OREXIN SIGNALING AND THE PREVENTION OF DIET-INDUCED OBESITY

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DEDICATION

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OREXIN SIGNALING AND THE PREVENTION OF DIET-INDUCED OBESITY

by

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OREXIN SIGNALING AND THE PRVENTION OF DIET-INDUCED OBESITY

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The hypothalamic neuropeptide orexin acutely promotes appetite, yet orexin deficiency in humans and mice is associated with obesity. Prolonged effects of orexin signaling upon energy homeostasis have not been fully characterized. In this study, I utilized both genetic and pharmacologic approaches to characterize metabolic effects of orexin gain of function. *CAG/orexin* transgenic mice confer resistance to high-fat diet-induced obesity

and insulin insensitivity by promoting energy expenditure and reducing food consumption. Genetic studies indicated that orexin receptor-2 (OX2R), rather than orexin receptor-1 (OX1R) signaling, predominantly mediates this phenotype. Likewise, prolonged central infusion of an OX2R selective peptide agonist prevents diet-induced obesity. While orexin overexpression enhances the anorectic-catabolic effects of central leptin administration, obese leptin-deficient mice (*ob/ob*) are completely resistant to the metabolic effects of orexin overexpression or OX2R selective agonist administration. I conclude that enhanced orexin-OX2R signaling confers resistance to diet-induced features of the metabolic syndrome through promoting a negative energy homeostasis and improving leptin sensitivity.

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PRIOR PUBLICATIONS

Original Reports

Funato H*, Tsai AL*, Willie JT, Kisanuki Y, Williams SC, Sakurai T, Yanagisawa M. (2009). Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. *Cell Metabolism* **9**(1): 64-76. (*Co-First Authors).

Kim KW, Zhao L, Lee C, Xu Y, Choi M, Lauzon D, Tsai AL, Yanagisawa M, Lutter M, Parker KL, Elmquist JK. (In submission). Steroidogenic factor 1 in the ventromedial hypothalamus is required for diet-induced thermogenesis and mood regulation.

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LIST OF DEFINITIONS

Agouti-related protein (AGRP)- A neuropeptide that binds to MC3R and MC4R as an inverse agonist and stimulates food intake and excess weight gain. Expressed exclusively in the ARH and is localized in NPY expressing neurons.

Arcuate nucleus (ARH) – Most ventral portion of the hypothalamus and surrounds the third ventricle. One of the most important feeding centers in the brain, contains many neurons involved in the regulation of food intake and energy balance.

Hypocretins- See Orexins.

Insulin- A hormone released from the pancreas β-cells that regulates glucose homeostasis through its ability to stimulate glucose uptake and glycogen synthesis in peripheral tissues. Serves as a peripheral indicator and binds to receptors in the ARH.

Lateral hypothalamic area (LHA) - Reticular region of the dorsal and lateral regions of the hypothalamus that is classically associated with regulation of feeding behavior, metabolic rate, and arousal in response to various stimuli. Intrinsic neurons project throughout the neuroaxis, and it also contains crucial fibers of passage including the median forebrain bundle

and the fornix. The perifornical nucleus is often considered to be part of the LHA.

Leptin - An endocrine hormone that is primarily produced by peripheral adipose tissues, and the circulating levels are proportional to body fat stores. Leptin deficiency causes hyperphagia, reduced energy expenditure, and results in obesity.

Metabolic syndrome - A complex disorder with a cluster of interconnected factors that increase the risk of coronary heart disease (CHD), cardiovascular atherosclerotic diseases (CVD), and diabetes mellitus type-2 (DMT2). Obesity and insulin resistance are the underlying core manifestations of the syndrome.

Neuropeptides - Peptides acting as neurotransmitters or hormones that are released by neurons or neuroendocrine cells and mediating slow activity via active G-protein coupled receptors.

Neuropeptide Y (NPY) - Expressed widely throughout the brain, but in the arcuate nucleus, stimulates food intake and is expressed in the same neurons as AGRP.

Obesity- A medical condition in which excess body fat accumulation present a risk to health. Clinically defined by using the body mass index (BMI)

which is a person's weight (kg) divided by the square of his or her height (m). An individual with a BMI greater or equal to 30 is obese.

Orexins - Neuropeptides (orexin A and orexin B, also known as hypocretin 1 and hypocretin 2) expressed in the lateral hypothalamic area, especially the perifornical nucleus. Products of the *prepro-orexin* gene activates two G-protein coupled receptors; orexin receptor-1 and orexin receptor-2, in mice and humans.

Perifornical nucleus- Cluster of neurons in the LHA that surround the fornix and have been implicated functionally in arousal and autonomic regulation. The majority of orexin neurons are found here in mice and humans.

Transgenic mice- Line of genetically engineered laboratory mice in which additional copies of a gene of interest have been randomly inserted in the genome, resulting in gain of function of the gene of interest.

CHAPTER ONE

Introduction and historical perspective

ENERGY BALANCE REGULATION IN THE HYPOTHALAMUS

Metabolic Centres in the Hypothalamus

Historically, it was long thought that there were specific “centres” or brain regions that controlled hunger and satiety. Based on lesion studies performed in the late 1940s, bilateral lesions of the lateral hypothalamic area (LHA) in rats caused anorexia and weight loss, whereas lesions of the hypothalamic ventromedial nucleus (VMN) caused profound obesity (Hetherington 1940). These findings led to the theory that the LHA and VMN were hunger and satiety centres, respectively; this theory ultimately dominated the field of food intake regulation for many decades.

The caveat of lesion experiments is that they were based solely on neuroanatomy. Therefore positive data from lesions of the LHA and VMN were easy to interpret, but misleading conclusions could be drawn from negative data. Based on lesion studies of the arcuate nucleus (ARH), there was mild effect on food intake and body weight regulation, therefore the ARH was considered irrelevant in the control of food intake for decades.

Neuropeptide Y and the Arcuate Nucleus

It was not until the hypothalamic neuropeptide Y (Npy) was discovered that it became evident the arcuate nucleus is actively involved in food intake (Tatemoto, Carlquist et al. 1982). *Npy* is globally expressed in the brain, and was identified based on its similarities to pancreatic polypeptide and peptide YY. Surprisingly, investigators that were studying reproductive biology found that Npy had a powerful stimulatory effect on food intake when centrally administered to rodents (Clark, Kalra et al. 1984). Although mRNA levels cannot precisely resemble neuronal synaptic activity, there is a strong correlation between *Npy* mRNA levels and food intake; specifically, during compensatory hyperphagia due to food deprivation and hyperphagia associated to uncontrolled diabetes, *Npy* mRNA levels are increased in the arcuate nucleus (Sahu, Sninsky et al. 1990; Baskin, Breininger et al. 1999). *Ob/ob* mice are deficient in leptin; a circulating protein secreted by the adipose tissue, and is known to reflect body fat stores (Zhang, Proenca et al. 1994; McGregor, Desaga et al. 1996; Ostlund, Yang et al. 1996; Rosenbaum, Nicolson et al. 1996). *Ob/ob* mice have many phenotypes resembling the human metabolic syndrome; notably, they are obese, hyperphagic and diabetic.

As expected, *Npy* mRNA levels are elevated in the arcuate nucleus in *ob/ob* mice since they are hyperphagic (Wilding, Gilbey et al. 1993); consistently, central leptin administration reduced hyperphagia and *Npy* mRNA levels in the arcuate nucleus (Stephens, Basinski et al. 1995). Importantly, injection of NPY into other brain regions had minimum effect on food intake (Stanley, Chin et al. 1985).

Agouti Related Protein

Besides NPY, the agouti related protein (AGRP) is another neuropeptide that is known to stimulate feeding in the arcuate nucleus. Approximately a century ago, it was known that mice carrying a rare dominant mutation of the agouti gene known as A^y, developed yellow hair coating, increased body length and severe obesity (Castle and Little 1910). The agouti protein is a paracrine molecule that is normally expressed in melanocytes to control hair color through melanocortin 1 receptor (MC1R) signaling; in A^y mice however, agouti is expressed ubiquitously and signals in the brain through melanocortin 4 receptor (MC4R), which are melanocortin receptors expressed specifically in the brain (Barsh, Ollmann et al. 1999). Agouti protein is an inverse agonist of melanocortin receptors; therefore instead of activating adenylate cyclase and

increasing intracellular concentrations of cAMP, agouti actually decreases intracellular concentrations of cAMP, thus inhibiting melanocortin signaling (Lu, Willard et al. 1994). Later on, the agouti related protein (AGRP) was discovered based on the sequence similarity of Agouti protein (Ollmann, Wilson et al. 1997; Shutter, Graham et al. 1997). AGRP is also an inverse agonist of Mc3r and Mc4r, but is endogenously expressed in the hypothalamus as opposed to melanocytes. AGRP's role in food intake became even more appealing when neuroanatomical findings indicated that *AGRP* and *NPY* were expressed in the same neurons in the arcuate nucleus, known as the AGRP/NPY neurons (Hahn, Breininger et al. 1998). Similar to NPY, AGRP increased food intake upon central administration in rodents and *AGRP* mRNA levels were also increased in *ob/ob* mice (Mizuno and Mobbs 1999; Korner, Savontaus et al. 2001; Takahashi and Cone 2005). Gene targeted mutant mice that have inactivated Mc4r signaling also developed obesity and overgrown phenotypes similar to the overexpression of Agouti and AGRP (Huszar, Lynch et al. 1997). All of these data collectively indicate that the arcuate nucleus has a neuronal population that can stimulate feeding through the activation of NPY receptors and the inhibition of MC3R and MC4R.

Preopiomelanocortin

The arcuate nucleus was further established as the feeding centre in the hypothalamus upon the discovery of preopiomelanocortin (POMC) neurons. POMC is the precursor of α-melanocyte stimulating hormone (α-MSH), which also signals through melanocortin signaling(Mains and Eipper 1979). As anticipated, activation of MC3R and MC4R in the hypothalamus by α-MSH decreases food intake. Central administration of a-MSH decreases food intake, body weight gain, and adiposity, while increasing energy expenditure, body temperature and brown adipose tissue activity (Haynes, Morgan et al. 1999; Murphy, Nunes et al. 2000; Hwa, Ghibaudi et al. 2001; Jonsson, Skarphedinsson et al. 2002; Li, Zhang et al. 2004). *POMC* mRNA levels is decreased upon fasting and is increased by leptin injection (Brady, Smith et al. 1990; Ahima, Kelly et al. 1999; Hagan, Rushing et al. 1999; van Dijk, Seeley et al. 1999; Korner, Savontaus et al. 2001; Bertile, Oudart et al. 2003).

Cocaine-and-Amphetamine-Regulated Transcript

Furthermore, investigators found that POMC neurons also co-express cocaine-and-amphetamine-regulated transcript (CART), which is a strong

anorexigenic neuropeptide that has similar effects to melanocortin agonists (Kristensen, Judge et al. 1998). *CART* mRNA levels are decreased upon fasting and increased by leptin stimulation in rodents, acting similarly as POMC (Ahima, Kelly et al. 1999; Bertile, Oudart et al. 2003; Wortley, Chang et al. 2004). Intracerebroventricular injection of CART decreases food intake (Kristensen, Judge et al. 1998; Abbott, Rossi et al. 2001; Stanley, Small et al. 2001); whereas chronic injections of CART reduced body weight and increased lipid oxidation (Larsen, Vrang et al. 2000; Rohner-Jeanrenaud, Craft et al. 2002). However, unlike NPY's specificity in the hypothalamus, CART has been shown to increase or decrease food intake in distinct hypothalamic nuclei injection, suggesting that CART might play a more modulatory role depending on its localization (Wang, Billington et al. 2000; Abbott, Rossi et al. 2001; Stanley, Small et al. 2001; Kong, Stanley et al. 2003). Therefore, not only the orexigenic NPY/AGRP neurons are localized in the arcuate nucleus, but anorexigenic POMC/CART neurons are as well. This explains why lesion studies in the arcuate nucleus had limited effects on food intake and body weight regulation.

Genetic studies in feeding

The physiological and pharmacological data compiled above suggests that NPY and AGRP potentially are the main hypothalamic mediators of feeding and body weight which are regulated by leptin signaling. This hypothesis was challenged however due to the fact that *NPY* knockout mice maintain a normal amount of food intake and show a normal amount of body weight increase (Erickson, Clegg et al. 1996), although they did show hyperphagic behavior upon fasting. However, when these mice were backcrossed to a C57BL/6 background, food intake was significantly reduced after 24 or 48 hours of food deprivation (Bannon, Seda et al. 2000; Qian, Chen et al. 2002), indicating that NPY does play an important role in feeding behavior but mouse genetic background can significantly impact these metabolic studies. One logical explanation would be that AGRP could compensate for the loss of function of NPY; unfortunately this hypothesis was denied because *NPY*, *AGRP* double knockout mice also have normal food intake and body weight increase (Qian, Chen et al. 2002). When *NPY* knockout mice were crossed into an *ob/ob* background, there was a significant decrease in body weight and food intake, increased energy expenditure and improved serum parameters influencing diabetes, indicating that NPY's central actions are downstream of leptin (Erickson, Hollopeter et al. 1996).

It is very intriguing that the arcuate nucleus has two distinct groups of sensing neurons; the NPY/AGRP orexigenic neurons and the POMC/CART anorexigenic neurons; these two sets of neurons perhaps are the key to the hypothalamus maintaining proper body weight regulation and energy homeostasis. *NPY* mRNA is upregulated upon fasting, but is not affected by overfeeding. However, *POMC* mRNA is both upregulated by fasting and downregulated by feeding (Seeley, Matson et al. 1996). This might serve as the underlying reason why gaining weight is easier than losing weight; energy deficiency is sensed by both NPY/AGRP and POMC/CART neurons whereas energy excess is only sensed by POMC/CART neurons in the arcuate nucleus.

Orexin, Melanin-Concentrating Hormone, and the Lateral Hypothalamic Area

Downstream the arcuate nucleus, NPY/AGRP and POMC/CART neurons signal to the lateral hypothalamic area (LHA) which contains the orexigenic orexin (also known as hypocretin) and melanin-concentrating hormone (MCH) neurons; both have been shown to be important factors in regulating feeding behavior in the hypothalamus. Orexin will be discussed in detail shortly.

The melanin-concentrating hormone is a cyclic neuropeptide that was

originally isolated from the pituitary of salmon, and exhibits skin color pigmentation lightening by antagonizing the effects of melanocyte-stimulating hormone (MSH) (Kawauchi, Kawazoe et al. 1983). A similar peptide was identified in the rat brain (Skofitsch, Jacobowitz et al. 1985) and later on the homologous peptide of MCH was purified from the rat hypothalamus (Vaughan, Fischer et al. 1989). By comparing *ob/ob* and *ob/+* mice in fed states, MCH was one of the overexpressed mRNA in *ob/ob* mice; fasting further increased MCH expression in both genotypes (Qu, Ludwig et al. 1996). Acute injection of MCH into rat lateral ventricles also stimulated food intake (Qu, Ludwig et al. 1996). While many other studies have also shown that MCH expression is increased upon fasting (Presse, Sorokovsky et al. 1996; Tritos, Mastaitis et al. 2001) and stimulates food intake when injected centrally (Rossi, Choi et al. 1997; Ludwig, Mountjoy et al. 1998; Abbott, Kennedy et al. 2003), MCH neurons appear to be inhibited by NPY neurons (van den Pol, Acuna-Goycolea et al. 2004). MCH peptide knockout and MCH receptor knockout mice are leaner, hypophagic and have increased activity, supporting the idea that MCH is an orexigenic neuropeptide (Shimada, Tritos et al. 1998; Marsh, Weingarth et al. 2002). However, when MCH was chronically ICV administrated to mice, food intake effects of MCH faded and body weight remained normal (Rossi,

Choi et al. 1997), suggesting that pharmacology and genetics do not always agree and that the mechanisms of feeding regulation is indeed a very complex system.

Corticotropin-Releasing Hormone, Thyroid-Stimulating Hormone, and the Paraventricular Nucleus

Besides the arcuate nucleus, another region in the hypothalamus important for energy balance and food intake regulation is the paraventricular nucleus (PVN) which expresses the anorexigenic corticotropin-releasing hormone (CRH) and thyroid-stimulating hormone releasing hormone (TRH) neurons (Lechan and Jackson 1982; Swanson, Sawchenko et al. 1983).

CRH is primarily known for its central role in the hypothalamic-pituitary adrenal (HPA) axis in response to stress, but is also involved in energy balance. PVN CRH mRNA levels were increased by 40% when leptin was ICV injected into rat brains but not in leptin-resistant Zucker animals (Schwartz, Seeley et al. 1996). CRH peptide levels also increased as soon as 2 hours after ICV injection of recombinant leptin, whereas leptin's anorexic effect was attenuated when a CRH antagonist and recombinant leptin were simultaneously ICV injected, indicating a role for CRH in food intake (Uehara,

Shimizu et al. 1998). Consistently, fasting also decreased CRH peptide expression (Suemaru, Hashimoto et al. 1986). Additionally, injections of α-MSH and CART attenuated the fasting-induced suppression of CRH levels, suggesting that the anorexigenic effects of CRH could be downstream to α-MSH and CART (Fekete, Legradi et al. 2000; Smith, Vaughan et al. 2004). ICV injection of CRH has been shown to reduce food consumption and body weight gain and simultaneously increasing locomotor activity and brown adipose tissue activity (Britton, Koob et al. 1982; LeFeuvre, Rothwell et al. 1987; Arase, York et al. 1988; Buwalda, Van Kalkeren et al. 1998). These findings coincide with the idea that PVN CRH has an anorexigenic and overall catabolic effect on energy balance.

TRH has known to be important for energy balance due to its role in the hypothalamic-pituitary-thyroid (HPT) axis (Lechan and Fekete 2006). TRH has innervations throughout the brain including the hypothalamus, median eminence and the pituitary. Through the pituitary, TRH stimulates the release of thyroid-stimulating hormone (TSH) to increase thyroid hormone secretion (Lechan and Jackson 1982); which is the primary factor responsible for regulating energy expenditure and thermogenesis (Lechan and Fekete 2006). TRH expression and release is reduced by fasting, NPY, and AGRP. In

contrast, TRH levels is increased by leptin, α-MSH, and CART (Fekete, Mihaly et al. 2000; Kim, Small et al. 2000; Fekete, Kelly et al. 2001; Fekete, Sarkar et al. 2002; Fekete, Singru et al. 2006). Acute central and peripheral injections of TRH decreased food consumption and increased body temperature (Vijayan and McCann 1977; Suzuki, Kohno et al. 1982; Choi, Hartzell et al. 2002). Chronic injection of TRH however, produced opposite results by increasing food intake without altering body weight (Iglesias, Llobera et al. 1986). Overall the current consensus is that TRH plays an anorexigenic and catabolic role in metabolism.

LEPTIN SIGNALING

Despite the numerous discoveries of neuronal control of metabolism, one of the most important of all was the cloning of the *ob* gene in 1994, which encodes the previously mentioned protein hormone leptin (Zhang, Proenca et al. 1994). Leptin is secreted by white adipose tissue and its circulating levels in plasma are an indicator of body fat mass (Frederich, Hamann et al. 1995; Maffei, Halaas et al. 1995). Leptin deficiency in mice homozygous for a mutant *ob* gene, also previously mentioned *ob/ob* mice have morbid obesity,

diabetes, and neuroendocrine abnormalities; replacement of leptin decreases food intake, normalized glucose homeostasis and increases energy expenditure (Halaas, Gajiwala et al. 1995; Pelleymounter, Cullen et al. 1995; Tartaglia, Dembski et al. 1995; Chua, Chung et al. 1996). Numerous splice variants of the leptin receptor have been identified; the short form receptors are expressed throughout multiple tissues (Tartaglia, Dembski et al. 1995), whereas the long form receptors (OB-Rb) which have a longer cytoplasmic domain are highly expressed in specific CNS regions including the ventral premammillary nucleus of the hypothalamus (PMV), ARH, dorsal medial hypothalamus (DMH), ventral medial hypothalamus (VMH) and the medial preoptic nucleus (MEPO) (Chua, Chung et al. 1996; Elmquist, Bjorbaek et al. 1998). The long form leptin receptors are sufficient for leptin's metabolic actions in the CNS (Kowalski, Liu et al. 2001; de Luca, Kowalski et al. 2005). Although leptin is known to signal in the peripheral as well, by using genetically modified mice, it has been shown that leptin signaling in the CNS is sufficient to regulate body weight, feeding, energy expenditure and glucose metabolism (Cohen, Zhao et al. 2001; Morton, Niswender et al. 2003; Coppari, Ichinose et al. 2005; de Luca, Kowalski et al. 2005).

Physiological roles of leptin

Upon starvation, serum leptin levels are rapidly decreased. This decrease of leptin levels have been hypothesized to act as a starvation signal for energy conservation and a response to lower energy availability (Ahima, Prabakaran et al. 1996). Endocrine, behavioral, and autonomic responses induced by fasting can be blunted by leptin during the fast. Thus circulating serum leptin acts as a key signal regarding energy storage. Low leptin levels can be the result of rare genetic disorders such as lipodystrophies and congenital leptin deficiency (Montague, Farooqi et al. 1997; Haque, Shimomura et al. 2002). Leptin administration corrects many metabolic anomalies seen in lipodystrophic patients including dyslipidemia, diabetes, and hepatic steatosis, and reverses the obese phenotype of leptin-deficient individuals (Farooqi, Matarese et al. 2002; Licinio, Caglayan et al. 2004).

Obese individuals systemically show elevated serum leptin levels. However, for reasons not fully understood yet, obese individuals do not have diminished food consumption or elevated energy expenditure, as would be predicted with elevated serum leptin (Maffei, Halaas et al. 1995; Considine, Sinha et al. 1996). Therefore the theory of leptin resistance was elaborated by researchers, which is the concept where obese individuals and high-fat fed

animals fail to respond to endogenous or exogenous leptin (Myers, Leibel et al. 2010). Leptin resistance is still currently an active area of research in the metabolic field.

Leptin and the regulation of energy balance

Leptin has been suggested to possess antidiabetic actions through the regulation of glucose homeostasis. Insulin-clamp studies have indicated that leptin can modify hepatic glucose production by simultaneously increasing gluconeogenesis and decreasing glycogenolysis in rats and mice (Kamohara, Burcelin et al. 1997; Rossetti, Massillon et al. 1997; Liu, Karkanias et al. 1998). Leptin can also dramatically improve glycemic control in animal models of type-1 diabetes (Yu, Park et al. 2008; Wang, Chen et al. 2010). Chronic ICV injection of leptin also exhibits beneficial effects on glucose homeostasis (Fujikawa, Chuang et al. 2010). Mice lacking OB-Rb in both POMC and AgRP neurons develop hyperinsulinemia. Strikingly, restoring leptin receptor expression solely in the ARH completely normalizes glucose and insulin levels, improves their hepatic insulin sensitivity, and reduces gluconeogenesis (German, Kim et al. 2009). Collectively, this suggests that ARH is the critical site of central leptin regulation of glucose and insulin homeostasis.

Leptin has been shown to modulate many aspects of feeding, including meal size (Flynn, Scott et al. 1998; Kahler, Geary et al. 1998), food reward (Figlewicz, Bennett et al. 2006; Hommel, Trinko et al. 2006), and food preference (Wetzler, Jean-Joseph et al. 2005; Licinio, Ribeiro et al. 2007). This suggests that the neural circuitry of leptin signaling is very complex. Indeed, injection of leptin directly into many brain sites such as the ARH, DMH, LHA, VT, and the NTS can all reduce food intake (Jacob, Dziura et al. 1997; Satoh, Ogawa et al. 1997; Grill, Schwartz et al. 2002; Leinninger, Jo et al. 2009).

Body weight regulation is significantly more complex, which involves multiple regulatory mechanisms including body temperature, energy expenditure, locomotor activity, cardiorespiratory parameters, and lipogenesis, all which will be examined in this study. These parameters are all under the control of the sympathetic nervous system; therefore it is very difficult to dissociate the reciprocal effects one has on the other. Several lines of evidence have shown that leptin regulates energy expenditure directly through stimulating the sympathetic tone (Haynes, Morgan et al. 1997; Overton, Williams et al. 2001; Hausberg, Morgan et al. 2002; Buettner, Muse et al. 2008). Genetic studies have also demonstrated that body temperature,

locomotor activity, and energy expenditure are changed by modified expression of OB-Rb (Coppari, Ichinose et al. 2005; Hill, Williams et al. 2008; van de Wall, Leshan et al. 2008; Al-Qassab, Smith et al. 2009).

In conclusion, regulation of energy balance homeostasis indeed is a complex system. Nevertheless, leptin plays an essential role in all aspects of energy balance including glucose homeostasis, food consumption, and body weight control. As I will demonstrate in this study, leptin's presence is crucial for orexin's prevention of diet-induced obesity.

THE OREXIN SYSTEM

Orexin Neuropeptides

Our group discovered the orexin neuropeptides through high-resolution high-performance liquid chromatography fractions from brain extracts by stimulating signaling pathways in cell lines expressing orphan G-protein-coupled receptors (GPCRs) (Sakurai, Amemiya et al. 1998).

The *prepro-orexin* gene is composed of two exons and an intervening intron, which encodes a 130-residue and 131-residue polypeptide in rat and humans, respectively (Sakurai, Amemiya et al. 1998; Sakurai, Moriguchi et al.

1999). The polypeptide has secretory signal sequences which are proteolytically cleaved into the 33 amino acid orexin-A (OXA) of 3562 Da or 28 amino acid orexin-B (OXB) of 2037 Da which has a 46% (13/28) amino acid sequence homology to OXA. Post-translational modifications include N-terminal pyroglutamyl cyclization and two sets of intrachain disulfide bonds in OXA and C-terminal amidation in both OXA and OXB. The two disulphide bridges, Cys2-Cys12 and Cys7-Cys14 together with the 19 C-terminal residues are essential for functional OXA (Darker, Porter et al. 2001; Okumura, Takeuchi et al. 2001). OXA is conserved among human, rat, mouse, cow, and pig genera (Sakurai, Amemiya et al. 1998; Dyer, Touchette et al. 1999). OXB is conserved among rat and mouse, but has two amino-acid substitutions in humans. Orexins in the amphibian *Xenopus laevis* also have high sequence homology to the mammalian counterparts, especially at the carboxyl terminus (**Fig. 1.1**) (Shibahara, Sakurai et al. 1999).

Utilizing a differential cloning approach from a hypothalamus enriched cDNA library, a messenger RNA encoding the same neuropeptide precursor was named hypocretins (de Lecea, Kilduff et al. 1998). Although nucleotide sequence alignment indicated that hypocretin-1 and hypocretin-2 are identical to orexin-A and orexin-B, respectively, the mature peptides in this study had

predicted additional amino acids that are not present in native orexins. Thus the hypocretin peptides synthesized from this study were markedly less potent agonists than orexins when tested with transfected cells expressing orexin receptors (Smart, Jerman et al. 2000). This literature will be primarily using the name orexins although the names are interchangeable.

Orexin Receptors

The two orexin receptors orexin receptor-1 (OX1R) and orexin receptor-2 (OX2R) are G-protein-coupled receptors that were also identified by our group (Sakurai, Amemiya et al. 1998). OX1R is the orphan G-protein-coupled receptor used for ligand hunting and eventually led to the identification and purification of the orexin peptides. Two candidate ESTs were found in the Genebank dbEST using the OX1R amino acid sequence. Utilizing PCR and primers designed using these ESTs, OX2R was discovered with a 64% amino acid sequence identity to OX1R. Competitive radio-ligand binding assays revealed that orexin peptides had different affinities for orexin receptors. OX1R has one-order magnitude higher affinity for OXA [50% Inhibitory Concentration (IC_{50}= 20nM)] compared to OXB (IC_{50}= 250 nM). In contrast, OXA and OXB bind to OX2R with equal affinity (IC_{50}= 20nM). Thus OX1R is

more selective for OXA, whereas OX2R is non-selective for both OXA and OXB. OX1R is coupled exclusively to the G_q subclass of heterotrimeric G proteins, whereas OX2R has been shown to couple to $G_{i/o}$ and/or G_q (Sakurai, Amemiya et al. 1998; van den Pol, Gao et al. 1998). Most studies up to date have demonstrated that orexin peptides exhibit neuroexcitatory activities by using multiple methods (Sutcliffe and de Lecea 2000).

Neuroanatomy of the Orexin System

Orexin neurons are restricted to the lateral and posterior hypothalamus and perifornical areas in the rodent CNS (**Fig 1.2**) (Peyron, Tighe et al. 1998; Sakurai, Amemiya et al. 1998). Although the neuronal bodies are highly restricted in localization, it has been shown by immunohistochemistry that orexin neuronal fibers project widely throughout the CNS. Projections to areas including cerebral cortex, olfactory bulb, hippocampus, amygdala, septum, diagonal band of Broca, bed nucleus of the stria terminalis, thalamus, anterior and posterior hypothalamus, midbrain, brainstem, and the spinal cord (Peyron, Tighe et al. 1998; Date, Ueta et al. 1999; Nambu, Sakurai et al. 1999; van den Pol 1999). Orexin immunoreactivity has also been reported in the enteric nervous system and the pancreas (Kirchgessner and Liu 1999), orexin

mRNA expression has also been detected in the testes by our group (Sakurai, Amemiya et al. 1998).

Orexin receptor expression has been studied by *in situ* hybridization and although resembles orexin fiber projections, the two receptors have very distinct distributions (Trivedi, Yu et al. 1998; Marcus, Aschkenasi et al. 2001). *OX1R* mRNA is highly expressed in the prefrontal cortex, hippocampus, paraventricular thalamus, ventromedial hypothalamus (VMH), arcuate nucleus (ARH), dorsal raphe nucleus, and locus coeruleus (LC). In contrast, *OX2R* mRNA is expressed in the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, dorsal and median raphe nuclei; and many hypothalamic nuclei including the tuberomammillary nucleus (TM), dorsomedial hypothalamus (DMH), paraventricular hypothalamic nucleus (PVN), and ventral premammillary nucleus. Orexin receptor mRNA has also been reported in peripheral tissues such as the adrenal gland (Malendowicz, Tortorella et al. 1999), enteric nervous system, and pancreas (Kirchgessner and Liu 1999).

Intracerebroventricular (ICV) injection of orexin peptides and utilization of c-Fos as an indicator of neuronal activation has been used to demonstrate that orexin-A and orexin-B activate a similar population of neurons (Date, Ueta et al.

1999). Areas of activation as indicated by c-Fos included the ARH, PVN, supraoptic nucleus, paraventricular thalamic nucleus, LC, central gray, dorsal raphe, nucleus of the solitary tract, dorsal motor nucleus of the vagus, and the suprachiasmatic nucleus. However, this data is not sufficient to represent actual orexin receptor specific activation. Furthermore, the possibility of orexin-mediated inhibition of neuronal activity could not be assessed through this method. Orexins have also been shown to increase the inhibitory neurotransmitter γ-aminobutyric acid (GABA), as well as the excitatory neurotransmitter glutamate by acting directly on the axon terminals in the ARH nucleus.

Orexin neurons also produce glutamate, the orexigenic opiod dynorphin (Chou, Lee et al. 2001), the secretory marker secretogranin II (Risold, Griffond et al. 1999), and angiotensin II (Hara, Beuckmann et al. 2001); and the biosynthesis of these peptides might be similarly regulated. The appetite stimulating neuropeptide galanin has also been identified in orexin neurons (Hakansson, de Lecea et al. 1999).

OREXIN AND METABOLISM

Upon the original discovery of the orexin neuropeptides, orexin was

described to acutely promote food intake (Sakurai, Amemiya et al. 1998), thus given the name “orexin”, which stands for appetite in Greek. Later studies, however, with transgenic mice that ablated orexin neurons in the LHA developed late onset obesity (Hara, Beuckmann et al. 2001); whereas according to the original report, one would predict the opposite, mice should be leaner without orexin neuropeptides present in the CNS. This really raises the question: is the acute effect of orexin just a temporary or secondary effect that is eventually overpowered by an overall negative energy balance? This is the basis of this thesis, and the following experiments will attempt to answer what the actual effect of orexin chronically is on energy balance.

Feeding

Early lesion studies have established the thought that LHA is one of the “feeding centres”. Lesions of the LHA caused decreased food and water intake and resulted in 75-80% of body weight compared to sham-operated controls (Bernardis and Bellinger 1996). Complimentary electrical stimulation of the LHA with acute stimulation resulted in hyperphagia, whereas chronic stimulation caused obesity. The LHA also participates in a dopaminergic dependent rewards system that is facilitated by food deprivation as

demonstrated by electrical self-stimulation of the LHA. Thus energy balance may significantly affect the excitability of the LHA feeding circuitry.

Due to the fact that orexin neurons reside in the LHA, it was very logical to hypothesize that orexin plays an essential role in feeding. Acutely injected OXA at early light phase induced a significant, dose-dependent stimulation of food consumption (Sakurai, Amemiya et al. 1998; Edwards, Abusnana et al. 1999; Haynes, Jackson et al. 1999; Yamanaka, Kunii et al. 2000). Similar experiments using OXB however, produced inconsistent results; studies have found that OXB had little or no effect, or even inhibits feeding (Haynes, Jackson et al. 1999). Even when OXB does stimulate feeding, OXA seems to have a longer half-life effect. This could be due to the disulfide bonds that make OXA more resistant to peptidases or that OXB can only activate partial signaling pathways primarily through OX2R, whereas OXA signals through both OX1R and OX2R. It is also important to note that the orexigenic effect of OXA is significantly weaker than NPY under the same conditions, although its duration does appear to be longer than NPY (Sakurai, Amemiya et al. 1998). However, the orexigenic effects of OXA is still within the same magnitude with other orexigenic peptides such as MCH and galanin (Edwards, Abusnana et al. 1999).

Evidence that endogenous orexin has a physiological effect on feeding was first demonstrated by Yamada et al. An anti-orexin antibody that was centrally injected dose-dependently suppressed feeding in fasted rats (Yamada, Okumura et al. 2000). Furthermore, OXA stimulates gut-motility, insulin secretion, and gastric secretion when given centrally and with an intact vagus nerve, suggesting that OXA plays a role in autonomic functions such as the cephalic phase of digestion (Kirchgessner and Liu 1999; Takahashi, Okumura et al. 1999; Nowak, Mackowiak et al. 2000). It can still be argued that the effect of orexin on feeding is secondary to other unmeasured behaviors such as wakefulness or circadian. Moreover, orexin neurons project widely throughout the CNS; many areas which have not been implicated in feeding.

Circadian rhythms is known to have an impact on feeding behavior and disruptions of normal circadian patterns have been described previously in LHA lesion studies (Bernardis and Bellinger 1996). In the study conducted by Haynes *et al.*, the effect of OXA on feeding was highly regulated by the time of the day (Haynes, Jackson et al. 1999). OXA increased feeding in satiated rats 3 hours into light phase and 6 hours into dark phase, but not at the beginning of the dark phase. Similarly, OXA was ineffective at increasing

food intake in the first hour of re-feeding after fasting. Possible speculation could be that beginning of the dark phase and immediately after fasting, orexin-stimulated feeding pathways are already over-activated and therefore unresponsive to additional pharmacologic stimulation of OXA.

Chronic infusion of OXA experiments have also been performed previously (Haynes, Jackson et al. 1999; Yamanaka, Sakurai et al. 1999). OXA was chronically infused using an osmotic pump for 8 consecutive days, daytime feeding was increased, whereas nocturnal feeding was decreased, however, there was no increase in daily average food intake, adiposity or body weight, which is similar to my results with an OX2R selective agonist on a low-fat diet in chapter 5.

Metabolic, Autonomic, and Endocrine Effects

The LHA is also known to be critical in regulating metabolic rate. Animal models with lesions in the LHA become hypercatabolic (Bernardis and Bellinger 1993). The hypermetabolic phenotype of *MCH* knockout mice is consistent with this phenotype. In contrast, decreased food intake and normal body weight suggests that *orexin* knockout mice are hypometabolic. This is further supported by indirect calorimetry of OXA ICV injected mice.

OXA ICV injection during the light phase increased oxygen consumption and respiratory quotient (Lubkin and Stricker-Krongrad 1998). Oxygen consumption is still increased during the dark phase, however, respiratory quotient is decreased, and again supporting the idea that circadian can affect orexin's metabolic involvement.

Orexin projections to the nucleus of the solitary tract, dorsal motor nucleus of the vagus, and sympathetic neurons in the intermediolateral column of the spinal cord suggest that orexin could be involved in autonomic function and stress response (Date, Ueta et al. 1999; van den Pol 1999). Both central and peripheral administration of orexin increased plasma levels of corticosterone, and orexin can stimulate corticosterone release directly from adrenocortical cells in vitro (Date, Ueta et al. 1999; Hagan, Leslie et al. 1999). Additionally, *OX1R* and *OX2R* mRNA are expressed in the adrenal medulla, suggesting that orexin might influence systemic epinephrine secretion and modulate vascular tone (Lopez, Senaris et al. 1999). This is consistent with reports finding that orexin dose-dependently increases blood pressure and heart rate (Samson, Gosnell et al. 1999; Shirasaka, Nakazato et al. 1999; Chen, Hwang et al. 2000); notably however, I did not find any significant increase in blood pressure or heart rate in our orexin overexpression transgenic mice, indicating there could

be complementary mechanisms that can compensate in regulating sympathetic tone homeostasis.

Regulation of Orexin Neurons

The LHA receives numerous direct and indirect innervations from the neuroaxis and is regulated by actively transported peripheral factors, such as leptin, insulin, as well as diffusible factors including glucose, amino acids, electrolytes, and peptides (Bernardis and Bellinger 1996). Thus the regulation of orexin neurons is a complex and sophisticated system.

Orexin neurons are activated by acute hypoglycemia. Insulin, when given to acutely lower blood glucose level rats, induces neuronal activity as indicated by c-Fos in the LHA that expresses orexin (Bahjaoui-Bouhaddi, Fellmann et al. 1994; Moriguchi, Sakurai et al. 1999). However, insulin receptor mRNA is not expressed in the LHA, indicating that insulin does not activate orexin neurons through a direct mechanism (Marks, Porte et al. 1990).

By utilizing different physiologic and pharmacologic manipulations to stimulate food intake, increased *orexin* mRNA expression was associated with subnormal plasma glucose levels and the absence of food intake (Cai, Widdowson et al. 1999).

Extensive neuroanatomic evidence of reciprocal innervation between orexin neurons and leptin sensitive feeding pathways of the ARC has been reported (Broberger, De Lecea et al. 1998; Elias, Saper et al. 1998). Leptin receptor immunoreactivity has also been detected in orexin neurons (Hakansson, de Lecea et al. 1999; Horvath, Peyron et al. 1999). Our group has demonstrated that orexin neurons directly monitor indicators of energy balance and mediate adaptive augmentation of arousal in response to fasting (Yamanaka, Beuckmann et al. 2003). Activity of isolated orexin neurons is stimulated by reduced glucose levels and reciprocally inhibited by increased glucose levels within physiological concentrations. Orexin neurons are inhibited by leptin, but stimulated by ghrelin which is released by the stomach in response to nutrition depletion (Nakazato, Murakami et al. 2001; Cummings, Weigle et al. 2002; Lee, Wang et al. 2002). Furthermore, orexin expression in wild-type and ob/ob mice negatively correlates with food intake, leptin, and blood glucose levels. All together, the regulation of orexin neurons is a complex system and tightly involved with key metabolic factors.

OBESITY AND THE METABOLIC SYNDROME

Obesity has become increasingly prevalent in the modern society,

whereas WHO estimated that clinically obese individuals have doubled since 1980, and it has been estimated that individuals considered clinically obese will represent 10% of the global adult population by 2015 (WHO 2011). In 2008, 1.5 billion adults above the age of 20 were overweight; among these 200 million men and 300 million women were obese. Additionally, 65% of the world's population lives in countries where overweight and obesity kills more people than underweight individuals. This trend of obesity not only affects adults, but nearly 43 million children under the age of five were overweight in 2010.

Overweight and obesity is the fifth leading risk for global deaths. At least 2.8 million adults die each year as a result of obesity. 44% of diabetes, 23% of ischaemic heart disease burden and between 7 to 41% of certain cancers is additional health risks associated with obesity.

The connection between orexin and obesity has been mentioned above, but this connection is not limited to rodent models. Narcoleptic humans that are orexin deficient also exhibit a greater body mass index and a higher incidence of the metabolic syndrome (Nishino 2007). Orexin deficiency could be the primary cause underlining this metabolic effect due to the fact that clinically indistinguishable narcoleptic patients with normal orexin levels have a

lower body mass index than orexin-deficient narcoleptic patients (Nishino, Ripley et al. 2001).

The metabolic syndrome is also a complex disorder on the rise and increasing prevalence among the human population. It is complex to the extent that various definitions currently exist, and has been difficult to identify a uniform criteria to define the metabolic syndrome (Kassi, Pervanidou et al. 2011). In general, the metabolic syndrome consists of a cluster of interconnected factors that increase the risk of coronary heart disease (CHD), cardiovascular atherosclerotic diseases (CVD), and diabetes mellitus type-2 (DMT2). Its main components are dyslipidemia (elevated triglycerides and apolipoprotein B-containing lipoproteins, and low high-density lipoproteins (HDL)), elevation of arterial blood pressure (BP) and dysregulated glucose homeostasis. Obesity and insulin resistance are the underlying core manifestations of the syndrome. Recently, chronic proinflammatory and prothrombotic states, non-alcoholic fatty liver disease and sleep apnea have been added to the entity to the syndrome, making it even more complex. Thus, besides testing for orexin's anti-obesity effect, many factors contributing to the metabolic syndrome are also examined in this study. I will be showing lines of evidence in this study that orexin also contributes to protective effects

against the metabolic syndrome, suggesting the importance of orexin signaling as a potential therapeutic target.

DISPLAY ITEMS

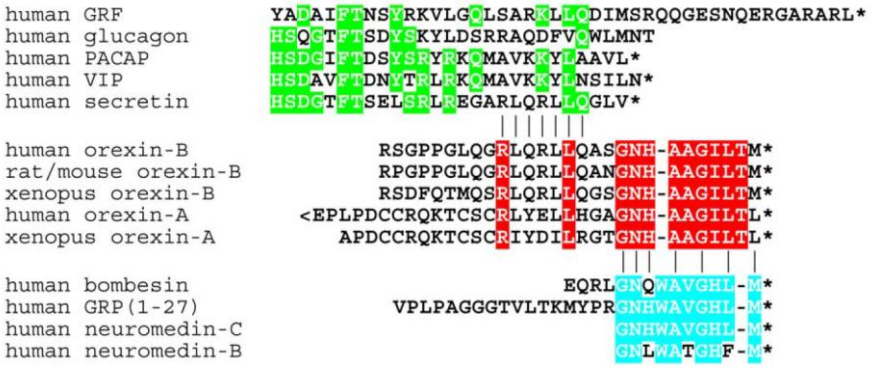

Figure 1.1 Comparisons of Orexin Sequences with the Secretin and Bombesin Families

Signature peptide sequences characteristic of secretins (green highlights) are found primarily at the amino-terminus. Neither orexin-A nor orexin-B shares significant identity with the secretin family. In contrast, characteristic sequences of the bombesin family (blue highlights) reside in the carboxy-terminus. Orexin-A and orexin-B both share significant identity with the bombesin family. Red highlights depict absolute interspecific and inter-isopeptide identity among orexins. There are two interchain disulfide bonds in orexin-A (Cys6-Cys12 and Cys7-Cys14) but none in orexin-B. Abbreviations and symbols: GRF, growth-hormone releasing factor; PACAP, pituitary adenylyl cyclase-activating peptide; VIP, vasoactive intestinal peptide; GRP, gastrin-releasing peptide; *, C-terminal amide; <E, pyroglutamyl residue.

Modified with permission J. Willie (2005).

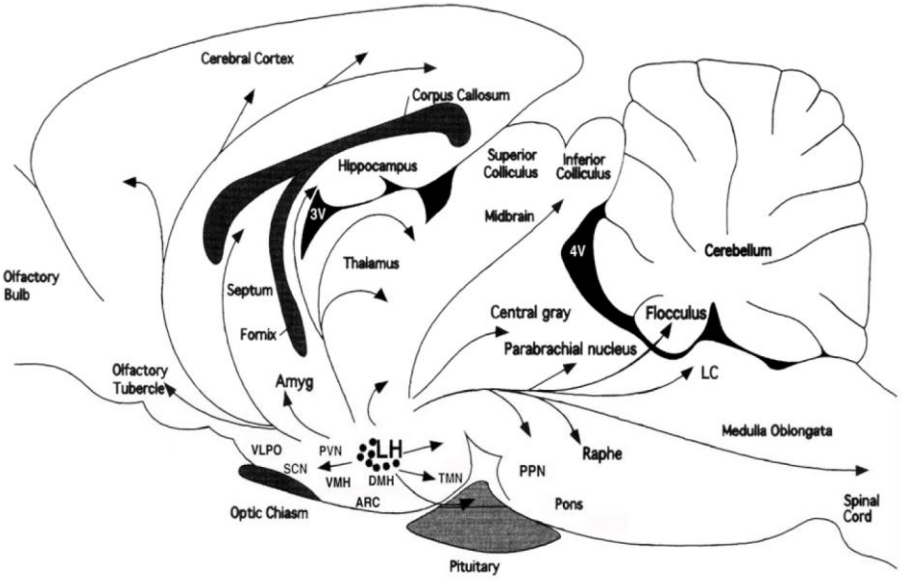

Figure 1.2 Neuroanatomy of the orexin neuropeptide system

Orexin neurons are found in the lateral hypothalamic area (LHA) and project widely throughout the CNS. Schematic drawing of a sagittal section through the right brain summarizes the organization of the orexin neuronal system. Abbreviations: 3V, third ventricle; 4V, fourth ventricle; Amyg, amygdala; VLPO, ventrolateral preoptic area; SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; TMN, tubermamillary nucleus; PPN, pedunculopontine nucleus; LC, locus coeruleus. Modified with permission from T. Sakurai (1999).

CHAPTER TWO

Orexin's protective effect against diet-induced obesity

SUMMARY

Evidence from orexin loss-of-function studies suggest that orexin promotes an overall negative energy balance. *CAG/orexin* transgenic mice that ectopically overexpress the orexin neuropeptide were used to analyze the effect of constitutively increased orexin on body weight regulation. The orexin transgene was overexpressed in many brain regions implicated in energy homeostasis and food intake. *CAG/orexin* transgenic mice had similar body weight compared to wild-type littermates on a low-fat diet, but were significantly leaner when challenged with a high-fat diet. To determine which orexin signaling pathway mediates this protective effect, *CAG/orexin* mice were mated with *OX1R*$^{-/-}$ and *OX2R*$^{-/-}$ mice to generate *OX1R*$^{-/-}$*; CAG/orexin* and *OX2R*$^{-/-}$*; CAG/orexin* mice respectively. *OX1R*$^{-/-}$*; CAG/orexin* mice also conferred resistance to diet-induced obesity compared to *OX1R*$^{-/-}$ mice, suggesting OX1R signaling pathway does not play an essential role in the prevention of diet-induced obesity. In contrast, *OX2R*$^{-/-}$*; CAG/orexin* mice became similarly obese compared to *OX2R*$^{-/-}$ mice when fed a high-fat diet,

indicating that orexin-OX2R signaling is critical for the prevention of diet-induced obesity.

Consistently, the *CAG/orexin* transgene also prevented hyperleptinemia in *CAG/orexin* and *OX1R$^{-/-}$; CAG/orexin* mice, but not in *OX2R$^{-/-}$; CAG/orexin* mice. Energy expenditure was significantly elevated in *CAG/orexin* and *OX1R$^{-/-}$; CAG/orexin* mice, but not in *OX2R$^{-/-}$; CAG/orexin* mice. These results collectively suggest that enhanced orexin receptor-2 signaling prevents diet-induced obesity.

INTRODUCTION

Orexin neuropeptides are produced in the lateral and posterior hypothalamic area and are endogenous ligands for the cell membrane receptors orexin receptor-1 (OX1R) and orexin receptor-2 (OX2R) (Sakurai, Amemiya et al. 1998). OX1R exhibits selective affinity for orexin-A, whereas OX2R displays equal affinity for both orexin-A and orexin-B. Both receptors are expressed in the hypothalamus and MPT but still have differential distribution throughout the CNS (Marcus, Aschkenasi et al. 2001). This suggests potential distinct functions of orexin receptors in aspects of vigilance

state, muscle tone, and energy balance. Specifically, OX2R expression is more prominent in the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei, and many hypothalamic nuclei including the histaminergic tuberomammillary nucleus (TM).

Studies by our group have shown that acute central administration of orexin-A induces food intake in rats (Sakurai, Amemiya et al. 1998). Our group has also produced a transgenic mouse line where the orexin expressing neurons were specifically ablated. Paradoxically, this orexin-ablated transgenic line developed narcolepsy, obesity and were hypoactive despite being hypophagic; opposite to what was expected for the ablation of orexigenic neurons (Hara, Beuckmann et al. 2001). This led to the hypothesis that orexin's physiological effect is to promote a catabolic effect on energy balance. To test this hypothesis, the *CAG/orexin* mouse line that ectopically overexpresses orexin was generated by our group (Mieda, Willie et al. 2004). With this mouse line in the combination with orexin receptor-1 knockout mice (Kisanuki, Chemelli et al. 2001) and orexin receptor-2 knockout mice (Willie, Chemelli et al. 2003), I will demonstrate how orexin prevents diet-induced obesity by promoting a negative energy balance.

RESULTS

Production of *CAG/orexin* mice

The *CAG/orexin* mice used in this study were generated in our laboratory by Mieda *et. al* (Mieda, Willie et al. 2004). The rat *prepro-orexin* gene was expressed under the control of β-actin/ cytomegalovirus (CAG) hybrid promoter which enables global expression (Niwa, Yamamura et al. 1991). Whole brain levels of orexin-A and orexin-B were increased by 30 and 80 fold respectively in the transgenic line chosen. The *CAG/orexin* transgene produced functional peptides in the brain because it was sufficient to rescue the narcolepsy/cataplexy phenotype of mice lacking endogenous orexin neurons (Mieda, Willie et al. 2004).

Expression of orexin peptide in *CAG/orexin* mice

Expression of orexin-A in *CAG/orexin* transgenic mice was examined carefully by immunohistochemistry (IHC). Orexin-A is ectopically expressed in many regions of the brain including the medial, basal, lateral, and suprachiasmatic hypothalamic nuclei, nucleus accumbens, globus pallidus, hippocampal formation, ventral tegmental area, and locus coerulus (**Fig. 2.1**

and 2.2; Table 2-1). All of these brain regions have previously been implicated in homeostatic, circadian, food intake regulation, taste preference and energy balance homeostasis (Saper, Chou et al. 2002). Orexin's role in peripheral tissues if any still remains controversial (Heinonen, Purhonen et al. 2008). Despite the fact that prepro-orexin was expressed by a global promoter, expression of orexin-A was rather limited to the peripheral tissues including the thyroid gland, adrenal cortex, and few pancreatic islets (**Fig. 2.3**). On an important note, no expression was detected in other important metabolic tissues such as brown and white adipose tissues, liver, or skeletal muscle (**Fig. 2.2; Tables 2-2 and 2-3**).

CAG/orexin mice are resistant to diet-induced obesity

To determine the effect of increased orexin expression on body weight regulation, *CAG/orexin* transgenic mice were compared to C57BL/6J wild-type littermates fed on a low-fat diet (standard normal chow) or high fat diet. A low-fat diet provided 4.1 kcal/g (67% carbohydrate, 20% protein, and 13% fat), whereas a high-fat diet provided 4.7 kcal/g of energy (35% carbohydrate, 20% protein, and 45% fat). When fed a high-fat diet, wild-type littermates showed a significant increase in body weight compared to low-fat diet controls as soon

as eight weeks in males and fourteen weeks in females (**Fig. 2.4 A and B**). However, *CAG/orexin* transgenic mice did not show a significant increase in body weight when fed a high-fat diet compared to low-fat diet, suggesting that the overexpression of orexin provides resistance to diet-induced obesity.

The orexin signaling system signals through two GPCR receptors: orexin receptor-1 (OX1R) and orexin receptor-2 (OX2R). Our lab has previously generated orexin receptor-1 knockout (*OX1R*$^{-/-}$) (Kisanuki, Chemelli et al. 2001) and orexin receptor-2 knockout mice (*OX2R*$^{-/-}$) (Willie, Chemelli et al. 2003). These receptor knockout mice enable us to examine which signaling pathway underlies the protective effect against diet-induced obesity of orexin overexpression in the *CAG/orexin* mice. *CAG/orexin* transgenic mice were crossed with *OX1R*$^{-/-}$ and *OX2R*$^{-/-}$ mice to generate *OX1R*$^{-/-}$*; CAG/orexin* and *OX2R*$^{-/-}$*; CAG/orexin* mice respectively. Body weight was also monitored under both low-fat and high-fat diet conditions for both genotypes. *OX1R*$^{-/-}$*; CAG/orexin* mice have isolated enhanced OX2R signaling, and they also had a significantly lower body weight under high-fat diet conditions when compared to *OX1R*$^{-/-}$ mice in both sexes (**Fig. 2.4 C and D**), thus enhanced orexin receptor-2 signaling is sufficient for orexin's protective effect against diet-induced obesity. On the other hand, *OX2R*$^{-/-}$*;*

CAG/orexin mice which have isolated enhanced OX1R signaling had a similar increase of body weight when compared to *OX2R*$^{-/-}$ mice, and weighed significantly more than *OX2R*$^{-/-}$*; CAG/orexin* mice on low fat diet (**Fig. 2.4 E and F**), thus enhanced OX1R signaling is not sufficient in preventing diet-induced obesity. These results indicate that OX2R but not OX1R signaling is critical for orexin's prevention of diet-induced obesity.

Despite most body weight phenotypes being similar in both sexes, there are still some discrepancies between sexes. Male *CAG/orexin* mice seem to have a protective effect against age-related adiposity. Body weight curves are similar up to 18 weeks in *CAG/orexin* and wild-type mice fed a low-fat diet; between 19-30 weeks of age however, wild-type male mice weighed significantly more than *CAG/orexin* male transgenic mice (P=0.0016) (**Fig. 2.4 A**). Similarly, *OX1R*$^{-/-}$ male mice also weighed significantly more than *OX1R*$^{-/-}$*; CAG/orexin* male mice from 17-30 weeks of age fed a low-fat diet (P=0.036) (**Fig. 2.4 C**). *OX2R*$^{-/-}$ male mice weighed significantly more than *OX2R*$^{-/-}$*; CAG/orexin* male mice throughout the whole observation period on low-fat diet (p=0.005) (**Fig. 2.4 E**); although fat mass and serum leptin levels were similar between *OX2R*$^{-/-}$ and *OX2R*$^{-/-}$*; CAG/orexin* male mice (**Fig. 2.5 A and C**).

CAG/orexin mice have reduced fat mass and leptin

To understand the nature of the protective effect of orexin overexpression; fat mass, lean mass and leptin were analyzed in all genotypes above. Consistent with body weight studies; *CAG/orexin* transgenic male mice had significantly less fat mass than wild-type male mice even under low-fat diet at 28 weeks of age (**Fig. 2.5 A**). Both male and female *CAG/orexin* and *OX1R*$^{-/-}$*; CAG/orexin* mice had significantly less fat mass than wild-type and *OX1R*$^{-/-}$ mice respectively under high-fat diet conditions (**Fig. 2.5 A and B**). As expected, both male and female *OX2R*$^{-/-}$*; CAG/orexin* mice did not have significantly less fat mass than *OX2R*$^{-/-}$ mice under both low-fat or high-fat diet conditions (**Fig.2.5 A and B**). An important result from this experiment is that *OX2R*$^{-/-}$ male mice on high-fat diet and *OX2R*$^{-/-}$ female mice on low-fat diet have a significant increase in fat mass compared to wild-type male mice on high-fat diet and wild-type female mice on low fat diet respectively, these results coincide with increased adiposity in narcoleptic mice (Hara, Beuckmann et al. 2001) and suggests a physiological role of OX2R signaling in preventing diet-induced obesity.

As mentioned previously, serum leptin level is known to correlate with

total amount of adipose tissue in the body. Similar to fat mass results, *CAG/orexin* male mice had significantly lower levels of serum leptin than wild-type mice under low-fat conditions. Consistently, both male and female *CAG/orexin* and *OX1R*$^{-/-}$*; CAG/orexin* mice had significantly less serum leptin than wild-type and *OX1R*$^{-/-}$ mice respectively under high-fat diet conditions (**Fig. 2.5 C and D**). No significant differences in serum leptin level were detected between *OX2R*$^{-/-}$*; CAG/orexin* and *OX2R*$^{-/-}$ mice under low-fat or high-fat diet conditions (**Fig. 2.5 C and D**). Female *OX2R*$^{-/-}$ mice had significantly more serum leptin than female wild-type mice under low-fat diet (**Fig. 2.5 D**).

Lean mass was also analyzed in all genotypes under both diets. Mild but statistically significant differences were detected compared to fat mass. Specifically, males only had mild less lean mass under high-fat diet conditions for *CAG/orexin* and *OX1R*$^{-/-}$*; CAG/orexin* mice when compared to wild-type and *OX1R*$^{-/-}$ mice. Females had mild differences in lean mass for *OX2R*$^{-/-}$*; CAG/orexin* mice compared to *OX2R*$^{-/-}$ mice under low-fat diet and *CAG/orexin* mice compared to wild-type under high-fat diet conditions.

CAG/orexin mice have increased energy expenditure

To examine the underlying cause of protection against diet-induced obesity by enhanced orexin signaling, all genotypes were subjected to core body temperature measurement and metabolic cages to measure oxygen consumption, carbon dioxide production, sources of metabolic energy and locomotor activity.

Core body temperature had a trend of being slightly higher in *CAG/orexin* mice compared to wild-type controls on both low-fat and high-fat diet, but neither reached significance. The core body temperatures of mice on a low fat diet for wild-type and *CAG/orexin* mice were 36.6 ± 0.1℃ and 36.7 ± 0.1℃ respectively; core body temperatures of mice on a high-fat diet for wild-type and *CAG/orexin* mice were 36.8 ± 0.1℃ and 37.0 ± 0.1℃ respectively with an n= 5-6.

Effective mass-corrected energy expenditure was significantly elevated in *CAG/orexin* mice and *OX1R*$^{-/-}$*; CAG/orexin* mice under high-fat diet compared to wild-type controls and *OX1R*$^{-/-}$ mice respectively (**Fig. 2.6 A and C**). Consistent with body weight data, *OX2R*$^{-/-}$ mice and *OX2R*$^{-/-}$*; CAG/orexin* mice under high-fat diet closely resembled each other in effective mass-corrected energy expenditure (**Fig. 2.6 E**). The respiratory quotient (RQ) which identifies the main source of metabolic energy (carbohydrates:

RQ= 1; protein: RQ= 0.8-0.9 or lipids: RQ= 0.7) was similar in all genotypes ranging from 0.80 to 0.85, indicating the primary source of energy utilized is proteins (**Fig. 2.6 B, D and F**). Effective mass-corrected energy expenditure for low-fat diet fed mice were also analyzed, however the *CAG/orexin* transgene did not induce an increase in energy expenditure or changes in RQ in any genotypes regardless if orexin receptors were present or not (**Fig. 2.7 A, B, C, D, E, F, G and H**). Of note, low-fat fed *OX1R*$^{-/-}$ mice showed a reduced energy expenditure compared to wild-type controls (**Fig. 2.7 C and G**). Importantly, the *CAG/orexin* transgene did not induce hyperactivity regardless of the presence or not of orexin receptors or diet (**Fig. 2.8 A, B, C, D, E, F and G**). Low-fat fed *OX2R*$^{-/-}$ mice had reduced activity compared to wild-type controls (**Fig. 2.8 G**) which is also consistent with previous data from narcoleptic mice (Hara, Beuckmann et al. 2001; Hara, Yanagisawa et al. 2005).

Daily high-fat food intake was also measured for 14 days. *CAG/orexin* mice had significantly less food intake compared to wild-type controls (**Fig. 2.6 I**). Critically, this was not due to genotypic specific food preference; both *CAG/orexin* mice and wild-type controls had higher preference for high-fat diet over low-fat diet (**Fig. 2.9 A**) and higher preference

for 10% sucrose over 1% sucrose (**Fig. 2.9 B and C**).

DISCUSSION

The *CAG* promoter is a universal, constitutively active promoter, yet ectopic orexin expression was limited to certain tissues. One possible explanation for this could be that tissues lack the lineage specific enzymes necessary to generate the functional orexin neuropeptide. Indeed, ectopic orexin expression was not present throughout brain parenchyma, but instead limited to specific brain regions. In the *CAG/orexin* LHA, dense orexin-A immunopositive cells (endogenous orexin cells) and the density of orexin-A positive fibers closely resemble those seen in wild-type mice (**Fig. 2.2**). Additionally, orexin-A positive fibers were detected in the medial basal hypothalamus and abundantly in the ARH (**Fig. 2.1 and 2.2**).

Nevertheless, skeptics might argue that the caveat of an overexpression system is that the phenotypes observed are not physiologically relevant. The argument against that was established previously by our group when Mieda *et al.* rescued narcoleptic mice with the *CAG/orexin* transgene (Mieda, Willie et al. 2004), indicating that the transgene does produce physiological functioning

orexin neuropeptides. Furthermore, the anti-obesity effect of the *CAG/orexin* transgene is abolished when orexin receptor-2 is not present. This indicates that *CAG/orexin* transgene's anti-obesity effect is not an artifact, but the physiological effect of increased orexin-OX2R signaling. Additionally, *OX2R*$^{-/-}$ female mice showed significantly more fat mass than wild-type mice even on a low-fat diet. This data supports two critical facts: the first one is that endogenous orexin-OX2R also confers resistance to late-onset obesity even on a low-fat diet; the second important fact to point out is that both orexin neurons and orexin overexpression neurons are not conferring resistance to obesity through change of lipid or nutritional sensing due to change of diet.

Increased energy expenditure in *CAG/orexin* mice and *OX1R*$^{-/-}$*; CAG/orexin* mice, but not *OX2R*$^{-/-}$*; CAG/orexin* mice fed a high-fat diet is consistent with body-weight and fat mass data. Locomotor activity, however, is not affected regardless of diet or genotype, supporting the idea that *CAG/orexin* mice and *OX1R*$^{-/-}$*; CAG/orexin* mice internally have a higher metabolic rate.

Perhaps one of the most confusing pieces of data is the decreased high-fat diet food intake of *CAG/orexin* mice, due to the fact the orexin is thought to act as an orexigenic factor. The significance of this study is that

orexin is produced chronically as opposed to most studies that are acute injections of orexin. It seems that the orexigenic effect of acutely injected orexin is just temporary and eventually is counterregulated by other energy balance mechanisms. Surprisingly, acute and chronic effects of factors often differ significantly in food consumption regulation. Chronic infusion of orexin-A was able to increase daytime food intake, but average daily food intake was unchanged due to a decreased food intake in the dark cycle (Haynes, Jackson et al. 1999; Yamanaka, Sakurai et al. 1999).

EXPERIMENTAL PROCEDURES

Breeding and Maintenance of Mice

The generation of *CAG/orexin* transgenic mice (Mieda, Willie et al. 2004), *OX1R*$^{-/-}$ mice (Kisanuki, Chemelli et al. 2001), and *OX2R*$^{-/-}$ mice (Willie, Chemelli et al. 2003) have been described before. All mice used were backcrossed to C57BL/6J strain for more than ten generations. Each genotypic group was compared with littermate controls as follows: wild-type with *CAG/orexin*, *OX1R*$^{-/-}$ with *OX1R*$^{-/-}$*; CAG/orexin*, *OX2R*$^{-/-}$ with *OX2R*$^{-/-}$*; CAG/orexin*, *ob/ob* (Zhang, Proenca et al. 1994) with *ob/ob; CAG/orexin* mice.

Ob/ob mice were obtained from the Jackson Laboratory. Mice were provided with ad libitum food and water, maintained on a 12 hr light/dark cycle at all times, and housed at 2 to 3 mice per cage under controlled temperature and humidity at all times unless otherwise specified. All procedures were approved by the appropriate institutional animal care and use committees and were carried out in strict accordance with NIH guidelines.

Immunohistochemistry

Immunohistochemistry for orexin-A was performed as previously described (Chemelli, Willie et al. 1999) with a few modifications. 3-5 month old male mice fed a low-fat diet were deeply anesthetized with ketamine and xylazine and transcardially perfused with PBS, then subsequently with 4% paraformaldehyde. Brains and peripheral tissues were dissected and postfixed for 12 hrs in 4% paraformaldehyde overnight before equilibrating in 30% sucrose. Free-floating sections (35um thickness) and peripheral tissues were blocked by normal goat serum before incubated with rabbit anti-orexin-A antibody (Chemicon, AB3704) at a 1:1000 dilution, subsequently incubated with an anti-rabbit IgG secondary antibody (Vector Labs) at a 1:400 dilution. Diaminobenzadine (DAB, Vector Labs) was used to visualize orexin-A

immunoreactivity.

Body Weight Study

For the body weight study, all mice were fed a low-fat diet (standard chow 8664 F6 Rodent Diet; Harlan Teklad) until 8 weeks of age. At 8 weeks of age, mice were randomly assigned to either a low-fat or high-fat diet (D12451; Research Diets). Body weight was monitored weekly until 30 weeks of age. At 28 weeks of age, mice were subjected to NMR (Minispec NMR Analyzer, Bruker) to measure fat mass and lean mass per the manufacture's instructions. Mice were euthanized at 30 weeks of age to collect blood and measure blood glucose. Serum was collected from centrifuged blood and stored at -80 ℃ until use.

Blood Analysis

Mouse Leptin ELISA kit (Crystal Chem) was used for analysis of serum for leptin levels per the manufacturer's instructions.

Metabolic Cage Studies

Indirect calorimetry and locomotor activity were simultaneously measured

using the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments). 16-20 week old mice were housed individually in calorimeter chambers, with 4 days of an acclimation period and 3 days of data collection. Metabolic parameters were based on the following equations:

RQ= CO_2 production/ oxygen consumption

Raw Energy Expenditure (REE) = (3.815+ 1.232x RQ) x oxygen consumption

Energy Expenditure with Effective Mass Correction

= REE/ (weight/mass unit) $^{\text{effective mass factor}}$; Effective mass factor= 0.75

Food Consumption

Food consumption was determined by the average of daily food consumption for 14 consecutive days for mice fed on a high-fat diet. High-fat food was grinded into powder-like sizes by a blender and stored in clear glass jars with a metal open lid. Daily food consumption was measured by subtracting current day total weight (food+ food jar) from previous day's total weight.

Food and Sucrose Preference Test

For the food preference test, two food jars: one containing low-fat diet, the

other containing high-fat diet was placed with an individually housed male mouse and food intake was measured for 14 consecutive days for both diets. Male mice were fed a low-fat diet prior to the food preference test.

For the sucrose preference test, two drinking bottles: one containing water, the other containing sucrose-water was placed with an individually housed male fed a low-fat diet. For the first 3 consecutive days, 3-5 month old male mice were given the choice of water or 1% sucrose solution. The following 3 consecutive days, the same mouse was given the choice of water or 10% sucrose solution. The positions of the 2 bottles were switched daily and the daily drink volume was measured. Sucrose preference was determined by the daily total fluid consumption. Tests were conducted with 5-10 mice per group. All data are expressed as means ± SEM.

DISPLAY ITEMS

Immunohistochemistry of Orexin-A

Region	wild type		*CAG/orexin*	
	cell body	nerve fiber	cell body	nerve fiber
Cerebral cortex				
Cerebral cortex	-	+	-	+
Anterior olfactory nucleus	-	±	-	±
Olfactory tubercle	-	±	-	±
Piriform cortex	-	±	-	±
Endopiriform nucleus	-	±	-	±
Basal ganglia				
Accumbens nucleus, shell	-	±	+	+
Accumbens nucleus, core	-	-	±	-
Lateral septal nucleus	-	+	-	+
Medial septal nucleus	-	+	-	+
Nucleus of the diagonal band	-	+	±	+
Caudate putamen	-	-	-	-
Globus pallidus	-	-	-	-
Bed nucleus of the stria terminalis	-	++	±	++
Anterior cortical amygdaloid nucleus	-	-	-	-
Postetolateral cortical amygdaloid nucleus	-	-	-	-
Postetomedial cortical amygdaloid nucleus	-	-	-	-
Basolateral amygdaloid nucleus	-	±	-	±
Basomedial amygdaloid nucleus	-	±	-	±
Central amygdaloid nucleus	-	+	-	+
Lateral amygdaloid nucleus	-	±	-	±
Hippocampal formation				
CA1	-	-	+	-
CA2	-	-	+	-
CA3	-	-	+	-
Dentate gyrus	-	-	+	-
Subiculum	-	-	-	-
Thalamus				
Anteriodorsal thalamic nucleus	-	-	-	-
Anteriomedial thalamic nucleus	-	-	-	-
Intermediodorsal thalamic nucleus	-	±	-	±
Mediodorsal thalamic nucleus	-	-	-	-
Laterodorsal thalamic nucleus	-	-	-	-
Paraventricular thalamic nucleus	-	++	-	++
Reuniens thalamic nucleus	-	+	+	+
Central medial thalamic nucleus	-	++	+	++
Lateral posterior thalamic nucleus	-	-	-	-
Ventrolateral thalamic nucleus	-	-	-	-
Ventromedial thalamic nucleus	-	-	-	-
Reticular thalamic nucleus	-	-	-	-
Zona incerta	-	+	-	+

Region	wild type		*CAG/orexin*	
	cell body	nerve fiber	cell body	nerve fiber
Epithalamus				
Medial habenular nucleus	-	-	-	-
Lateral habenular nucleus	-	+	-	+
Hypothalamus				
Medial preoptic nucleus	-	++	+	++
Paraventricular hypothalamic nucleus	-	++	+	++
Suprachiasmatic nucleus	-	-	++	-
Anterior hypothalamic area	-	++	+	++
Arcuate hypothalmic nucleus	-	++	+	++
Ventromedial hypothalamic nucleus	-	++	+	++
Dorsomedial hypothalamic nucleus	-	++	+	++
Lateral hypothalamic area	+++	+++	++++	+++
Posterior hypothalamic area	-	++	+	++
Lateral mammillary nucleus	-	-	-	-
Medial mammillary nucleus	-	-	-	-
Supramammillary nucleus	-	++	-	++
Tuberomammillary nucleus	-	++	±	++
Brain stem and cerebellum				
Substance nigra, compact part	-	+	+	+
Substance nigra, reticular part	-	-	-	-
Ventral tegmental area	-	++	+	++
Superior colliculus	-	±	-	±
Inferior colliculus	-	±	-	±
Interpeduncular nucleus	-	+	-	+
Red nucleus	-	-	-	-
Cerebellum	-	-	-	-
Vestibular nucleus	-	±	-	±
Oculomotor nucleus	-	-	-	-
Motor trigeminal nucleus	-	-	-	-
Facial nucleus	-	±	-	±
Dorsal motor nucleus of vagus	-	±	-	±
Hypoglossal nucleus	-	±	-	±
Nucleus of the solitary tract	-	++	+	++
Area postrema	-	+	+	+
Parabrachial nucleus	-	++	±	++
Spinal trigeminal nucleus	-	-	-	-
Dorsal raphe nucleus	-	++	±	++
Median raphe nucleus	-	++	±	++
Dorsal tegmental nucleus	-	-	±	-
Laterodorsal tegmental nucleus	-	++	+	++
Locus coeruleus	-	++	+	++
Periaqueductal gray	-	++	±	++
Pontine nucleus	-	-	-	-
Pontine reticular nucleus	-	±	-	±
Inferior olivary nucleus	-	-	-	-
Gigantocellular reticular nucleus	-	+	±	+

Table 2.1 Immunohistochemistry of Orexin-A Localization

Immunohistochemistry of Orexin-A in the peripheral tissues

	Wild-type	*CAG/orexin*
Brain	+++	+++
Liver	-	-
Kidney	-	-
Thyroid	-	++
Adrenal	-	+
Pancreas (Exocrine)	-	-
Pancreatic islet	-	++
Skeletal muscle	-	-
White fat tissue	-	-
Brown fat tissue	-	-

Table 2.2 Immunohistochemistry of Orexin-A in Peripheral Tissues

Tissue	Method	OX1R	OX2R	Species	Reference
Heart	Northern	−	−	Rat	Sakurai (1998)
	RT-PCR	−	−	Rat	Johren (2001)
Stomach	RT-PCR, IHC	+	−	Rat	Kirchgessner (1999)
	RT-PCR	−	−	Rat	Johren (2001)
Jejunum	qPCR	−	−	Mouse	This study
Liver	Northern	−	−	Rat	Sakurai (1998)
	RT-PCR	−	−	Rat	Johren (2001)
	qPCR	−	−	Mouse	This study
Pancreas	RT-PCR	−	−	Rat	Johren (2001)
	RT-PCR	+	+	Rat	Kirchgessner (1999)
	RT-PCR	+	+	Rat	Nowak (2005)
	qPCR	−	−	Mouse	This study
Pancreatic islet	IHC	+	−	Rat	Kirchgessner (1999)
	qPCR	3% of brain expression level	30% of brain expression level	Mouse	This study
Thyroid gland	RT-PCR	+	ND	Rat	Johren (2001)
	qPCR	3% of brain expression level	2% of brain expression level	Mouse	This study
Adrenal cortex	RT-PCR, IHC	−	+	Human	Randeva (2001)
	IHC	+	−	Human	Blanco (2002)
	RT-PCR, IHC	+	+	Human, rat	Spinazzi (2005)
	RT-PCR	−	+	Rat	Johren (2001)
Adrenal medulla	RT-PCR	+	+	Rat	Lopez (1999)
	RT-PCR	−	−	Rat	Johren (2001)
	IHC	−	+	Human	Blanco (2002)
White adipose tissue	RT-PCR	−	−	Rat	Johren (2001)
	RT-PCR, IHC	+	+	Human	Digby (2006)
	qPCR	−	−	Mouse	This study
Skeletal muscle	Northern	−	−	Rat	Sakurai (1998)
	RT-PCR	−	−	Rat	Johren (2001)

Table 2.3 Distribution of Orexin Receptors in Peripheral Tissues

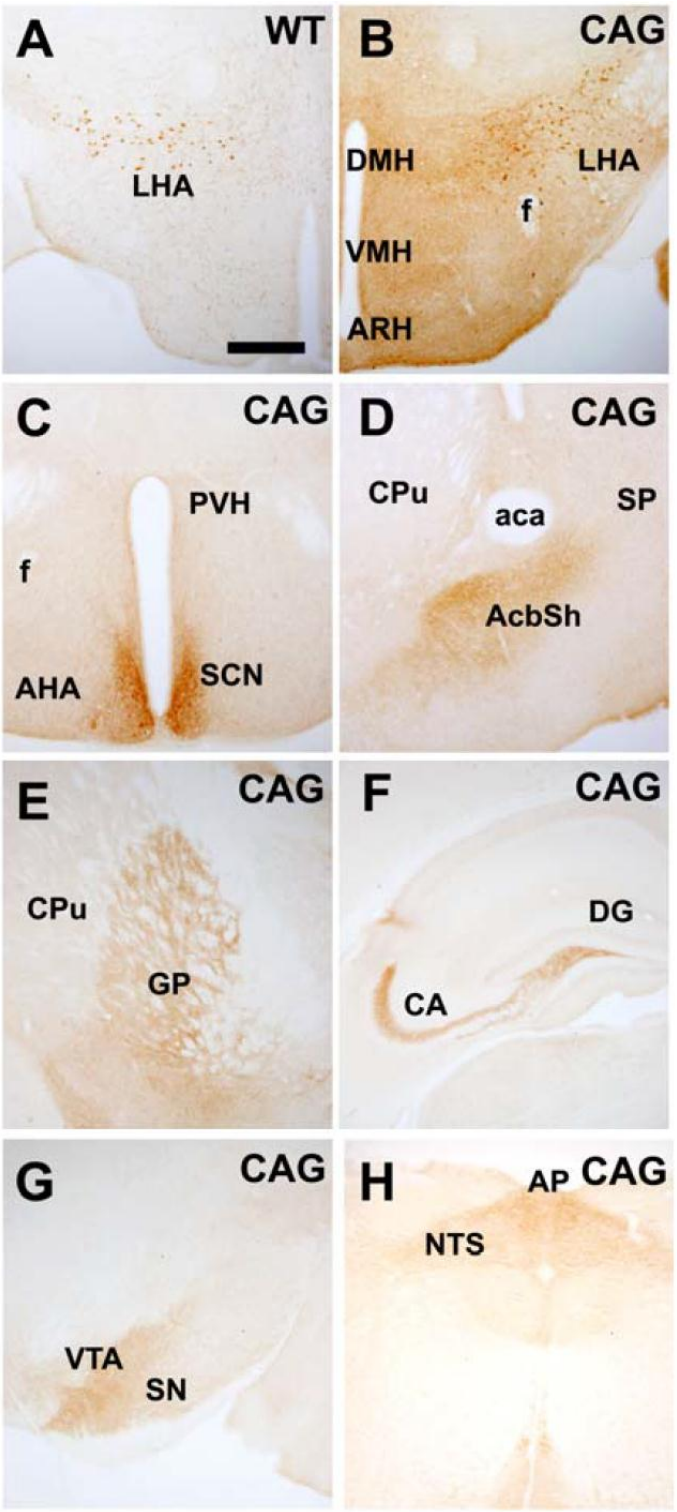

Figure 2.1 Immunohistochemistry of Orexin-A in the Mouse Brain

(A) Wild-type mouse exhibits orexin-A in the lateral hypothalamic area (LHA).

(B) *CAG/orexin* mouse shows a strong orexin-A immunoreactivity in the

arcuate nucleus (ARH), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), and LHA in addition to native orexin-A positive neurons in the LHA.

(C) *CAG/orexin* mouse shows orexin-A immunoreactivity in the suprachiasmatic nucleus (SCN).

(D) *CAG/orexin* mouse shows orexin-A immunoreactivity in the nucleus accumbens shell (AcbSh).

(E) *CAG/orexin* mouse shows orexin-A immunoreactivity in the globus pallidus (GP).

(F) *CAG/orexin* mouse shows orexin-A immunoreactivity in the stratus moleculare of Cornu Ammonis (CA) and the hilus of the dentate gyrus (DG).

(G) *CAG/orexin* mouse shows orexin-A immunoreactivity in the substantia nigra (SN).

(H) *CAG/orexin* mouse shows orexin-A immunoreactivity in the area postrema (AP) and the nucleus of the solitary tract (NTS). Abbreviations: aca, anterior branch of the anterior commisure; AHA, anterior hypothalamic area; CPu, caudate putamen; f, fornix; LV, lateral ventricle; PVH, paraventricular nucleus; SP, septum; VTA, ventral tegmental area. Scale bars: 400 μm.

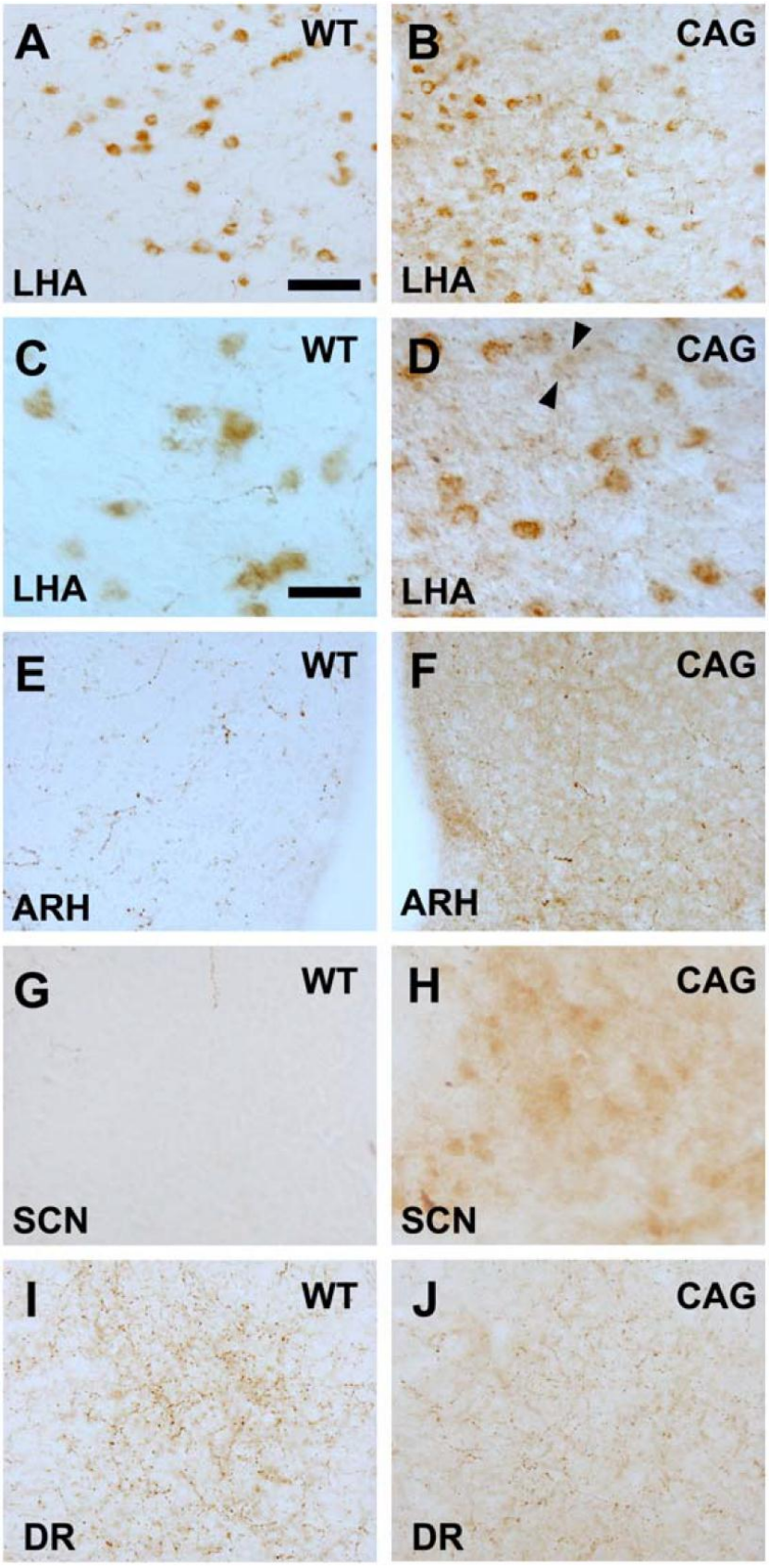

Figure 2.2 Orexin-A Positive Neurons and Fibers

(A) Orexin-A positive neurons and fibers in the LHA of wild-type mice.

(B) *CAG/orexin* mouse shows a similar number of strong orexin-A positive neurons and fibers in the LHA. Additionally, weak immunoreactivity of orexin-A is distributed throughout the LHA.

(C) Higher magnification of orexin-A positive neurons and fibers in the LHA of wild-type mice with negligible background staining.

(D) In addition to strong orexin-A positive neurons, weak immunoreactivities are observed in many cell bodies (indicated by arrowheads) in *CAG/orexin* mice.

(E) Orexin-A positive fibers in the ARH of wild-type mice.

(F) Orexin-A positive fibers and additional weak orexin immunoreactivity is observed in the ARH of *CAG/orexin* mice.

(G) SCN of wild-type mice is free of orexin-A immunoreactivity.

(H) SCN of *CAG/orexin* mice shows weak to moderate orexin-A immunoreactive cell bodies.

(I) Dense orexin-A positive fibers are observed in the dorsal raphe (DR) of wild-type mice.

(J) Dense orexin-A positive fibers are observed in the dorsal raphe (DR) of *CAG/orexin* mice. Scale bars on (A) and (C) are 100 μm and 40 μ

m ,respectively. The magnifications of (A) and (C) are the same with (B, E, F, I, J) and (D, G, H), respectively. (C, D, G, and H) were observed using Nomarski differential-interference contrast.

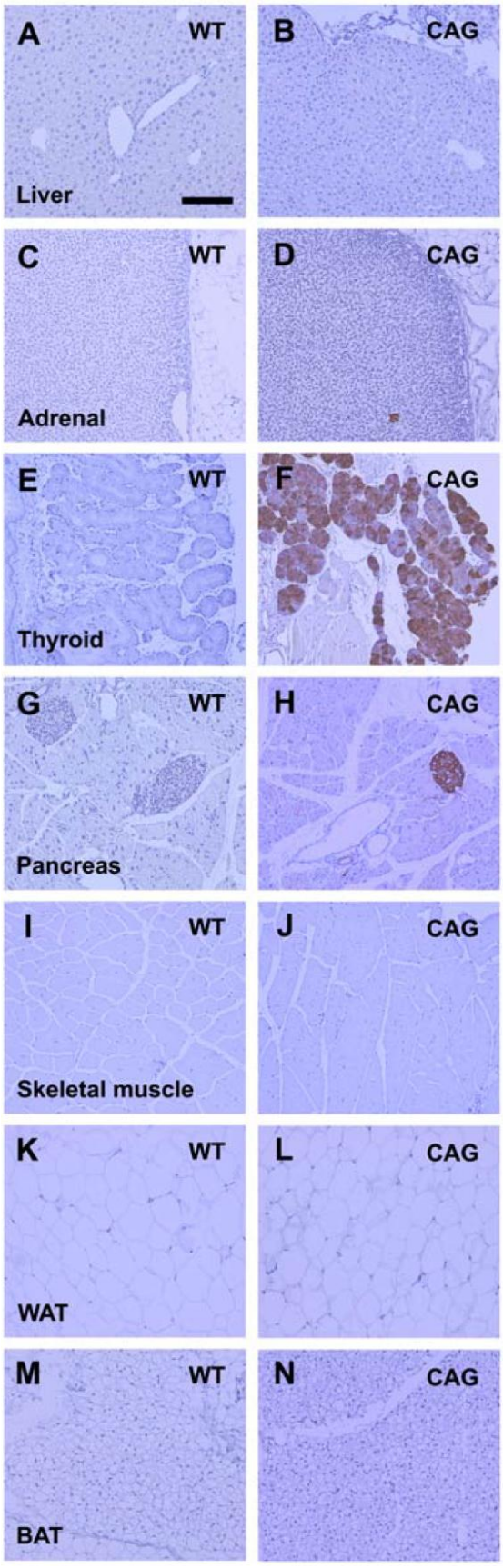

Figure 2.3 Orexin-A in the Peripheral Tissues

Wild type mice did not exhibit orexin-A immunoreactivity in the liver (A), adrenal gland (C), thyroid (E), pancreas (G), skeletal muscle (I), white adipose tissue (K), and brown adipose tissue (M). CAG/orexin mice exhibited orexin-A immunoreactivity in the thyroid (F), islet of the pancreas (H), and scattered areas of the medulla of the adrenal gland (D), while no immunoreactivity was detected in the liver (B), skeletal muscle (J), white adipose tissue (L), and brown adipose tissue (N). Scale bar: 400 μm.

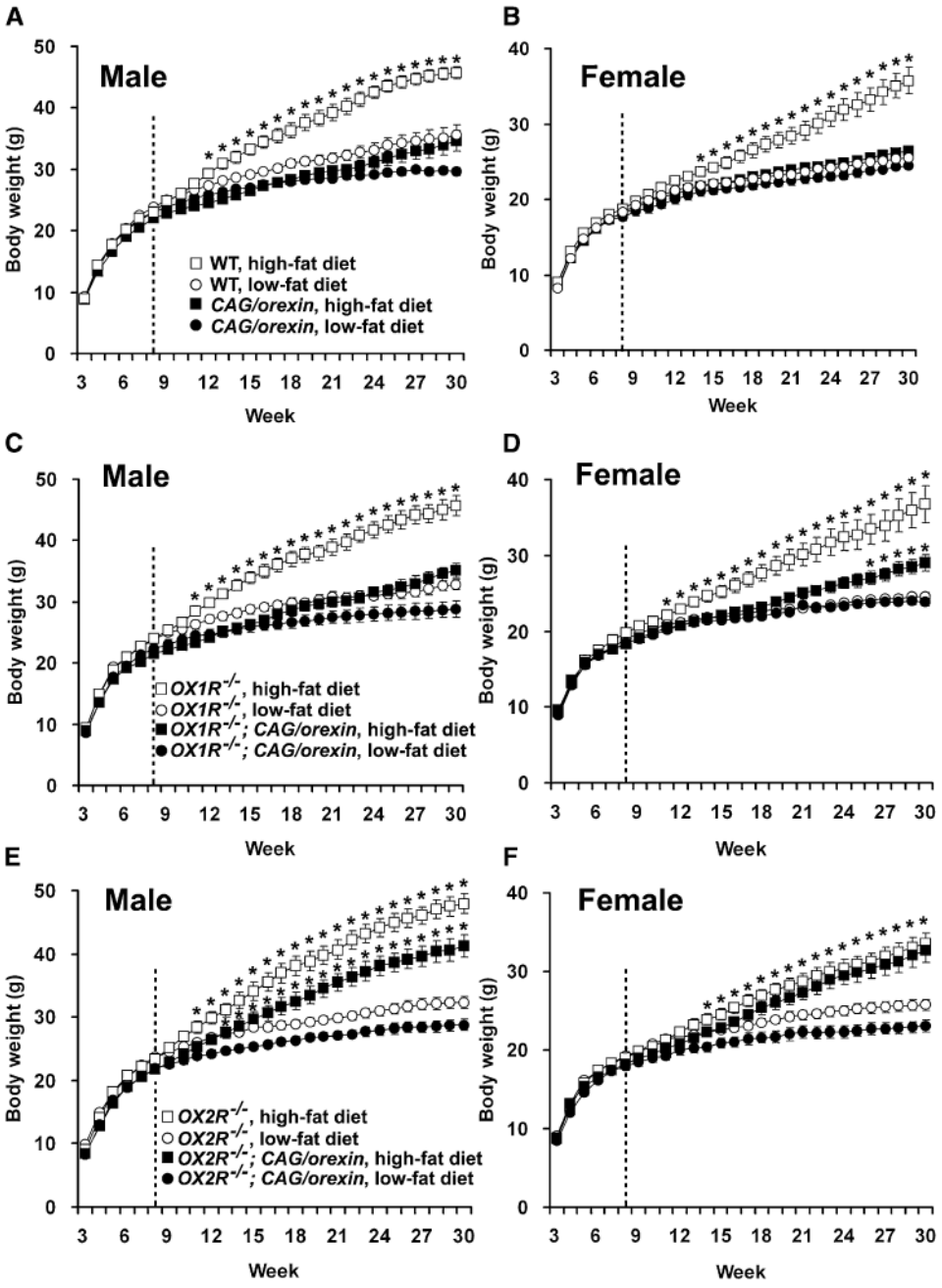

Figure 2.4 Growth Curves of Orexin Genetically Modified Mice Fed a Low-or High-Fat Diet

(A-F) Body weights of mice measured weekly from the age of 3 weeks to 30 weeks. A high-fat diet started at the age of 8 weeks (dotted line). The

numbers of mice are 10-14 per group. * indicates significant differences between different diet conditions for each genotypic group according to post hoc analysis at each time point. Significant differences in (F) for *OX2R*$^{-/-}$ mice and *OX2R*$^{-/-}$*; CAG/orexin* mice are indicated by one asterisk. Data are expressed as mean ± SEM.

Body weight curves of wild-type male (A) and female (B) mice on a high-fat diet were significantly higher than those of wild-type mice on a low-fat diet ($p<0.0005$, both sexes). CAG/orexin mice did not differ significantly under low-fat and high-fat diet conditions ($p=0.51$ male; $p=0.13$ female).

Body weight curves of *OX1R*$^{-/-}$ male (C) and female (D) mice on a high-fat diet were significantly higher than those of *OX1R*$^{-/-}$ mice on a low-fat diet ($p<0.0001$ male; $p<0.01$ female). *OX1R*$^{-/-}$*; CAG/orexin* male mice did not differ significantly between low-fat and high-fat dietary conditions ($p=0.21$). The body weights of *OX1R*$^{-/-}$*; CAG/orexin* female mice on a high-fat diet were significantly less than those of *OX1R*$^{-/-}$ mice on a high-fat diet ($p<0.01$), in spite of no body weight difference between *OX1R*$^{-/-}$*; CAG/orexin* and *OX1R*$^{-/}$ female mice on a low-fat diet ($p=0.50$).

The body weight growth curves of *OX2R*$^{-/-}$ male (E) and female (F) mice on a high-fat diet were significantly higher than those of *OX2R*$^{-/-}$ mice on a low-fat

diet (p<0.0001 male; p<0.005 female). Likewise, *OX2R$^{-/-}$; CAG/orexin* male (E) and female (F) mice showed significant weight gain on a high-fat diet compared to those on a low-fat diet (p<0.0005 male; p<0.001 female).

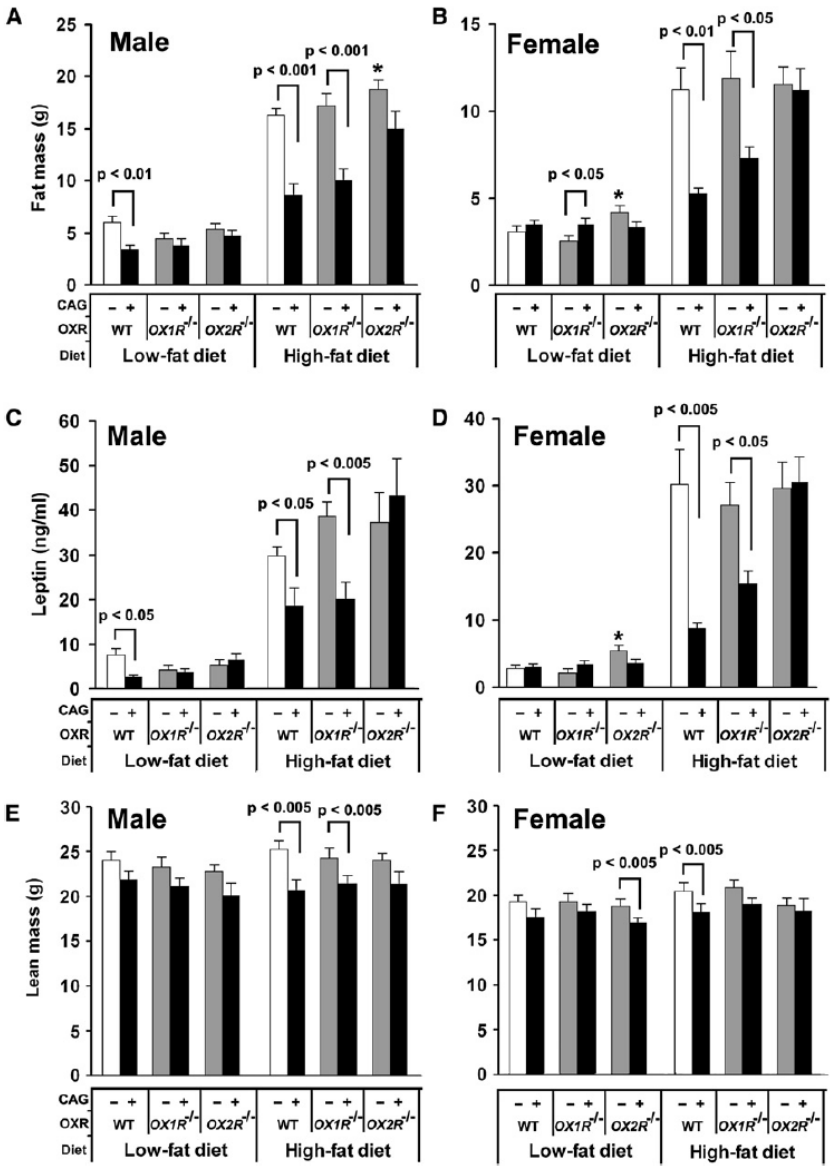

Figure 2.5 Fat Mass, Serum Leptin Levels, and Lean Mass of Orexin Signaling Modified Mice

(A and B) The fat masses of male (A) and female (B) mice at 28 weeks of age on different diets.

(C and D) The serum leptin levels of male (C) and female (D) mice at 30 weeks of age on different diets.

(E and F) The lean masses of male (E) and female (F) mice at 28 weeks of age on different diets. * indicates significant ($p<0.05$) increase compared to wild-type mice under the same food condition. The numbers of mice are 8-14 mice per group. Data are expressed as means ± SEM.

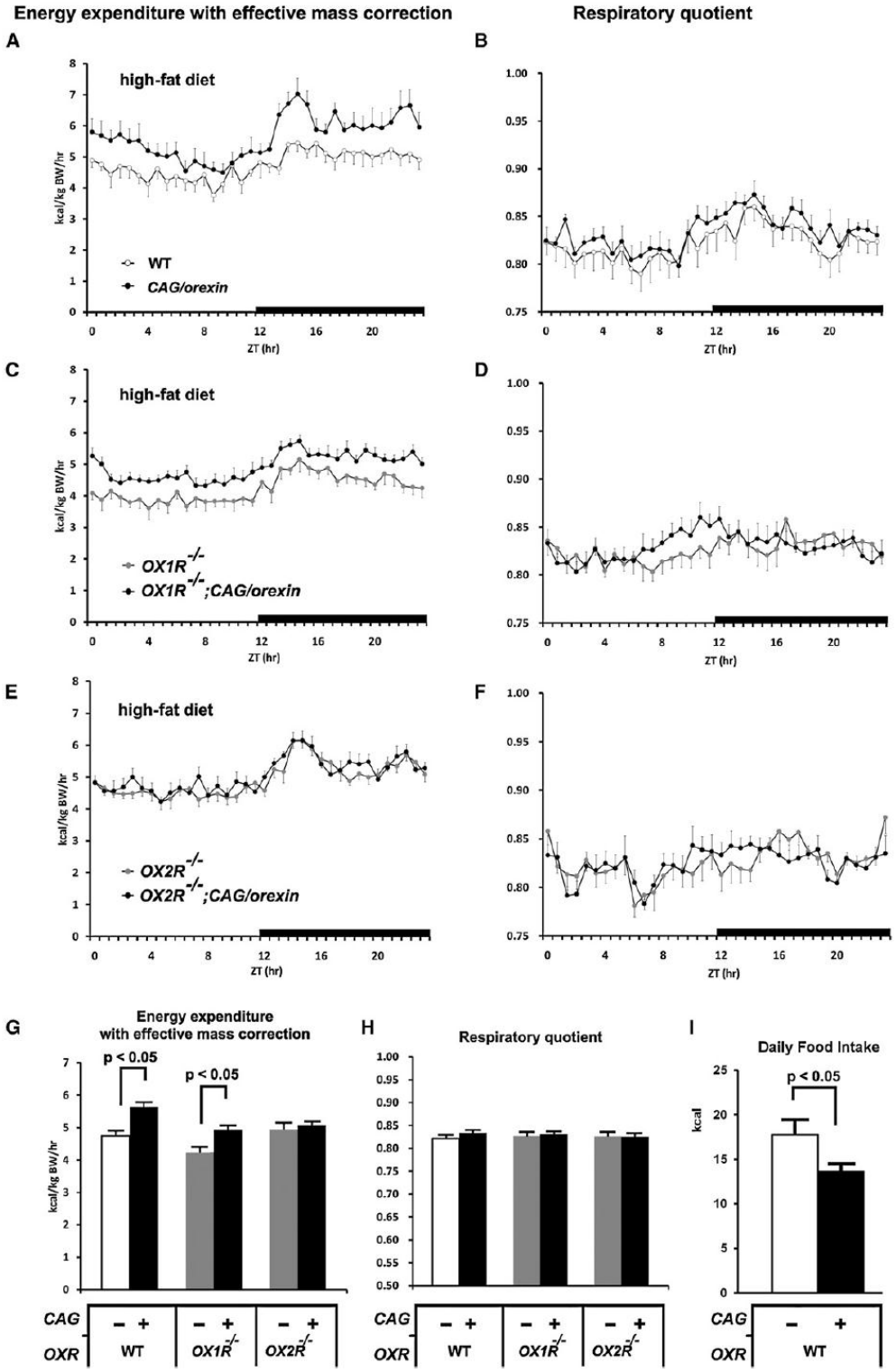

Figure 2.6 Metabolic Parameters of Orexin Signaling-Modified Mice on a High-Fat Diet

(A and B) The energy expenditure with effective mass correction (A) and respiratory quotient (B) sampled every 40 min over 24 hr of *CAG/orexin* mice and wild-type at 16-20 weeks of age.

(C and D) The energy expenditure with effective mass correction (C) and respiratory quotient (D) over 24 hr of *OX1R*$^{-/-}$*; CAG/orexin* mice and *OX1R*$^{-/-}$ mice.

(E and F) The energy expenditure with effective mass correction (E) and respiratory quotient (F) over 24 hr of *OX2R*$^{-/-}$*; CAG/orexin* mice and *OX2R*$^{-/-}$ mice.

(G and H) The averaged energy expenditure with effective mass correction (G) and respiratory quotient (H).

(I) Averaged daily high-fat diet intake of *CAG/orexin* mice and wild-type mice for 14 days. The numbers of mice are 6-9 mice per group. Data are expressed as means ± SEM.

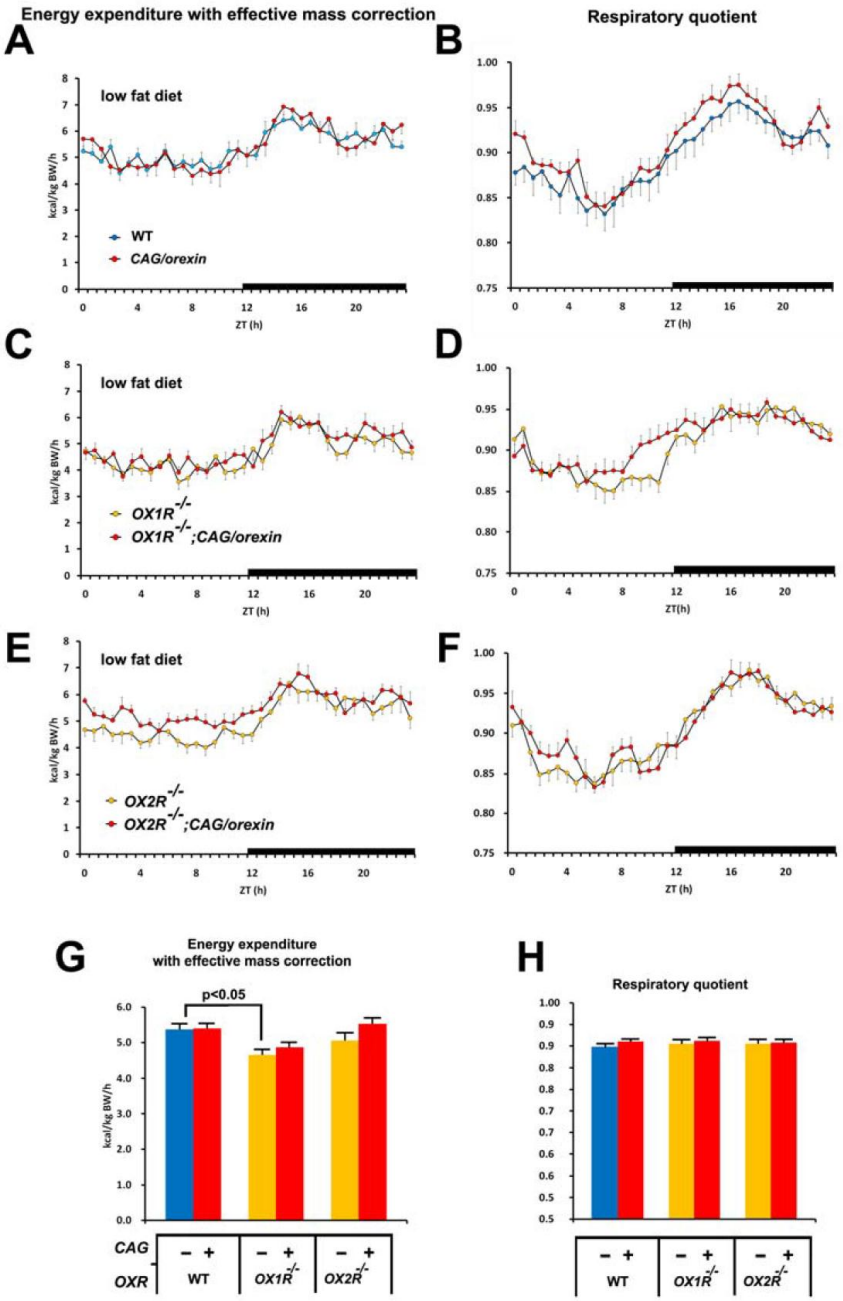

Figure 2.7 Energy Expenditure with Effective Mass Correction and Respiratory Quotient of Orexin-Signaling Modified Mice on a Low-Fat Diet

(A and B) The energy expenditure with effective mass correction (A) and

respiratory quotient (B) over 24 hrs of *CAG/orexin* and wild-type mice.

(C and D) The energy expenditure with effective mass correction (C) and respiratory quotient (D) over 24 hrs of *OX1R*$^{-/-}$*; CAG/orexin* and *OX1R*$^{-/-}$ mice.

(E and F) The energy expenditure with effective mass correction (E) and respiratory quotient (F) over 24 hrs of *OX2R*$^{-/-}$*; CAG/orexin* and *OX2R*$^{-/-}$ mice.

(G and H) The averaged energy expenditure with effective mass correction (G) and respiratory quotient (H).

Data were sampled every 40 min. Mice were 16-20 weeks of age. The numbers of mice were 6-9 per group. Data are expressed as means ± SEM.

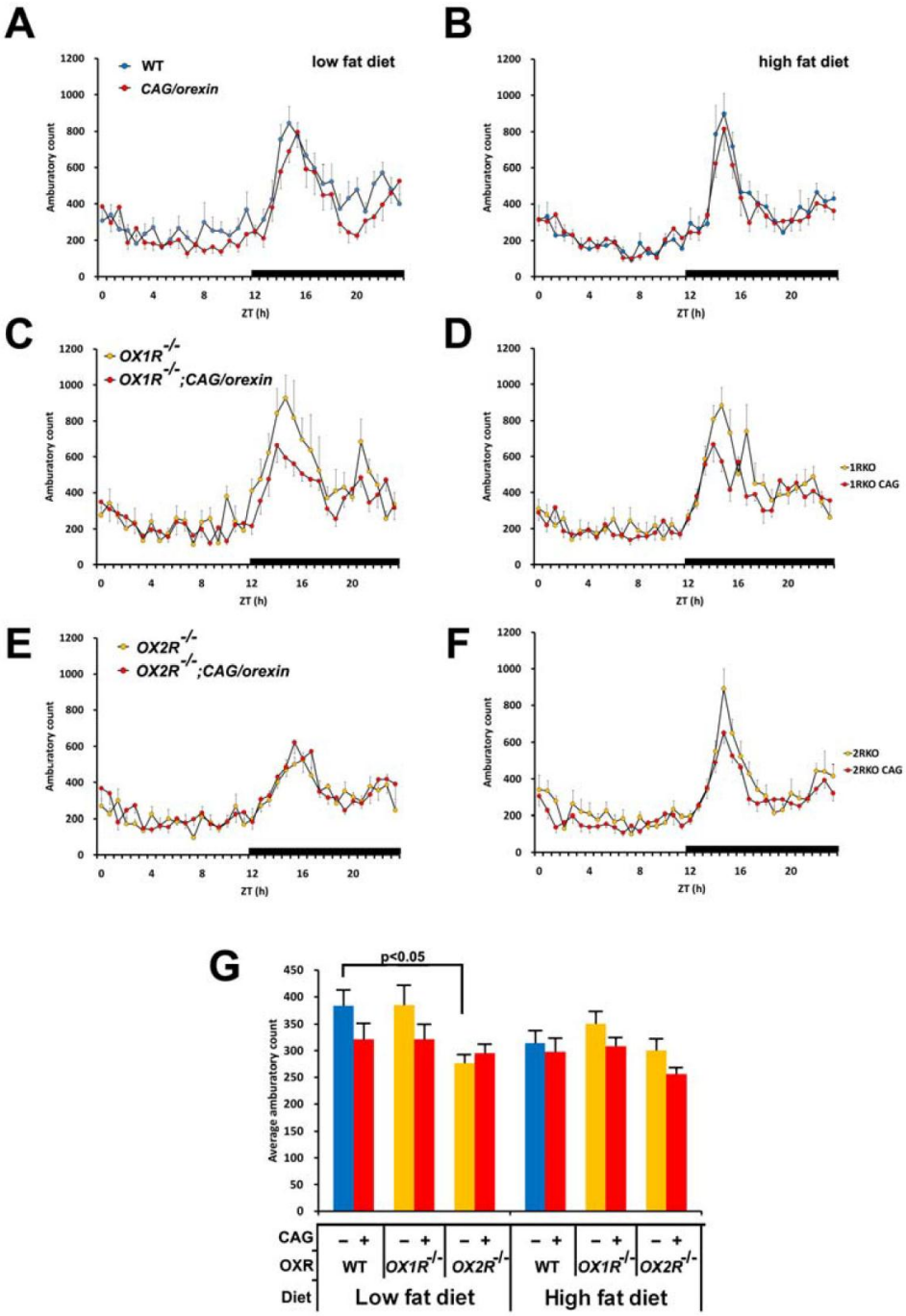

Figure 2.8 Locomotion of Orexin-Signaling Modified Mice

(A and B) The locomotor activity over 24 hr of *CAG/orexin* and wild-type mice on a low-fat (A) and a high-fat diet (B).

(C and D) The locomotor activity over 24 hr of *OX1R*$^{-/-}$*; CAG/orexin* and

OX1R$^{-/-}$ mice on a low-fat (C) and a high-fat diet (D).

(E and F) The locomotor activity over 24 hr of *OX2R*$^{-/-}$*; CAG/orexin* and *OX2R*$^{-/-}$ mice on a low-fat (E) and a high-fat diet (F).

(G) The averaged locomotor activity of each genotypic group under different fat diet conditions.

Data were sampled every 40 min. Mice used are the age of 16-20 weeks. The numbers of mice used were 6-9 mice per group. Data are expressed as means ± SEM.

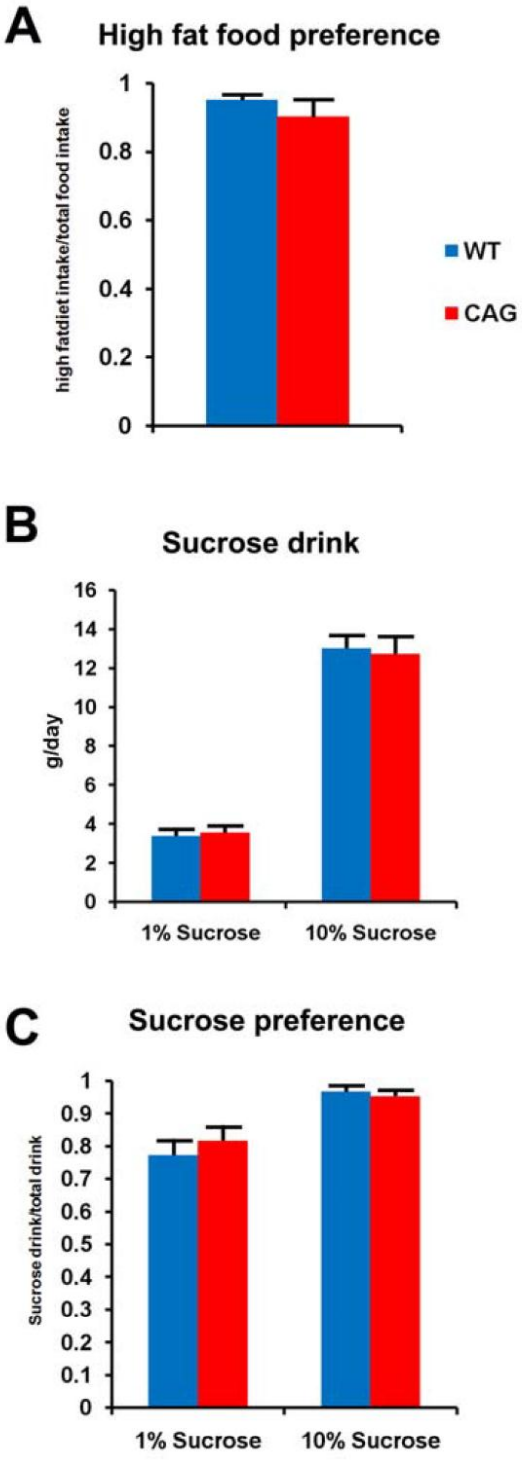

Figure 2.9 Similar Preferences for High-Fat Diet and Sucrose Containing Water

(A) Both *CAG/orexin* and wild-type mice showed higher preference for a high-fat diet. Data is plotted as food intake of high-fat diet divided by total

food intake for 14 consecutive days.

(B) *CAG/orexin* and wild-type mice consumed similar amounts of sucrose-containing water for the two bottle choice test.

(C) *CAG/orexin* and wild-type mice both showed preference to 10% sucrose water.

5-10 mice were used per group. Data are expressed as means ± SEM.

CHAPTER THREE

Orexin and glucose metabolism

SUMMARY

Glucose metabolism in *CAG/orexin* mice was analyzed by measuring blood glucose levels, serum insulin levels and utilizing the glucose tolerance test. The *CAG/orexin* transgene prevented hyperglycemia and hyperinsulinemia on a high-fat diet. Surprisingly, *OX1R*$^{-/-}$ mice itself prevented hyperglycemia and hyperinsulinemia on a high-fat diet without the presence of the *CAG/orexin* transgene, indicating that endogenous OX1R signaling could participate in deleterious effects of high-fat diet on glucose metabolism. Glucose tolerance test results were consistent with body weight curves; specifically, *CAG/orexin* and *OX1R*$^{-/-}$*; CAG/orexin* mice had improved glucose metabolism under high-fat diet conditions, but *OX2R*$^{-/-}$*; CAG/orexin* mice did not show any improvement compared to *OX2R*$^{-/-}$ mice.

INTRODUCTION

Glucose metabolism plays an important part in energy balance

regulation and its dysregulation is critical in the development of diabetes mellitus type 2; one of the main factors contributing towards the metabolic syndrome. Obesity and insulin resistance have gained attention as the core manifestations of the syndrome (Kassi, Pervanidou et al. 2011). Therefore, since the *CAG/orexin* transgene confers resistance to diet-induced obesity, this chapter is set out to examine if the transgene serves potential anti-diabetic functions as well by measuring glucose metabolism and serum insulin levels.

RESULTS

Glucose and insulin levels were measured in all genotypes fed both a low-fat or high-fat diet. There were no significant differences in glucose levels in each genotype compared to their respective controls when fed a low-fat diet (**Fig. 3.1A**). When fed a high-fat diet, wild-type male controls had significantly higher blood glucose levels compared to *CAG/orexin* male mice (**Fig. 3.1A**). There were no significant differences in blood glucose level between *OX1R*$^{-/-}$ and *OX1R*$^{-/-}$*; CAG/orexin* mice or *OX2R*$^{-/-}$ and *OX2R*$^{-/-}$*; CAG/orexin* mice even fed a high-fat diet (**Fig. 3.1A**). Interestingly though, *OX1R*$^{-/-}$ mice had significantly lower blood glucose levels compared to wild-type male mice when fed a high-fat diet.

In contrast, insulin levels were significantly higher in wild-type male mice compared to *CAG/orexin* male mice even under low-fat diet conditions (**Fig. 3.1 B**). When subjected to a high-fat diet, wild-type mice developed hyperinsulinemia, and were significantly higher than *CAG/orexin* mice (**Fig. 3.1 B**). *$OX1R^{-/-}$; CAG/orexin* and *$OX2R^{-/-}$; CAG/orexin* mice both had significantly lower insulin levels compared to *$OX1R^{-/-}$* and *$OX2R^{-/-}$* mice respectively (**Fig. 3.1 B**).

Furthermore, *CAG/orexin* transgene's effect on glucose homeostasis was tested by the glucose tolerance test. On a low-fat diet, *CAG/orexin* transgene did not significantly affect glucose homeostasis (P=0.47, **Fig. 3.2**). On a high-fat diet, however, *CAG/orexin* transgene not only induced a significantly lower basal fasted glucose level, but also improved glucose tolerance at all time points tested (**Fig. 3.1 C**). Although *$OX1R^{-/-}$; CAG/orexin* had similar basal fasted glucose levels compared to *$OX1R^{-/-}$* controls, *$OX1R^{-/-}$; CAG/orexin* had improved glucose tolerance at 90 and 120 minutes after injection of glucose (**Fig. 3.1 D**). *$OX2R^{-/-}$* and *$OX2R^{-/-}$; CAG/orexin* had both similar levels of fasted glucose and glucose tolerance at all time points tested (**Fig. 3.1 E**).

DISCUSSION

The *CAG/orexin* transgene had a protective effect against hyperglycemia and hyperinsulinemia. Interestingly, *OX1R*$^{-/-}$ also confers resistance to hyperglycemia without the presence of the *CAG/orexin* transgene on a high-fat diet, despite being similarly obese compared to wild-type mice. This suggests that endogenous orexin-OX1R signaling may contribute to the deleterious effect of high-fat diet on glucose metabolism. Indeed, OX1R is expressed in the solitary tract nucleus and dorsal motor nucleus of the vagus (Marcus, Aschkenasi et al. 2001), which participates in the regulation of hepatic glucose production (Pocai, Obici et al. 2005).

Although I did not detect orexin-A in wild-type mice in this study, previous studies have shown immunoreactivity of orexin-A and OX1R in the β cells of pancreatic islets (Kirchgessner and Liu 1999) and glucagon-secreting α-cells in rats (Ouedraogo, Naslund et al. 2003). Nevertheless, *in vitro* studies conducted in isolated rat islets so far have yielded conflicting data. 80 nM OXA did not have any effect but 100 and 150 nM OXA significantly decreased glucose stimulated insulin secretion in isolated rat islets (Ouedraogo, Naslund et al. 2003). In contrast, Nowak *et al.* found that 10 nM OXA was sufficient to

stimulate insulin secretion in basal (6.66 nM) and high glucose (26.4 nM) concentrations in rat islets. The significance of circulating OXA *in vivo* remains uncertain, because the plasma levels of OXA is remarkably lower (10^{-12} to 10^{-11} M) compared to the minimal concentrations of OXA eliciting biological effects in peripheral tissues *in vitro* (10^{-10} to 10^{-8} M) (Spinazzi, Andreis et al. 2006).

In vivo data of orexin's regulation in glucose metabolism was also inconsistent. Ouedraogo *et al.* found that intravenous injection of OXA significantly increased plasma glucagon and decreased plasma insulin levels (Ouedraogo, Naslund et al. 2003). In contrast, when Ehrstrom *et al.* infused OXA into the jugular vein of 18-hr fasted rats, insulin levels were not affected, however, plasma glucagon was suppressed (Ehrstrom, Gustafsson et al. 2005). These discrepancies in reports suggests that responses to orexin might be modulated by glucose availability and some unknown factors, therefore the specific role of orexin and OX1R signaling on glucose metabolism merits further investigation.

Importantly, under orexin overexpression, the OX2R mediated effects prevail, and the presence or absence of OX1R does not affect the improvement of glycemia or insulinemia.

EXPERIMENTAL PROCEDURES

Blood Analysis

Whole blood glucose level was measured using a standard clinical glucometer (Elite; Bayer). Whole blood insulin level was measured using the Ultra Sensitive Rat Insulin ELISA kit with the Mouse Insulin Standard (Crystal Chem) per manufacturer's instructions.

Glucose Tolerance Test

21-25 week old male mice fed low-fat and high-fat diet were fasted for 12 hours starting from ZT16 and then injected intraperitoneally with glucose (1.5g/kg of body weight) at ZT4. Tail blood was then collected at 0, 15, 30, 60, and 90 minutes after injection. Glucose levels were assessed by a standard clinical glucometer (Elite; Bayer)

DISPLAY ITEMS

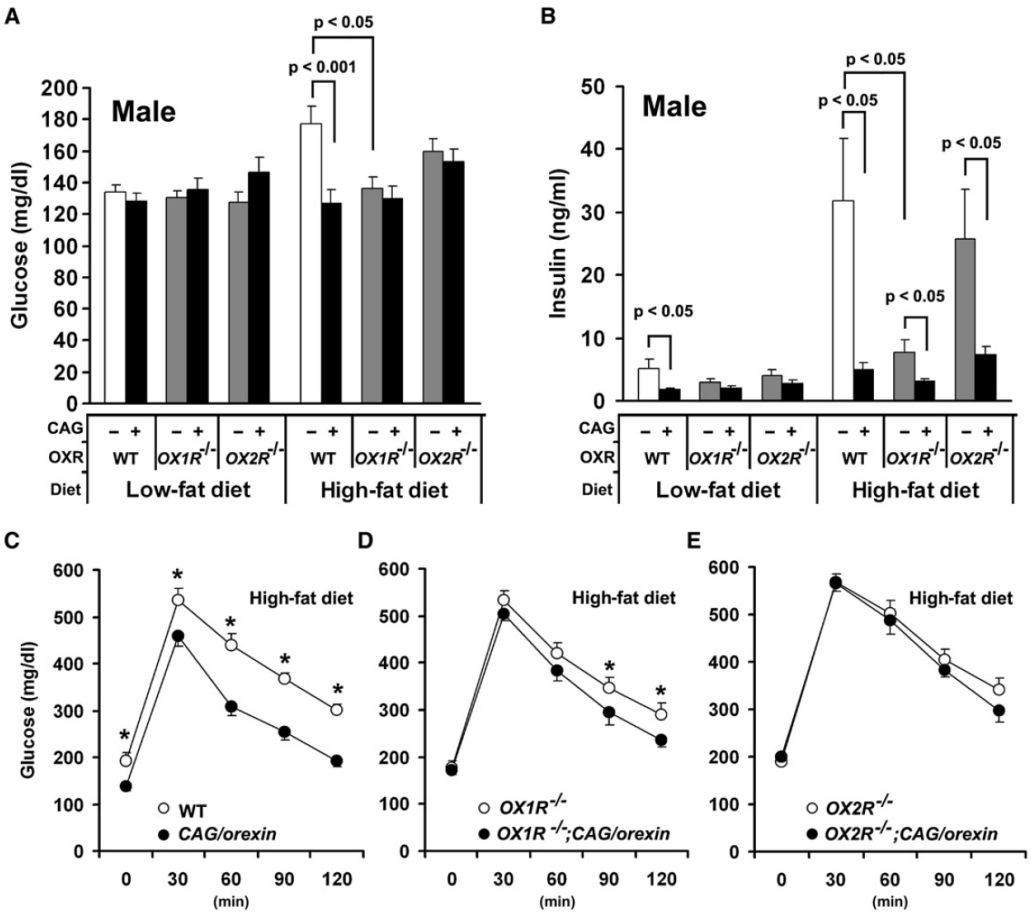

Figure 3.1 Glucose Metabolism of *CAG/orexin* Mice on Different Fat Diets

(A) Blood glucose levels of orexin-related gene mutant mice on different fat diets.

(B) Serum insulin levels of orexin-related gene mutant mice on different fat diets.

(C) Glucose tolerance test showed that blood glucose levels of *CAG/orexin* mice were significantly lower than wild-type littermate mice on a high-fat

diet after the administration of glucose. Data are expressed as data ± SEM.

(D) Glucose tolerance test showed that blood glucose levels of *OX1R*$^{-/-}$*; CAG/orexin* mice were significantly lower than *OX1R*$^{-/-}$ mice on a high-fat diet after the administration of glucose. Data are expressed as data ± SEM.

(E) Glucose tolerance test showed that there is no significant difference in blood glucose levels between *OX2R*$^{-/-}$*; CAG/orexin* mice and *OX2R*$^{-/-}$ mice on a high-fat diet after the administration of glucose (p=0.33).

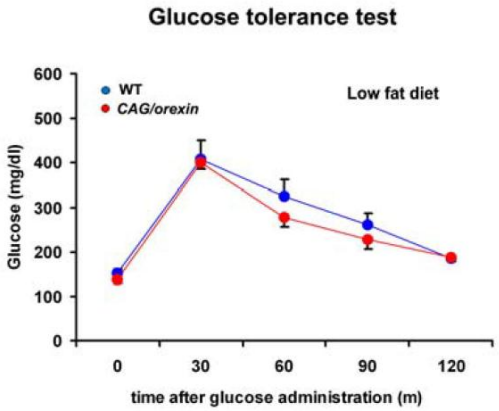

Figure 3.2 Glucose Tolerance of *CAG/orexin* Mice on a Low-Fat Diet

CAG/orexin male mice fed a low-fat diet has fasted glucose levels similar to wild-type mice. Likewise, after the administration of glucose, glucose levels were similar between *CAG/orexin* and wild-type mice at all time points. Data

are expressed as means ± SEM.

CHAPTER FOUR

Orexin has no detectable effects on peripheral tissues

SUMMARY

Ectopic expression of the orexin transgene in several peripheral tissues including the thyroid, adrenal gland, and pancreatic islets (**Fig. 2.3 C, D, E, F, G, and H**) raises the question whether or not peripheral tissues are what govern this anti-obesity effect or contribute to this phenotype. Consistent with the fact that orexin's effect on peripheral tissues still remains controversial (Heinonen, Purhonen et al. 2008); I did not detect any significant effects on peripheral tissues ectopically expressing orexin. Specifically, for the thyroid secreted hormones, there were no significant differences in levels of TSH, T4, and T3 hormones between wild-type and *CAG/orexin* mice (**Fig 4.1 A, B, and C**). No significant differences were detected for urinary epinephrine, urinary norepinephrine, or serum corticosterone between genotypes on a low-fat diet (**Fig. 4.3 A, B, and C**). *UCP1*, *2*, and *3* mRNA levels were unchanged in both brown adipose tissue and skeletal muscle regardless of low or high-fat diet (**Fig. 4.2 A and B**). Last but importantly, systolic blood pressure was not altered regardless of low or high-fat diet

between both genotypes (**Fig. 4.3 D**).

INTRODUCTION

Orexin ligand and receptors have been identified in many peripheral tissues; however, most of the present studies either have conflicting results or are inconclusive (Heinonen, Purhonen et al. 2008). Therefore, it was essential to examine if the orexin transgene had any adverse effects on key metabolic peripheral tissues.

TRH is produced in the PVN (as described in the introduction) and controls the release of thyroid-stimulating hormone (TSH) from the pituitary. TSH then stimulates the thyroid gland which produces the thyroid hormones triiodothyronine (T3) and thyroxine (T4), which regulates growth, development, and metabolism in almost all tissues (Flier, Harris et al. 2000). Thyroid hormone levels are highly regulated by nutritional state. In rodents, starvation is known to rapidly suppress thyroid hormone levels (Blake, Eckland et al. 1991; Ahima, Prabakaran et al. 1996).

Metabolic uncoupling refers to a state where nutrient fuels are oxidized but the resultant energy does not generate ATP but is dissipated as

heat, thus uncoupling activity potentially affects energy balance. Uncoupling is governed mainly by mitochondrial inner membrane uncoupling proteins *UCP 1, 2,* and *3*. *UCP1* is expressed in the brown adipose tissue of rodents, and is important for whole-body energy expenditure (Enerback, Jacobsson et al. 1997; Florez-Duquet and McDonald 1998). Although thermoregulation is disrupted in *UCP1* knockout mice, they were neither hyperphagia nor obese on low or high-fat diets (Enerback, Jacobsson et al. 1997). It was hypothesized that there could be redundancy due to the fact that a homologue *UCP2* was discovered. Unlike *UCP1*, *UCP2* is widely expressed in the heart, white adipose tissue, lung, skeletal muscle, and kidney in both mice and humans (Gimeno, Dembski et al. 1997). *UCP1* knockout mice are cold-intolerant despite strong induction of *UCP2* expression. Additionally, *UCP2* knockout mice displayed normal body temperature even when cold-exposed; collectively suggesting that *UCP2* does not participate in thermoregulation. However, UCP2 has been associated to type 2 diabetes. *UCP2* knockout mice have lower fasted blood glucose levels and elevated serum insulin when compared to wild-type mice on a high-fat diet (Joseph, Koshkin et al. 2002). The questioning of the involvement of uncoupling is not just limited to *UCP2*, but *UCP3* as well. *UCP3* knockout mice are normophagic, not obese, have

normal energy expenditure, and is not more pre-disposed to diet-induced obesity compared to wild-type mice (Gong, Monemdjou et al. 2000).

Orexins have been reported to affect the sympathetic tone upon central administration (Hagan, Leslie et al. 1999), and modulation of the sympathetic tone could serve as the underlying mechanism *CAG/orexin*'s anti-obesity effects. After the sympathetic tone neurotransmitter was characterized as norepinephrine, clinical studies of the sympathetic nervous system were performed by measuring the urinary excretion of norepinephrine in patients with hypertension (Verrier and Lown 1984). Sympathetic tone also regulates heart rate and blood pressure, thus these factors were measured to determine the effect of *CAG/orexin* on the sympathetic tone.

RESULTS

For peripheral tissue tests, only wild-type and CAG/orexin mice were the two genotypes compared.

Serum thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) were measured from mice fed a low-fat or high-fat diet. T3 and T4 levels were significantly increased in mice fed a high-fat diet to a similar

extent in both *CAG/orexin* and wild-type control mice despite significant differences in adiposity and energy expenditure between the two groups (**Fig. 4.1 B and C**). There were no significant differences between *CAG/orexin* and wild-type under either diet. TSH levels were significantly higher in *CAG/orexin* mice fed a high-fat diet, however, were similar to wild-type controls on a high-fat diet (**Fig. 4.1 A**). Overall, TSH, T3, and T4 levels are similar between genotypes on low-fat diet.

I also analyzed expression levels of the mitochondrial uncoupling proteins (UCP) in brown adipose tissue and skeletal muscle. High-fat diet increased *UCP1* expression in brown adipose tissue (**Fig. 4.2 A**), but not skeletal muscle (**Fig. 4.2 B**), in both genotypes; however, was not significantly different between genotypes. *UCP2* and *UCP3* were not altered by diet in brown adipose tissue or skeletal muscle in both genotypes (**Fig. 4.2 A and B**), which is consistent with previous findings.

Even with the ectopic expression of orexin in adrenal glands, no significant differences were detected for urinary epinephrine and norepinephrine for mice on a low-fat diet (**Fig. 4.3 A and B**). Serum corticosterone levels were also similar (**Fig. 4.3 C**).

Since orexin overexpression might prevent diet-induced obesity through

elevating overall sympathetic tone, I also analyzed if there were any differences in systolic blood pressure. However, I did not detect any significant differences in systolic blood pressure between genotypes in both low and high-fat diet (**Fig. 4.3 D**).

DISCUSSION

Despite ectopic expression of orexin-A in multiple peripheral tissues in *CAG/orexin* mice, I did not detect any abnormalities induced in peripheral tissues by the transgene. *CAG/orexin* mice have normal levels of serum TSH, T3, T4, uncoupling proteins, corticosteroid, urinary catecholamines, and systolic blood pressure. Although we cannot completely exclude the possibility of altered sympathetic tone, at least from the experiments performed in this chapter, I did not find any evidence of an elevated sympathetic tone.

EXPERIMENTAL PROCEDURES

Serum Thyroid Hormones

Blood was collected at the late light phase at the age of 30 weeks. Serum TSH, T3, and T4 was determined by Dr. A.F. Parlow at the National

Hormone and Peptide program using radioimmunoassays. 8-10 mice were used per group. Kruskal-Wallis test was used to assess TSH data because there were some samples below the detecting limit (48ng/ml).

Quantitative PCR

3-4 month old male mice were fed 2 weeks of high-fat diet then harvested for brown adipose tissue and skeletal muscle. Total RNA was isolated using RNeasy Mini Kit (QIAGEN) and used for cDNA synthesis by random hexamer and Omniscript Reverse Transcriptase (QIAGEN). Real-time quantitative PCR reactions were performed on cDNA with ABI Prism 7000 Sequence Detection System using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's manual. *UCP* mRNA expression levels are normalized by GAPDH mRNA levels and further normalized by the average *UCPs* mRNA expression levels of wild-type mice on a low-fat diet.

Adrenaline Related Hormones

Urine was collected for 3 consecutive days from male mice fed a low fat diet. Urinary epinephrine and norepinephrine concentrations were measured using the CatCombi ELISA kit (IBL) and normalized to body weight. Urine

was collected into 0.1N hydrochloric acid solution per the manufacturer's instructions. 5 mice per group were used.

Systolic Blood Pressure

Tail-cuff method was used to measure systolic blood pressure for both genotypes on both diets using the BP-2000 Blood Pressure Analysis System Series II (Visitech Systems) per the manufacturer's instructions. Male mice were acclimated for 4 consecutive days and actual data was collected on the 5^{th} day. 6 mice were used per group.

DISPLAY ITEMS

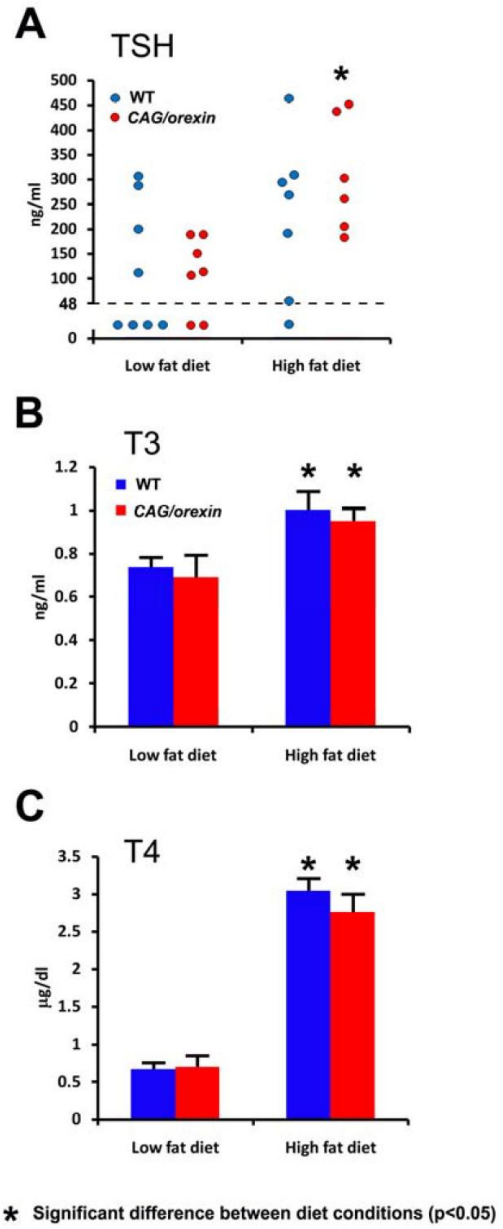

Figure 4.1 Serum Thyroid Hormone Levels

(A) Serum TSH levels of *CAG/orexin* mice were significantly elevated on a high-fat diet compared to low-fat diet while there was no significant difference in wild-type mice between low and high-fat diet.

(B) Both *CAG/orexin* and wild-type mice showed increased levels of T3 under high-fat diet conditions. No significant differences between genotypes.

(C) Both *CAG/orexin* and wild-type mice showed increased levels of T3 under high-fat diet conditions. No significant differences between genotypes.

Data are expressed as means ± SEM.

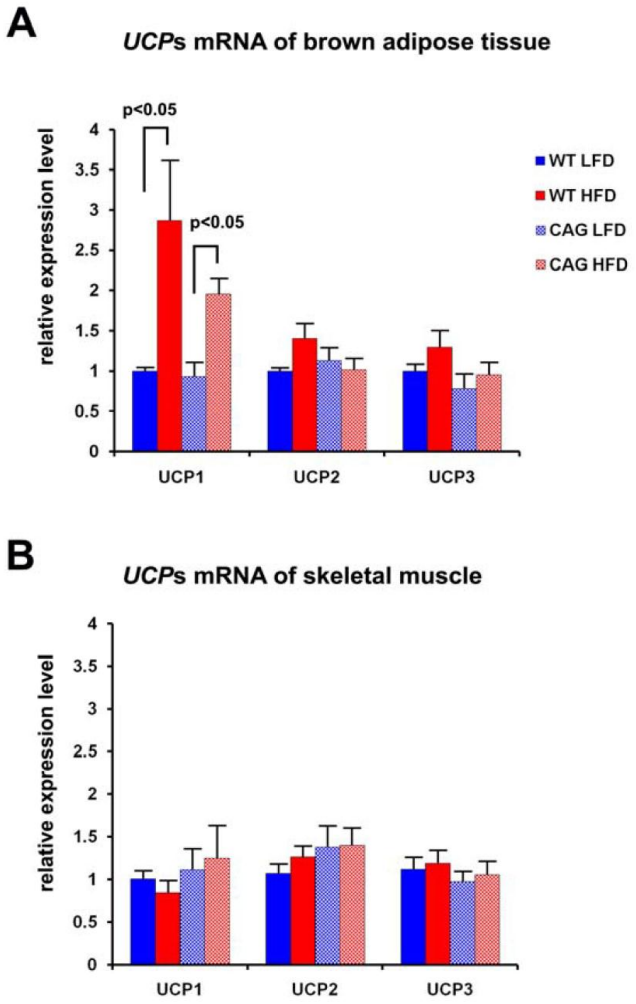

Figure 4.2 *UCP* mRNA Expression in Brown Adipose Tissue and Skeletal Muscle

(A) High-fat diet increased *UCP1* mRNA expression in wild-type and *CAG/orexin* mice. No significant differences between *CAG/orexin* mice and wild-type under both diet conditions. Neither *UCP2* nor *3* were affected by diet or *CAG/orexin* transgene.

(B) Neither *UCP1*, *2*, nor *3* mRNA expression were affected by diet or *CAG/orexin* transgene. Data are expressed as means ± SEM.

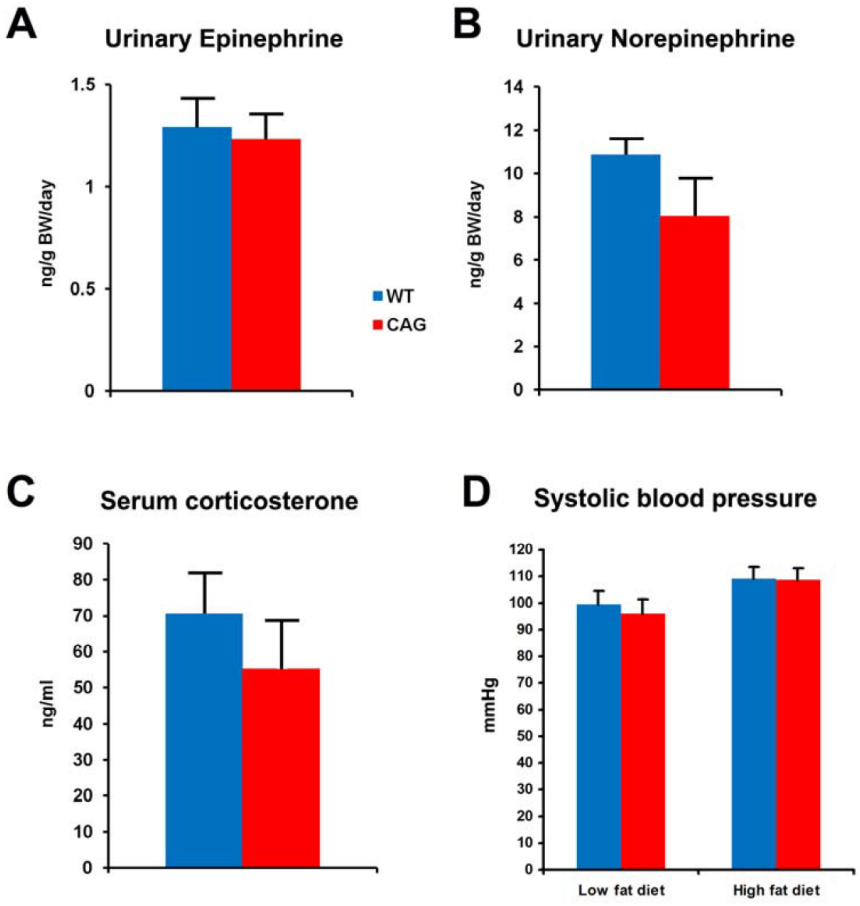

Figure 4.3 Adrenaline Related Hormone Levels and Blood Pressure Measurement

(A) Urinary epinephrine was not significantly different between *CAG/orexin* and wild-type mice on a low-fat diet.

(B) Urinary norepinephrine was not significantly different between *CAG/orexin* and wild-type mice on a low-fat diet.

(C) Serum corticosterone was not significantly different between *CAG/orexin* and wild-type mice on a low-fat diet.

(D) Systolic blood pressure was not significantly different between *CAG/orexin* and wild-type mice on a low-fat or high-fat diet. Data are expressed as means ± SEM.

CHAPTER FIVE

Orexin receptor-2 selective agonist prevents diet-induced obesity

SUMMARY

In this chapter a pharmacological approach was used to study the effect of orexin-OX2R signaling in the prevention of diet-induced obesity. In agreement with genetic studies, chronic central infusion of an OX2R selective agonist prevented diet-induced obesity, decreased fat mass, elevated energy expenditure, decreased food intake, and decreased orexigenic factors NPY/AGRP on a high-fat diet. Importantly, neuronal activity in the dorsal hypothalamic area of the ARH (where NPY/AGRP neurons reside) was decreased, consistent with the decreased expression of NPY and AGRP.

INTRODUCTION

From the genetic studies of *CAG/orexin* mice, I have found that *CAG/orexin* confers protection against diet-induced obesity primarily through an OX2R signaling mechanism. The transgene was also able to confer elevated energy expenditure; prevention of hyperleptinemia, hyperglycemia,

and hyperinsulinemia when challenged with a high-fat diet. Additionally, no obvious effects were induced in peripheral tissues ectopically expressing the orexin transgene. All data lead to the hypothesis that the anti-obesity and anti-diabetic effects of the orexin transgene are mediated centrally through an OX2R signaling mechanism. To test this hypothesis, I chronically ICV infused an OX2R selective agonist to see if similar metabolic results were attainable.

RESULTS

The OX2R selective agonist [Ala11, D-Leu15] Orexin-B used in this study has been described before (Asahi, Egashira et al. 2003). OX2R agonist or PBS vehicle was chronically infused into the lateral ventricle of mice brains for 14 consecutive days. Similar to genetic studies, OX2R agonist infused mice gained significantly less weight than vehicle infused mice within 14 days of observation when fed a high-fat diet (**Fig. 5.1 A**). Low-fat fed diet mice maintained a similar body weight homeostasis in both vehicle and OX2R agonist infusion (**Fig. 5.1A**). Fat mass was analyzed by NMR as described previously. Low-fat fed mice had no significant differences in fat mass between vehicle and OX2R agonist infusion. High-fat fed mice, however,

gained significantly less fat mass infused with OX2R agonist compared to vehicle (**Fig. 5.1 B**). Critically, OX2R selective agonist had no obvious effect upon OX2R-deficient mice on a high-fat diet (n=4, weight gain 3.33 ± 0.61g, p=0.67), verifying the specificity of the selective agonist *in vivo*. High-fat fed OX2R agonist infused mice also had significantly elevated energy expenditure when subjected to metabolic chambers (**Fig. 5.1 C**). Consistently, RQ and locomotor activity were not significantly different between the two groups (**Fig. 5.1 D** and data not shown).

OX2R signaling is known to be important for sleep/wake regulation (Lin, Faraco et al. 1999; Willie, Chemelli et al. 2003), thus disturbances of the sleep/wake cycle by the infusion of an OX2R selective agonist could be the primary reason why energy expenditure and food intake is altered. To rule out these possibilities, EEG/EMG was recorded for vehicle and OX2R agonist infused mice. Total wake and sleep time was not affected by the OX2R agonist compared to vehicle infusion regardless of diet (**Fig. 5.2 A**). As expected from previous studies (Willie, Chemelli et al. 2003), continuous OX2R infusion promoted consolidation of wake and NREM sleep under low-fat diet conditions (**Fig. 5.2 B**). However, this consolidation of sleep/wake states was not prominent under high-fat diet conditions; therefore sleep/wake

disturbances cannot be the primary cause in metabolic effects of the OX2R agonist which is only evident under high-fat diet conditions.

Daily food intake was reduced in high-fat fed mice as shown in previous studies (West, Boozer et al. 1992), and further exacerbated by the infusion of OX2R selective agonist (**Fig. 5.1 E**). Several metabolic related genes in the hypothalamus were assessed for mRNA expression level change after 14 days of OX2R agonist infusion. Specifically, the orexigenic *NPY* and *AGRP* mRNA levels were decreased by OX2R agonist infusion under high-fat diet compared to low-fat diet; whereas there was also a trend in vehicle infused mice between high-fat and low-fat diet, it did not reach significance (**Fig. 5.1 F**). Critically, I performed immunohistochemistry for c-Fos to assess neuronal activity in the ARH of the hypothalamus. C-Fos positive cells were significantly reduced after 14 days of OX2R agonist infusion in the ARH region of high-fat fed mice (**Fig. 5.1 G, and H**). The reduction of c-Fos staining was highly notable in the ventromedial aspect of the ARH (**Fig. 5.1 G**), which is the main region where orexigenic NPY/AGRP neurons reside, this result further supports the data that food intake is suppressed and *NPY/AGRP* mRNA expression is reduced by infusion of the OX2R selective agonist.

DISCUSSION

Utilizing a pharmacological approach, I was able to demonstrate that orexin confers resistance to diet-induced obesity through an OX2R dependent mechanism in agreement with the genetic studies. Importantly, OX2R agonist central infusion increased energy expenditure and further suppressed daily consumption of a high-fat diet. Although this initially seems at odds with the original findings of acute pharmacologic orexigenic activity of orexin (Sakurai, Amemiya et al. 1998), chronic central administration of orexin-A does not support increased food consumption or anabolism in rats (Yamanaka, Sakurai et al. 1999). This suggests that the acute appetite promoting effects of orexin may be temporary, or progressively overwhelmed by counterregulatory mechanisms that oppose weight gain.

EXPERIMENTAL PROCEDURES

Chronic ICV Injection

Three- to four- month old male C57BL/6J mice were single-housed 1 week before surgery and fed a low-fat diet. Mice were anesthetized with

ketamine and xylazine (100mg/kg and 10mg/kg, respectively, i.p.). A cannula (Brain Infusion Kit III; Alzet) was implanted into the right lateral ventricle (0.3 mm posterior for the bregma, 0.9 mm lateral from the midline, and 2.4 mm from the surface of the skull) using standard stereotactic techniques. An osmotic minipump (model 2001; Alzet) was attached to the cannula and implanted in the subcutaneous space during the same surgical session. The OX2R selective agonist [Ala11, D-Leu15] Orexin-B (American Peptide) (Asahi, Egashira et al. 2003) or vehicle (Dulbecco's PBS; Sigma) was continuously infused into the lateral ventricle for 14 days (0.5 nmol/day). The agonist was diluted with vehicle immediately before use. On the day of surgery, the implanted mice were randomly assigned to a low-fat or a high-fat diet. Body weight and food intake were monitored daily for 14 consecutive days, and fat mass was detected by NMR immediately after surgery and again on day 14.

Metabolic Cage Studies

Metabolic cage studies were performed as previous described in chapter 2 beside the following modifications. ICV chronic infusion of OX2R agonist and vehicle surgery was performed as described above. Mice were then individually housed for 7 days prior to housing in the metabolic chambers.

This step provided time for mice to recover from surgery since the metabolic cages are a new environment and could be stressful for mice post-surgery. This also allowed me to collect data after the 4 days of acclimation in metabolic cages on day 12-14 which should be the maximum impact time frame of chronic infused OX2R agonist.

Quantitative PCR

The hypothalamus was dissected coronally under the microscope from the optic chiasm to the mamillary bodies. The thick coronal section was further trimmed bilaterally at 1 mm from the midline and dorsally at 1.5 mm from the ventral surface. This dissected tissue included the ARH, VMH, DMH, PVN, anterior hypothalamic area, and a part of LHA. RNA preparation and quantitative PCR methods have been described before in chapter 4. *GAPDH* mRNA level was used as the standard for normalization.

Immunohistochemistry

Immunohistochemistry methods have been described before in chapter 2. The c-Fos antibody (Ab-5; Oncogene) was used in this study at 1:50,000 and visualized by DAB. Fos-positive cells in the ARH region were quantified by

counting 2 sections per animal containing the ARH region by an observed blinded-to-treatment group.

EEG/EMG surgery

EEM/EMG surgical procedures have been described previously (Chemelli, Willie et al. 1999; Willie, Chemelli et al. 2003). The EEG/EMG electrode and osmotic-pump connected ICV cannula was implanted at the same time on day 0. Mice were maintained on a low-fat diet prior and post-surgery. From day 9 to day 10, EEG/EMG recording for a low-fat diet was performed as previously described (Chemelli, Willie et al. 1999; Willie, Chemelli et al. 2003). The diet was then switched to high-fat diet on day 11 and further recorded to day 14. OX2R agonist and vehicle were continuously infused throughout the 14 days of EEG/EMG recording.

DISPLAY ITEMS

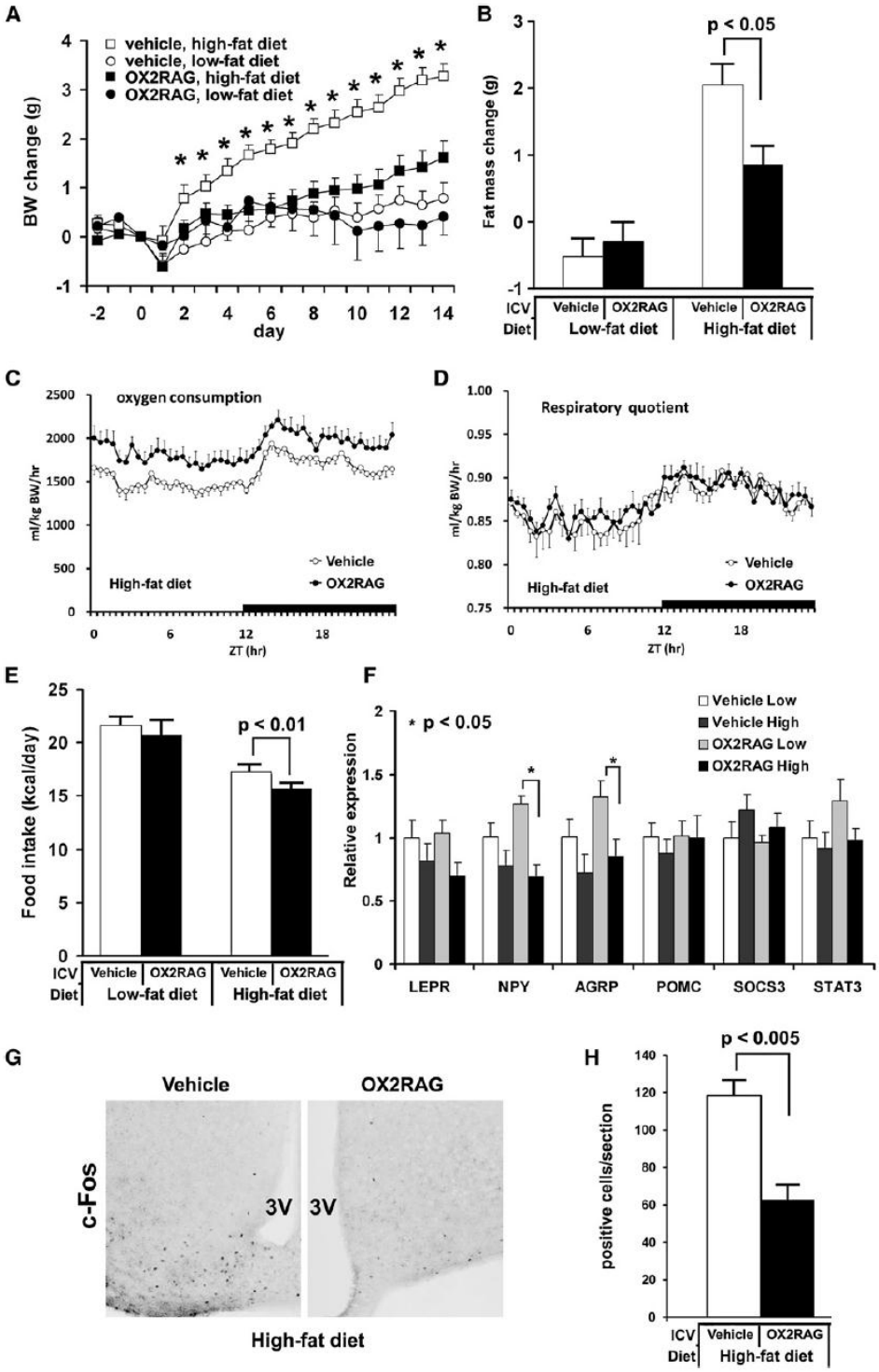

Figure 5.1 Effects of OX2R Selective Agonist on Diet-Induced Obesity

(A) Daily body weight changes of chronically ICV-injected mice. ICV and high-fat diet began at day 0. Body weight growth curve of OX2R selective agonist injected mice (0.5nmol/day) are significantly lower than vehicle injected mice on a high-fat diet ($p<0.0005$), whereas no significant differences were detected in body weight between them on a low-fat diet ($p=0.45$).

(B) Fat mass change after 14 days of OX2R selective agonist infusion under low or high-fat diet.

(C) Oxygen consumption with effective mass correction of OX2R agonist infused mice on a high-fat diet was higher than vehicle infused mice ($p<0.0005$, repeated ANOVA). Data was sampled every 30 minutes.

(D) Respiratory quotient of OX2R agonist infused mice on a high-fat diet was similar to vehicle infused mice.

(E) Average daily food intake of mice infused with OX2R selective agonist or vehicle on different diets for 14 days.

(F) Hypothalamic gene expressions at the end of OX2R selective agonist infusion determined by q-PCR. Gene expressions are normalized by GAPDH.

(G) Immunostaining for c-Fos in ARH region of mouse on a high-fat diet during

central administration of OX2R agonist or vehicle; 3V, third ventricle.

(H) The number of c-Fos positive cells in ARH region. The numbers of mice per group are 7-14 for (A), (B) and (C); 6-7 mice for (D), (G), and (H); and 5-6 mice for (E) and (F). Data are expressed as means ± SEM.

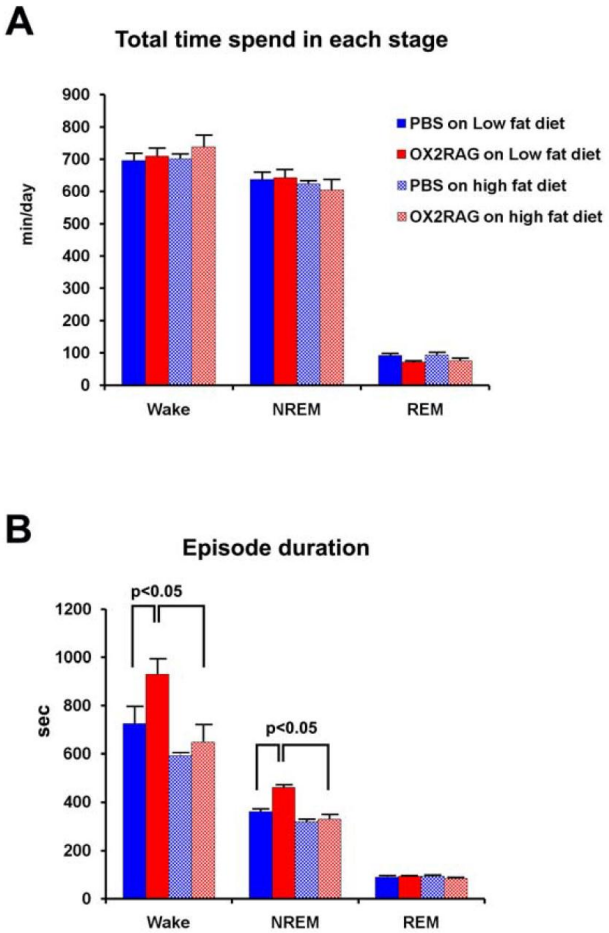

Figure 5.2 Sleep/wake Analysis of Mice During ICV Infusion of OX2R Agonist

(A) Total time spent in each stage. No significant differences in daily total time spent in wake, NREM, or REM.

(B) Episode duration in each stage. OX2R agonist infused mice fed a low-fat diet showed significantly longer duration for wake and NREM compared to low-fat fed PBS infused and high-fat diet OX2R agonist infused mice.

CHAPTER SIX

Orexin and leptin

SUMMARY

The effect of orexin overexpression on leptin sensitivity was examined in this chapter. Surprisingly, the *CAG/orexin* transgene and OX2R selective agonist had no significant effects on ob/ob mice body weight or food intake, suggesting that leptin is essential in the phenotypes characterized previously. However, central leptin infusion into *CAG/orexin* mice resulted in increased body weight loss and decreased food consumption, indicating orexin overexpression enhances leptin sensitivity.

INTRODUCTION

Leptin decreases food intake by inhibiting the orexigenic NPY/AGRP neurons, negatively regulated body weight homeostasis, has anti-diabetic effects and also increases energy expenditure. Diet-induced obesity is associated with leptin resistance resulting from signal transduction abnormalities in the ARH (Myers, Cowley et al. 2008). OX2R is highly expressed in the ARH, which is one of the key regions of leptin signaling. The similarities between leptin's effects on metabolism and the prevention of

diet-induced obesity through orexin-OX2R signaling led to the hypothesis that orexin's protective effects could be mediated through leptin. To test this hypothesis, *ob/ob* mice were mated with *CAG/orexin* mice to generate *ob/ob*; *CAG/orexin* mice and body weight curves were analyzed.

RESULTS

Surprisingly, the *CAG/orexin* transgene had no impact on *ob/ob* mice body weight curve for 30 weeks or fat mass analyzed at 30 weeks of age on a low-fat diet (**Fig. 6.1 A and B**). I then administered the OX2R selective agonist to *ob/ob* mice chronically for 14 days as in previous experiments on both low-fat and high-fat diet. Unfortunately, I still did not detect any significant differences in terms of body weight gain over the 14 days of observation on either diet (**Fig. 6.1 C** and data not shown).

Since leptin seems to be essential for orexin's anti-obesity effects, there is the possibility that orexin modulates this effect by changing leptin's sensitivity. Thus leptin was chronically ICV injected for 14 days into *CAG/orexin* and wild-type littermate pairs. All male mice were 3 to 4 months at the time of surgery and fed a low-fat diet to maintain a similar body weight (WT 29.7 ±

3.6g and CAG/orexin 27.0 ± 3.0g). Both wild-type and *CAG/orexin* mice lost weight upon initial infusion of leptin, but *CAG/orexin* mice showed significantly enhanced weight loss and anorexia compared to wild-type mice on a low-fat diet (**Fig. 6.2 A and B**). Profiling of orexigenic and anorexigenic gene expression was performed similar to the previous chapter. *NPY* and *AGRP* mRNA expression maintained basal levels upon leptin infusion in wild-type on a low-fat diet. *NPY* and *AGRP* mRNA basal levels were higher in *CAG/orexin* compared to wild-type mice and were both decreased upon leptin infusion (**Fig. 6.2 C**). As expected, *POMC* levels were increased in both wild-type and *CAG/orexin* mice when leptin was centrally infused on a low-fat diet (**Fig. 6.2 C**).

DISCUSSION

Antiadipogenic effects orexin-OX2R signaling requires the presence of leptin, suggesting that leptin mediates the suppressive effect of enhanced OX2R signaling on diet-induced obesity. Leptin-responsive neurons are found in the ARH, VMH, DMH, LHA, and tuberomamillary nucleus of the hypothalamus (Elmquist 2000). All of these nuclei receive orexin innervations,

express high levels of OX2R, and exhibit ectopic orexin-A immunostaining in *CAG/orexin* transgenic mice. Among these areas, the ARH is a critical nexus for body weight regulation and energy balance that monitors peripheral energy storages and enteral feeding status through integration of circulating leptin and insulin, metabolites, and vagal relays. The ARH modulates the thresholds, triggering drives to eat and expend energy, and it influences insulin secretion and sensitivity (Coppari, Ichinose et al. 2005; Horvath 2005; Myers, Cowley et al. 2008). Moreover, ARH harbors cellular abnormalities underlying acquired leptin resistance (Kievit, Howard et al. 2006), and reduced leptin sensitivity in ARH has been linked with diet-induced obesity (Enriori, Evans et al. 2007).

While ARH neurons project to orexin neurons of the LHA, ARH receives dense reciprocal orexin fiber innervation and expresses mainly OX2R receptor (Peyron, Tighe et al. 1998; Marcus, Aschkenasi et al. 2001; Cluderay, Harrison et al. 2002). Acute microinjections of orexin-A into ARH increase oxygen consumption and body temperature under anesthesia (Wang, Osaka et al. 2003).

The mechanism however, by which orexin and leptin signals interact remains unclear. Long form leptin receptors are also highly expressed in the LHA. Although they appear to be a distinct neuronal population from orexin

and MCH neurons, dense neurites are localized in the LHA itself, suggesting that Ob-Rb neurons in the LHA could have cross-talk with orexin neurons in the LHA (Leinninger, Jo et al. 2009). Neurons expressing both OX2R and Ob-Rb may have convergent intracellular second messenger signaling, including extracellular factor-regulated kinase (ERK) and the Janus kinase JAK2/STAT3 pathways (Zhu, Miwa et al. 2003; Myers, Cowley et al. 2008). In the ARH, leptin-responsive neurons such as those expressing NPY/AGRP are directly excited by orexin while POMC neurons are directly inhibited by orexin (Muroya, Funahashi et al. 2004), but inhibitory GABAergic interneurons in ARH may also be activated via postsynaptic OX2R (Burdakov, Liss et al. 2003), predicting complexity in up- or downregulation of these circuits. Thus, the relationship between orexin and leptin signaling prompts further investigation.

EXPERIMENTAL PROCEDURES

Body Weight and Fat Mass

Body weight measurement was performed as described in chapter 2. *Ob/ob* and *ob/ob*; *CAG/orexin* mice were fed on a low-fat diet and monitored for 30 weeks. Fat mass was analyzed as described in chapter 2, *ob/ob* and

ob/ob; *CAG/orexin* mice were subjected to NMR at 30 weeks of age.

Chronic ICV infusion

Chronic ICV infusion of OX2R surgery was described previously in chapter 5. Twelve-week-old *ob/ob* male mice were used for chronic ICV infusion of OX2R agonist for 10 days. For leptin administration experiments, weight-matched 3 to 4 month old CAG/orexin and wild-type littermates were continuously infused with leptin (2ug/day; PreproTech), as described above, while maintained on a low-fat diet. Body weight and food intake were monitored daily for 14 consecutive days.

DISPLAY ITEMS

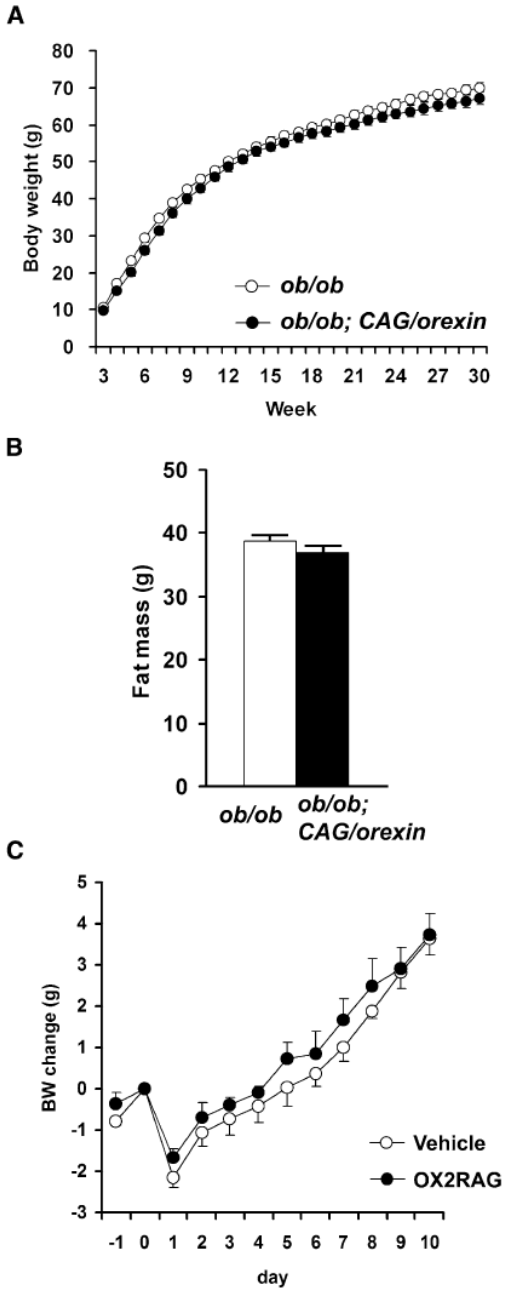

Figure 6.1 Orexin Overexpression has no Effect on Weight Gain of *ob/ob* Mouse

(A) Body weight growth curve was similar between *ob/ob; CAG/orexin* and *ob/ob* male mice (p=0.76).

(B) No significant difference for fat mass of 28 week old *ob/ob; CAG/orexin* and *ob/ob* male mice. There were 13-20 mice per group.

(C) Body weight growth curve of OX2R agonist-infused *ob/ob* mice was similar to that of vehicle infused *ob/ob* mice maintained on a low-fat diet.

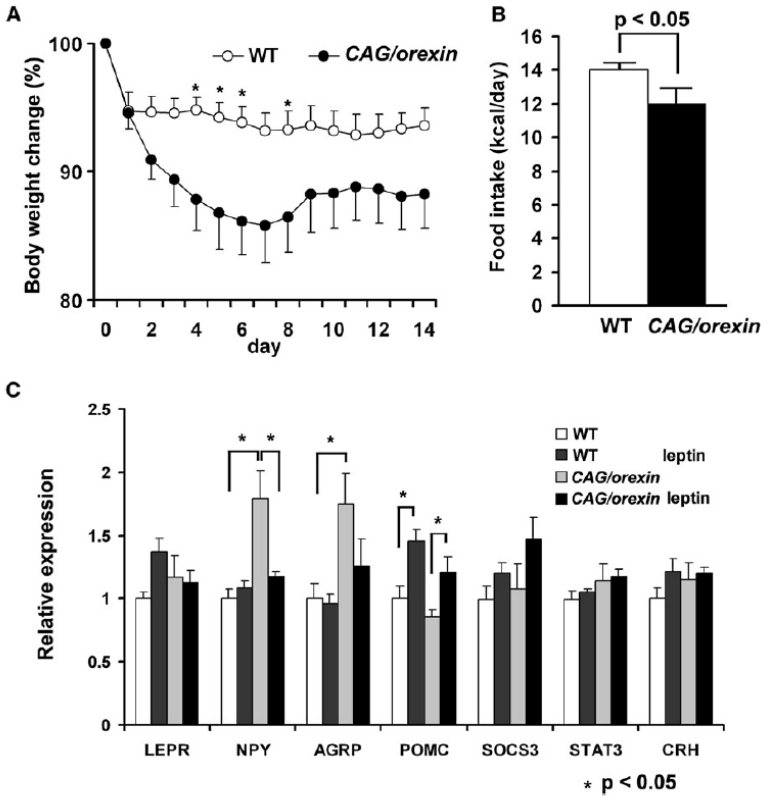

Figure 6.2 Increased Sensitivity of *CAG/orexin* Mouse to Leptin

(A) Body weight change during chronic ICV infusion of leptin (2ug/day) for 14 days. *CAG/orexin* mice showed significantly larger weight loss than wild-type littermates ($p<0.05$).

(B) Daily food intake during chronic infusion of leptin is decreased in

CAG/orexin mice compared to wild-type littermates.

(C) Hypothalamic gene expression at the end of chronic leptin administration. POMC was increased in both wild-type and *CAG/orexin* mice upon leptin infusion. There were 6-8 mice per group. Data are expressed as means ± SEM (*p<0.05).

CHAPTER SEVEN

Conclusion

Despite differences in orexigenic effects across different experimental paradigms in various studies, consistent and unifying results from pharmacologic and genetic studies here indicate that orexin gain of function promotes energy expenditure while loss of function promotes energy conservation. In conclusion, this study shows that orexin gain of function prevents diet-induced obesity through increasing energy-expenditure and decreasing food intake. Orexin also prevents hyperleptinemia, hyperglycemia, and hyperinsulinemia. Finally, these effects are dependent on leptin, and are achieved through modulating leptin sensitivity.

Just as the orexin system is believed to orchestrate disparate circuits of the ascending arousal system to maintain a consolidated state of arousal, it may also normally serve to consolidate the activity in parallel reward and metabolic networks that control behavioral and homeostatic responses to support energy expenditure. The exact peripheral or downstream mechanisms for the orexin-mediated increases of energy consumption remain unclear. Although I did not detect significant increases in urinary catecholamines or basal blood pressure, the data do not exclude the possibility

of subtly increased sympathetic tone in certain peripheral tissues unexamined. Indeed, I speculate that the sympathetic pathways are one of the likely downstream mechanisms for the increased metabolic rate under enhanced orexin signaling.

The robust innervation by the orexin system of the whole brain and the multiple phenotypic aspects of orexin-deficient animals such as narcolepsy/cataplexy, attenuated morphine dependence, and diminished stress response has led to conceptualization of the orexin system as a hypothalamic output pathway controlling arousal, motivational behavior, and autonomic responses (Chemelli, Willie et al. 1999; Sakurai 2007). Results from this study demonstrate that orexin signaling also has the capacity to primarily promote energy expenditure via leptin sensitization. Augmentation of OX2R signaling or its downstream targets beneficially alters hypothalamic set-points controlling metabolic rate, food intake, and insulin and leptin sensitivity. Similar interventions in humans might prevent or reverse the effects of calorie-dense food consumption that promote pathological adiposity and the metabolic syndrome. From a therapeutic standpoint, it is critical that orexin gain of function did not overtly alter the basal blood pressure, thyroid, glucocorticoid and catecholamine statuses in our model. Therefore, the

orexin system has emerged as a key target for therapeutic intervention in disorders associated with hypothalamic dysfunction, including not only narcolepsy and hypersomnia, but now also the metabolic syndrome.

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