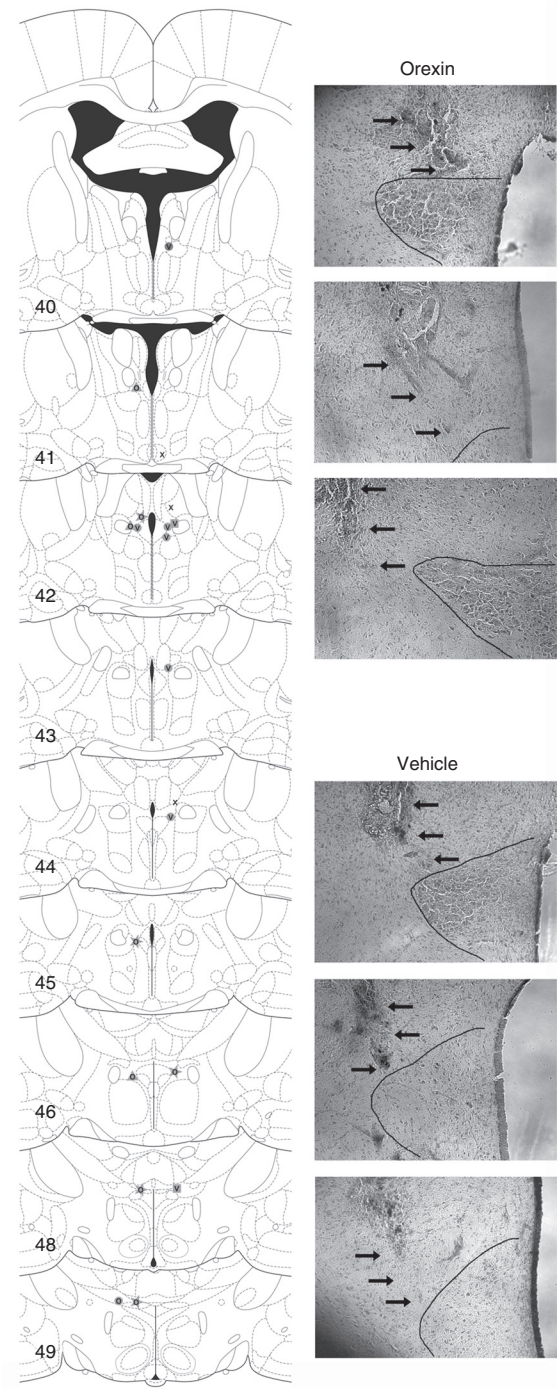

Figure 3 Injection placements in relation to the paraventricular nucleus of the hypothalamus (PVN) in rats, indicating incorrect cannula placement (X); weight loss of 20–30 g (star), 10–20 g (triangle), or 0–10 g (circle); or weight gain (square) in rats injected with orexin ("o") or vehicle ("v"). Orexin-A injections are shown on the left, and vehicle on the right, with one exception each. Atlas figure number is indicated on the far left (9). Photomicrographs of injection sites (arrows) in rats injected with orexin-A or vehicle into the PVN (outlined) are illustrated on the right.

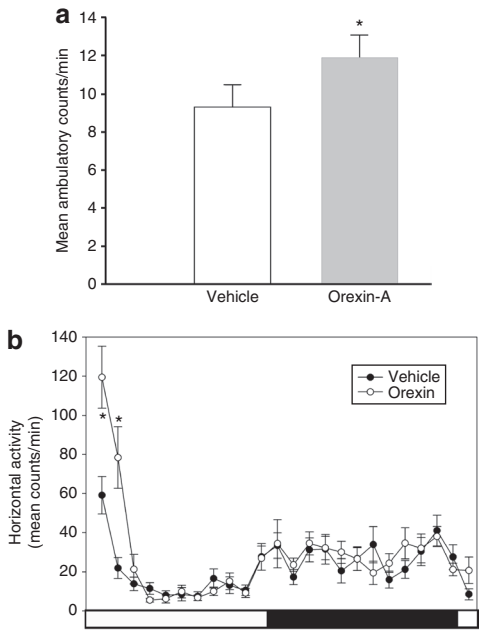

Figure 4 (**a**) *Orexin-A (0.5 nmol) induced more daily ambulatory activity compared to vehicle (aCSF) in the same rats. (**b**) Activity analysis showed that, compared to vehicle injection, orexin-A induced significant horizontal activity in the first 2 h after injection (through 1000 hours). No compensatory dip in activity was detected in orexin-injected rats in the remaining hours of the day (lights on at 0700 hours, lights off at 1900 hours; average injection time = 0819 hours).

and weight loss. In fact, central orexin increases thermogenesis, and the increased energy expenditure is only partially accounted for by physical activity (14,15). Altogether, these studies suggest that agents such as orexin that increase physical activity levels and energy expenditure have the potential to induce weight loss.

It is seemingly paradoxical that orexin-A, a peptide that is known to increase food intake (6), acts to decrease body weight. In fact, the ability of orexin to promote food intake is confined to the light phase of the cycle (13,16,17), when rats and mice are less active and eat less. Orexin-A, particularly in the PVN, induces very little food intake, even at the optimal time of day (18). In contrast, orexin increases activity and energy expenditure at all times of day (11,13). Orexin might be best conceptualized as a circadian output peptide (19), promoting behavioral and physiological states typical of the active phase such as food intake, physical activity, increased energy expenditure, and general wakefulness. When orexin is ablated using orexin-specific expression of ataxin in mice, the animals eat less but also become less active and obese, further emphasizing the importance of orexin in the energy expenditure of activity (20). Thus, orexin provides a unique route to pharmacologically affect energy balance. Here, we focused on the role of the PVN in orexin-induced physical activity because of the importance of the PVN in the regulation of energy expenditure and physical activity levels. The ability of intra-PVN orexin to increase energy expenditure is robust, especially when compared to the modest increase in food intake (11,17,21); significant effects of intra-PVN orexin-A on food intake are reliably seen only at high doses (22). In our study, we found no significant effect of daily intra-PVN orexin on 24-h food intake (**Figure 2c**).

Other studies found no effect of daily orexin treatment on body weight. Russell *et al*. administered orexin-A into the PVN region twice daily (0.3 nmol) for 3 days and found no significant effect on food intake or body weight (17). We found that the effects of orexin on body weight approached significance by the fifth morning of injection (**Figure 1**). Second, Yamanaka *et al.* found no effect of orexin-A on food intake or body weight when given intracerebroventricular using osmotic minipumps over 7 days (16). Reduced sensitivity due to repeated or chronic treatment may hinder an agent's long-term effectiveness. In this study, orexin-induced weight loss leveled off after 5 days (see **Figure 1**), possibly due to reduced sensitivity from repeated injections. Finally, a large sample size may be needed to reveal significant effects on body weight using microinjections (16), which have a limited duration of action and may be stressful to the animal; injection stress can influence body weight independent of the agent being injected (see **Figure 1**).

Our data do not indicate which receptor—OR1 or OR2—mediates the heightened activity and weight loss seen here (**Figure 1**). An earlier study demonstrated that an OR1 antagonist significantly decreased, but did not abolish, the increase in activity after intra-PVN orexin-A (11). We therefore surmise that activation of OR1 may partially account for the orexin-induced weight loss reported here (**Figure 1**). There remains the possibility, however, that OR2 also contributes to orexin-induced NEAT. Orexin-A acts on both OR1 and OR2 (6). Moreover, OR2 signaling may be a key factor in maintaining leptin sensitivity as well as in resisting diet-induced obesity in mice (23). Absence of OR2, but not OR1, results in decreased daily activity (23). The relative contribution of OR1 and OR2 to orexin-induced NEAT is unresolved and is a source for future investigation.

Daily intra-PVN orexin-A resulted in a small decrease in fat mass that was also seen in the vehicle-treated group. The rats receiving orexin-A also showed a decrease in fat-free mass and lean mass (**Table 1**). The ability of an intervention that alters energy balance to affect both fat and lean mass is not surprising given recent data concerning human energy balance. In a recent clinical study, participants that spent 6 months on either 25% caloric restriction or 12.5% caloric restriction plus 12.5% increase in energy expenditure through exercise both showed weight loss through equal amounts of fat and fat-free mass (24). This implies that the change in energy balance, rather than the route taken to achieve negative energy balance, is the key factor in weight loss and associated changes in body composition. Although this idea would have explained a small loss of lean mass in addition to fat mass, it does not explain the loss of lean mass seen here in the orexin-treated rats, which was double that of the vehicle-treated rats (**Table 1**).

Brain orexin has been linked to physical activity, energy expenditure (7,8), and body weight (20), but evidence that orexin treatment directly alters body weight has been lacking. Here, we demonstrate that increasing activity through activation of brain orexin has the potential to influence energy balance and decrease body weight. The duration of orexin treatment was short and was targeted to a specific region of the brain important in the regulation of physical activity and energy expenditure. Nonetheless, our results suggest that orexin has the potential to meaningfully affect energy balance and body weight through the modulation of energy expenditure rather than energy intake. This further highlights the importance of physical activity and NEAT in the prevention of obesity. A systemic orexin agonist has the potential to be used in the treatment of obesity, a previously unexploited option.

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DISCLOSURE

The authors declared no conflict of interest.

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