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The Effect of Hindbrain Orexin A Signaling on Brown Adipose Tissue Thermogenesis and Physical Activity

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Abstract

Obesity (body mass index (BMI) ≥ 30) and overweight (BMI 25 - 29) are major health concerns in the United States, with the incidence of obesity affecting nearly one third of the US population. Previous work has emphasized the importance of neuropeptides (protein neurotransmitters) in the control of behaviors associated with ingestion and energy expenditure (EE). The orexins (orexin A and orexin B) are a family of neuropeptides important in promoting physical activity, and have also recently been shown to increase EE through effects on thermogenesis, specifically via the raphe pallidus (RPa) and dorsomedial hypothalamic nucleus. The aim of this project is to explore brain-mediated defense against obesity via control of EE, specifically focusing on thermogenesis as regulated by hindbrain orexin A (OXA) signaling. While this pathway has been studied in a model using anesthetized rats, similar findings have not yet been duplicated in freely moving animals. Our overall hypothesis is that orexin signaling in the RPa modulates energy balance by increasing brown adipose tissue (BAT) thermogenesis, thereby increasing EE with limited effects on physical activity. Rats received fourth ventricle OXA injections and were monitored for 24 h for food intake, activity, and calorimetry data. Results show that OXA significantly increased heat production (kCal/h) and ambulatory activity in the first two h post-treatment ($p = 0.0019$, and $p = 0.0174$, respectively). While ambulatory activity did increase post-treatment, this difference is not believed to be large enough to fully explain the difference in EE during the first 1-2 h. Despite no significant difference in UCP1 expression (a marker of BAT thermogenesis), a lack of difference in food intake suggests that BAT (rather than diet-induced) thermogenesis is contributing to the difference in EE. BAT thermogenesis is an important component of EE in rodents, and has recently been confirmed to increase EE in humans as well. Therefore, data from this and ongoing studies may contribute to the development of orexin-based therapies to increase energy expenditure and reduce body weight in obese humans.

Keywords: Orexin, BAT, Thermogenesis

1. Introduction

Obesity (body mass index (BMI) ≥ 30) and overweight (BMI between 25 and 29) are major health concerns in the United States [1]. Centers for Disease Control data show that the incidence of obesity has greatly increased over the past 20 years and now affects nearly one third of the US population [2]. Obesity is the result of a long-term imbalance between energy intake and energy expenditure (EE). Previous work has emphasized the importance of neuropeptides – small protein molecules capable of acting as neural transmitters within the brain – in the control of behaviors associated with ingestion and EE. Specifically, the hypothalamic neuropeptide orexin has been shown to alter feeding and arousal, and orexin effects on non-exercise activity thermogenesis (NEAT) have been implicated in the regulation of energy balance and expenditure [3]. With the use of a polygenic rat model of obesity, it has been shown that obesity-resistant rats have higher endogenous orexin sensitivity and orexin receptor levels, and that intra-hypothalamic injection of orexin increases NEAT and reduces weight gain [4].

The orexins (hypocretins) are a family containing two peptides, the 33 amino acid orexin A (OXA, hypocretin-1) and the shorter 28 amino acid orexin B (OXB, hypocretin-2), both derived from a single precursor protein, preproorexin, through proteolytic processing [5, 6]. In the central nervous system, orexin expression is primarily limited to cell bodies in the perifornical region (PeF) and adjacent areas of the lateral hypothalamus (LH) [7, 8]. The orexins bind to two G-protein coupled receptors, orexin receptor 1 and 2 (OX1R and OX2R, respectively). OX2R is nonselective, while OX1R shows 30- to 100-fold greater affinity for OXA over OXB [5, 9]. Because OXA and OXB are produced in the same neurons and have largely identical distribution in the brain [8], and because OXB appears to have similar (but less salient) physiological effects as OXA [10, 11], most studies using orexin focus solely on OXA. While orexin neurons form a diffuse rather than compact cell group, synaptic connections are present between orexin neurons, suggesting that subpopulations of orexin cells might coordinate functions using these recurrent collateral connections [12]. The total number of orexin neurons is fairly small, but axonal projections from these cells extend from the LH to many regions of the brain and spinal cord [13-17], and the distribution of these neurons and axonal projections is very similar across rodent strains and species [8]. The overall distribution of orexin fibers in the brain and spinal cord allows this small population of neurons to play roles in integrating multiple autonomic and behavioral functions, primarily feeding, sleep/wake behavior, and arousal [18]. Disruptions or deficiencies in orexin signaling have been linked to a number of sleep/wake and endocrine disorders in humans and in animal models [19-23], and Nixon and colleagues have previously shown that rats with higher orexin signaling are protected against obesity [18].

While prior research on orexin-mediated mechanisms of obesity has largely focused on the activity-promoting effect of OXA, recent publications show that in addition to effects on food intake and physical activity, orexins can also increase EE through effects on thermogenesis [24-27]. Data suggest orexins may protect against obesity both by effects on physical activity and via brown adipose tissue (BAT) thermogenesis. BAT thermogenesis is an important component of EE in rodents that has recently been shown to exist in adult humans [28, 29], and can significantly affect human energy expenditure [30]. Consequently, brown adipose tissue may represent one of the most promising physiological targets for treatment of obesity [31, 32]. Orexin protects against body weight gain by stimulating increased EE in part via modulation of BAT thermogenesis [18, 33, 34], and orexin deficiency in development causes impaired BAT thermogenesis [27].

The orexin cell fields in the PeF and DMH (dorsomedial hypothalamus) are two of several hypothalamic areas known to promote thermogenesis via projections to BAT depots [24, 26, 33]. Specifically, orexin fibers from the PeF project to the DMH and raphe pallidus (RPa), and orexinergic input stimulates RPa neurons, which in turn project to BAT to promote thermogenesis [8, 24, 33]. A schematic illustrating these pathways can be seen in **Fig. 1**.

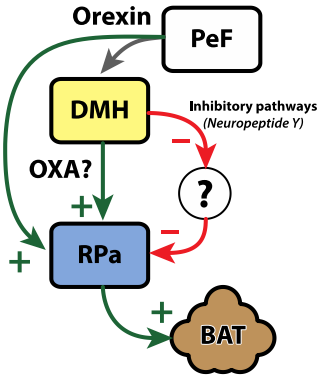

Figure 1. Schematic illustrating pathways and mechanisms of thermogenesis.
Orexin from perifornical region (PeF) or dorsomedial hypothalamus (DMH) activates raphe pallidus (RPa) and brown adipose tissue (BAT) thermogenesis.

The raphe pallidus is a brainstem nucleus known to play a critical role in BAT thermogenesis, and RPa orexin influences this process. Injection of orexin into the RPa was shown to significantly increase BAT thermogenesis in anesthetized rats [24]. Stimulation of the lateral hypothalamic field also increases thermogenesis, and this effect can be blocked by inhibition of either the DMH or RPa [26], indicating that interaction between these sites is needed for BAT thermogenesis. Retrograde tracer studies using a pseudorabies virus injected into BAT show that the RPa is consistently labeled before expression of the virus is seen in the DMH [33, 35], suggesting that the RPa is downstream

of DMH in the pathway for orexinergic stimulation of BAT thermogenesis. OXA injection to the 4V has also been shown to induce c-Fos expression in the RPa and parapyramidal nucleus [25], providing a potential indirect method of stimulating RPa orexin neurons to promote BAT thermogenesis.

Brown adipose tissue is physiologically distinct from white adipose tissue (WAT) in that it is specialized to dissipate energy as heat via non-shivering thermogenesis. Conversely, WAT is used for energy storage. Brown adipocytes contain uncoupling protein-1 (UCP1), a mitochondrial uncoupling protein expressed predominantly in BAT, which, when activated, releases energy as heat by uncoupling oxidative phosphorylation from ATP synthesis [36-38]. Therefore, an increase in UCP1 is indicative of increased thermogenesis. Activators of BAT thermogenesis include stimuli such as cold exposure, thiazolidinediones, natriuretic peptides, thyroid hormone, fibroblast growth factor 21, and orexin [37, 39]. In addition to brown adipocytes, UCP1-expressing adipocytes, termed beige or "brite", have also been shown to develop in WAT in response to various stimuli [37]. However, while UCP1-positive cells have been identified in several classical WAT tissues, studies have shown that traditional BAT depots are still the primary contributors to non-shivering thermogenesis [40, 41]. In rodents, the primary BAT depot is found in the interscapular region, whereas in adult humans, BAT is found mostly in the supraclavicular and paravertebral regions. Due to its thermogenic properties, BAT has increasingly become a therapeutic target for obesity and related metabolic disorders [42, 43].

Overall, obesity and associated comorbidities greatly increase the risks of other chronic diseases and mortality, thus increasing the long-term costs of medical care and reducing longevity [1]. This study aims to provide information regarding neural mechanisms mediating EE, specifically through hindbrain orexin signaling, and to test whether these mechanisms have the potential to be exploited to protect against weight gain. Ultimately, data from this and ongoing studies aim to contribute to the development of orexin-based therapies to increase EE and reduce body weight in obese humans.

2. Hypothesis

This experiment will test the hypothesis that orexin signaling in the RPa modulates energy balance by increasing BAT thermogenesis in freely moving rats. We predict that rats receiving hindbrain-targeted OXA will demonstrate increased total energy expenditure and decreased weight gain, with little or no effect on physical activity and food intake. Following analysis of intrascapular BAT samples, we expect to see increased UCP1 expression, indicating increased BAT thermogenesis.

3. Methodology

3.1 Animals

Adult male Sprague-Dawley (SD) rats (Charles River, Chicago, IL USA; n = 16) were housed in accordance with the National Institutes of Health guidelines in a temperature-controlled room (21-22°C) with an alternating 12 h light/12 h dark photoperiod (lights on at 0600 h). Rodent chow (8604, Harlan Teklad, Madison, WI USA) and water were allowed *ad libitum*. All studies were approved by the local Institutional Animal Care and Use Committee at the Minneapolis Veterans Affairs Health Care System.

3.2 Surgery And Injections

Rats with surgically implanted unilateral stainless steel guide cannulae (Plastics One, Roanoke, VA USA) targeting the fourth ventricle (AP: -12.0; L/M: ±0.0; DV: -9.8 mm relative to bregma) were used for this study. A dummy stylet extending to the tip of the cannula was placed in the guide cannula following surgery and between injections. Animals were allowed at least 1 week of recovery from surgery prior to initiation of experiments.

For several days prior to beginning injections, rats were habituated by gentle handling and removal of cannula caps. For injections, rats were gently restrained and cannula caps were removed. A stainless steel injector was inserted into the cannula and a volume of 0.5 µl was delivered manually over 30 s using a Hamilton syringe attached to the injector. Injectors were left in place for an additional 15 s following injectate delivery; after injection, the injector was removed and cannula caps with dummy stylets were replaced. Animals were then weighed and returned

to the cage/monitoring system. Prior to beginning experimental treatments, rats received at least 1 d of artificial cerebrospinal fluid (aCSF) injections for acclimation purposes and to resolve any potential blockage in the cannulae.

3.3 Food Intake, Body Weight, And Body Composition Measurements

Food intake (total food removed from hopper minus spillage, in grams) and body weight were measured daily during each experiment phase. Spillage was defined as any food removed from the hopper but not ingested. Food intake and body weight measurements were collected between 0830-0930 h on all days, unless otherwise indicated. Direct measurement of body composition, including total fat and lean mass, was obtained approximately 24 h prior to euthanasia using a quantitative magnetic resonance (QMR) body composition analyzer (EchoMRI, Houston, TX USA) as described in (44).

3.4 Physical Activity And Energy Expenditure Measurements

Physical activity, defined as time ambulatory (beam breaks in x and y axes) was measured using an open-field activity system (Med Associates, St. Albans, VT USA). EE was determined by thermogenesis calculated from O_2 consumption and CO_2 production measured in individual-chamber indirect calorimeters (CLAMS, Columbus Instruments, Columbus, OH USA). A standard CLAMS set-up (using 7 ⅜" x 12 ⅛" x 6 ¾" acrylic chambers) was used. The system was customized to incorporate the Med Associates open-field activity beam array, allowing for simultaneous collection of physical activity and indirect calorimetry data. Animals were allowed 3 d of acclimation to the test chambers prior to data collection. Data was collected every 30 s and analyzed in 1 h bins. During the first acclimation period, one animal become ill and had to be removed from the study. Animals were given access to food and water *ad libitum* while in the chambers.

3.5 Collection Of Brain And Adipose Tissue

Upon conclusion of the study, ink was injected into the cannula to aide in placement verification, and rats were euthanized by decapitation. Brains were removed and stored in 10% buffered formalin. Intrascapular BAT depots were collected, minced, and divided into samples of ≤ 250 mg. Samples were then flash frozen in liquid nitrogen.

3.6 Verification Of Cannula Placement

Cannula placement was verified by the presence of cerebrospinal fluid at the injection site and/or using histological evaluation. Brains were sectioned at 40 µm onto glass slides using a cryostat. Slides were examined under a light microscope, and the anatomical location of the end of the cannula tract was identified with the aid of a rat brain atlas [(45)]. Only rats with correctly placed cannulas (within a 1 mm diffusion distance of the 4V) were included in the analysis (OXA: n = 7, aCSF: n = 7).

3.7 Gene And Protein Expression Analysis

RNA was isolated from BAT samples using the RNeasy Micro Kit (Qiagen) for column-based isolation, following tissue homogenization with the aid of TRIzol (Invitrogen) and a Bullet Blender (Next Advance, BBX24). UCP1 expression, normalized to GAPDH and tissue weight, was tested using qRT-PCR at a concentration of approximately 50 ng/µl, measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific). Samples were prepared using the LightCycler RNA Amplification Kit: SYBR Green I (Roche) and 10 µM primer mix; qRT-PCR was run using the Roche LightCycler Carousel-Based System (LightCycler 1.5). Primer sequences were generated using MacVector 12 and commercially obtained from Integrated DNA Technologies. The sequences of primers used were as follows: GAPDH, accession number NM_017008, forward 5'-GAC ATC AAG AAG GTG GTG AAG CAG-3', reverse 5'-AAG GTG GAA GAA TGG GAG TTG C-3'; UCP1, accession number NM_012682, forward 5'-TGG CAT CCA GAG GCA AAT CAG-3', reverse 5'-AGC ATT GTA GGT CCC AGT GTA GCG-3'.

Mitochondria were isolated from BAT samples using a commercially available kit (Pierce Thermo Scientific) as described by the manufacturer's provided protocol and in (46). Protein content was determined using a Bradford assay (BioRad). Mitochondrial fractions (7.5 µg) were mixed in SDS-loading buffer and separated on 10% polyacrylamide gels (BioRad). Membranes were incubated in primary antibody (UCP1, Santa Cruz, sc-6795) following incubation in blocking buffer (Superblocker, Pierce Thermo Scientific) at a final dilution of 1:1000 using

the SNAP i.d. system (Millipore) as described in (47). Secondary antibodies were used at a final concentration of 1:60,000 (rabbit anti-goat IgG HRP, Abcam, ab97100). Detection was determined following addition of the HRP substrate (West Pico-Super Signal, Pierce Thermo Scientific) by placing membranes on x-ray film and developing using an Agfa Developer CP1000 (Agfa Corporation). Band density was determined using Image Studio software (LI-COR Biosciences). The mitochondrial inner membrane protein prohibitin was used as a loading control as it is constitutively expressed in mitochondrial fractions and would not be present in a cytosolic fraction.

3.8 Experimental Design

Rats were singly housed in standard hanging wire rodent caging except when undergoing activity or calorimetry measurements. A single cohort of rats was used for all experiments, with rats randomly assigned to OXA or vehicle (aCSF) treatment groups following baseline measurements. Baseline body weight and 24 h food intake measures were taken for three days prior to beginning the experiments, and rats were randomly assigned treatments such that these measures did not differ between groups. Experimental groups were maintained across all studies, with the exception of one animal that was switched from the aCSF to OXA group following Experiment 1 in order to keep groups even after several animals had to be excluded from the study due to an equipment error. Treatments were delivered between 0900 and 0930 h, with data collection starting at 1000 h.

Experiment 1: Rats were placed in standard test chambers, treated with vehicle, and allowed to acclimate for 72 h. On the 4th day, rats received aCSF (0.5 µl, n = 7) or OXA (300 pmol/0.5 µl, n = 8), and 24 h indirect calorimetry and physical activity was recorded.

Experiment 2: Rats were kept in their standard home cages and treated with either OXA (n = 6) or vehicle (n = 5). Food intake was measured 1 h following injection, and again 24 h following injection.

Experiment 3: Approximately 24 h prior to euthanasia, body composition data for each animal was obtained. The following day, rats received treatment with either OXA (n = 6) or aCSF (n = 5). At 90 min post-injection, rats were euthanized, and brain and adipose tissue samples were collected. Brains were used to verify cannula placement, while BAT samples were tested for UCP1 expression following RNA isolation.

3.9 Statistics

Statistical analyses were performed and data was graphically represented using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA USA). Timecourse data from all experiments were analyzed using repeated measures two-way ANOVA, followed by Bonferroni post-tests. Calorimetry and food intake data were analyzed using unpaired two-tailed *t* tests. Real-time RT-PCR data were analyzed for relative changes in gene expression using the Δ-ΔCT method [48] and unpaired two-tailed *t* tests. Western blot data was analyzed using Image Studio (LI-COR Biosciences) software and unpaired two-tailed *t* tests.

4. Results

4.1 Experiment 1

Injection of OXA into the 4V significantly increased EE in the first two h following treatment (**Fig. 2A**, $p = 0.0019$). Data show that OXA treatment did not significantly affect 24 h EE (**Fig. 2B**), total EE during the light or dark periods, or food intake. However, when EE was examined hourly, a significant interaction (treatment x time) was observed (**Fig. 2C**, $p < 0.0001$). Specifically, orexin-treated animals had significantly higher EE at 1000 h and decreased EE throughout the dark period (with this difference being significant at 0300 h), when compared to controls. Examination of ambulatory time revealed a significant interaction (treatment x time) for both cumulative ($p = 0.0374$) and non-cumulative ($p = 0.0048$) data (**Fig. 2D**). Time spent moving was significantly increased for treated animals at 1000 h, and significantly decreased at 0300 h, compared to controls. A significant effect of treatment on ambulatory time was also observed in orexin-treated animals during the first two h following treatment ($p = 0.0174$).

4.2 Experiment 2

In an attempt to explain the observed increase in EE and ambulatory activity in orexin-treated animals in the first 1-2 h following injection, food intake was measured 1 and 24 h following treatment with OXA or vehicle. The data show no significant difference in food intake between orexin- or aCSF-treated animals at either 1 or 24 h following injection (**Fig. 2E**). While food intake appears to be slightly higher in orexin-treated animals 1 h following injection, at 24 h post-injection, food intake is equal among groups, if not slightly higher in animals treated with vehicle.

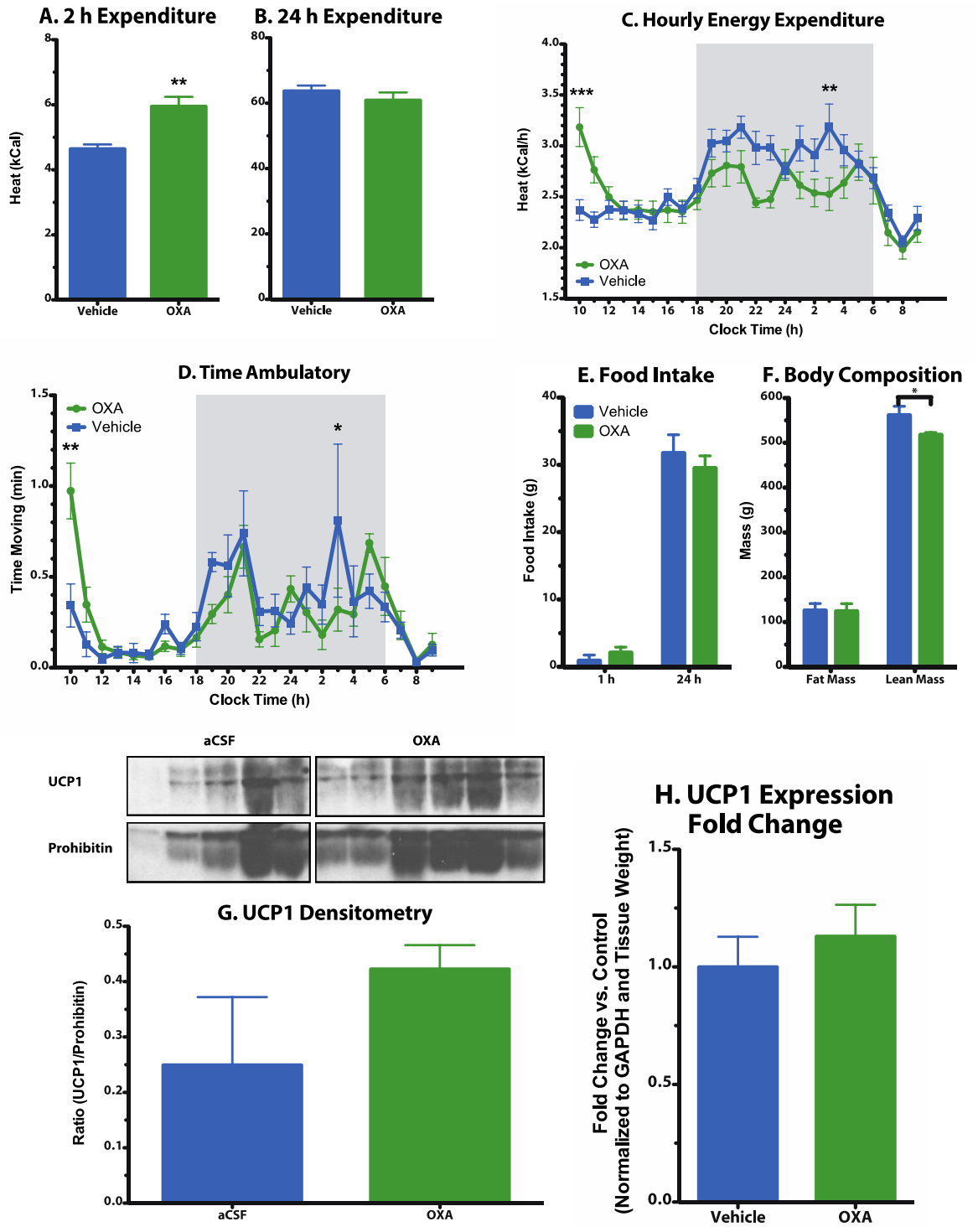

Figure 2. Observed effects of hindbrain orexin A (OXA).

A. OXA (300 pmol/0.5 µl) significantly increased energy expenditure (EE) in the first 2 h following treatment ($p = 0.0019$). **B.** OXA did not alter 24 h EE. **C.** Significant interaction (treatment × time, $p < 0.0001$) was found in hourly EE. OXA significantly increased EE in the first 1 h ($p < 0.001$), and significantly decreased EE at 0300 h ($p < 0.01$). **D.** Significant interaction (treatment × time, $p = 0.0374$) was observed in ambulatory data. OXA significantly increased ambulatory time in the first 1 h post-treatment ($p < 0.01$) and increased ambulation at 0300 h ($p < 0.05$). **E.** No significant difference in food intake was observed. **F.** Fat mass did not differ between OXA and control animals, though OXA animals had slightly but significantly less lean mass than controls ($p = 0.0463$). **G,H.** While

there was a trend for increased BAT UCP1 protein expression (G) and mRNA expression (H) in OXA-treated animals, this difference was not significant.

4.3 Experiment 3

Prior to euthanasia and tissue collection, body composition from all animals was obtained. Data show no difference in fat mass between treated and control animals, though treated animals had slightly, but significantly, less lean mass than controls (**Fig. 2F**, $p = 0.0463$). This, however, is believed to be a result of difference in baseline body composition. An analysis of UCP1 mRNA expression in BAT showed that while UCP1 expression was slightly higher in orexin-treated animals, this difference was not significant. Protein levels of UCP1 expression were also examined via Western blot. Data show that UCP1 expression, normalized to prohibitin, was not significantly different between treatment groups (**Fig. 2G**).

5. Discussion

The goal of this study was to provide information regarding neural mechanisms mediating EE via hindbrain OXA signaling, and to test whether these mechanisms might ultimately have the potential to be exploited to protect against weight gain. A previous study showed that injection of orexin A into the rostral RPa of anesthetized rats significantly increased BAT thermogenesis [24]. To determine whether these findings could be duplicated in freely moving animals, a series of experiments was performed in which rats were injected into the 4V with either OXA or vehicle (aCSF) and monitored for feeding, activity, and indirect calorimetry. At the end of the experiments and following a final injection, intrascapular brown adipose tissue was collected and used for analysis of gene and protein expression. Ultimately, OXA treatment was shown to increase EE in the first 1-2 h following treatment. Data from gene and protein expression analyses, however, showed no significant change in UCP1 expression, as would be expected with an increase in BAT thermogenesis [49].

The significant increase in EE observed in treated animals in the first 1-2 h following treatment in Experiment 1 suggests increased SNS-induced BAT activation and a possible increase in BAT thermogenesis. While ambulatory activity also increased post-treatment, the observed difference (1 min versus 30 s over 1 h) does not appear large enough to fully explain the difference in EE in the first h. However, it was necessary to determine whether BAT thermogenesis or an alternative source of thermogenesis – such as a thermic effect of food – was responsible for the increase in EE. As the orexins are known to stimulate feeding and activity when administered centrally to areas such as the lateral hypothalamus or paraventricular hypothalamic nucleus [3-6], food intake was monitored at both 1 h and 24 h post-treatment to see if an increase in feeding may have caused the increases in EE and SPA (spontaneous physical activity). At 1 h post treatment, although OXA-treated animals appeared to have eaten slightly more than their untreated counterparts, there was no significant difference in food intake between the treatment groups. After 24 h, OXA animals appeared to have eaten slightly less than the vehicle group, though, again, this difference was not significant. These results suggest that the observed increases in EE in the first 1-2 h post-treatment cannot be solely attributed to feeding and feeding-related activity, but that another source of EE – such as BAT thermogenesis – is also playing a role.

Injection of OXA into the hindbrain also appears to elicit a possible rebound effect on EE during the dark phase. These findings appear to be in line with data from other orexin studies [18, 50] in which a rebound effect was also observed. In Experiment 1, OXA significantly decreased EE at 0300 h ($p < 0.05$), and overall EE during the dark phase is dampened. While a significant decrease in ambulatory activity at 0300 h ($p < 0.05$) was also observed, based on overall activity data, this appears to be simply a random fluctuation. Essentially, OXA-treated animals are overcompensating during the dark phase for their increased EE early on, due to the short-acting effects of OXA, though the reasoning for this remains unclear. Further exploration into this phenomenon would be necessary to understand the exact mechanisms involved and to determine whether the rebound effect can be prevented in order to maintain an overall increase in EE (and thus a net weight decrease) for OXA-treated animals. To do so would require looking at downstream targets of orexin or other orexin agonists and seeing if the effects seen here could be duplicated. Based on current literature, possible targets could include glutamate or melanin-concentrating hormone [39, 49]. An alternative approach could utilize subchronic administration of orexin via osmotic minipump, as this method, combined with biotelemetry probes to monitor BAT, has been shown to produce profound changes in BAT thermogenesis [49]. The hindbrain is an important area for this research as it acts as a site of neural integration for

signals to brown adipose tissue coming from other areas of the brain including the hypothalamus and brainstem nuclei such as the preoptic area, arcuate hypothalamic nucleus, paraventricular hypothalamic nucleus, periaqueductal gray matter, DMH, and RPa [51, 52].

While there was a trend for increased BAT UCP1 expression in OXA-treated animals from both PCR and Western blot data (**Fig. 7**), this difference was not significant, as would be expected if the increase in EE were a result of BAT thermogenesis [36]. It should be noted that one of the control/aCSF samples was not included in analysis of the Western blot data as a loading or transfer error seems to have occurred, and not enough sample was present to perform a densitometry analysis. One possible explanation for the observed data is that the hindbrain OXA signaling was not strong enough to elicit a significant increase in UCP1. This theory would be consistent with data showing that OXA-induced increases in BAT thermogenesis require an ongoing, basal level of BAT sympathetic nerve activity which would not have been present in the warm (i.e. room temperature and unaltered body/skin temperature) conditions used [24]. While OXA injection to the 4V has also been shown to induce neuronal activation in the RPa, 4V OXA administration is not a direct method of stimulating RPa orexin neurons and has been shown to activate OXA neurons in other areas, including the parapyramidal nucleus, that are involved in the modulation of cardiovascular and gastrointestinal functions in addition to brown fat thermogenesis [25]. Thus, it is possible that direct targeting of the RPa may be necessary for OXA stimulation of BAT thermogenesis. While direct cannulation of the RPa may have resulted in a more robust increase in thermogenesis and UCP1 expression, the decision to target the RPa indirectly stemmed from concern over cannulating an area in such close proximity to brainstem centers responsible for basic cardiovascular and respiratory functions. The indirect method, using 4V administration of OXA, offers a safer and more clinically feasible approach for the study of orexin-based therapies.

Overall, data from this study suggests that hindbrain OXA signaling has the potential to increase EE via BAT thermogenesis, though perhaps not without also increasing ambulatory activity. It also appears that direct cannulation of the RPa may be necessary for more salient effects of OXA on BAT thermogenesis and UCP1 expression to be seen. While no effect of OXA treatment on body weight was observed, when body composition was analyzed by QMR, it was noted that OXA treated animals did have slightly, yet significantly, less lean mass than control animals ($p = 0.0463$). This is believed to be a result of baseline difference in the treatment groups, and not an effect of treatment itself. Therefore, it would be advised to perform a body composition assessment pre- and post- treatment in future experiments. Similarly, as baseline EE and activity measurements were not taken, it is possible that differences observed between treated and control groups may have been pre-existing; however, because both groups were matched by weight and food intake, this is an unlikely possibility.

Future directions would include repeating the experiment described here with more animals, comprehensive baseline measurements, and direct cannulation of the RPa. As poor yield from isolation of BAT mitochondria was an issue, a larger *n* would allow for treatment groups to be divided and BAT samples to be analyzed by either qPCR or Western blot, rather than performing both analyses on a single BAT depot. Future experiments may also involve looking at the effects of OXA signaling in the PeF and DMH on BAT thermogenesis and UCP1 expression, as these areas are also known to promote thermogenesis via projections to BAT depots [24, 26, 33] but evidence of this in freely moving animals is lacking. Evidence of this nature is particularly important in obesity research, as while volitional exercise is one of the first recommended actions for individuals aiming to lose weight, we are an increasingly sedentary society and many obese individuals suffer from limited mobility. In addition to volitional activity, NEAT and other alternative forms of EE have been shown to have significant potential for increasing weight loss [53, 54]. Ultimately, data from these and ongoing studies aim to contribute to the development of orexin-based therapies with the potential to increase EE and reduce body weight in obese humans.

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