NEUROMODULATION OF OREXIN NEURONS IN THE LATERAL HYPOTHALAMUS REGULATES SPONTANEOUS PHYSICAL ACTIVITY, ENERGY EXPENDITURE, AND DIET-INDUCED OBESITY

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ANASTASIA N. ZINK

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CO-ADVISER: CATHERINE M. KOTZ, PHD
CO-ADVISER: CHARLES J. BILLINGTON, MD

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DEDICATION

TO SANDRA. AN INSPIRATION IN LIFE AND IN SCIENCE.

TO WADE. FOR THE LESSON THAT EVERYTHING IS FINITE.

TO SAM. AND THE FUTURE.

ABSTRACT

Spontaneous physical activity (SPA) is a promising therapeutic target for improving multiple clinical outcomes in obesity and metabolic syndrome. Low levels of the neuropeptide, orexin, are correlated with reduced SPA and pathological body weight in humans and animals. The dissertation research described here used a pharmacosynthetic approach (Designer Receptors Exclusively Activated by Designer Drugs; DREADDs) to modulate orexin neurons activity in adult male and female mice. Selective cellular and anatomic targeting was achieved by bilateral virus injection aimed at the lateral and caudal lateral hypothalamic area of transgenic mice expressing the DNA-recombinase, Cre, in orexin neurons. In the presence of Cre, viruses expressed the excitatory, Gq-coupled DREADD, hM3Dq, or the inhibitory, Gi-coupled DREADD, hM4Di. Expression and functional activation were validated via histology. A single *systemic* dose of the Designer Drug, Clozapine-N-Oxide (CNO; 5mg/kg; IP) induced cFos in the lateral hypothalamus, increased SPA, and energy expenditure (indirect calorimetry) 4hrs post-injection compared to vehicle. Females exhibited higher sensitivity to the lowest dose of CNO (1mg/kg) tested, leading to a significant sex-difference. However, no difference between sexes was observed at the dose (5mg/kg) selected for use in subsequent experiments. Under standard chow conditions, acute CNO treatment did not affect body weight. Eating and drinking patterns were altered in a complimentary fashion such that total food and water intake were unchanged. To test if elevated SPA protects against an obesogenic diet, males were housed on 45% high fat diet (HFD) and given repeated daily injections of CNO or vehicle. After 10d, adult mice (4.5M) showed significant weight loss and improved adiposity, while aged mice (9M) exhibited similar effects after only 5d. This study demonstrates the ability of SPA to counteract HFD-induced weight gain. Many medical conditions, in addition to obesity, stand to benefit from enhanced physical activity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1:

INTRODUCTION AND OVERVIEW OF CONTRIBUTIONS TO THE FIELD

The work described in this dissertation fills a gap in the scientific understanding of how the brain regulates physical activity and presents unique research uncovering a refined therapeutic target for treating obesity, specifically orexin neuropeptide producing neurons. First, a literature review (Ch.2) introduces the neurobiology of the orexin signaling system. Briefly, orexin neurons are located in the Lateral Hypothalamus (LH) and exhibit a medial to lateral functional gradient. Medial cells are more strongly associated with circadian and stress effects compared to orexin neurons in the lateral LH, which are shown here (Ch. 3) to have robust effects on physical activity (Harris and Aston-Jones, 2006). Effects of gender, age, and environment on orexin and physical activity levels are also discussed. Next, Ch. 3 describes original experimental research investigating the functional output and clinical relevance of orexin neurons in LH of mice. Pharmacosynthetic neuromodulation selectively excited or inhibited orexin neurons in the lateral LH. Stimulation of orexin neurons increased physical activity and caloric output whereas inhibition reduced activity and energy expenditure. Importantly, total food and water intake remained unchanged. Daily orexin neuron stimulation was sufficient to mitigate the effects of a high fat diet on weight gain and adiposity. Isolated effects on energy *output* and no change in caloric *intake* make orexin neurons in the lateral LH an extremely promising target for counteracting the deleterious effects of sedentary lifestyles and calorie dense diets.

When this dissertation research was first initiated in 2013, the field lacked direct evidence that orexin neuron activity drives spontaneous physical activity and can do so in a clinically meaningful way. Selective pharmacosynthetic targeting and modulation of orexin neurons was based on a similar approach previously published by Dr. Takeshi Sakurai and colleagues (Sasaki et al., 2011). Differences in experimental findings are compared to previously published results utilizing similar methods of orexin neuron neuromodulation (Sasaki et al., 2011; Inutsuka et al., 2014). The technique reported here targeted a more spatially restricted population of orexin neurons in the lateral and caudal extremes of the lateral hypothalamus. Additional experiments explore the influence of environment, sex, age, and time of day on the functional output of orexin neuron neuromodulation. The novel,

preclinical data presented here provide strong evidence in support of developing orexin-neuron based therapies humans. In full, this dissertation identifies a promising biological target for treating obesity and describes approaches for developing orexin-based therapies in the lab and clinic.

CHAPTER 2:

NEUROBIOLOGY AND ENVIRONMENTAL REGULATION OF THE OREXIN SYSTEM

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PHYSICAL ACTIVITY AND THE OREXIN NEUROPEPTIDE SYSTEM

Physical activity can improve overall health. For example, it can prevent obesity and reduce age-associated cognitive decline. There is wide variation between individuals in their drive to be physically active. The drive for physical activity is operationally defined as spontaneous physical activity (SPA). In humans, SPA includes time spent standing and ambulating, but not voluntary exercise. The energy expended by SPA is termed "nonexercise activity thermogenesis" or NEAT. Exercise is a necessary part of a healthy lifestyle but many people cannot or do not exercise. New treatments to target exercise-independent aspects of achieving and maintaining a healthy weight are greatly needed. Spontaneous physical activity is an excellent candidate, but our understanding of the brain mechanisms driving SPA is incomplete. Therapies that enhance SPA will contribute to better clinical outcomes for obesity and metabolic syndrome, diseases of high prevalence in the developed world. This review describes recent advances in our understanding of neuronal processes that regulate SPA, with a specific focus on changes that occur in the orexin neuropeptide system during normal and pathological aging.

The orexin (hypocretin) neurons are a group of hypothalamic neurons defined by expression of the orexin peptides. The orexin signaling system regulates a variety of complex behaviors, including sleep/arousal, reward, food intake and SPA, with an overall effect of increasing energy expenditure. Orexin neuron activity is affected by multiple environmental and physiological variables like fasting and circadian rhythms. Function of the orexin system varies with lifestyle and age, as does its ability to influence factors that contribute to pathological weight gain in humans and animals. Clarifying how these two variables impact orexin-induced SPA will facilitate development of improved obesity prevention and treatment programs.

Orexin Neuropeptides and Receptors

The orexin signaling system consists of two orexin peptides (orexin A and orexin B) and two G-protein coupled receptors (orexin receptor 1, OXR1 and orexin receptor 2, OXR2) (de Lecea et al., 1998; Sakurai et al., 1998). Orexin A and orexin B are 33- and 28-amino acid peptides cleaved from a single gene product, prepro-orexin (Sakurai et al., 1998).

Orexin A has equal affinity for both orexin receptors, while orexin B preferentially binds to OXR2 (Sakurai et al., 1998; Ammoun et al., 2003). Both OXR1 and OXR2 couple to the $G_{q/11}$-alpha subunit to activate phospholipase C and induce cation influx, thereby depolarizing neurons and increasing excitability (de Lecea et al., 1998; Zhu et al., 2003). When overexpressed in cultured cells, OXR2 also signals through the pertussis-toxin sensitive $G_{i/o}$-alpha subunit to reduce cAMP production (van den Pol et al., 1998; Zhu et al., 2003). Electrophysiological studies of cell types that endogenously express a single OXR subtype *in vivo* confirm that orexin receptors are generally excitatory in nature and can affect neuronal activity via both presynaptic and post-synaptic mechanisms (Zhu et al., 2003; Aracri et al., 2013; Schöne et al., 2014). Like the other neuropeptide systems lacking known reuptake transporters, it is believed that orexin signaling is terminated through diffusion, receptor sequestration, and enzymatic degradation.

The expression pattern of the orexin receptors differs widely among brain sites but is often complimentary in nature. Most brain sites investigated thus far predominately express a single receptor subtype and those that express both subtypes typically do so in separate cell types (Trivedi et al., 1998; Marcus et al., 2001). Functional differences between the two orexin receptor subtypes are not clearly delineated. Many studies are limited by the use of methods that affect both receptor populations, as is the case with exogenous administration of orexin A and genetic manipulations of the prepro-orexin gene. Direct comparison of OXR1 and OXR2 knockout mice report contributions of both subtypes to body weight and sleep patterns, albeit with one receptor subtype typically displaying a greater effect (Funato et al., 2009; Mieda et al., 2011).

Orexin signaling takes on a modulatory nature in many experimental paradigms. Behavioral or physiological effects differ depending on the brain site of action. In other words, the function of the brain area in which orexin signaling is being manipulated is the primary determinant of the particular orexin-dependent effects that are observed at both the behavioral and cellular levels. For example, orexin A signaling via OXR1 in the periaquaductal gray area induces analgesia through cannabinoid-mediated retrograde inhibition whereas OXR1 signaling in the dorsal hippocampus facilitates excitatory LTP

and formation of new associative memories (Ho et al., 2011; Riahi et al., 2013; Yang et al., 2013). Thus, while it is tempting to assign distinct functions to each receptor subtype, the currently available body of data does not fully support a simple, dichotomous characterization. A more refined understanding is needed of functional dissociations in brain-site specific receptor subtypes and the molecular mechanisms underlying them.

Orexin Neurons

In the mammalian brain, orexin neurons are concentrated in the lateral hypothalamus (LH), perifornical area, and dorsomedial hypothalamus (Peyron et al., 1998). Orexin fibers are found throughout the central nervous system (CNS), including nuclei in cortical and limbic areas, basal ganglia, midbrain, brainstem, and spinal cord (de Lecea et al., 1998; Peyron et al., 1998; Taheri et al., 2001; Colas et al., 2014). In addition to orexin, these neurons synthesize glutamate, as well as other neuropeptides, notably dynorphin (Chou et al., 2001; Rosin et al., 2003; Torrealba et al., 2003). Orexin neuron activity is affected by a variety of metabolic signaling molecules (i.e., glucose, leptin, amino acids) and environmental factors which will be discussed in more detail below (Yamanaka et al., 2003; Karnani and Burdakov, 2011; Karnani et al., 2011; Leinninger et al., 2011). For example, activity levels of orexin neurons, as measured by the immediate early gene Fos, increased during the waking phase of the circadian cycle and during fasting or caloric restriction (Sakurai et al., 1998; Estabrooke et al., 2001). Orexin neurons, in turn, regulate physiological and behavioral processes that have major impacts on energy balance and metabolic state, physical activity, blood glucose levels, and food intake (Sakurai et al., 1998; Akiyama et al., 2004; Alam et al., 2005; Kotz et al., 2006; Inutsuka et al., 2014).

As the orexin neurons are known to modulate multiple behaviors, it has been suggested there are functionally specialized subpopulations of orexin neurons, yet this critical issue remains unresolved. The most well-known hypothesis proposes that orexin neurons located in the lateral portion of the LH mediate reward behaviors and those located more medially in the perifornical/dorsomedial areas are involved in arousal and stress (Harris and Aston-Jones, 2006; Harris et al., 2008). This theory is in part supported by the observation that circadian fluctuations in Fos expression in orexin neurons are most pronounced in the

medial LH and less so in the more lateral portions, as well as, by differential activation of orexin neurons in reward behavioral paradigms (Estabrooke et al., 2001; Harris and Aston-Jones, 2006; Harris et al., 2008). However, orexin neurons send collateral projections throughout the CNS, indicating that anatomical location of orexin cell bodies is unlikely to be the most informative criterion when attempting to identify or predict functional specialization of orexin neurons. Accordingly, subpopulations of orexin neurons have been described based on electrophysiological and morphological variables (España et al., 2005; Oldfield et al., 2007; Schöne et al., 2011). Analysis of orexin neuron projections to the ventral tegmental area and locus coeruleus revealed that differences in electrophysiological properties and neuronal architecture are better parameters compared to location of soma when attempting to categorize distinct subpopulations of orexin neurons (Schöne et al., 2011; González et al., 2012). While there is some degree of specialization of orexin neurons, the characteristics that define specific subpopulations and whether they have overlapping or unique functions remain poorly defined.

OREXIN AND ENERGY EXPENDITURE

Orexin peptides modulate energy metabolism, arousal, and physical activity (Chemelli et al., 1999; Hara et al., 2001; Kiyashchenko et al., 2002; Mileykovskiy et al., 2005; Adamantidis et al., 2007; Takahashi et al., 2008; Sasaki et al., 2011; Inutsuka et al., 2014). Orexin system activity is positively associated with activity levels in animals and humans (Kiyashchenko et al., 2002; Wu et al., 2002; Kok et al., 2003). Orexin signaling promotes obesity resistance via enhanced SPA and energy expenditure (Perez-Leighton et al., 2012). Animal models lacking a functional orexin system develop obesity despite consuming fewer calories than their wildtype counterparts (Hara et al., 2001, 2005). Pathological weight gain in these animals is most likely due to energy imbalance resulting from reduced physical activity. Animals in which there is progressive loss of orexin neurons display more severe obesity phenotypes than mice who are only deficient in prepro-orexin, indicating that multiple factors and signaling systems coalesce in orexin neurons to regulate body weight (Hara et al., 2005). To complement genetic ablation approaches, pharmacological studies of repeated orexin A injection into the brain result in

body weight loss and protection against obesity (Novak and Levine, 2010; Perez-Leighton et al., 2012; Teske et al., 2013). Indeed, selective activation of orexin neurons in the LH via Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) stimulates SPA, food intake, and energy expenditure (Inutsuka et al., 2014).

Orexin-dependent modulation of SPA involves several brain sites with site-specific participation of OXR subtypes (Kiwaki et al., 2004; Thorpe and Kotz, 2005; Kotz et al., 2006). Data from our laboratory and others show that a major effect of orexin A signaling is to promote SPA and NEAT (Kotz et al., 2006;Inutsuka et al., 2014). Increased SPA and NEAT are observed following injection of the orexin peptides directly into the rostral LH, hypothalamic paraventricular nucleus, nucleus accumbens, locus coeruleus, dorsal raphe nucleus, tuberomammillary nucleus, and substantia nigra (Kotz et al., 2002, 2006; Kiwaki et al., 2004; Thorpe and Kotz, 2005; Novak and Levine, 2010; Perez-Leighton et al., 2012; Teske et al., 2013). Of these sites, our work suggests that orexin A in the rostral LH has the greatest effect on SPA. As this brain area has been the focus of previous reviews the reader is referred to those reviews for additional information (Kotz et al., 2008, 2012; Teske et al., 2010). It is worth emphazing that the effect of orexin A on SPA is a primary outcome that occurs within minutes whereas effects on body weight are considerably more delayed (Teske et al., 2010; Perez-Leighton et al., 2012).

Orexin, energy expenditure, and obesity

The strong correlation between orexin signaling, SPA, and NEAT, makes orexin an attractive anti-obesity target. Indeed, selective activation of orexin neurons is sufficient to drive increased SPA and energy expenditure in mice (Inutsuka et al., 2014). Many reports exist implicating reduced physical activity and NEAT in the etiology of obesity in humans (Levine et al., 1999, 2005). Our work using two different animal models of obesity reveals a strong link between endogenous orexin function, SPA, and body weight. In rats selectively bred for their weight gain in response to high-fat diet (HFD), obesity resistant rats have higher sensitivity to the behavioral effects of orexin A (Levin, 1991; Teske et al., 2006, 2013). Over time, HFD decreases SPA in obesity prone animals, whereas obesity

resistant rats maintain pre-HFD levels of SPA and sensitivity to orexin-induced SPA (Perez-Leighton et al., 2012, 2013). Additionally, higher SPA in obesity resistant rats predicts lower fat mass gain throughout their lifetime (Teske et al., 2012). Consistent with these findings, non-selectively bred rats that display greater levels of SPA are significantly more resistant to pathological weight gain induced by a HFD compared to animals with naturally lower SPA (Perez-Leighton et al., 2012, 2013). Animals who are resistant to diet induced obesity also exhibit higher expression of prepro-orexin in the LH and enhanced sensitivity to effects of orexin A in rostral LH on SPA (Perez-Leighton et al., 2012, 2013). Importantly, 10 daily treatments of orexin A administration into the rostral LH prevented HFD induced obesity without altering caloric intake (Perez-Leighton et al., 2012). Together, these data implicate orexin signaling in determining sensitivity to diet induced obesity and provide clear evidence that orexins regulate energy expenditure through SPA and NEAT.

Animal models of diet-induced-obesity consistently display attenuated levels of orexin signaling molecules in both the CNS and peripheral tissues (Kotz et al., 2005; Zhang et al., 2005a,b; Sellayah and Sikder, 2014). Similarly, obese humans have lower circulating levels of orexin and impaired orexin receptor activity in adipose tissue (Adam et al., 2002; Digby et al., 2006). No comparable studies have been performed investigating differences in the orexin system in the CNS of obese and healthy humans. Unlike in animal studies, we are unable to distinguish between the contributions of individual differences in orexin signaling that predispose humans to develop obesity, and the consequences of environmental effects of calorie-rich diets and sedentary lifestyles (Kotz et al., 2006; Perez-Leighton et al., 2012, 2013). Nonetheless, physical activity is a promising candidate for improving clinical outcomes in aged humans at both the metabolic and neurological levels (Castaneda et al., 2002; Larson et al., 2006).

Orexin, energy expenditure, and narcolepsy

There is a near complete loss of central orexin production in human narcolepsy with cataplexy, as measured by orexin immunoreactivity in post-mortem brain slices (Nishino

et al., 2000; Peyron et al., 2000). Human narcoleptic patients suffer from extreme episodes of daytime sleepiness. In both humans and animals, narcolepsy is accompanied by higher BMI, increased prevalence of obesity, and reduced physical activity levels (Daniels, 1934; Hara et al., 2001; Kok et al., 2003; Heier et al., 2011). It should be noted that some research groups have attempted to correlate BMI with orexin levels in blood or CSF, samples which can be relatively easily obtained in a clinical setting. However, studies of circulating orexin, either in serum or CSF, should be interpreted with caution, as one study reported no correlation between orexin A concentrations in serum and CSF samples in either control or narcoleptic patients (Dalal et al., 2001). Here, narcoleptic individuals had normal serum levels of orexin A yet CSF levels were below detectable levels, in agreement with post-mortem tissue analysis showing a widespread loss of orexin production in the hypothalamus (Nishino et al., 2000; Dalal et al., 2001). Perhaps of greater consequence is the issue that measures of freely available orexin neuropeptides do not effectively capture orexin neuropeptide concentrations at important sites of action in the CNS or peripheral tissues nor will this approach fully appreciate the dynamic changes that may be occurring in the signaling system as a whole, including changes in receptor efficacy and cellular excitability (Estabrooke et al., 2001; Kiyashchenko et al., 2002; Wu et al., 2002). Despite these methodological limitations, selective optogenetic or DREADD stimulation of orexin neurons unmistakably rescues deficits in sleep and wake patterns in mouse models of narcolepsy (Adamantidis et al., 2007; Hasegawa et al., 2014).

Central orexin and peripheral physiology

As described above, a critically important function of the orexin system is its ability to maintain a healthy energy balance by driving physical activity. Orexins act at sites both in the brain and peripheral tissues to regulate physiological processes that contribute to body weight, notably, glucose mobilization, utilization, and adipocyte differentiation (Cai et al., 1999; Sellayah et al., 2011; Tsuneki et al., 2012). The overwhelming majority of orexin production occurs in the hypothalamus, yet orexin signaling is not limited to the CNS (Sakurai et al., 1998). Small amounts of orexins produced by the enteric nervous system and secretory organs result in circulating plasma levels that are a fraction of those observed

in the brain (Sakurai et al., 1998; Kirchgessner and Liu, 1999). Importantly, orexin A given intravenously or intranasally to non-human primates is able to rescue cognitive impairments due to sleep-deprivation, indicating central action of systemically administered neuropeptides and viability of clinical applications (Deadwyler et al., 2007).

Orexin receptors are found in a number of tissues outside of the brain, including adipose tissue, gonads, and the gut (Jöhren et al., 2001; Digby et al., 2006; Ducroc et al., 2007). While most tissues display relatively low levels of orexin receptor expression there is approximately four-fold higher expression of OXR2 in the adrenal glands of rats than of that in the brain (Jöhren et al., 2001). This is consistent with our understanding of the orexin system being involved in HPA-activation and the responses to physiological and environmental stressors. Although the functional significance is unclear, it is worth noting that orexin receptor levels in the adrenal cortex are dysregulated in an animal model of diabetes (Jöhren et al., 2006).

Numerous studies indicate a clear relationship between central orexin signaling and pathological changes in peripheral physiology. Selective loss of orexin neurons in the hypothalamus of mice increases susceptibility to diet-induced obesity and age-related weight gain, despite having an intact orexin system in peripheral tissues (Hara et al., 2001, 2005). As expected, transgenic mice engineered to over-express prepro-orexin, thereby increasing orexin signaling tone, exhibit improved insulin-sensitivity and protection against the negative effects of a HFD on adiposity (Funato et al., 2009). Furthermore, DREADD-dependent activation of orexin neurons in food-deprived mice promoted glucose mobilization into the blood stream, suggesting enhanced ability to access energy stores during a state of energy imbalance (Inutsuka et al., 2014). As a whole, the studies described above demonstrate the importance of orexin signaling in promoting healthy energy balance through coordinated mechanisms in both the CNS and in the periphery.

Effects of Life-Style Choices on Physical Activity and the Orexin System[1]

Evidence that moderate, aerobic physical activity has positive effects on health and body weight is well established. One of the most well characterized phenomena is the ability of physical activity to improve cognitive performance (Colcombe et al., 2004, 2006; Lindwall et al., 2008; Erickson et al., 2011; Miller et al., 2012). This is a two-way interaction, as choices made throughout life and aging, either directly or indirectly, impact physical activity levels. This section focuses on how excessive calorie consumption (i.e., over-nutrition), which commonly results in obesity and metabolic syndrome, affects physical activity, in particular, SPA, and the orexin system.

In the current climate of rising obesity trends, a great deal of focus has been given to the deleterious effects of sedentary lifestyles on body weight and overall health. Studies have reported that obese individuals spend significantly less time engaged in physical activity. Lean people spend an extra 150 min per day moving compared to obese people, while obese patients sat for 2 h longer per day than lean individuals (Levine et al., 2005). This difference in SPA equates to an additional energy expenditure of 5 kcal/kg in non-obese participants, indicating excellent therapeutic potential for treating pathological body weight (Levine et al., 2005). Severity of obesity (measured as accumulation of fat mass) is negatively correlated with NEAT, although this effect only appears in humans after long-term overfeeding (Levine et al., 1999; Schmidt et al., 2012). These data reinforce the view that obesity decreases physical activity, but there is large inter-individual variability in this effect.

Animal studies support the idea that higher SPA prior to overfeeding, as well as increased SPA during overfeeding, protects against obesity (Teske et al., 2006; Perez-Leighton et al., 2012, 2013). Similarly, development and maintenance of obesity is associated with decreased levels of physical activity in rodents (Bjursell et al., 2008). The question then becomes, what brain mechanisms contribute to obesity via regulation of physical activity

[1] Section (pg12-13) authored by CPL. Revised by AZ.

levels? Different lines of evidence support the orexin peptides as key modulators of physical activity, especially in response to nutrition levels and energy availability.

The orexin system is well-placed to both modulate and be influenced by metabolic state. Overall, orexin signaling is suppressed in an obese state (Kok et al., 2003; Perez-Leighton et al., 2012). Caloric restriction, as occurs during food deprivation in animals or dieting in humans, increases orexin mRNA and orexin receptor expression (Mondal et al., 1999; Komaki et al., 2001; Alam et al., 2005). Furthermore, orexin neurons act as adaptive glucosensors and are inhibited directly at higher glucose concentrations, suggesting that hyperglycemia results in decreased orexin signaling (Burdakov et al., 2006; Williams et al., 2008; Gonzàlez et al., 2009). This would promote lower SPA and energy expenditure, contributing to the development of obesity, but there are currently no reported electrophysiological studies comparing orexin neuron activity in lean and obese states.

The short- and long-term consequences of diet and lifestyle on orexin neuron activity merit further investigation. It must be emphasized that orexin neurons are part of a local (intra-hypothalamic) and global (across the brain) network involved in the control of behavior and energy balance (Peyron et al., 1998; Burt et al., 2011; Kotz et al., 2012). Thus, when considering specific mechanisms that contribute to obesity, orexin signaling is but one part of an interconnected system influenced by multiple genetic and environmental factors.

AGING AND THE OREXIN SYSTEM

A number of physiological functions controlled by the hypothalamus vary with age, including SPA, circadian rhythms, and cognitive function. Weight is typically gained throughout early and middle age, followed by gradual, age-associated anorexia (Chumlea et al., 1988; Schoenborn et al., 2002;Sullivan et al., 2002). The evidence reviewed above indicates orexin signaling is an important driver of energy expenditure and modulates energy metabolism via blood glucose levels and food intake. Simply put, increases in physical activity are generally accompanied by greater energy needs. Anecdotally, one might consider the diet of a professional athlete when training compared to off-season calorie consumption. In line with this reasoning, reduced physical activity levels observed in studies of aged humans and animals may underlie decreased appetite and changes in

body weight observed in these populations (Meijer et al., 2001; Schoenborn et al., 2002; Kotz et al., 2005; Bordner et al., 2011). Many patients near the end of life undergo precipitous weight loss, suggesting severe dysregulation of mechanisms that normally maintain a healthy body weight (Aziz et al., 2008). Moreover, elderly populations experience a greater prevalence of sleep disturbances and cognitive decline/dementia (Foley et al., 2004; Corrada et al., 2010). The diminished physical activity, blunted circadian rhythms, and cognitive deficits associated with aging could be readily explained by compromised orexin signaling in the aged brain.

Aging in Humans

Reductions in the orexin system are observed in humans under a variety of conditions in which symptom onset and severity are strongly tied to aging (Drouot et al., 2003; Fronczek et al., 2007, 2012; Karakus et al., 2012). Dramatic drops in body weight often precede the rapid cognitive and physical decline seen in age-related neurodegenerative diseases, clearly indicating disruption of neurological and physiological processes that promote healthy energy balance (Fronczek et al., 2007, 2012; Aziz et al., 2008). While it is clear that patients with Parkinson's and Alzheimer's disease display significant loss of orexin neurons in post-mortem exams, analysis of CSF levels in living patients do not always bear this pattern, suggesting there may be a progressive and possibly sudden loss of central orexin synthesis or compensatory peripheral production (Ripley et al., 2001; Drouot et al., 2003; Baumann et al., 2005; Fronczek et al., 2007, 2012). Some animal studies suggest a tentative link between neurodegenerative disease symptoms and deficits in orexin signaling in monoaminergic and cholinergic neurons in the brainstem and forebrain (Drouot et al., 2003; Wu et al., 2004; Sakurai et al., 2005; Zhang et al., 2005a,b; Downs et al., 2007;Stanley and Fadel, 2012; Fadel et al., 2013; Yang et al., 2013).

Orexin plasma levels are correlated with body weight in postmenopausal females, such that individuals with more circulating orexin A in their blood have lower BMI (Karakus et al., 2012). However, other studies have failed to identify a clear relationship between changes in orexin CSF and plasma levels. For instance, in narcolepsy, where there is a well-known loss of orexin-producing neurons in the brain, there are reports of patients with low orexin

CSF, yet normal orexin plasma levels (Peyron et al., 2000; Dalal et al., 2001). It should be noted that assessments of circulating orexin neuropeptides provide very limited insight into the orexin system as a whole, as they do not accurately reflect the complex minutia of events occurring at vital sites of action in the CNS (see Section Orexin, Energy Expenditure, and Narcolepsy for further discussion). Measuring absolute levels of orexin peptide also fails to capture dynamic changes in orexin receptor signaling or changes in somatodendritic excitability of orexin neurons, which are important factors when considering the overall function of the orexin signaling system. Evidence from non-human primates is in line with this reasoning. There was no detectable difference in orexin B labeling in the LH or serum levels of aged rhesus macaques (25–32 years old) compared to mature adults (9–13 years old), yet there was significantly reduced innervation of orexin B fibers in the locus coeruleus (Downs et al., 2007). Increased levels of orexin in the periphery may be a compensatory response to reduced production in the brain. Therefore, even if peripheral levels of orexin do not decline in aged humans, there may be undetected alterations in prepro-orexin production and/or efficacy of orexin receptor activation in the brain. Unfortunately, given the present lack of investigations using post-mortem human brain tissue or functional imaging, it is still unknown whether age-dependent alterations in physical activity and body composition observed in humans can be attributed to decreased orexin signaling in the CNS.

Aging in Animal Models

Animal models exhibit clear age-related reductions in the orexin system in the hypothalamus and other brain regions (Brownell and Conti, 2010; Sawai et al., 2010; Kessler et al., 2011). Aging appears to have a uniform effect on orexin production throughout the hypothalamus as orexin A labeling is reduced to a similar degree in both medial and lateral portions of the hypothalamus (Kessler et al., 2011). Although there is no overt neuronal loss or degeneration in the hypothalamus of aged rats, there is a substantial age-related decrease of both orexin A and orexin B peptides (Sawai et al., 2010; Kessler et al., 2011). Aging also results in reduction of one or both of the orexin receptors in the brain, with some species-specific differences in orexin receptor expression throughout life (Terao

et al., 2002; Zhang et al., 2002; Porkka-Heiskanen et al., 2004; Takano et al., 2004). As expected, transgenic mice with enhanced orexinergic tone exhibit resistance to both age-related weight gain and diet-induced obesity (Funato et al., 2009; Willie et al., 2009).

Research groups consistently report reduced behavioral efficacy of orexin-neuropeptides in aged rodents. Intraventricular and intrahypothalamic administration of orexin A increased food consumption in adult rats less than 1 year old, but not in aged, 2-year old rats (Kotz et al., 2005; Akimoto-Takano et al., 2006). The ability of both orexin A and orexin B to alter circadian rhythms and increase time-spent awake was also diminished in aged animals (Morairty et al., 2011). Furthermore, age-related loss of prepro-orexin mRNA production in the LH of rodents is accompanied by reduced orexinergic innervation in the hippocampus, basal forebrain, and locus coeruleus, brain regions associated with cognitive decline in neurodegenerative diseases (Zhang et al., 2005a,b; Downs et al., 2007; Stanley and Fadel, 2012).

Central orexin signaling modulates aspects of peripheral physiology (e.g., blood sugar regulation and adipocity), which are critically linked to obesity and often become dysregulated with age (Cai et al., 1999;Tsuneki et al., 2008, 2012; Sellayah et al., 2011; Inutsuka et al., 2014). Animals that do not produce prepro-orexin in the brain develop insulin sensitivity, hyperglycemia, and increased susceptibility to diet-induced obesity, all of which escalate in severity with age (Cai et al., 1999; Hara et al., 2005; Tsuneki et al., 2008, 2012; Sellayah et al., 2011). Age-associated impairments in brown adipose tissue thermogenesis, which contribute to energy imbalance and weight gain, can be rescued by systemic orexin administration (Sellayah and Sikder, 2014). Aging-dependent reductions in brown adipose tissue thermogenesis are further exacerbated in mice lacking orexin neurons (Sellayah and Sikder, 2014). Importantly, dysregulation of insulin signaling is detected in the hypothalamus of prepro-orexin knockout mice *before* abnormal metabolic symptoms occur in the periphery (Tsuneki et al., 2008). Together, these studies indicate that central orexin neuron dysfunction precedes development of overt changes in peripheral tissues that result in metabolic disorders and pathological weight gain.

The studies described above indicate that orexin release and receptor activation in the brain declines with age, but additional studies are needed to determine if this occurs in a consistent, uniform fashion or if some projections are spared or possibly increased in a compensatory manner (Zhang et al., 2002, 2005a,b;Stanley and Fadel, 2012). This will be an important factor to consider when developing therapies that target orexin signaling, as some treatments may be more or less effective with age.

SUMMARY

The hypothalamus is an important regulator of energy balance. Orexin neuropeptide-producing neurons in the hypothalamus integrate metabolic cues (energy availability) and physical activity (energy expenditure). Orexin neurons alter their activity in response to metabolic signals from the periphery, including leptin, glucose, and insulin (Håkansson et al., 1999; Moriguchi et al., 1999; Tsuneki et al., 2002, 2012; Yamanaka et al., 2003; Burdakov et al., 2006; Karnani and Burdakov, 2011; Leinninger et al., 2011). Orexin signaling is positively correlated with physical activity and negatively correlated with adiposity in both humans and animals (Hara et al., 2001; Adam et al., 2002; Perez-Leighton et al., 2012). Aging has an overall inhibitory effect on orexin signaling, which is likely exacerbated by unhealthy lifestyle choices (Kok et al., 2003; Hara et al., 2005; Brownell and Conti, 2010; Sawai et al., 2010; Kessler et al., 2011).

While much has been done in animal models and in humans to show that SPA significantly impacts body weight, metabolic and cognitive health, more work is needed to fully understand the neurocircuitry and molecular mechanisms which regulate SPA, in particular, what happens to this network during aging. Given our current knowledge, therapies should be developed that aim at producing behavioral and lifestyle changes that prevent or ameliorate age-associated declines in physical activity. There is a clear need for multifaceted approaches to altering SPA that include targeted manipulations of the neural systems that drive SPA. Knowing that aging is associated with an altered metabolic and hormonal milieu, an important future research direction is to understand how these molecular changes directly impact orexin signaling and SPA.

In summary, hypothalamic orexin activity fluctuates over the lifespan to impact physical activity and body weight throughout the aging process. Aged animals have reduced levels of orexin peptides and receptors, although the magnitude is species dependent. Consistent with a loss of signaling molecules are diminished behavioral, cognitive, and metabolic responses to administration of OXR agonists; a significant issue to consider when developing therapeutics to enhance orexinergic tone. Elevating orexin system activity during aging has the potential to improve both physiologic and cognitive status. A significant strategy in moving forward will be to focus on developing treatments that selectively enhance orexin neuron activity and/or receptor function.

CHAPTER 3:

NEUROMODULATION OF OREXIN NEURONS REGULATES SPONTANEOUS PHYSICAL ACTIVITY, ENERGY EXPENDITURE, AND DIET-INDUCED OBESITY

Anastasia N. Zink, Amelia A. Holm, Charles J. Billington, and Catherine M. Kotz. Neuromodulation of Orexin Neurons Regulates Spontaneous Physical Activity, Energy Expenditure, and Diet-Induced Obesity. In preparation for Cell Metabolism (submission expected August 2015).

**Ms. Zink designed experiments, collected data, analyzed results and produced the following text in full. Dr.s Billington and Kotz provided mentorship and resources. Ms. Holm assisted with data collection.*

OVERVIEW

Sedentary lifestyles are a major risk factor for metabolic disease and negatively influence overall health. Low levels of the neuropeptide, orexin (hypocretin), are associated with reduced activity levels and obesity in both humans and animals. Similarly, higher orexin

sensitivity predicts protection against diet induced weight gain. Orexin and its receptors regulate energy balance through whole organism behavior (e.g. eating, sleeping, and locomoting) and sympathetic innervation of peripheral tissues. Historically, studies of the orexin neuropeptide system have relied on administration of exogenous orexin or genetic ablation to probe the role of orexin signaling in the brain. These approaches do not allow investigations of the functional consequences of *endogenous orexin release* resulting from orexin neuron activity *in vivo.* The recently developed pharmacosynthetic neuromodulation technique, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), was used to selectively activate orexin neurons in the lateral hypothalamic area (LH) of mice and measure the effects on Spontaneous Physical Activity (SPA).

1. INTRODUCTION

Sedentary lifestyles are associated with obesity, metabolic syndrome, and poor health outcomes (Buscemi et al., 2014). Restoring healthy activity levels throughout the day would have a tremendous impact on rising social and medical costs of obesity, particularly in populations that cannot or do not engage in sustained exercise. The neuropeptide, **orexin (hypocretin),** presents a promising therapeutic target in the brain for achieving and maintaining a healthy body weight. Orexin modulates a number of physiological and metabolic processes that are intricately connected to body weight; notably **Spontaneous Physical Activity (SPA**) and caloric intake (Kotz et al., 2002; Sellayah et al., 2011; Hara et al., 2001; Inutsuka et al., 2014). Humans and animals deficient in orexin exhibit reduced physical activity levels and elevated rates of obesity (Nishino et al., 2000; Schuld et al., 2000; Hara et al., 2001; Perez-Leighton et al., 2012).

Experimentally amplifying orexinergic tone, either through intracranial infusions of the peptide or stimulating orexin neuron activity, increases spontaneous locomotor activity and calories burned (Kotz et al., 2002, 2006; Inutsuka et al., 2014). When administered daily for ten days, intracranial orexin protected rats against weight gain while eating a high fat

diet (Perez-Leighton et al., 2013, 2012). While very promising, the explicit roles of physical activity and energy expenditure in this protective effect remain untested. Moreover, the specific contribution of *endogenous* orexin neuron activity under obesogenic conditions is presently unknown.

Orexin is made by neurons located in the lateral hypothalamus (LH). The LH is a highly heterogeneous brain region with mixed cell populations, complex connectivity patterns, and functional attributes (Peyron et al., 1998; Kotz et al., 2002). The two primary neuron types in the LH are the orexin- and melanocortin concentrating hormone (MCH)-producing cells (Burt et al., 2011). Orexin and MCH neurons are often implicated in opposing effects on cellular, circadian, and metabolic activities (Steininger et al., 2004; Sasaki et al., 2011). Orexin neurons are generally excitatory (glutamatergic) but also produce multiple neuropeptides, including dynorphin and neurotensin; whereas MCH neurons have inhibitory effects through gamma-aminobutyric acid (GABA) signaling (Rosin et al., 2003; Alam et al., 2005; Steininger et al., 2004; Leinninger et al., 2011; Chou et al., 2001; de Lecea et al., 1998). Orexin cells in the LH are theorized to exhibit a medial to lateral functional gradient such that neurons in the medial portions are more involved in circadian and arousal responses while lateral areas are most strongly associated with physical activity (Estabrooke et al., 2001; Clifford et al., 2015; Harris and Aston-Jones, 2006; Harris et al., 2008). In an effort to refine the potentially diverse effects of orexin neuron manipulations, we chose to target the most lateral portion of the LH.

Historically, investigations of orexin neuron function *in vivo* have been limited by an inability to control neural activity in a cell-type dependent manner. Technical advancements in genetics and pharmacology now offer tools to achieve minimally invasive neuromodulation with cellular and anatomic selectivity. Given the chronic, slow developing nature of weight gain and obesity pathophysiology, we selected a pharmacosynthetic or chemogenetic form of neuromodulation, Designer Receptors Exclusively Activated by Designer Drugs **(DREADDs),** which produces sustained alterations in neuronal activity lasting on the order of minutes to hours. DREADDs rely on modified G-protein coupled receptors that have lost affinity for their biological ligand and

gained the ability to be potentially activated by the synthetic, designer drug, **Clozapine-N-Oxide (CNO)** that is physiologically inert in rodents**.** Importantly, DREADD approaches allow for within-subjects experimental designs, which are preferred for studies measuring dependent variables with known individual variation, like SPA (Perez-Leighton et al., 2012, 2013).

Cellular and anatomic targeting was achieved by stereotactic injection of virus containing the DREADD construct, encoded in an inverted open reading frame and flanked by lox-p recombination sites, into the LH of mice engineered to express the DNA-recombinase, Cre, driven by the orexin promoter. The Gq-coupled DREADD, hM3Dq, was chosen for its ability to produce burst-like firing similar to the type of sustained cellular activity observed with neuropeptide release (Inutsuka et al., 2014). A complimentary set of experiments used the Gi-coupled DREADD, hM4Di, for prolonged neuronal silencing. Importantly, *systemically* administered CNO *selectively* activates or inhibits DREADD-expressing orexin neurons in the lateral LH.

We targeted the most lateral and caudal portions of the LH in an effort to focus on an anatomically distinct orexin neuron population. Our manipulations yielded a behavioral and metabolic profile that favored energy expenditure while leaving total caloric intake unchanged. Repeated treatments significantly improved body weight and adiposity outcomes in an obesogenic environment. These results strongly implicate orexin neurons and the lateral/caudal LH as promising therapeutic targets for enhancing SPA and warrant investigation in other metabolic disorders.

2. MATERIALS & METHODS

Mice: Animal use was reviewed and approved by the IACUC of the Minneapolis Veterans Affairs Health Care System (Minneapolis, MN). Adult (8-40 wks) male and female mice, C57/B6J background, were maintained on a 12 h light/dark cycle with food and water *ad libitum*. Generation and initial phenotyping of Orexin-Cre heterozygous (Orexin-Cre) mice and wildtype (Wt) littermates has been described previously (Matsuki et al., 2009). A

subset of male Cre::hM3Dq mice was housed on High Fat Diet (45% kCal from fat; D12492; Research Diets; New Brunswick, NJ) for 10 d or 22 d.

Viral Injection: Animals were anesthetized and placed in a stereotactic apparatus (Kopf Instruments). DREADD targeting was achieved by stereotaxic injection of a Cre-dependent AAV vector expressing double-floxed inverted open reading frame (DIO) around the DREADD transcript and fluorescent tag, all under control of Elongation Factor 1-alpha (EF_{1alpha}) promoter. Vectors (University of North Carolina Gene Therapy Core) were injected into the LHA (AP-1.8/DV-5.0/ML+/-0.8 mm from bregma; 333 nl/5min) of Orexin-Cre or Wt mice. Excitatory neuromodulation was achieved via Gq-coupled AAV2-EF1a-DIO-hM3Dq-mCherry ($1.4x10^{12}$ v.u./ul). Inhibition was achieved via Gi-coupled AAV2-hSyn-DIO-hM4Di-mCherry ($2.0x10^{12}$ v.u./ul). Animals recovered for at least two weeks prior to testing.

Drug Administration: Clozapine-N-Oxide (CNO; 1 or 5 mg/kg; .1ml/20g mouse; IP; Enzo, City, State) or saline vehicle (Veh) was injected via small gauge 3/10CC insulin syringe once per day between 4 and 5 hrs post-lights-ON (Light cycle) or 15 min before LightsOFF (Dark cycle). All CNO and Veh treatments were counter-balanced; with the exception of the HFD intervention in aged (9M) animals, where CNO+HFD occurred prior to Veh+HFD treatments. Acute tests were followed by a 48 hr rest or 'washout' period. To reduce day to day variability in acute experiments, data points are the average of two repeated exposures to each test condition. Repeated treatments in the HFD intervention studies consisted of a CNO or Veh injection once per day for 5 or 10 consecutive days; data points were not averaged in intervention studies.

Histology: Mice were perfused with ice-cold saline and 4% buffered formaldehyde. For cFos analysis, subjects received an i.p. injection of saline or CNO 90 min before transcardial perfusion. Brains were extracted, postfixed overnight and then cryoprotected with ~10% sucrose in 3% formaldehyde (replaced 1/3 of fix with 30% sucrose solution). Brain sections (40 um, sliced on vibratome) were incubated with blocking solution (0.01M PBS, 3% Normal Horse Serum; 0.05% Tween20) for 2 hrs at room temp. Slices were transferred to goat anti-Orexin antibody (1:1000, SantaCruz) and rabbit anti-mCherry antibody (1:2000, Abcam) or rabbit anti-cFos antibody (1:2000; SantaCruz) for 72hrs at

4°C, washed six times (20min each, at RT) in wash buffer and then incubated with 488-conjugated donkey anti-goat IgG antibody and Cy3-conjugated donkey anti-rabbit IgG (1:1000, Jackson ImmunoResearch) overnight at 4°C. Slices were washed five times (20 min each at RT) in wash buffer and one time in 0.01M PBS before being mounted (GoldBio ProLong/Antifade with DAPI mounting media). Semiquantitative cell counts were collected using the classic visual enumeration technique aided by software algorithms for image processing and counting. Digital images were acquired with a Leica SPE3fluorescent microscope and the Leica Application Suite (LAS 3.3.1 build 8976). Bilateral images were collected from every sixth slice containing the lateral hypothalamus. Using Imaris 8.0 Spot Function software, fluorescent soma and/or nuclei were identified by size (12 um and 7 um, respectively) and intensity (difference from background). Colocalization was determined by an experimenter based on overlapping or adjacent spots. Spots were counted using the internal Spot Function algorithms of the Imaris 8.0. Final data analysis was performed using Microsoft Excel and graphs were rendered in GraphPad Prism 5.

Spontaneous Physical Activity (Activity Chambers, 17x17in, MedAssociates™): SPA, or time spent moving (time moving horizontal + rearing), post-injection was quantified via infrared beam breaks in three-axes, X+Y+Z. Test sessions start 3-4 hr post-LightsON. Mice were weighed and placed in the activity chambers for 2 hrs, removed briefly for IP injection and returned to the testing chamber for an additional 4.25 hrs. Animals were given at least three daily acclimation sessions (saline injections) before testing (saline and/or CNO). To eliminate disturbances in SPA caused by handling/injection procedures, data collection started 15min post-injection. Total food intake and fecal boli were determined by collecting waste at the end of the 6hr session.

Metabolic & Behavioral Profiling (Mouse Promethion, SableSystem™): Mice were housed in home-cage conditions with hanging food and water hoppers connected to inverted laboratory balances. An empty plastic tube was suspended from a third laboratory balance and was used to monitor body weight through the test period and Light/Dark cycle. Mice were acclimated to housing conditions for at least 7 d and habituated to handling and injection procedures for at least 3 d prior to testing. SPA, or distance traveled, was

quantified via infrared beam breaks in three-axes, X+Y+Z. Ambient air was passed through the cages (2 L/min) and gases were sampled from multiple points within the cage (250 ml/min). Raw data was collected by SableScreen v2.2 every sec and extracted using Expedata v1.8.2 (SableSystem ™). Indirect Calorimetry was calculated using the respiratory quotient, Vmax O2/Vmax CO2, converted to kCal.

Body Composition (Adiposity): Direct measurements of total body fat, lean mass, free water, and total body water were collected from live animals via ultrasound-based NMR (EchoMRI™). Data collection required brief restraint (~3 min). Reported data are the average of three consecutive scans (~1 min/scan) taken 8-9 hrs post-LightsON, approximately 26-28 hrs following the last treatment. Adiposity index was calculated as the ratio of Fat Mass to Lean Mass. Repeated body composition data collection (and restraint) was separated by 5-10 d. Animals were returned to their home cages for at least 20 hrs before subsequent testing ensued.

Data analysis: All statistical comparisons were within-subjects using Repeated Measures analyses. Differences between normally distributed means were evaluated by a one-tailed Student's t-test for two group comparisons. Parametric one-way and two-way analysis of variance (ANOVA) with the Bonferroni post-hoc correction was performed for pairwise comparisons amongst multiple data sets. Data were processed in Microsoft Excel. Statistical analyses and graphing was carried out using GraphPad Prism 5. All data are expressed as mean values± S.E.M.

3. RESULTS

3.1 Expression and functional activation of DREADD-mCherry in lateral LH orexin neurons

Genetic and stereotaxic approaches were used to refine the cellular and anatomic targeting of DREADDs; restricting expression to orexin cells in the most caudal and lateral portions of the LH (Fig. 1). We employed a transgenic Orexin-Cre mouse line, which produces the Cre-recombinase transgene driven by the prepro-orexin-promoter, previously described

(Matsuki et al., 2009; Sakurai et al., 2011). The presence of Cre is necessary for the DREADD-containing genetic sequence in the virus to undergo recombination at the Dual Inverted Open Reading Frame (DIO); enabling cell-type selective DREADD expression. Adult male and female Orexin-Cre (Cre) or wildtype mice received AAV2-EF1a-DIO-hM3Dq-mCherry (hM3Dq) injection, one site per hemisphere, aimed at the caudal lateral LH (Fig. 1B).

Histological analysis of LH tissue confirmed selective expression of hM3Dq-mCherry in orexin neurons (Fig. 1C, F). Low magnification images capture nearly the entire orexin neuron field and show clear colocalization between immunoreactive channels labeling orexin (green) and hM3Dq-mCherry (red; Fig. 1C, D). High magnification images show expected cytoplasmic labeling of mCherry and orexin (orange arrowheads, middle row; Fig1C). mCherry staining was absent in Wt::hM3Dq controls (Fig. 1C, E).

Functional activation of hM3Dq by CNO (5 mg/kg) *in vivo* was verified by immunofluorescent labeling of the immediate early gene, cFos (red; Fig. 1F). Animals were treated with CNO or vehicle (saline) 5-6 hrs post-LightsON (a timepoint that corresponds with subsequent behavioral experiments during the light cycle and sacrificed 90min later. Photomicrographs show cFos staining in both orexin (red arrowheads; Fig. 1C) and non-orexin positive cells (white wedges; Fig. 1C). High magnification images reflect the expected intracellular distribution patterns with orexin being cytoplasmic and cFos exhibiting nuclear labeling (middle row; Fig. 1F). Some endogenous cFos expression was observed in vehicle treated Cre::hM3Dq mice (Fig. 1H).

Coronal slices of the LH were imaged at low magnification for visual enumeration (Imaris 8.0; Spot Function) to determine the percentage of orexin neurons infected (Fig. 1D, E, F, G). Absolute cell counts and percentages are summarized in Table 1. Similar numbers of orexin cells were observed in all genotype and treatment conditions (Table 1). Our approach infected 69.9% +/-3.18 of orexin neurons in Orexin-Cre mice (Fig. 1D). A few mCherry positive, orexin negative cells were observed in both Wt::hM3Dq and

Cre::hM3Dq near the injection site, likely attributable to cellular and DNA damage sustained during virus infusion or needle movement.

CNO (5 mgkg) induced extensive expression of cFos in the LH. Our treatments achieved activation in 73.76%+/-2.35 of orexin labeled cells. The number of cFos immunoreactive cells (1770.33+/-55.75) was two to three times greater than the number of orexin neurons observed (565+/-19.22). This suggests the presence of a high degree of excitatory intercellular connections between orexin neurons and other cells in the LH. Given the time of day, orexin neurons were expected to be quiescent and exhibited low levels of endogenous cFos (5.01%+/-0.7) in Cre::hM3Dq mice that received a control vehicle (saline) injection.

Table 1 – Cre-dependent transfection efficiency and functional DREADD activation *in vivo.*

Genotype::Virus	Rx	Stain	Red+	Green+	Red+/Green+	% orexin cells
Cre::hM3Dq	–	mCherry (red) Orexin (green)	409.0 (+/-22.11)	567.7 (+/-18.46)	387.7 (+/-24.55)	68.19 (+/-2.56)
Wt::hM3Dq	–	mCherry (red) Orexin (green)	1.0 (+/-0.57)	557.3 (+/-9.74)	0.0 (+/-0.0)	0.0 (+/-0.0)
Cre::hM3Dq	CNO	cFos (red) Orexin (green)	1770 (+/-55.74)	565.3 (+/-19.22)	416.7 (+/-15.93)	73.76 (+/-2.36)
Cre::hM3Dq	Saline	cFos (red) Orexin (green)	188.3 (+/-20.04)	550.0 (+/-12.00)	27.67 (+/-4.26)	5.012 (+/-0.70)

Table 1. Cells with colocalized expression of orexin and mCherry or orexin and cFos. Red+ cells immunoreactive for mCherry (top two rows) or cFos (lower two rows). Green+ cells immunoreactive for orexin. Cells were counted using Spot Function in Imaris, 8.0. in both hemispheres of every sixth slice containing the lateral hypothalamus. Data reported as mean (+/-SEM) ; N=3 per condition.

3.2 Pharmacosynthetic activation of orexin neurons increases Spontaneous Physical Activity but not Food Intake in male and female mice.

The research described here extends and refines previously published findings utilizing pharmacosynthetic neuromodulation to investigate functional contributions of orexin neurons (Sasaki et al., 2011; Inutsuka et al., 2014). Notably, this is the first instance to directly compare female and male subjects.

Initial investigations of Spontaneous Physical Activity (SPA) were performed in large activity chambers (MedAssociates, St. Albans, VT) and time spent moving (locomotion and rearing) was recorded for 4 hrs following CNO or Veh treatments. Lateral LH orexin neuron activation during the Light cycle (inactive period) dramatically enhanced SPA in both male and female Cre::hM3Dq mice (Fig. 2A, D, E) but not Wt::hM3Dq control animals (Fig. 3A). Sex differences in CNO-induced SPA appeared between male and female mice. This was not driven by differences in basal SPA, as were observed in Wt::hM3Dq mice. The sex difference was most dramatic at the lowest dose (1 mg/kg) tested, which significantly enhanced SPA in females but not males (Fig. 2A). The largest CNO dose tested (5 mg/kg) significantly enhanced SPA to a similar degree in both sexes. For this reason, 5 mg/kg was selected for subsequent experiments.

When tested during the Light (inactive) period, acute pharmacosynthetic activation of orexin neurons results in a sustained, reversible, and dosable effect on SPA (Fig. 2D, E). CNO had no effect on total calories consumed or gut motility, as measured by number of fecal boli passed during the test session (Fig. 2B, C). The size and duration of effects on physical activity levels are comparable to existing reports despite slight differences in methodological approach; specifically a more lateral injection site and a smaller infusion volume (Inutsuka et al., 2014). Intriguingly, our manipulations did not produce gross alterations in food intake, warranting further investigation with higher resolution data capabilities.

3.3 Pharmacosynthetic silencing of orexin neurons decreases Spontaneous Physical Activity and Energy Expenditure during the active (Dark) period.

The well-established relationship between orexin signaling and locomotor activity led us to hypothesize that orexin neuron activation and silencing would modulate SPA in opposing directions. To achieve pharmacosynthetic silencing, we employed the same

approach described above but replaced hM3Dq with injections of virus containing the inhibitory DREADD, *AAV2-hSyn-DIO-hM4Di-mCherry* (hM4Di).

In large activity chambers (17in x 17in; MedAssociates, St. Albans, VT), CNO reduced SPA in a circadian-dependent manner; with significant reductions observed only during the Dark cycle and not during the Light cycle (Fig. 4A, B). CNO-induced inhibition of SPA was explored further under home-cage conditions (SableSystem; Reno, NV), which enabled additional quantification of energy expenditure (EE). Analysis of EE and SPA is presently limited to the first 2hrs of the Dark cycle due to technical limitations in software analysis. CNO treatments given just prior to LightsOFF decreased SPA to a similar magnitude (~50%) in both an open-field environment (Fig. 4A) and home-cage setting (Fig. 4C). Reduced SPA was accompanied by a significant attenuation in energy expenditure (Fig. 4D).

Acute DREADD-dependent inhibition of orexin neurons induced a sustained, reversible effect on SPA and Energy Expenditure (Fig. 4). As with hM3Dq stimulation, hM4Di-induced inhibition treatments had no effect on total calories consumed or gut motility (fecal boli) during the test sessions (Fig. 4E, F).

3.4 Pharmacosynthetic activation of orexin neurons increases Energy Expenditure.

The metabolic impact of enhanced SPA was investigated further under home-cage conditions (SableSystem; Reno, NV), which enabled quantification of energy expenditure (EE; Fig. 5) and other metabolically relevant behaviors (Fig. 6, Fig. 7). SPA and total EE were significantly increased in the 24 hrs following pharmacosynthetic activation (Fig. 5B). Higher resolution analysis identified increased EE during physical activity (ActiveEE) and a slight but not significant increase in EE during inactivity (RestEE; Fig. 5B). All of the 24 hr effects were driven by alterations that were restricted to the 6 hr period following CNO treatment (Fig. 5C). Acute stimulation of orexin neurons in the lateral LH did not impact body weight (Fig. 5).

3.5 Pharmacosynthetic activation of orexin neurons alters consumatory patterns but not overall intake.

Orexin has a long history of involvement in food intake, generally stimulating energy intake as well as energy expenditure and SPA (Kotz et al., 2002; Inutsuka et al., 2014). Unexpectedly, total consumption of chow and water was not affected by pharmacosynthetic stimulation of orexin neurons (Fig. 6, Fig. 7). However, the pattern of intake was altered in the 6 hr period following CNO treatment (Fig. 6B, Fig. 7B). The data show a complementary pattern of increased number and frequency of trips (bouts) to the food and water hoppers (Fig. 6B, Fig. 7B). There is notable reduction in the amount of food and water consumed per trip yet the duration of each bout remained unchanged (Fig. 6B, Fig. 7B). The pattern of consumatory behaviors is consistent with enhanced SPA.

3.6 Repeated stimulation of orexin neurons rescues high fat diet-induced weight gain and improves adiposity

The isolated effects on SPA and EE further strengthened the appeal of testing orexin-neuron based interventions to induce weight loss under obesogenic conditions. Physiological measures were improved in adult (4.5 M) Cre::hM3Dq mice fed a high fat diet (HFD; 45%kcal from fat; Research Diets) in conjunction with 10 consecutive daily CNO treatments. Animals with elevated SPA and EE gained approximately half as many grams (CNO, 1.40+/-0.33; Veh+HFD, 2.55+/-0.43) of body weight (Fig. 8A). Ultrasound-based body composition analysis revealed reduced adiposity levels following the 10 d intervention (Fig. 8B). The ability of DREADD-dependent activation of orexin neurons to induce SPA and EE was preserved in animals fed HFD (Fig. 8C, D). CNO did not alter total HFD or water intake (Fig. 8E, F). Similar behavioral profiles were observed in aged (9M) mice (Fig. 9), except that aged animals required fewer treatments to reach similar amounts of weight loss (Fig. 9A) and adiposity (Fig. 9B).

These findings clearly indicate that orexin neuron stimulation is a viable, effective intervention for diet-induced weight gain. Furthermore, some patient populations may be more sensitive to orexin-based treatments. In total, the results point to developing orexin-neuron and LH-based therapies for treating overweight and obese conditions in patients.

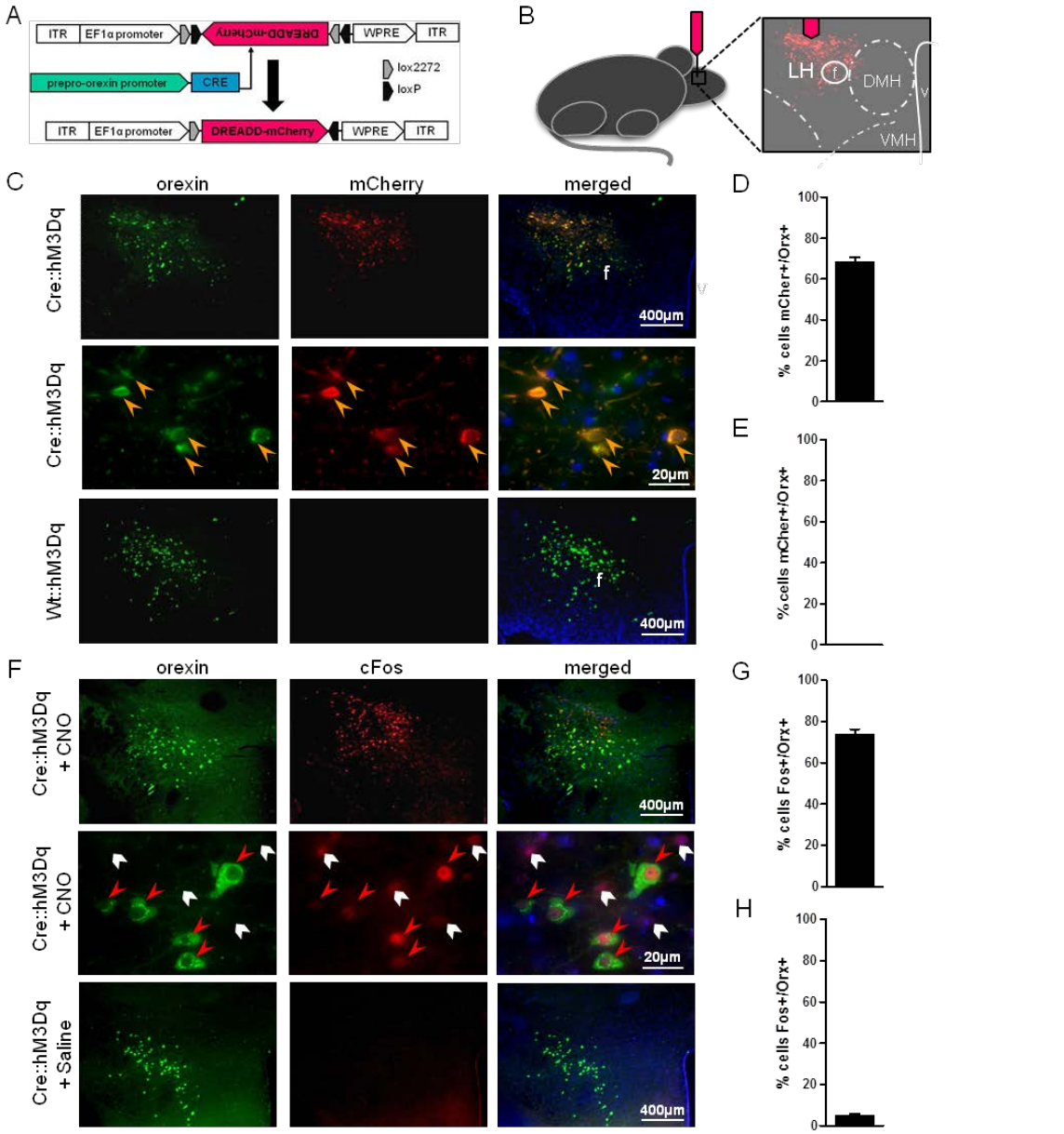

Figure 1. Cre-dependent genetic targeting and pharmacosynthetic activation of orexin neurons. (A) Schematic diagram of AAV vector encoding DREADD-mCherry drive by Elongation factor alpha (EFa1) promoter sequence and flanked by dual flox sites for recombination in the presence of Cre-recombinase. Cre is driven by the prepro-orexin-promoter of Orexin:Cre mice. (B) Schematic of anatomical targeting with stereotactic viral infusion. Photomicrographs of coronal sections containing immunofluorescent orexin neurons (green) and hM3Dq-mCherry (red; C) or cFos (red; F). Percent of orexin neurons expressing mCherry (orange arrow head) in Cre::hM3Dq (D) and Wt::hM3Dq (E) mice. Percentage of orexin neurons expressing Fos (red arrowhead) after CNO (G) or vehicle (E). DAPI (blue), nuclear label; Cre, Orexin-Cre; lateral hypothalamus (LH), fornix (f), dorsal medial hypothalamus (DMH), ventral medial hypothalamus (VMH); inverted terminal repeat (ITR); woodchuck postregulatory regulatory element (WPRE).

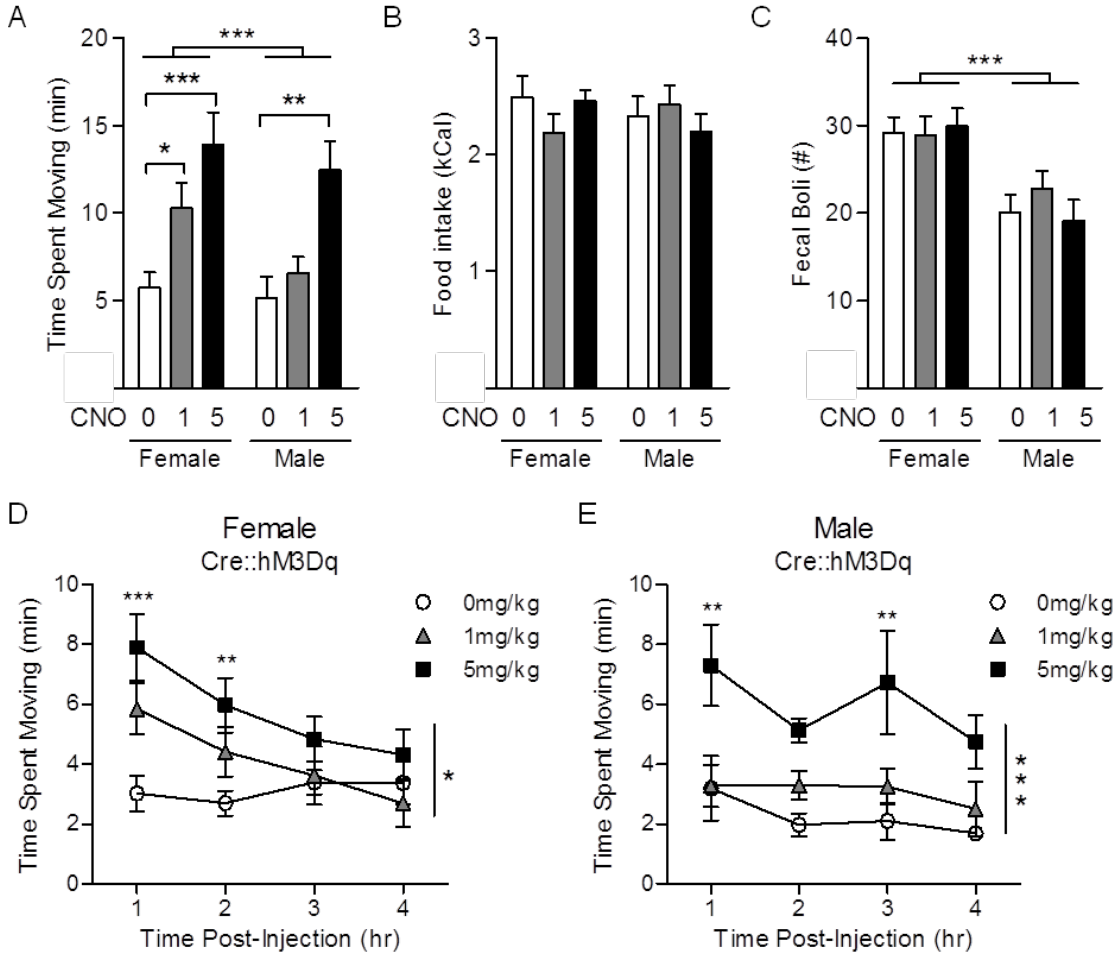

Figure 2. Acute pharmacosynthetic activation of orexin neurons increases Spontaneous Physical Activity in Female and Male Cre::hM3Dq mice (N=21). Behavioral activity in large activity chambers (MedAssociates; St. Albans, VT) 4 hrs after an injection of CNO (5 mg/kg; black bars) or vehicle (0 mg/kg; saline; white bars) 5 hrs into the Light Cycle. Time spent moving (A, D, E), chow intake (B), and fecal boli (C) in female (n=13) and male (n=8) mice. Flat brackets indicate significant main effect of treatment; individual asterisk and downward tick brackets represent post-hoc comparison; *p<0.05, **p<0.01, ***p<0.001.

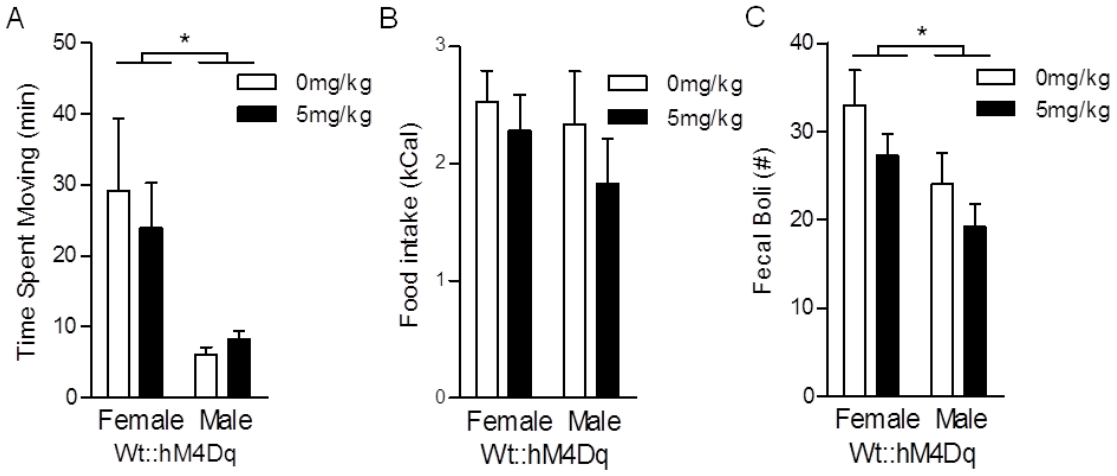

Figure 3. Spontaneous Physical Activity in control (wildtype::hM4Dq) animals (N=15). Physical and metabolic activity in large activity chambers (MedAssociates; St. Albans, VT) 4 hrs after an injection of CNO (5 mg/kg; black bars) or vehicle (0 mg/kg; saline; white bars) 5 hrs into the Light Cycle. Time spent moving (A), chow intake (B), and fecal boli (C) in female (n=7) and male (n=8) mice. Flat brackets indicate significant main effect; *p<0.05, **p<0.01, ***p<0.001.

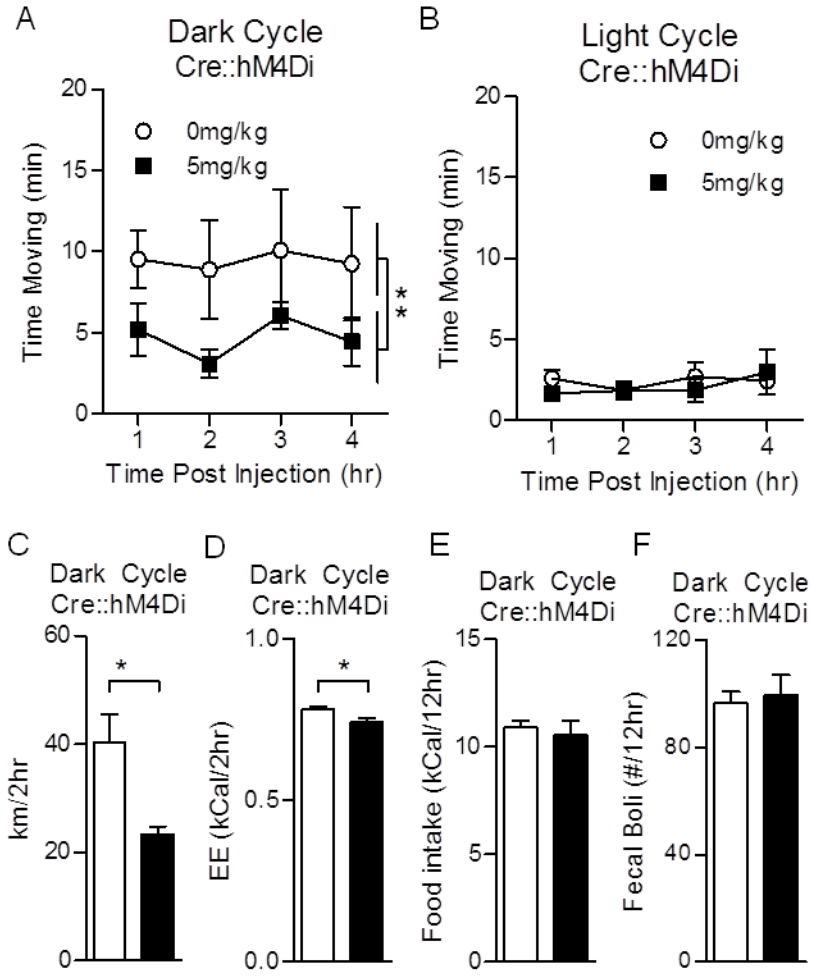

Figure 4. Spontaneous Physical Activity and Energy Expenditure following acute pharmacosynthetic inhibition in Female Cre::hM4Di mice. Time spent moving in large activity chambers (MedAssociates; St. Albans, VT) 4 hr after an injection of CNO (5 mg/kg; black boxes) or vehicle (veh; saline; white circle) 15 min before Dark Cycle onset (A) or 5 hrs into the Light Cycle (B); (N=4). SPA (C) and EE (D) 2 hrs post injection in a home-cage environment (Promethion™; SableSystem, Reno, NV; N=6). Total chow intake (E) and fecal boli (F) during the 12hr Dark Cycle (N=4). Flat brackets indicate significant main effect; downward tick brackets represent post-hoc comparison; *p<0.05, **p<0.01, ***p<0.001.

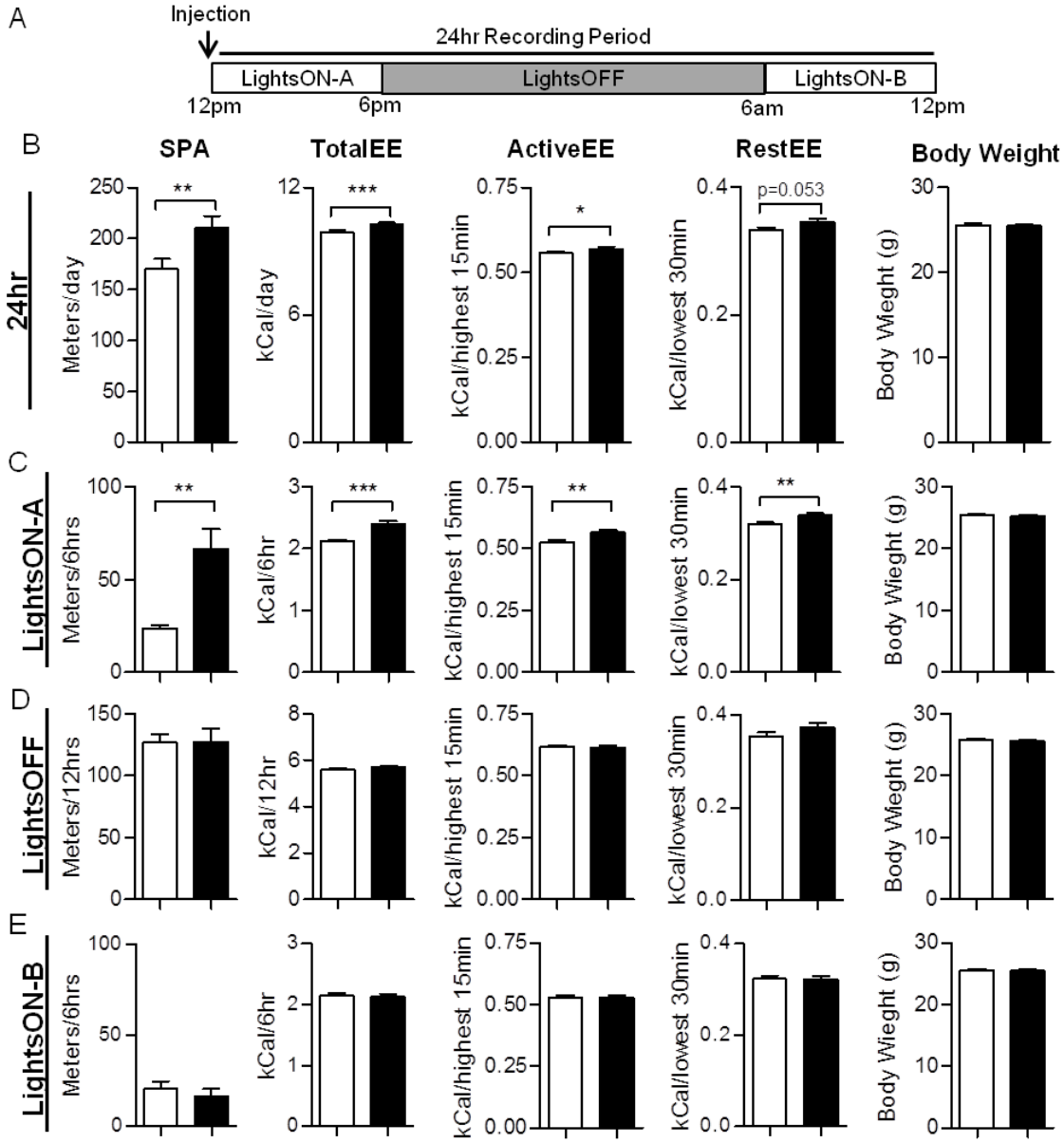

Figure 5. Pharmacosynthetic activation of orexin neurons increases energy SPA and energy expenditure (EE) in the hours following treatment (male, Cre::hM4Dq, N=6). (A) Schematic of treatment and recording periods. Metabolic and behavioral data collected in the home-cage (Sable Promethium; Reno, NV) following CNO (5 mg/kg; black bars) or vehicle (saline; white bars). SPA, TotalEE, ActiveEE, RestEE, and body weight are reported for (B) 24 hr post-injection and divided into (C) LightsON-A (post-injection; 6hr), (D) LightsOFF (12 hrs), and (E) LightsON-B. *p<0.05, **p<0.01, ***p<0.001.

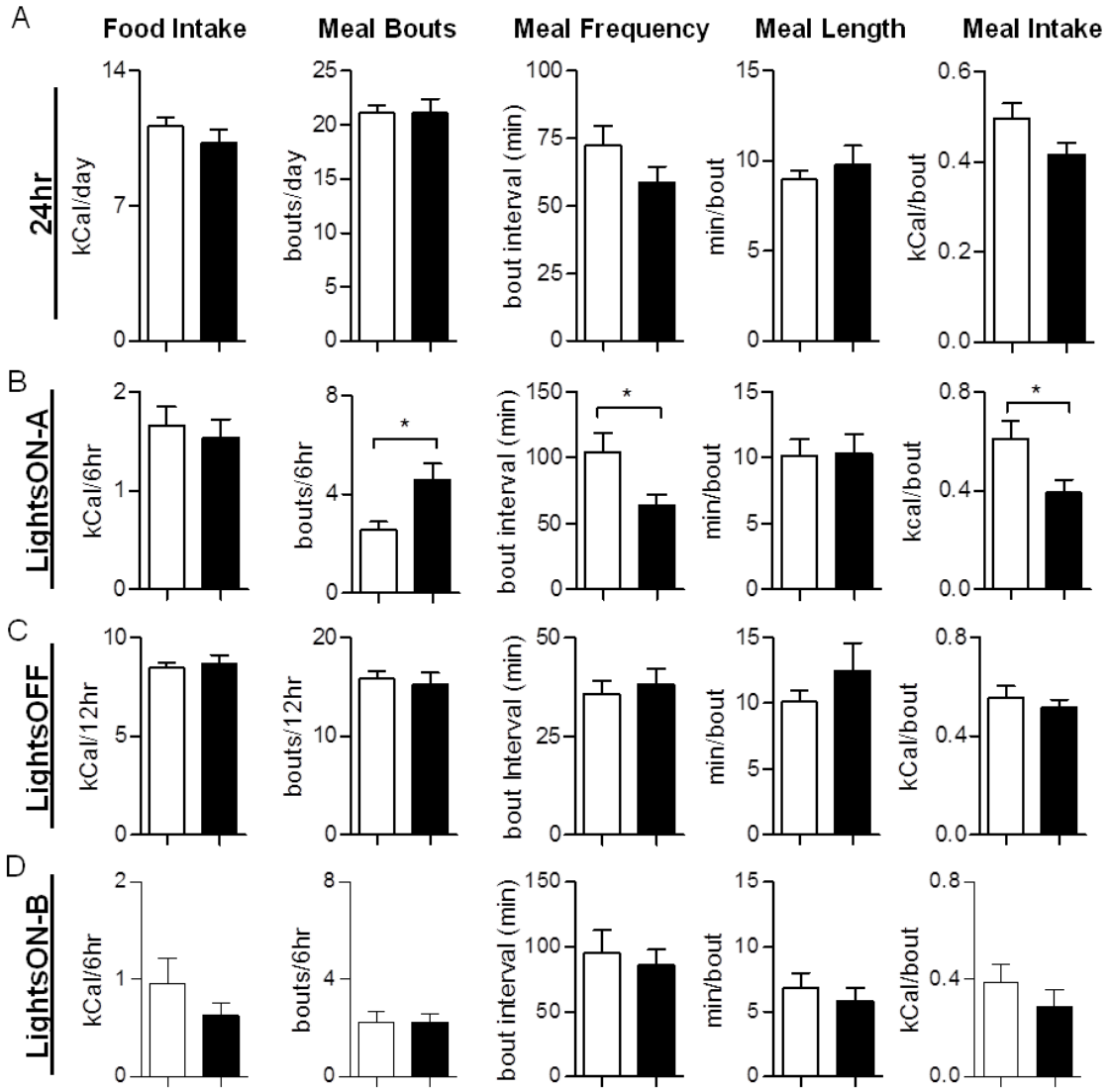

Figure 6. Food (chow) eating behaviors following acute pharmacosynthetic activation of orexin neurons (male, Cre::hM4Dq, N=6). Behavioral data collected in the home-cage (Sable Promethium; Reno, NV) following CNO (5mg/kg; black bars) or vehicle (saline; white bars). Total chow, meals (bouts), bout frequency, bout duration, and bout size are reported for (A) 24hr post-injection and divided into (B) LightsON-A (post-injection; 6hr), (C) LightsOFF (12hrs), and (D) LightsON-B. *p<0.05, **p<0.01, ***p<0.001.

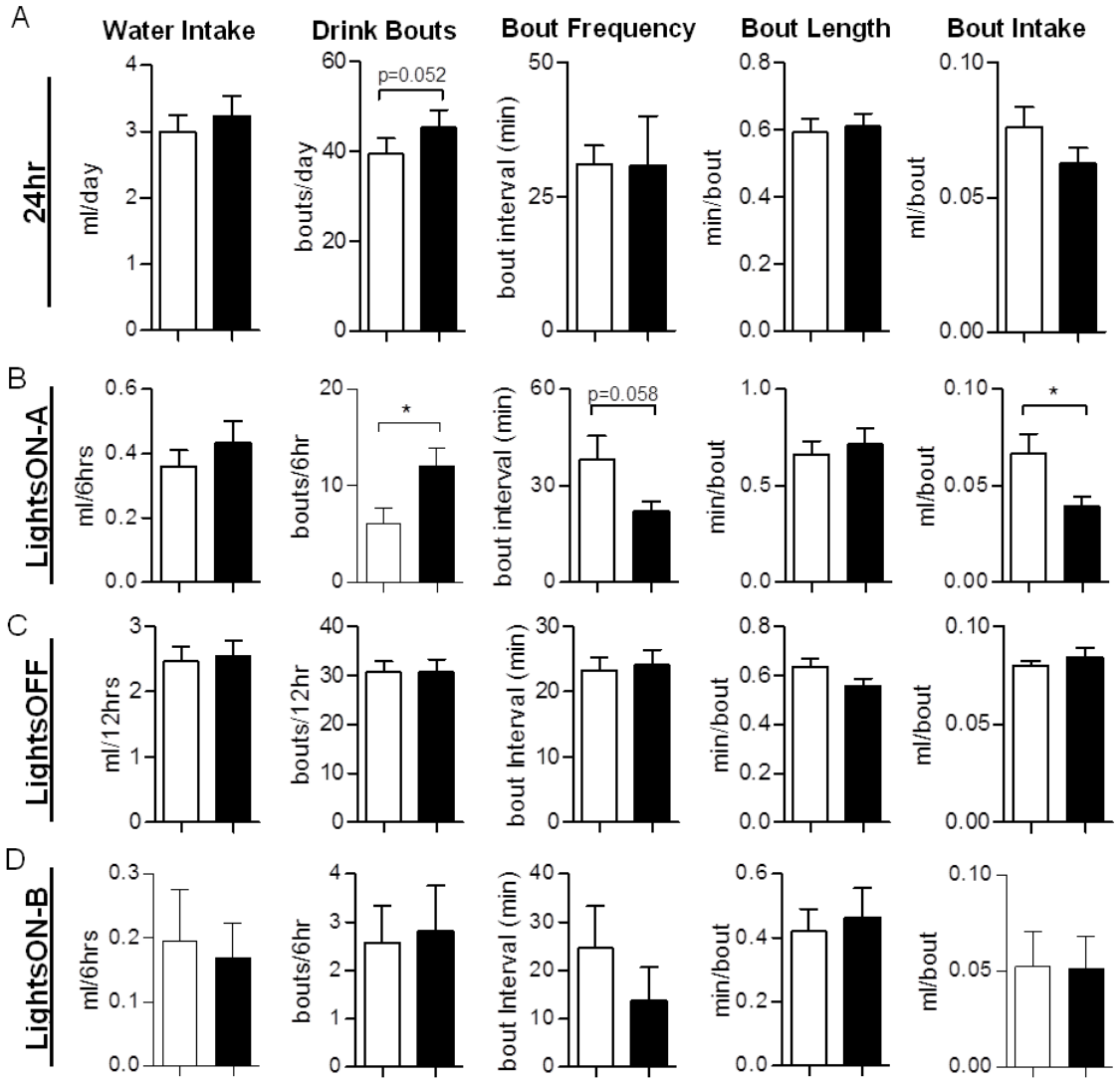

Figure 7. Drinking (water) behaviors following acute pharmacosynthetic activation of orexin neurons (male, Cre::hM4Dq, N=6). Behavioral data collected in the home-cage (Sable Promethium; Reno, NV) following CNO (5mg/kg; black bars) or vehicle (saline; white bars). Total water, drinks (bouts), bout frequency, bout duration, and bout size are reported for (A) 24hr post-injection and divided into (B) LightsON-A (post-injection; 6hr), (C) LightsOFF (12hrs), and (D) LightsON-B. *p<0.05, **p<0.01, ***p<0.001.

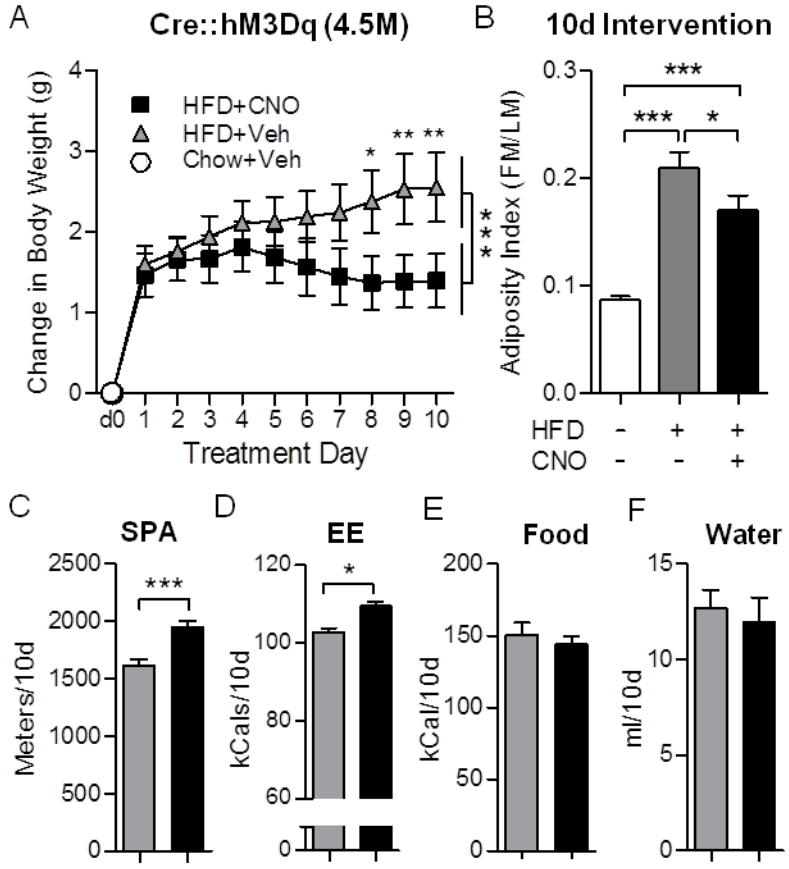

Figure 8. Repeated, daily (10d) pharmacosynthetic activation of orexin neurons protects against weight gain in adult mice on high fat diet (N=14). Metabolic and behavioral collected from adult (4.5 M) male Cre::hM3Dq mice. (A) Weight gain and (B) adiposity (FatMass/LeanMass; EchoMRI; Plano, TX) before (d0; Chow+Veh; HFD-/CNO-; white circle or bar) and after 10 d HFD+CNO (5 mg/kg; black boxes and bars) or HFD+vehicle (veh; saline; grey triangle) treatment. Total physical activity (C), energy expenditure (D), food intake (E), and water intake (F) during 10 d treatment period. Flat brackets indicate significant main effect (2way-ANOVA); individual asterisk and downward tick brackets represent Bonferroni post-hoc comparison (A, B); downward tick brackets represent t-test comparison (C, E); *p<0.05, **p<0.01, ***p<0.001.

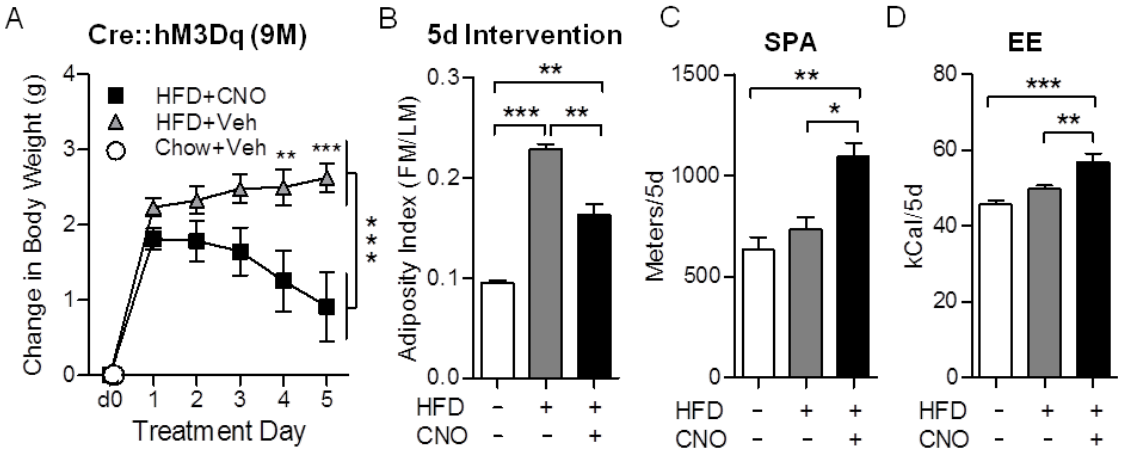

Figure 9. Repeated, daily (5 d) pharmacosynthetic activation of orexin neurons protects against weight gain on high fat diet in adult mice (9 M) male Cre::hM3Dq mice (N=4). Metabolic and behavioral collected from adult (4.5 M) male Cre::hM3Dq mice. (A) Weight gain and (B) adiposity (FatMass/LeanMass; EchoMRI; Plano, TX) before (d0; Chow+Veh; HFD-/CNO-; white circle or bar) and after 10d HFD+CNO (5mg/kg; black boxes and bars) or HFD+vehicle (veh; saline; grey triangle) treatment. Total physical activity (C), energy expenditure (D), food intake (E), and water intake (F) during 10 d treatment period. Flat brackets indicate significant main effect; downward tick brackets represent t-test comparison;*p<0.05, **p<0.01, ***p<0.001.

CHAPTER 4:

DISCUSSION & SUMMARY

Anastasia N. Zink, Amelia A. Holm, Charles J. Billington, and Catherine M. Kotz. Neuromodulation of Orexin Neurons Regulates Spontaneous Physical Activity, Energy Expenditure, and Diet-Induced Obesity. In preparation for Cell Metabolism (submission expected August 2015).

**Ms. Zink designed experiments, collected data, analyzed results and produced the following text in full. Dr.s Billington and Kotz provided mentorship and resources. Ms. Holm assisted with data collection.*

Sedentary lifestyles and high calorie diets are growing health problems, implicated as primary suspects in the rising rates of obesity and metabolic syndrome worldwide. Orexin neuropeptide signaling has been shown to have protective effects on body weight but the specific contribution of SPA is unknown (Perez-Leighton et al., 2012, 2013). We used minimally invasive pharmacosynthetic neuromodulation (DREADDs) to increase or decrease orexin neuron activity *in vivo*. A single, systemic injection of CNO enhanced physical activity and energy expenditure in Orexin-Cre mice expressing the excitatory DREADD, hM3Dq. In mice expressing the inhibitory DREADD, hM4Di, CNO reduced SPA and EE. Experiments uncovered significant differences of sex, time of day, and age on the effects of CNO. There were no effects on total food or water intake and gut function was unchanged. Repeated treatments, once per day, to enhance orexin neuron activity were sufficient to mitigate the effects of a high fat diet on weight gain and adiposity.

The behavioral and metabolic profile produced by DREADD-dependent activation of orexin neurons closely resembles the goals of clinical weight loss; sustained, elevated daily physical activity and energy expenditure without altering total food or water consumption. Furthermore, number of fecal boli was unchanged; suggesting overall GI motility and anxiety-like behaviors were unaffected (Hall, 1934). Inhibition of orexin neurons yielded opposite changes in SPA and EE. Repeated stimulation of orexin neurons produced clinically relevant improvements in a mouse model of diet-induced weight gain. Because there were no changes in caloric intake or gut motility, we can confidently attribute the weight loss and adiposity to enhanced metabolic output. CNO treatments increased SPA at least two-fold in the hours after injection. SPA undoubtedly accounts for a substantial portion of the additional calories burned. However, kCals burned during periods of inactivity (RestEE) was also higher; suggesting that other mechanisms for EE are being activated in addition to SPA. Thermogenesis from brown adipose tissue activation may be involved (Morrison, Madden, and Tupone, 2012). Or, increased RestEE could be a carryover effect from SPA and ActiveEE, akin to post-exercise thermogenesis. Additionally, altered patterns of food intake could result in changes in the thermic effects of food that are captured in RestEE.

4.1 Functional Dissociation of Neural Regulation of SPA, EE, and Food Intake

CNO was able to enhance SPA and EE to a similar magnitude and time course as previous reports (Inutsuka et al., 2014). However, the effects on food and water intake were strikingly different. Inutsuka and colleagues saw a dramatic increase in chow and water intake; whereas, we saw no difference in total consumption. Instead, the pattern of intake was significantly altered in the hours following CNO. Cre::hM3Dq animals interacted more often with the food and water hoppers but behaviorally compensated by reducing the amount consumed per bout.

A comparison of similar experiments suggests that anatomic targeting is essential for refining the behavioral output of orexin neuron stimulation. The methods and results reported here differ in a number of key ways from findings published by other groups who have used Orexin-Cre::hM3Dq mice (Sasaki et al., 2011; Inutsuka et al., 2014). We opted to inject a moderate volume (333nl) into a *single* site per hemisphere aimed at the lateral and caudal extremes of the orexin cell field. The injection site used in this study differed from the Yamanaka group's coordinates by an absolute distance of 0.32mm in the caudal and lateral direction (Inutskua et al., 2014). Furthermore, Yamanaka and colleagues (Inutsuka et al., 2014) injected twice the volume (600nl) per injection site, likely producing a larger infection radius. Similarly, Sakurai, and colleagues (Sasaki et al., 2011) injected two sites per hemisphere, one in the caudal LH and another in the rostral LH. These differences and others may account for the distinct behavioral and metabolic profiles observed in our studies.

The selective anatomic targeting may also explain the higher dose of CNO (5mg/kg) required to induce robust behavioral changes than the dose used by other groups (1mg/kg; Sasaki, et al., 2011; Inutsuka et al., 2014). With the higher dose, we induced cFos expression in a similar overall percentage of orexin neurons as groups that delivered hM3Dq to multiple sites within the LH or a larger infection radius (Sasaki et al., 2011; Inutsuka et al., 2014). The higher dose presumably achieved greater relative activation of lateral LH orexin neurons clustered in the lateral LH, as well as activation of cells receiving

projections from those neurons. However, the potential explanations discussed here will need to be tested empirically.

4.2 Therapeutic Applications: Optimal Environmental and Patient Populations

Neuromodulation of orexin neurons produced a highly desirable suite of behavioral and metabolic changes for achieving weight loss. Considering the slow-developing nature of obesity and metabolic syndrome, treatments will need to be used for an extended duration. The pharmacosynthetic method used here is attractive in the ability to induce relatively long-lasting effects with minimally invasive treatments compared to the chronic implants and fast temporal dynamics of electrical or optical stimulation.

Perhaps of greatest import to clinical developments is the dramatic CNO-induced cFos throughout the lateral and caudal LH, in both orexin and non-orexin cells. Additional studies need to be done to further characterize the anatomic and cellular identity of neurons driving the behavioral profile described above. There is presently no technology available to achieve cell-selective neuromodulation in humans. However, there are many investigational and commercially available devices for achieving selective neuromodulation in *spatially* defined populations.

Sex Differences. Sex and age are both critical variables contributing to weight gain and obesity. This is the first report of selective neuromodulation of orexin neurons in female subjects. We reported a sex-dependent difference in the effectiveness of orexin-neuron stimulation to enhance SPA. Females were more sensitive to acute CNO-dependent activation (Fig 2.); which is in line with known sex-differences in orexin neurobiology (Brownell and Conti et al., 2010). Together, these findings suggest that females may benefit more from orexin-based therapies; although, this was not tested directly. We selected 45% kcal from fat diet (Research Diets) in order to best model the macronutrient content of the ‘western’ diet commonly found in developed countries. Our studies did not assess the ability of SPA to rescue HFD-induced weight gain in females, as female mice do not consistently gain weight on the 45% HFD selected for this study.

Aging. Levels of orexin neuropeptide, physical activity, and body weight vary with age. The interplay between aging and orexin has been reviewed in depth recently (Zink et al., 2014). Multiple sources of evidence suggest that reduced orexin signaling associated with aging may contribute to reduced physical activity levels and gradual weight-gain throughout life (Funato et al., 2009; Willie et al., 2009; Kessler et al., 2011). A small pilot study in aged (9 M; Fig. 9A) mice showed significant weight loss four days before overt changes were observed in adults (4.5 M; Fig. 8A). These findings suggest that orexin-based treatments aimed at weight loss may be more effective in older populations. However, direct comparisons between ages are still needed.

Circadian Rhythms. The ability of orexin neuron inhibition to reduce SPA depends on circadian rhythms, well known to regulate both orexin neuron activity and SPA levels. Exploring the circadian regulation of orexin neuromodulation will be important for understanding how to optimize the treatment for individual patients and disease populations. This study is the first to report significant reductions of SPA and EE using pharmacosynthetic silencing of orexin neurons. We anticipated and observed a pronounced circadian effect. The lack of difference during the light cycle, a period of low intrinsic orexin activity (Fig. 1H), as well as, low physical activity (Fig. 2D) is likely due to a floor effect, such that lower levels of SPA cannot be achieved behaviorally. However, it is possible that the small amount of basal SPA observed during the light cycle is driven and regulated by orexin-independent signaling.

We stimulated orexin neurons in the lateral LH during periods of lowest physical activity in order to maximize potential effects on SPA. This manipulation was sufficiently powerful to overcome the negative effects of excess-calorie consumption. However, the magnitude of SPA induced during the active (dark) period may not be as dramatic since the stimulation occurs during a period of already high endogenous cellular activity, which may contribute to a ceiling effect. In other words, the therapeutic capacity may be diminished or occluded. It remains to be tested whether or not orexin neuron stimulation can further enhance SPA when SPA is at its natural peak during the active/dark period.

4.3 Limitations and Other Considerations

The research and methodological approaches presented here lay the groundwork for exploring the downstream neural output and molecular mechanisms responsible for carrying out the behavioral and physiological changes induced by orexin neuron stimulation. A number of genetic tools, e.g. DREADD-constructs with trafficking sequences for axonal or dendritic cell compartments, could be employed to characterize functional orexin neuron populations and parse projection site specific contributions. Critical follow-up studies are needed to determine the degree to which anatomical targeting explains the difference in food intake we observed from that of Yamanaka's group (Inutsuka et al., 2014).

Our findings suggest that SPA-based interventions work best during times of inactivity. The timing and patterns of sustained inactivity will vary for each person, as these are intricately woven in the complexity of modern day lifestyles and occupations that beget considerable variation in circadian rhythms. Potentially adverse effects on sleep could possibly be mitigated through timing and dosing, perhaps on an individual scale akin to deep brain stimulation tuning in Parkinson's patients.

As with any pharmacological treatment, the possibility of tolerance, dependence, and habituation is a concern. This is particularly true of a weight loss therapy that would need to be used over a long period of time. The likelihood of adverse or unwanted effects produced from orexin-based treatments is relatively high given the diverse physiological and behavioral functions associated with orexin signaling, e.g. sleep, attention, and cognition. Additionally, this study did not test effects of long term repeated DREADD-stimulation in animals with a healthy body weight. Ideally, weight loss therapies can be developed that induce *excess* weight loss but do not affect normal, healthy individuals. These are issues that need to be empirically tested before clinical applications are developed.

SUMMARY

Spontaneous physical activity (SPA) is a promising therapeutic target for improving multiple clinical outcomes in obesity and metabolic syndrome. Orexin neurons in the lateral hypothalamus drive spontaneous physical activity and regulate metabolic rate. Stimulation or inhibition of orexin neuron activity via pharmacosynthetic neuromodulation increased or decreased, respectively, physical activity and energy expenditure. Total food and water intake were unaffected. Repeated, daily stimulation of orexin neurons partially rectifies the metabolic imbalance in obesogenic, high-fat conditions.

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