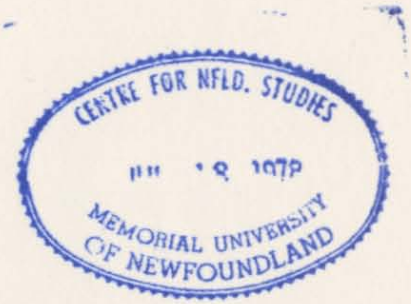
KATP CHANNEL-DEPENDENT REGULATION OF OREXIN NEURONS

MATTHEW P. PARSONS


CENTRE FOR NFLD. STUDIES
MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

KATP channel-dependent regulation of orexin neurons

by

Matthew P Parsons

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

Division of BioMedical Sciences

Faculty of Medicine

Memorial University of Newfoundland

2011

St. John's NL

Abstract

The hypothalamus performs many functions that are vital to an organism's survival. These include, but are not limited to, the regulation and coordination of basic functions such as energy metabolism, the sleep-wake cycle and motivation. As obesity, sleep disorders and/or addictions can result from the dysfunction of specific neural systems within the hypothalamus, it is important to understand the endogenous factors that regulate their activity. Neurons containing the orexin neuropeptides are located exclusively within the hypothalamus, send excitatory projections to arousal- and reward-related brain regions and have been implicated in numerous physiological and behavioral functions including feeding, sleep-wake regulation and reward and addiction. Neighbouring neurons containing melanin-concentrating hormone (MCH) also project throughout much of the neuroaxis and are implicated in similar functions. In the present thesis, I use electrophysiological recordings from acute hypothalamic slices as the main technique to investigate some of the endogenous regulators of orexin and MCH neurons. The present thesis shows that nociceptin/orphanin FQ (N/OFQ), an endogenous opioid, as well as lactate and temperature all act as regulators of orexin neuron activity. Interestingly, they all share a similar mechanism which involves the ATP-sensitive potassium (KATP) channel. These channels are metabolically-sensitive, are composed of a unique combination of subunits in orexin neurons and likely represent major contributors to the determination of orexin neuron activity. In contrast, MCH neurons were hyperpolarized by N/OFQ due to the activation of G-protein dependent inwardly rectifying potassium channels, while being insensitive to temperature changes. With regards to behavior, local injection of N/OFQ within the orexin and MCH field in vivo

inhibits reward-related feeding whereas the temperature regulation of orexin neurons appears to mediate the hypophagia associated with fever. Through the identification of orexin neurons as both temperature and energy sensors, it is suggested by the present thesis that these neurons can gate brain activity according to energy supply while possessing the ability to adapt to the increased temperatures associated with infection. These data shed new light on the function of these neuronal systems and introduce the KATP channel as a critical regulator of orexin activity.

Acknowledgements

First of all, I would like to thank my supervisor, Dr. Michiru Hirasawa. You have provided me with an excellent research environment in which to complete a thesis and with ample opportunities to present my work at national and international conferences. You have guided, encouraged and challenged me for the last number of years in a way which has fully confirmed my interest in pursuing a career in research. I cannot thank you enough for this.

Thanks to my former MSc supervisor Dr. Gilbert Kirouac. Your initial guidance was critical to the decisions that I have made.

I also thank the members of my supervisory committee, Drs. Xihua Chen and John McLean. Your input into my work has always been greatly appreciated.

I thank Christian Alberto for his technical assistance. You have helped me in so many ways and have made my time in the lab significantly more enjoyable. I consider you to be a good friend rather than a co-worker and wish you all the best in the future.

I also thank my fellow lab mates, in particular my good friends Robert Trask and Michelle Quinlan.

Many thanks go to those outside the lab whose support and guidance have been invaluable to my progress. I would like to specifically thank my parents, Don and Marie, as well as my beautiful wife, Amy.

I thank both NSERC and the Heart and Stroke Foundation of Canada for supporting me financially throughout my graduate work.

Table of Contents

List of Figures

List of abbreviations

4-CIN, alpha-cyano-4-hydroxycinnamate;

5-HT, 5-hydroxytriptamine (serotonin);

ACSF, artificial cerebrospinal fluid;

ACTH, adrenocorticotropic hormone

Arc, arcuate nucleus;

BF, basal forebrain;

CA, cell-attached;

CalC, calphostin C;

CCCP; carbonyl cyanide m-chlorophenyl hydrazone

CeA, central nucleus of the amygdala;

CRF, corticotropin-releasing factor;

CSF, cerebrospinal fluid;

CTL, control;

CWC, conventional whole-cell;

DA, dopamine;

DMN, dorsomedial nucleus of the thalamus;

DR, dorsal raphe;

DYN, dynorphin;

Dz, diazoxide;

FAC, fluoroacetate;

GABA, gamma-aminobutyric acid;

GE, glucose-excited;

GI, glucose-inhibited;

GIRK, G-protein dependent inwardly rectifying

GLP-1, glucagon-like peptide 1;

Glib, glibenclamide;

GLU, glucose

GLUT, glucose transporter;

HE, high energy;

HPA, hypothalamic pituitary adrenal;

IML, intermediolateral cell column;

KATP, ATP-sensitive potassium

K-Glu, potassium gluconate;

LAC, lactate

LC, locus coeruleus;

LDTg, laterodorsal tegmental nucleus;

LE, low energy;

LH, lateral hypothalamus;

LPS, lipopolysaccharide;

LT, long term;

MCH, melanin concentrating hormone;

MCHR1, melanin concentrating hormone receptor 1;

mEPSC, miniature excitatory postsynaptic currents;

met-Enk, met-Enkephalin.

mIPSC, miniature inhibitory postsynaptic currents;

NacSh, nucleus accumbens shell;

NE, norepinephrine;

N/OFQ, nociceptin/orphanin FQ;

NOP, nociceptin opioid peptide;

NPY, Neuropeptide Y;

ORX, orexin;

PFA, perifornical area;

PGE_2, prostaglandin E_2;

PKC, protein kinase C;

POAH, preoptic anterior hypothalamus;

POMC, proopiomelanocortin;

PTX, picrotoxin;

PVN, paraventricular nucleus of the thalamus;

PVT, paraventricular nucleus of the thalamus

REM, rapid eye movement

ST, short term;

SUR, sulphonylurea receptor;

TMN, tuberomammillary nucleus;

Tol, tolbutamide

TQ, tertiapin Q

TRH, thyrotropin-releasing hormone;

TTX, tetrodotoxin;

VTA, ventral tegmental area;

WC, whole-cell.

Chapter 1

Introduction and Overview

1.1 The hypothalamus

The hypothalamus maintains homeostasis by interacting with endocrine, autonomic and motivational systems. The significance of the hypothalamus cannot be overstated as this relatively small area of brain tissue critically regulates energy homeostasis, the sleep-wake cycle, the stress-response, body temperature, heart rate, blood pressure, blood osmolality, motivated behaviors and reproduction, amongst others. Overall, the hypothalamus integrates information related to an organism's internal and external environments, responds to this convergent information rapidly, and sends its output to target systems which influence the very environmental factors the hypothalamus responded to in the first place. Many of the processes mediated by the hypothalamus are regulated according to a set point; a desired basal value of a controlled physiological variable. It is the role of the hypothalamus to detect the polarity and magnitude of any deviations away from a particular set point and to induce an appropriate homeostatic response. Body temperature and fluid osmolality are good examples of physiological processes that are regulated in a hypothalamic-dependent manner according to a set point value.

The anatomy of the hypothalamus is critical to its homeostatic function. In terms of inputs, both synaptic and humoral information converge within the hypothalamus. Mono- and polysynaptic projections exist that connect much of the neuroaxis to the

hypothalamus and it is through such projections that the hypothalamus receives input from olfactory, visual and visceral sensory systems as well as from multimodal brainstem afferents and emotional limbic regions. Humoral factors can also reach the hypothalamus through mediated transport across the blood-brain barrier or via circumventricular organs; select regions of the brain that lack a significant blood-brain barrier. Many of these humoral factors are themselves influenced by hypothalamic activity and thus provide the necessary feedback to the hypothalamus that is critical to homeostasis.

Hypothalamic output occurs through three well-established routes: (1) Neural information from the hypothalamus can be converted to humoral signals which act upon target peripheral organ systems. In this case, specific hypothalamic transmitters are released into the portal capillary system in the median eminence where they are carried to the anterior pituitary gland. In the pituitary, these transmitters act to stimulate or inhibit the release of a corresponding hormone into the bloodstream which can then act upon specific target organs; (2) Neurotransmitters from the hypothalamus can be released directly into the bloodstream via the posterior pituitary. This system, the hypothalamic-neurohypophyseal system, consists of oxytocin and vasopressin neurons in the supraoptic and paraventricular nuclei projecting directly to the posterior pituitary where their contents are released into circulation. These peptides are critically involved in osmoregulation (vasopressin) and lactation in females (oxytocin); (3) Output from the hypothalamus can also function independently of the pituitary gland via extensive projections throughout much of the neuroaxis including autonomic and motivational systems in the brain and spinal cord. Thus, by integrating a host of information regarding an organism's internal and external environments and by communicating with the brain

and periphery through synaptic and humoral means, the hypothalamus is well-suited for its role in the regulation of physiology and behavior.

The hypothalamus consists of clusters of specific neuronal phenotypes with distinct transmitter systems, projection targets and endogenous regulators. As reflected by the different output pathways described above, the function of the hypothalamus is not carried out by the hypothalamus as a whole, but rather by the actions of multiple different neuronal ensembles which each subserve specific functions. These neuronal ensembles have the daunting task of integrating a host of central and peripheral information while sending appropriate responses to autonomic, endocrine and/or emotion/motivation systems that are essential to an organism's survival. For example, caloric homeostasis is maintained by the actions of multiple satiety signals on a variety of anabolic and catabolic hypothalamic neuronal ensembles whose outputs ultimately control subsequent feeding behaviour and energy expenditure. Gaining a full understanding of the regulatory mechanisms of hypothalamic functions could, in theory, alleviate countless disorders including obesity, addiction, anxiety and sleep disturbances to name a few. Such a level of understanding requires an appreciation of not only the effector systems upon which the hypothalamus acts but also the precise endogenous regulators that influence the activity of specific hypothalamic neuronal networks. The latter is the focus of the present thesis. More specifically, this thesis investigates the regulation of two major neuropeptide systems that have virtually come to define the lateral hypothalamus (LH): the orexins and melanin concentrating hormone (MCH).

1.2 Neuropeptides of the lateral hypothalamus/perifornical area

The LH was identified as the brain's feeding centre over 50 years ago. Dramatic hypophagia and weight loss was observed following LH lesions while electrical stimulation of this region could induce feeding behaviour (Stellar, 1994;Hetherington and Ranson, 1940;Anand and Brobeck, 1951). It is now known that the idea of a precise "feeding-centre" is outdated (Flier and Maratos-Flier, 1998) and that feeding is controlled by the coordinated actions of a growing number of neuropeptides and hormones acting in a number of different brain areas (Schwartz et al., 2000). Nonetheless, the role of the LH in the control of food intake and energy homeostasis is undisputed. Furthermore, self-stimulation sites within the LH were found to significantly overlap with sites that promoted feeding behavior upon stimulation (Margules and Olds, 1962), suggesting a role of this region in more than just the homeostatic control of food intake. Based upon the association of the LH with obesity and, more recently, addiction, it is of interest to determine the relevant neuronal phenotypes that exist within this area and how these neurons are regulated on a cellular level. Importantly, over the last decade or so, it has become evident that much of the known function of the LH can be attributed to the actions of the two known major neuropeptide systems whose cell bodies reside more-or-less exclusively within this region of the hypothalamus: the orexin system, residing in the lateral hypothalamus and perifornical area (LH/PFA) and the MCH system, residing in the LH/PFA and zona incerta. Roughly 10-15 years of research on these two peptide systems have shown promising results for the generation of not only anti-obesity pharmaceutical interventions but also medications that can alleviate stress and anxiety, drug addiction as well as sleep disturbances, amongst others. Given the multiple roles of

these neuropeptides, as will be discussed below, it is of utmost importance to understand how they are regulated at a cellular level. Therefore, the focus of the present thesis is on the regulation of these neuronal phenotypes, with emphasis on the orexin system.

1.2.1. The orexins

The orexin neuropeptides, orexin-A and orexin-B, were discovered simultaneously by two independent groups in 1998 (Sakurai et al., 1998;de Lecea L. et al., 1998). On the basis of their structural similarity to secretin and their hypothalamic localization, de Lecea et al. named the peptides "hypocretin-1" and "hypocretin-2". On the other hand, Sakurai et al. named the peptides "orexin-A" and "orexin-B" due to their ability to induce feeding when injected centrally. For the sake of simplicity, the present thesis will refer to these neuropeptides as the orexins.

The orexins are cleaved from a single precursor polypeptide prepro-orexin and bind to two G-protein coupled receptors, termed orexin receptor 1 and orexin receptor 2. Orexin receptor 1 is coupled to G_q proteins while orexin receptor 2 has been observed to be able to couple to either G_q or $G_{i/o}$ proteins (Zhu et al., 2003). Although prepro-orexin mRNA and orexin peptide expression is found localized to the LH/PFA, orexin varicosities and orexin receptor expression can be found throughout the brain (Peyron et al., 1998;Trivedi et al., 1998). For example, heavy orexin projections are found in most major arousal-related cell groups including the locus coeruleus, raphe nuclei and basal forebrain. Within the hypothalamus, orexin projections are seen in regions such as the ventromedial, tuberomammillary, arcuate and preoptic nuclei. Orexin fibers also appear in low density, but diffusely, throughout the cortex. Such projection patterns have

implicated the orexin system in a wide variety of physiological functions including the regulation of energy balance and the sleep-wake cycle as well as reward and addiction.

1.2.1.1 The orexins and energy homeostasis

Considering their restricted localization within the LH/PFA, it is not surprising that many early studies on the physiological roles of the orexins focused on feeding behavior. During the light phase, injections of orexin-A or B into the lateral ventricle dose-dependently induced hyperphagia within 1 hour post-injection, with the effect of orexin-A lasting longer than that of B (Sakurai et al., 1998). The hyperphagic effect of the orexins could be further demonstrated by injections directly into the paraventricular nucleus, dorsomedial nucleus, LH/PFA or nucleus accumbens (Dube et al., 1999;Sweet et al., 1999;Thorpe and Kotz, 2005), suggesting critical sites of action. An interaction with the orexigenic neuropeptide Y (NPY) neurons is demonstrated by the finding that orexin-induced feeding is blocked by a NPY receptor antagonist (Jain et al., 2000).

Additional data support a role of the endogenous orexin system in feeding behavior. Fasting is associated with an upregulation of orexin mRNA (Sakurai et al., 1998) and peptide (Park et al., 2004) as well as an increase in c-Fos expression in orexin neurons. Administration of an orexin receptor antagonist decreases food intake (Haynes et al., 2000) and mice with a specific ablation of orexin neurons are hypophagic, although they do develop late-onset obesity (Hara et al., 2001). This is explained by the fact that the orexins not only stimulate food intake but also energy expenditure.

1.2.1.2 The orexins and the sleep-wake cycle

A number of landmark studies have demonstrated the orexins' involvement in the maintenance of wakefulness and the stabilization of the sleep-wake cycle. The importance of orexin neurons to sleep-wake regulation is evidenced by the narcolepsy that results from a loss of orexin neurons. In just one year following the discovery of the orexins, it was recognized that canine narcolepsy that had showed up some 30 years previously had resulted from a mutation of the orexin receptor 2 gene (Lin et al., 1999). Subsequently, symptoms that strikingly resembled human narcolepsy could be seen in orexin neuron ablated (Hara et al., 2001) and knockout mice (Chemelli et al., 1999). Narcoleptic patients have lower CSF levels of orexin than controls (Nishino et al., 2000) which is caused by the selective death of orexin neurons (Thannickal et al., 2000;Crocker et al., 2005). Narcolepsy is characterized by the intrusion of rapid eye movement (REM) sleep into wakefulness, fragmented sleep/wake cycles and the inability to maintain vigilance. The narcoleptic phenotype in orexin-compromised animals suggests that the endogenous orexin system plays a critical role in the maintenance of wakefulness and aids in the stabilization of sleep/wake states. Interestingly, like orexin ablated mice (Hara et al., 2001), narcoleptic humans have a higher body mass index despite lower caloric intake (Schuld et al., 2000;Lammers et al., 1996), further confirming the role played by endogenous orexin neurons in both sleep/wake regulation and energy homeostasis.

The orexins have been shown to have an excitatory effect on the arousal-related noradrenergic locus coeruleus (Hagan et al., 1999;Horvath et al., 1999), serotonergic dorsal raphe (Liu et al., 2002), histaminergic tuberomammillary nucleus (Yamanaka et al., 2002) and cholinergic basal forebrain (Eggermann et al., 2001). Thus, it is not

surprising that central orexin administration increases arousal and locomotor activity (Hagan et al., 1999). Studies using c-Fos as a marker of neuronal activity have demonstrated that orexin activity in rats is increased during the dark phase, when arousal level is high (Estabrooke et al., 2001). In support of this, *in vivo* recordings from unanesthetized, unrestrained rats show that orexin neurons are most active during active waking, less active during quiet waking and virtually silent during sleep save for a few bursts in REM sleep (Mileykovskiy et al., 2005). Moreover, optogenetic control over orexin neurons using lentiviral-mediated expression of the light-sensitive channelrhodopsin-2 in orexin neurons demonstrated that specific activation of orexin neurons at 5 hertz or greater significantly decreased the latency from sleep to a waking state (Adamantidis et al., 2007). Thus, ample evidence exists demonstrating an undisputable role of the orexins in the maintenance of wakefulness and the control of the sleep/wake cycle.

1.2.1.3 The orexins and reward/addiction

The mesolimbic dopamine system, consisting of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens and prefrontal cortex, is considered to be the brain's reward system. Drugs of abuse all act to increase dopamine release and addiction is associated with plastic changes within this system (Kauer and Malenka, 2007). A role of the orexins in reward and addiction is suggested by their direct excitatory effect on dopamine neurons of the VTA (Nakamura et al., 2000;Korotkova et al., 2003) and was first confirmed by the demonstration of an orexin involvement in morphine addiction (Georgescu et al., 2003). Further studies show that orexin activity is

associated with cues predicting either food or drug reward and that intra-VTA orexin administration reinstates extinguished drug-seeking behavior (Harris et al., 2005). Orexin-A also appears to be critical to the cocaine-induced plastic changes in the VTA that contribute to behavioral sensitization (Borgland et al., 2006). Recently, it was shown that the orexins are involved in stress-induced reinstatement of nicotine-seeking (Plaza-Zabala et al., 2010). Supporting a role of the human orexin system in reward and addiction is the observation that narcoleptic patients, in which orexin neurons are compromised, show resistance to drug abuse (Guilleminault et al., 1974). Together, these data suggest that the orexin system, aside from a role in energy homeostasis and sleep/wake regulation, is critically important to the behavioral responses to both natural rewards and drugs of abuse.

1.2.1.4 Other functions of the orexins

Orexin activity has also been recently linked to stress and anxiety. The body's stress response, characterized by the activation of the hypothalamic-pituitary-adrenal (HPA) axis, is initiated by the release of corticotropin releasing factor (CRF) from the paraventricular nucleus of the hypothalamus (PVN), which acts in the anterior lobe of the pituitary to release adrenocorticotropic hormone (ACTH) into the circulation which subsequently acts on the adrenal cortices to increase corticosterone (cortisol in humans) release. That the orexins are involved in the stress response is evidenced by the finding that their central administration can induce c-fos activation in the PVN and increase plasma levels of both ACTH and corticosterone (Kuru et al., 2000). The orexin neurons themselves are depolarized by CRF, which is thought to contribute to the maintenance of

arousal during stress (Winsky-Sommerer et al., 2004). In terms of anxiety, an increased orexin-A cerebrospinal fluid level is seen in human subjects with panic anxiety when compared to control subjects and orexin signalling is required to induce a panic-prone state in a rat panic model (Johnson et al., 2010). Moreover, orexin infusion into the paraventricular nucleus of the thalamus (PVT) increases, whereas intra-PVT orexin antagonist infusion decreases, anxiety measures (Li et al., 2010), suggesting that endogenous orexin acts in the PVT to regulate anxiety levels.

The orexins also have well-documented roles in autonomic regulation. For example, central administration increases heart rate and blood pressure (Shirasaka et al., 2001) and basal blood pressure is lower in orexin-deficient mice (Kayaba et al., 2003). The orexins have been implicated in the stress response and have also been shown to potentiate sexual behavior in male rats (Gulia et al., 2003). Orexin neurons appear to be critical for stress-induced thermogenesis (Zhang et al., 2010) although their precise role in body temperature regulation is unclear as both an increase (Yoshimichi et al., 2001;Monda et al., 2001) and decrease (Balasko et al., 1999;Jaszberenyi et al., 2002) in body temperature has been observed following orexin administration. Pain regulation also appears to be mediated by orexin signaling as intrathecal orexin administration produced analgesia (Yamamoto et al., 2002) and stress-induced analgesia is absent in animals with a compromised orexin system (Xie et al., 2008). An overview of the key projections and related functional implications of the orexin system can be seen in Figure 1.1.

1.2.1.4 Endogenous regulators of orexin neuron activity

As orexin activity is linked to important physiological functions such as feeding and sleep-wake regulation, reward and addiction as well as stress and anxiety, it is important to understand how this system is regulated endogenously. As mentioned, orexin neuron activity shows a circadian rhythm, with higher levels of activity during the active phase (Mileykovskiy et al., 2005). Importantly, maximal activation was observed during active waking, suggesting that the orexins are regulated by more than just circadian influence from the suprachiasmatic nucleus (Zhang et al., 2004). In the past decade, much attention has been given to identifying the factors that regulate orexin activity and the mechanisms by which they do so. Contributing to this literature was of great interest to myself and thus became the overlying theme of the present thesis.

Glutamate and GABA have excitatory and inhibitory effects on orexin neurons, respectively. Glutamate antagonists were found to inhibit orexin neurons, suggesting that, at least in vitro, orexin neurons are under tonic stimulation by glutamate (Li et al., 2002). An important GABAergic projection has been noted from the sleep-promoting ventrolateral preoptic area (Sakurai et al., 2005). Orexin neurons also appear to be innervated by many of the monoaminergic and cholinergic systems that they themselves innervate. Noradrenaline, serotonin and dopamine neurons send negative feedback projections as these transmitters were each found to directly inhibit orexin neurons (Alberto et al., 2006;Li et al., 2002;Li and van den Pol, 2005). On the other hand, histamine appears to have no effect (Yamanaka et al., 2003) while inhibitory, excitatory and null effects have been observed with the cholinergic agonist carbachol (Sakurai et al.,

2005). Such reciprocal projections are likely to play a critical role in stabilizing the sleep-wake cycle (Saper et al., 2005).

Various endogenous neuropeptide regulators of the orexin system have also been observed. Vasopressin and oxytocin, peptides critical for water homeostasis, as well as social, sexual and maternal behavior, both have a direct excitatory effect on orexin neurons (Tsunematsu et al., 2008). The same authors demonstrated that water deprivation, which normally induces locomotor activity, has no effect on locomotion in orexin knockout mice, suggesting a role for the orexins in thirst-induced arousal. Thyrotropin releasing hormone (TRH) was also found to have a direct excitatory effect on orexin neurons and may play a role in the increased arousal observed following central injections of TRH (Hara et al., 2009) (Gonzalez et al., 2009a). The endogenous opioids dynorphin and met-enkephalin both have direct inhibitory effects that involve the activation of a potassium current (Li and van den Pol, 2008;Li and van den Pol, 2006), the functional consequences of which have yet to be fully elucidated. Furthermore, CRF has an excitatory effect on orexin neurons, suggesting a positive feedback relationship which likely functions to aid in the maintenance of cognitive arousal during stressful events (Winsky-Sommerer et al., 2004).

Aside from the numerous aforementioned neurotransmitters that influence the orexin system, specific humoral factors appear to be critical regulators of orexin activity. For example, leptin, which circulates in proportion with adipose mass, has a direct inhibitory effect on orexin neurons (Yamanaka et al., 2003). The orexins have also been reported to be inhibited by glucose (Burdakov et al., 2006) and excited by ghrelin, an appetite-stimulating hormone (Yamanaka et al., 2003). Thus, circulating factors

signalling energy status can influence orexin neurons. A summary of the endogenous regulators of orexin neurons are shown in figure 1.2.

1.2.1.5 Overall function of the orexins

The aforementioned roles of orexin neurons in energy balance, sleep/wake regulation and reward-based behavioral responding are likely not entirely independent functions. In other words, the orexins likely do not have a primary feeding-stimulatory (orexigenic) or wake-promoting role. Rather, it has been proposed that this system is critical to the orchestration of appropriate systems required for the execution of relevant behaviors when faced with a particular environmental challenge (Boutrel et al., 2010). Rather than being involved specifically in drug-seeking behavior, the orexins are viewed as a system that can induce arousal/vigilance and increase motivation at appropriate times (e.g. during hunger or thirst) to help direct goal-oriented behaviors and, ultimately, aid in the organism's survival. Thus, the orexins coordinate multiple external and internal signals and respond by increasing or decreasing arousal and motivation accordingly. This is well-exemplified by a study showing that the anticipatory increases in locomotor activity observed in animals on a food-restricted diet is lost after orexin neuron ablation (Akiyama et al., 2004).

1.2.2 Melanin-concentrating hormone

MCH was originally isolated in the salmon pituitary (Kawauchi et al., 1983) and its expression in the hypothalamus was demonstrated almost two decades ago (Bittencourt et al., 1992). MCH-containing cell bodies, like the orexins, are restricted to the hypothalamus. They are found to co-exist (but do not co-localize) with orexin neurons in the LH/PFA but also extend slightly more dorsally into the zona incerta. An excellent figure showing the distribution of orexin versus MCH neurons was recently published (Hahn, 2010). Like the orexins, MCH fibers and varicosities are found throughout much of the neuroaxis (Bittencourt et al., 1992) as is its G-protein coupled receptor MCHR1 (Saito et al., 2001). Although the main theme of the present thesis regards orexin neuron regulation, MCH neurons are viable in our experimental setup and were also of interest due to their own physiological and behavioral implications. Nonetheless, as the majority of the present thesis focuses on the orexin system, the following introduction of the MCH system is brief in comparison.

1.2.2.1 MCH and energy homeostasis

As with the orexins, the rather restricted localization of MCH neurons within the lateral hypothalamus prompted investigation of the role of this peptide in the control of food intake. It was found that MCH activity has a stimulating effect on appetite (Qu et al., 1996) and MCH overexpression causes obesity (Ludwig et al., 2001). MCH knockout mice are hypophagic and lean and also display increased locomotor activity, a high basal metabolic rate and are resistant to diet-induced obesity (Shimada et al., 1998;Kokkotou et al., 2005). Food deprivation increases both orexin and MCH mRNA expression (Qu et al.,

1996) via a nuclear translocation of the transcription factor foxa2 (Silva et al., 2009). The precise projection targets of MCH neurons that mediate the feeding effect are not entirely understood, although there appears to be at least a partial role of inhibitory MCH projections to medium spiny neurons in the nucleus accumbens shell (Georgescu et al., 2005;Sears et al., 2010). Nonetheless, it is well-accepted that MCH neuron activity promotes energy intake while decreasing energy expenditure. This is in contrast with orexin neurons, which increase both energy intake and energy expenditure.

1.2.2.2. MCH neurons and the sleep-wake cycle

MCH neurons are quiet during active or quiet wakefulness but are active during REM sleep (Hassani et al., 2009). This pattern of activity across the sleep-wake cycle is different from that of the orexin neurons as well as that of the wake-promoting noradrenergic, serotonergic and histaminergic neurons in the locus coeruleus, dorsal raphe and tuberomammillary nucleus, respectively (Gervasoni et al., 1998;Gervasoni et al., 2000;Takahashi et al., 2006). Central MCH administration increases both slow-wave and REM sleep whereas MCH knockout mice sleep less during both the active and inactive periods (Willie et al., 2008), suggesting a role of endogenous MCH in sleep-wake regulation. Although speculative, MCH is likely to exert its effect on the sleep-wake cycle through the inhibition of wake-promoting areas such as the locus coeruleus, dorsal raphe and tuberomammillary nucleus (Peyron et al., 2009).

1.2.2.3 Other functions of MCH

Like the orexins, MCH neurons have also been implicated in anxiety and drug addiction. MCH release has been shown to activate the HPA axis (Kennedy et al., 2003) while MCH antagonists are consistently found to have potent anxiolytic effects (Borowsky et al., 2002;Chaki et al., 2005;Smith et al., 2006;Georgescu et al., 2005). Mice lacking the MCH receptor subtype 1 display anxiolytic behaviors in a number of tests routinely used to quantify fear and anxiety in rodents (Roy et al., 2006). MCH has also been implicated in the consumption of rewarding substances including palatable food (Morens et al., 2005), sucrose (Sakamaki et al., 2005) and ethanol (Duncan et al., 2006) and can increase the reward value of cocaine via projections to the nucleus accumbens (Chung et al., 2009). An additional role of MCH in increasing learning and memory has been suggested (Adamantidis and de, 2009). This was recently supported by the observation of hippocampal synaptic plasticity deficits in MCHR1 knockout mice (Pachoud et al., 2010). It is suggested that MCH projections to the hippocampus may promote memory consolidation during sleep (Pachoud et al., 2010).

1.2.2.4 Endogenous regulators of MCH neuron activity

Much less is known regarding the regulators of MCH neurons in comparison to orexin neurons. One report (van den Pol et al., 2004) demonstrated that MCH neurons are excited by glutamate, ATP and orexin-A whereas GABA, norepinephrine, acetylcholine, serotonin and NPY all inhibit MCH neurons. MCH neurons are also inhibited by the opioid dynorphin (Li and van den Pol, 2006). Cannabinoids, which increase feeding and decrease arousal, depolarize MCH neurons through a reduction in presynaptic GABA

release (Huang et al., 2007). Our overall comprehension of MCH function would benefit greatly from studies addressing some additional endogenous regulators of this system.

1.2.3 Summary of the orexin and MCH systems

The orexin and MCH systems reside side-by-side within the LH/PFA with MCH neurons extending dorsally into the zona incerta. In terms of energy homeostasis, orexin neurons stimulate food intake as well as energy expenditure whereas MCH neurons act like typical anabolic peptides by increasing food intake and decreasing energy expenditure. The orexins are linked to a net negative energy balance whereas MCH is linked to a net positive energy balance. As these peptides also stimulate classic reward pathways in the brain, their role in hedonic feeding has not been overlooked (Zheng et al., 2007;Morens et al., 2005). They have also been shown to regulate stress and anxiety levels. Given the obesity, addiction and anxiety that can result from the manipulation of either one of these systems, it is of interest to understand how orexin and MCH neurons are normally regulated at the cellular level. When I began my PhD, the newly-defined roles of the orexin and MCH systems in reward were becoming of great interest to the field. At the time, very little was known regarding how orexin and MCH neurons are regulated by endogenous opioids, a neuropeptide family intimately tied to reward. One particular opioid, termed nociceptin/orphanin FQ (N/OFQ), was of particular interest due to its functional overlap with a number of aspects of both the orexin and MCH systems including food intake, reward and addiction as well as stress and anxiety. The regulation of orexin and MCH neurons by N/OFQ became the initial focus of my thesis and is presented as separate manuscripts in chapters 2 and 3.

1.3 Nociceptin/Orphanin FQ

N/OFQ is an endogenous opioid (Meunier et al., 1995;Reinscheid et al., 1995) that binds to its own receptor, termed the nociceptin opioid peptide (NOP) receptor. N/OFQ-containing cell bodies as well as N/OFQ fibers and NOP receptors are found throughout the brain and spinal cord, which is in contrast to the orexin and MCH neurons whose soma are found exclusively in the hypothalamus. Electrophysiological studies of the cellular actions of N/OFQ generally include potassium current activation and/or calcium current inhibition (Meis, 2003). N/OFQ has been shown to activate an inwardly-rectifying potassium current, thereby exerting a direct inhibitory effect, in cells from a number of brain regions including the locus coeruleus (Connor et al., 1996b), paraventricular nucleus (Shirasaka et al., 2001) and VTA (Zheng et al., 2002) amongst others.

Nociceptin is so-named due to initial observations of the hyperalgesia produced when the peptide was injected centrally (Meunier et al., 1995). However, further investigation demonstrated a potent analgesic effect when injected into the spinal cord and the central effect has largely been reinterpreted as a result of a decrease in stress-induced analgesia (Mogil et al., 1996).

Interestingly, N/OFQ has also been described as the brain's anti-opioid (Mogil et al., 1996), which is especially true in terms of reward and addiction. Central infusion of N/OFQ itself does not result in a conditioned place preference or avoidance (Ciccocioppo et al., 2000). It does, however, abolish the place preference induced by cocaine (Sakoori and Murphy, 2004), amphetamine (Kotlinska et al., 2003), morphine (Ciccocioppo et al., 2000) and alcohol (Ciccocioppo et al., 1999). An interaction between N/OFQ and the mesolimbic dopamine system has been shown, suggesting a mechanism for N/OFQ's

inhibitory effect on the brain's reward circuitry. For example, VTA dopamine neurons contain NOP receptor mRNA (Maidment et al., 2002), N/OFQ application directly inhibits VTA dopamine neurons (Zheng et al., 2002) and intra-VTA infusion of N/OFQ decreases dialysate levels of dopamine in the nucleus accumbens (Murphy and Maidment, 1999).

Aside from nociception and addiction, additional studies have demonstrated a role of N/OFQ in food intake, locomotor activity, stress and anxiety (Civelli, 2008). For example, N/OFQ has a hyperphagic effect when injected either into the ventricles (Pomonis et al., 1996), the arcuate (Polidori et al., 2000) or ventromedial hypothalamic nuclei or the nucleus accumbens shell (Stratford et al., 1997). Central N/OFQ also dose-dependently decreases locomotor activity (Reinscheid et al., 1995), although the role of endogenous N/OFQ in locomotion is less clear as NOP receptor knockout mice have no apparent change in basal activity levels (Nishi et al., 1997). A clear role of N/OFQ in stress and anxiety has emerged based on studies showing that central N/OFQ induces a potent anxiolytic effect (Jenck et al., 1997) while N/OFQ knockout animals have higher plasma corticosterone levels and exhibit increased anxiety-like behaviors (Reinscheid and Civelli, 2002;Koster et al., 1999). Thus, N/OFQ, and its interactions with orexin and MCH neurons, may play important functional roles in energy homeostasis, reward and addiction as well as stress and anxiety.

1.4 Rationale and objectives (relevant to chapters 2 and 3)

Objective 1: To determine the effect of N/OFQ on orexin and MCH neurons

Due to N/OFQ's close association with the physiological functions of the orexin and MCH systems, it was of initial interest to determine whether N/OFQ exerted an effect on either of these neuronal phenotypes at a cellular level. Chapter 2 of the present thesis describes the cellular effect of N/OFQ on MCH neurons and discusses the results in the form of a potential cellular mediator of anxiety and/or addiction. Chapter 3 of the present thesis describes the cellular effect of N/OFQ on orexin neurons. As I was completing this work, Xie et al (2008) reported that N/OFQ inhibited mouse orexin neurons by the activation of a potassium current and inhibition of calcium currents and implicated the inhibition as a means by which N/OFQ can decrease stress-induced analgesia. My work demonstrates the molecular mechanism involved in N/OFQ-induced orexin inhibition. We also took an *in vivo* approach to help understand the effects of N/OFQ actions within the LH/PFA. N/OFQ is typically thought of as an orexigenic peptide based on central injection studies (Polidori et al., 2000;Pomonis et al., 1996;Stratford et al., 1997), which does not fit with inhibitory effects on the feeding-stimulatory orexin and MCH neurons in the LH/PFA. Thus, it was of further interest to determine how the local actions of N/OFQ within the LH/PFA affect food intake. This is also presented in Chapter 3.

1.5 KATP channels

The results of chapter 3 generated an interesting piece of information that shifted the focus of my PhD work. As you will see, the effect of N/OFQ in orexin neurons was dependent on the activation of ATP-sensitive potassium (KATP) channels; ion channels gated by intracellular ATP which were yet to be shown on orexin neurons. The properties of these channels (see below) raised some interesting thoughts on additional potential regulators of orexin neurons, including energy substrate availability (Chapter 4) and ambient temperature (Chapter 5). Thus, I decided to focus solely on the role of KATP channels in orexin neuron regulation.

1.5.1 Overview of KATP channel structure and function

KATP channels were discovered in cardiac myocytes and have since been described in many excitable cells including neurons. These channels are hetero-octamers composed of four identical pore-forming subunits (Kir6.1 or Kir 6.2) along with four identical regulatory sulphonylurea subunits (SUR1, SUR2A or SUR2B). KATP channels in pancreatic beta cells are composed of Kir6.2 and SUR1 subunits whereas Kir6.2/SUR2A channels are present in cardiac and skeletal muscle and Kir6.1/SUR2B channels are present in vascular smooth muscle. It appears that most neurons contain KATP channels of the beta-cell type; Kir6.2/SUR1 (Karschin et al., 1997;Thomzig et al., 2005). Although Kir6.1 subunits are mainly found in astrocytes (Thomzig et al., 2001), a Kir6.1/SUR1 combination has been observed in certain hypothalamic neurons (Lee et al., 1999). The activity of KATP channels is principally determined by a complex interaction with intracellular ATP and Mg^{2+}-bound nucleotides (Nichols, 2006). Generally speaking,

a decrease in the ATP/ADP ratio will increase the KATP channel's activity whereas an increase in the ratio inhibits channel activity. Due to their sensitivity to intracellular ATP, KATP channels act as molecular sensors of cellular metabolism (Nichols, 2006). This is best exemplified by the canonical beta-cell model where an increase in extracellular glucose results in an increase in the intracellular ATP/ADP ratio which triggers KATP channel inhibition, cellular depolarization and subsequent calcium influx and the triggering of insulin release (Fig. 1.3) (Miki and Seino, 2005).

The inhibitory effect of ATP is determined by an interaction with the cytoplasmic side of the Kir subunit whereas Mg^{2+}-bound nucleotides can activate these channels via interaction with nucleotide binding folds found on the cytoplasmic side of the SUR subunits (Nichols, 2006). Different channel compositions have different sensitivities to inactivation and activation by ATP and Mg-bound nucleotides, respectively (Takano et al., 1998;Gribble et al., 1997;Tucker et al., 1997;Liss et al., 1999) and sulphonylurea drugs, such as tolbutamide and glibenclamide, inhibit KATP channels via interaction with the SUR subunits.

As the model in Fig. 1.3 demonstrates a role of KATP channels in the release of insulin in response to glucose availability, it is not surprising that knockout models have demonstrated a clear role for KATP channels in the control of glucose homeostasis. As predicted by the model, insulin secretion is not properly regulated by glucose in Kir6.2 knockout mice, a dysfunction due to the loss of functional KATP channels in pancreatic beta-cells (Miki et al., 1998). These mice also have impaired recovery from insulin-induced hypoglycaemia, suggesting a role of KATP channels in counter-regulatory responses to abnormal glucose fluctuations. Interestingly, central neurons expressing

KATP channels can detect the level of extracellular glucose and respond with changes in cellular excitability. This ability of specific neurons, termed neuronal glucosensing, is critical to glucose homeostasis (Miki et al., 2001). As the work in Chapter 3 of the present thesis resulted in the identification of functional KATP channels on orexin neurons, it became of interest to investigate the glucosensing properties of orexin neurons.

1.5.2 Neuronal glucosensing

Glucosensing is a term used to describe an excitable cell that can monitor the level of extracellular glucose and alter its output accordingly. Glucosensing neurons are classified as either glucose excited (GE) or glucose inhibited (GI) based on whether they are excited or inhibited by a rise in glucose, respectively. It is thought that central glucosensing is responsible for detecting shifts in extracellular glucose and to, in turn, induce counter-regulatory mechanisms to help restore glucose to a particular set point. Hypothalamic glucosensing is KATP channel-dependent, at least in certain GE cell types, as the glucosensing abilities of neurons in the VMH is lost in Kir6.2 knockout animals (Miki et al., 2001). Disruption of KATP function specifically in POMC neurons resulted in a loss of POMC glucosensing abilities and also disrupted the animal's counter-regulatory response to a systemic glucose load (Parton et al., 2007). KATP involvement in glucosensing is dependent on the metabolism of glucose and it appears that a critical enzyme involved in metabolism-dependent glucosensing is glucokinase (Dunn-Meynell et al., 2002). Glucosensing in the VMH was absent following glucokinase RNA interference (Kang et al., 2006). These data suggest that central metabolism-dependent glucosensing is critical to whole-body glucose homeostasis.

1.5.2.1 Glucosensing in orexin neurons

Orexin neurons are known glucosensors. This was first shown in 2003, where mice orexin neurons were demonstrated to be inhibited by glucose elevations from 10 to 15 or 30 mM and excited by glucose decreases from 10 to 5 or 0 mM (Yamanaka et al., 2003), defining orexin neurons as GI. As these glucose concentrations are not physiologically relevant, subsequent studies have further characterized the glucosensing abilities of orexin neurons. For example, it was shown that orexin neurons are indeed GI neurons, which respond to glucose changes from 1 to 2.5 mM (Burdakov et al., 2006). The same study demonstrated that the glucosensing performed by orexin neurons was not based on the metabolism of glucose but instead was due to the metabolism-independent sensing of glucose from an unknown surface receptor. Considering our identification of KATP channels on these neurons and the previous report demonstrating a lack of glucokinase expression in orexin neurons (Dunn-Meynell et al., 2002), we hypothesized that orexin neurons may be capable of the direct metabolism of lactate, an alternate energy substrate (Pellerin and Magistretti, 1994). Although glucose has long been considered the main provider of fuel for active neurons, many lines of evidence support the idea that active neurons receive a significant amount of fuel in the form of lactate which is derived from the anaerobic glycolytic processing of glucose by astrocytes. The lactate is then released from astrocytes and taken up by neurons to be used as energy. This has been aptly named the "astrocyte neuron lactate shuttle hypothesis" (Pellerin and Magistretti, 1994). Chapter 4 of the present thesis investigates whether orexin neurons are sensitive to the level of cellular metabolism and whether they rely on lactate over glucose as their preferred energy substrate.

1.5.3 Temperature-sensing neurons

After investigating the effect of intracellular metabolism and extracellular energy substrate on orexin neurons, we then asked whether there are any additional potential regulators of KATP channels in orexin neurons. It was suggested that local temperature changes may be able to influence KATP channel activation (Qu et al., 2007b), raising the possibility that orexin neurons may be temperature-sensitive. Certain neurons in the brain, in particular in the preoptic anterior hypothalamus (POAH), are directly responsive to local temperature changes. Temperature sensitive neurons include warm-sensitive and cold-sensitive neurons which increase and decrease their firing rates, respectively, with an increase in temperature (Boulant, 2000). In the case of the POAH, these neurons detect changes in external and internal temperature and project to thermoeffector systems such as brown adipose tissue and skin vasculature in an effort to maintain a relatively stable internal temperature despite dramatic changes in external temperature (Romanovsky et al., 2005). For example, warm-sensitive neurons are depolarized by an increase in temperature and when active, these neurons promote heat loss through skin vasculature vasodilation while thermogenesis in brown adipose tissue is inhibited. Conversely, when cold-sensitive neurons are activated by low temperatures, an increase in body temperature is promoted by vasoconstriction and shivering while thermogenesis is induced in brown adipose tissue (Romanovsky, 2007).

Such body temperature regulation is offset by pyrogens. By inhibiting warm-sensitive neurons of the POAH, pyrogens can increase body temperature, resulting in fever. Interestingly, orexin neuron inhibition during fever is suggested by studies using c-Fos as a marker of neuronal activation (Gaykema and Goehler, 2009;Becskei et al.,

2008;Park et al., 2008) and such inhibition is fitting considering the physiological associations of the orexin system and the sickness behaviors that accompany fever. Thus, we asked whether orexin neurons are inhibited by temperature and, if so, could this contribute to sickness behaviors associated with fever. This is discussed in more detail in Chapter 5 of the present thesis.

1.6 Rationale and objectives (relevant to chapters 4 and 5)

Objective 2: To determine the effect of energy substrate on orexin neurons

As mentioned, the results of Chapter 2 determined that functional KATP channels exist on orexin neurons, channels which can link energy metabolism to firing frequency in other cell types. Thus, we sought to determine whether KATP channels in orexin neurons respond to metabolic manipulations and whether orexin neurons can act as metabolism-dependent sensors of extracellular energy. These data are presented in Chapter 4.

Objective 3: To determine the effect of temperature on orexin neurons

Orexin neuron activity promotes arousal, food intake and motivated behavioral responses. Fever and hyperthermia are associated with behavioral depression, anorexia and a lack of motivation. Based on a possible involvement of KATP channels in neuronal temperature-sensing, we decided to test the effect of temperature on orexin neurons. These data are presented in Chapter 5.

1.7 Objectives summary

The initial focus of my thesis involved the regulation of orexin and MCH neurons by N/OFQ. This is presented in chapters 2 and 3, respectively. The results of chapter 3 demonstrated a regulatory role of KATP channels in orexin neuron activity. This resulted in an investigation of the effects of cellular metabolism and energy substrate (Chapter 4) and ambient temperature (Chapter 5) on orexin neurons.

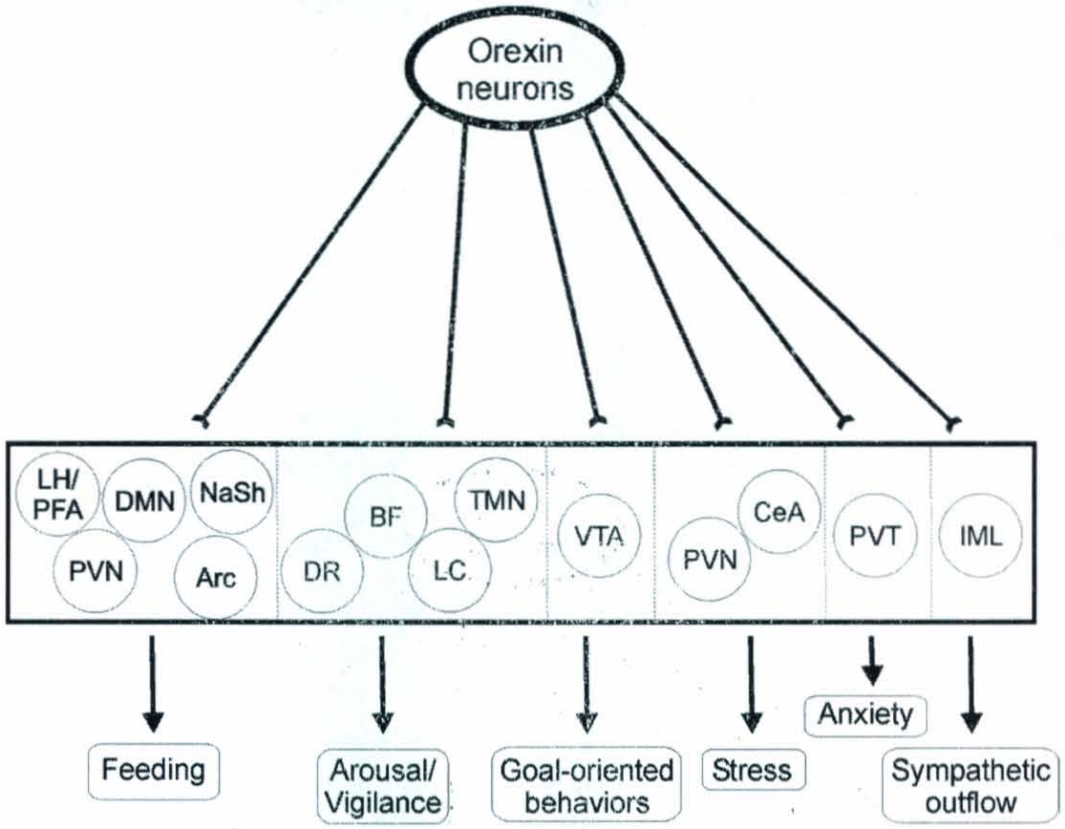


Figure 1.1 Overview of some of the critical projection targets and related functional implications of the orexin system. PVN, paraventricular nucleus of the thalamus; DMN, dorsomedial nucleus of the thalamus; Arc, arcuate nucleus; NacSh, nucleus accumbens shell; DR, dorsal raphe; BF, basal forebrain; LC, locus coeruleus; TMN, tuberomammillary nucleus; VTA, ventral tegmental area; CeA, central nucleus of the amygdala; PVT, paraventricular nucleus of the thalamus; IML, intermediolateral cell column.

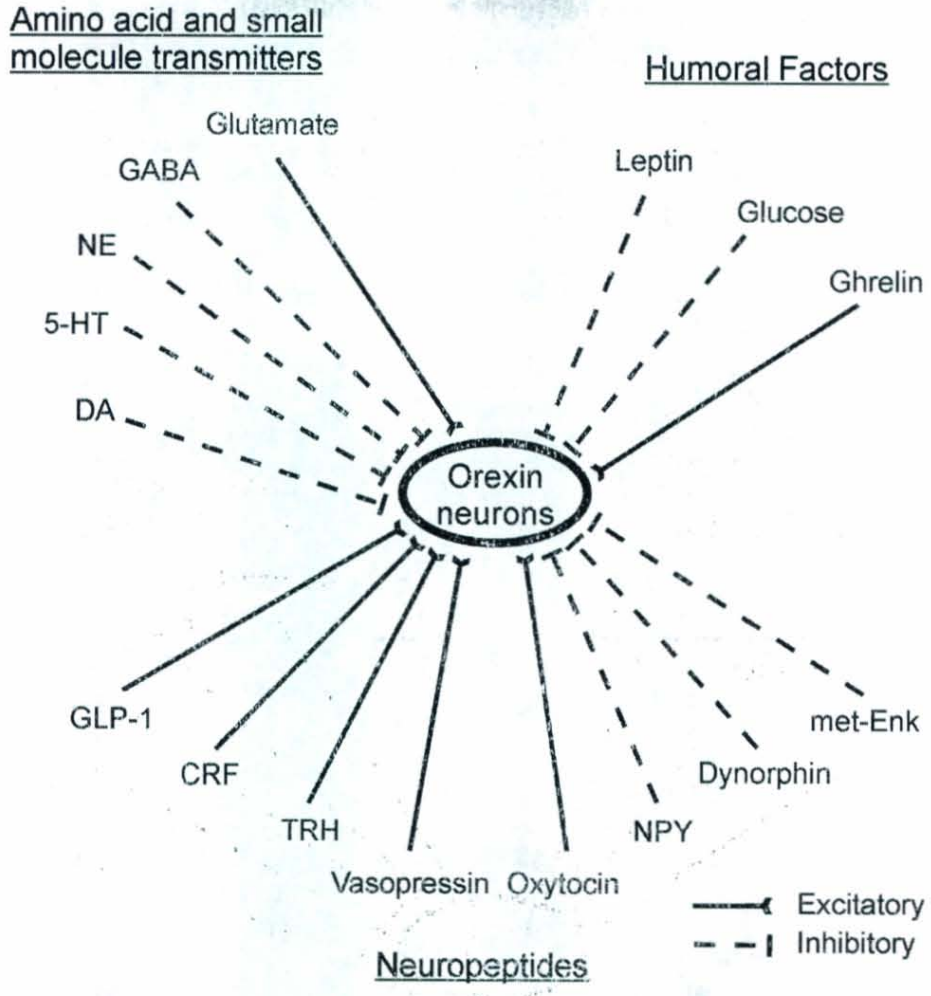


Figure 1.2 Overview of some of the critical endogenous regulators of the orexin system. GABA, gamma-aminobutyric acid; NE, norepinephrine; 5-HT, 5-hydroxytriptamine (serotonin); DA, dopamine; GLP-1, glucagon-like peptide 1; CRF, corticotropin-releasing factor; TRH, thyrotropin-releasing hormone; NPY, neuropeptide Y; met-Enk, met-Enkephalin.

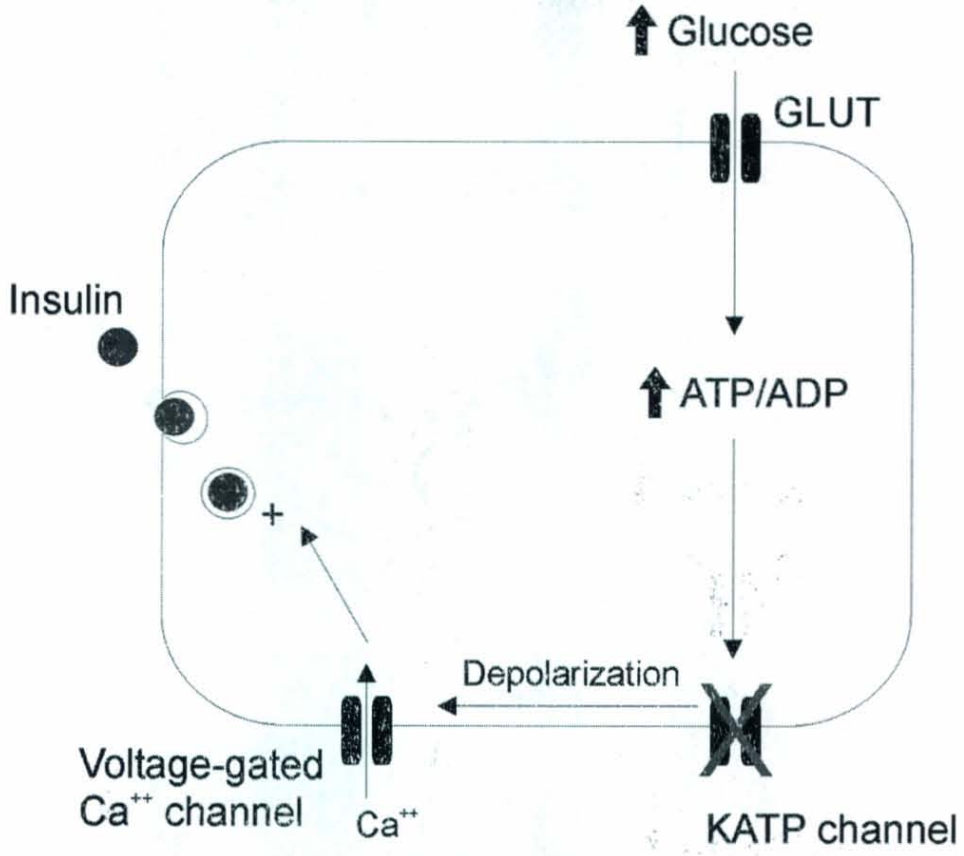


Figure 1.3 Overview of KATP channel regulation in the pancreatic beta-cell.

Increased glucose is sensed by the pancreatic beta-cell and insulin is released in response. The increase in extracellular glucose is transported inside the cell via glucose transporters (GLUT) where it is ultimately metabolized to generate ATP. This increases the ATP/ADP ratio which acts to close KATP channels. This leads to cellular depolarization, activation of voltage-gated calcium channels, calcium influx and the calcium-dependent exocytosis of insulin. Adapted from (Miki and Seino, 2005).

Co-authorship statement

I, Matthew Parsons, hold principle author status for all the manuscript chapters in this thesis (chapters 2-5). I am also the co-first author on a manuscript in preparation, second author on a published review article and second author on a review article in preparation that do not appear in this thesis. All manuscripts were written by me in their initial forms and were revised over time by myself and Dr. Hirasawa. Below, I have acknowledged additional contributors to each chapter.

Chapter 2, entitled "GIRK channel-mediated inhibition of melanin-concentrating hormone neurons" is published in the Journal of Neurophysiology. I appear as first author and Dr. Michiru Hirasawa appears as the other (and corresponding) author. I conducted all of the experiments in this chapter, with technical assistance from Christian Alberto.

Chapter 3, entitled "Hypophagia and KATP-dependent inhibition of orexin neurons induced by nociceptin/orphanin FQ" is a manuscript in preparation in which I appear as first author, followed by Julia Burt, Katrin Zipperlen and Michiru Hirasawa. In this chapter, I conducted all of the experiments with the exception of the food intake study (Figure 3.1). This was done by Julia Burt and Katrin Zipperlen under my supervision. Technical assistance (including sterotaxic surgery) was provided by Christian Alberto.

Chapter 4, entitled "KATP channel mediated lactate effect on orexin neurons: Implications for brain energetics during arousal" is published in the Journal of Neuroscience. I appear as first author and Dr. Michiru Hirasawa appears as the other (and corresponding) author. I conducted all of the experiments in this chapter with the exception of the immunofluorescence data, which was completed with help from Dr. Hirasawa. Christian Alberto provided technical assistance while Drs. Jackie Vanderluit and Quentin Pittman provided suggestions that helped improve the manuscript.

Chapter 5, entitled "KATP-dependent thermosensitivity of orexin neurons: Implications in lipopolysaccharide-induced anorexia" is a manuscript in preparation in which I appear as first author. Christian Alberto appears as second author and Michiru Hirasawa as last and corresponding author. Christian provided his technical skills for the stereotaxic surgeries and contributed significantly to the design of the LPS experiments. Dr. Hirasawa did the tracer injections into the locus coeruleus. I conducted the rest of the experiments in this Chapter.

CHAPTER 2

GIRK CHANNEL MEDIATED INHIBITION OF MELANIN-CONCENTRATING HORMONE NEURONS

(Published in the Journal of Neurophysiology)

2.1 Introduction

Melanin-concentrating hormone (MCH) neurons are located exclusively within the lateral hypothalamus/perifornical area (LH/PFA) and zona incerta. Despite this restricted localization, MCH-containing fibers and MCH receptors (MCH1R) can be seen in widespread regions throughout the central nervous system (Bittencourt et al., 1992;Hervieu et al., 2000). Likely owing to its broad central distribution, MCH has been implicated in many physiological functions. For example, MCH-deficient mice are hypophagic, lean (Shimada et al., 1998), hypermetabolic and resistant to diet-induced obesity (Kokkotou et al., 2005). On the other hand, overexpression of this peptide generates susceptibility to obesity (Ludwig et al., 2001). MCH has also been linked to reward and addiction as well as stress and anxiety. MCH was recently shown to potentiate cocaine reward while both cocaine-induced place preference and locomotor sensitization were diminished in mice lacking MCH1R (Chung et al., 2009). Furthermore, MCH has a stimulatory effect on the hypothalamic-pituitary-adrenal axis (Kennedy et al., 2003) and MCH antagonists act as potent anxiolytics when injected centrally (Borowsky et al., 2002). As increased MCH activity is associated with obesity, drug addiction and anxiety disorders, it is important to recognize the endogenous factors that regulate these neurons.

Nociceptin/orphanin FQ (N/OFQ) is the most recently discovered endogenous opioid and binds to the nociceptin opioid peptide (NOP) receptor which is expressed throughout the brain (Meunier et al., 1995;Reinscheid et al., 1995). N/OFQ is a product of preproN/OFQ, which is structurally related to the other opioid precursors, in particular preprodynorphin. Furthermore, the NOP receptor shares roughly 60% homology with the classic μ, δ and κ opioid receptors. Despite such similarities, the NOP receptor does not bind other endogenous opioids and N/OFQ has no significant affinity for the μ, δ and κ receptors (Meunier, 1997). However, unlike other opioid family members, N/OFQ has been referred to as the brain's "anti-opioid" as it can diminish the rewarding value of various drugs of abuse including cocaine, amphetamine, morphine and ethanol (Sakoori and Murphy, 2004;Kotlinska et al., 2003;Ciccocioppo et al., 2004). N/OFQ peptide also plays a role in feeding (Stratford et al., 1997) and behavioral responses to stress (Koster et al., 1999). Thus, many physiological functions of N/OFQ overlap with those of the MCH system. NOP receptor expression has been demonstrated to exist within the LH/PFA and zona incerta (Neal, Jr. et al., 1999a) and N/OFQ is co-expressed in orexin (hypocretin) neurons (Maolood and Meister, 2010) which are known to form synaptic appositions onto MCH neurons (van den Pol et al., 2004), suggesting that N/OFQ is released onto MCH neurons. However, whether or how N/OFQ regulates MCH neurons is unknown. Therefore, the present study used conventional whole cell patch clamp recordings from acute hypothalamic slices to investigate the cellular effect of N/OFQ on MCH neurons.

2.2 Materials and Methods

All experiments followed the guidelines set by the Canadian Council on Animal Care and were approved by the Memorial University Institutional Animal Care Committee. Male Sprague Dawley rats (60–70 g) were obtained from the breeding colony at Memorial University.

2.2.1 Electrophysiology

Animals were deeply anesthetized with halothane, decapitated and brains were quickly removed. Coronal hypothalamic slices (250 μm) were sectioned using a vibratome (Leica). Sectioning took place in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 25 $NaHCO_3$, 2 $CaCl_2$, 10 glucose, pH 7.3-7.35. Following dissection, slices were incubated in ACSF at 32-35°C for 30-45 min, then at room temperature until recording. ACSF was continuously bubbled with O_2 (95%)/CO_2 (5%).

Conventional whole-cell patch-clamp recordings were performed on brain slices perfused with ACSF at 1.5-2 ml/min, 26°C, using a Multiclamp 700B amplifier and pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). The internal solution contained (in mM): 123 K gluconate, 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10 HEPES, 4 Na_2-ATP, 0.3 Na-GTP, pH 7.3-7.35. Biocytin (1-1.5 mg/ml) was included in the internal solution to label a subset of cells for post-hoc immunohistochemical phenotyping and these sections were processed to visualize biocytin, MCH and orexin-A (Fig. 2.1A, B, orexin-A staining not shown). Targeted neurons, visualized using an infrared differential-interference contrast microscope (Leica), were located in the LH, PFA or zona incerta.

Upon attaining whole cell access, each neuron's electrophysiological characteristics were observed by a series of 300-ms hyperpolarizing (-200 and -100 pA) and depolarizing (100 and 200 pA) current injections in current clamp mode. Cells that did not display voltage responses typical of MCH neurons were not used in the present study. These include spike adaptation upon positive current injection and a lack of spontaneous action potentials, I_h and rebound currents (Fig. 2.1C) (Eggermann et al., 2003;Alberto et al., 2006). During the course of the present experiments, 27 cells displaying characteristic MCH electrophysiological properties were successfully filled with biocytin and identified immunohistochemically. Of these 27 cells, 26 were MCH-immunopositive suggesting that the detection of MCH neurons based on electrophysiological criteria is accurate (96%) in our hands. All cells displaying the aforementioned electrophysiological criteria were thus included in the present study. The 300-ms current injections described above were also performed every 30 seconds on cells recorded in current clamp to monitor input resistance as well as action potential responses to positive current injections. As MCH neurons rarely fire spontaneously *in vitro* (Eggermann et al., 2003), all analyses of action potentials represent responses to the 100 pA current injection. In some cases, during N/OFQ application, the 300-ms current injections were insufficient to fire action potentials in MCH neurons. For these cells, values of 0 Hz and 300 ms were given for action potential frequency and latency, respectively. All voltage clamp experiments were performed at a holding potential of -70 mV, with the exception of voltage ramps. To determine the effect of N/OFQ on current-voltage relationships, the membrane potential was ramped from -140 to -20 mV (600 ms) in the absence and presence of N/OFQ. To measure calcium currents, MCH neurons were first identified by their

electrophysiological characteristics using the aforementioned internal and ACSF solutions. When an MCH neuron was obtained, the pipette was carefully removed from the cell and the ACSF was switched to one containing (in mM): 100 NaCl, 40 TEA-Cl, 2.5 KCl, 2 $MgCl_2$, 5 $BaCl_2$, 10 HEPES, 10 Glucose, 0.001 tetrodotoxin (TTX). The same cell was then re-patched using an internal solution containing (in mM): 120 CsCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA, 4 Mg-ATP, 0.5 Na_2-GTP. In voltage clamp mode, voltage steps (100 ms) from -80 to 0 mV were applied every 5 seconds to activate voltage-dependent calcium channels. Cells in which a significant calcium current rundown was observed were not included for analysis.

2.2.2 Post-Hoc Immunofluorescence

Immunohistochemical phenotyping was performed as previously described (Alberto et al., 2006). Immediately following recording, the 250 μm sections were fixed in either 4% paraformaldehyde or 10% formalin for >18 hours at 4 °C before being washed (3 x 10-15 minutes) in 0.1 M PBS. Sections were incubated with a cocktail of goat anti-orexin A (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-MCH (1:2000; Phoenix Pharmaceuticals, Belmont, CA, USA) primary antibodies for 3 days at 4 °C. Sections were then washed in PBS and incubated in a cocktail of Cy3-conjugated donkey anti-goat, Cy2-conjugated donkey anti-rabbit and streptavidin-conjugated AMCA (1:500; Jackson ImmunoResearch, West Grove, PA, USA) secondary antibodies for 3 hours at room temperature. All antibodies were diluted in 0.1 M PBS with 0.05% triton X-100. Sections were washed, mounted, coverslipped and visualized using a fluorescence microscope to detect MCH (Cy2), Orexin-A (Cy3) and biocytin (AMCA).

2.2.3 Data analysis

Action potential frequency, membrane potential and holding current were measured using Clampfit 9.2 (Molecular Devices; Sunnyvale, CA). Data are expressed as mean ± S.E.M. The statistical tests used included one-way ANOVA with Dunnett's post test for multiple group comparisons and paired or unpaired Student t-tests for two-group comparisons. A value of $p < 0.05$ was considered significant. The reported n-values for all the electrophysiology data throughout this entire thesis represent the number of cells recorded which is not necessarily the same as the number of animals used. Although multiple experiments were sometimes carried out from different slices from the same animal, in no case did a single animal contribute to all n-values for a particular experiment set.

2.2.4 Drugs

1000x frozen aliquots of drugs were thawed and diluted with ACSF to their final concentration immediately prior to experimentation. TTX was obtained from Alomone Labs (Jerusalem, Israel) and picrotoxin (PTX) was obtained from Sigma-Aldrich (St. Louis, MO). N/OFQ (1-13)NH_2, UFP-101, tertiapin Q and glibenclamide were obtained from Tocris Bioscience (Ellisville, MO). N/OFQ (1-13)NH_2 is a bioactive metabolite of N/OFQ and was used in the present study as a potent NOP receptor agonist. N/OFQ (1-13)NH_2 is referred to as N/OFQ throughout the Results section.

2.3 Results

We first reasoned that if MCH neurons are influenced by N/OFQ, a response should be elicited by bath application of 1 μM N/OFQ as this concentration was shown to induce a near-maximal inhibition of neighbouring orexin neurons (Xie et al., 2008). Using current clamp recordings, we found that N/OFQ (1 μM, 2-4 minutes) resulted in the reversible hyperpolarization of MCH neurons (21.3 ± 2.2 mV, n = 4, Fig. 2.2A, B). This effect was accompanied by a significant decrease in input resistance (Fig. 2.2C), suggestive of postsynaptic channel activation. Upon injection of 100 pA-step currents, firing frequencies were significantly lower and latencies to fire were significantly longer during N/OFQ application (Fig. 2.2D, E).

To investigate the mechanism of inhibition, the N/OFQ effect was examined in voltage clamp mode at a holding potential of -70 mV. N/OFQ (2-4 minutes) induced a reversible outward current in all MCH neurons tested in a concentration-dependent manner (EC_{50} = 50.7 nM, Fig. 2.3B). An N/OFQ concentration of 0.3 μM, which induced a robust effect, was used for the remainder of the study unless otherwise noted. The N/OFQ-induced current was insensitive to TTX (Fig. 2.3A, n = 4), suggesting a direct postsynaptic effect. Outward currents also persisted in the presence of the $GABA_A$ blocker picrotoxin (50 μM, n = 8). When slices were pre-exposed (> 5 min) to 1 μM UFP-101 (Gompf et al., 2005), a peptide antagonist of the NOP receptor with a high affinity and selectivity over classic opioid receptors (Calo et al., 2005), N/OFQ's effect was significantly attenuated (n = 4; Fig. 2.3C, D). These data suggest that N/OFQ acts postsynaptically at NOP receptors to hyperpolarize MCH neurons.

Additional studies were carried out to determine the effector channel(s) involved in N/OFQ-induced hyperpolarization. The voltage ramp protocol revealed an N/OFQ-induced steady-state current with inward rectification that reversed near the potassium equilibrium potential (Fig. 2.4A, B). These are the characteristics of inwardly rectifying potassium channels; i.e. ATP-sensitive potassium (KATP) or G-protein dependent inwardly rectifying potassium (GIRK) channels. Thus, we decided to examine N/OFQ's effect in the presence of glibenclamide (0.2 μM) or tertiapin Q (0.1 μM), blockers of KATP and GIRK channels, respectively. Pretreatment with glibenclamide (> 5min) at a concentration shown to be effective at blocking KATP channels in the same hypothalamic preparation (Parsons and Hirasawa, 2010a) did not prevent the effect (n = 3; Fig 2.4C, E). However, in slices pre-exposed to tertiapin Q (> 5 min), N/OFQ's effect on MCH neurons was significantly attenuated (n = 7; Fig. 2.4D, E). We also found that N/OFQ (1 μM, 30s) reversibly inhibits voltage-gated calcium currents in MCH neurons (Fig. 2.4F, G). Thus, N/OFQ's effect on MCH neurons involves the activation of GIRK, but not KATP, channels as well as the inhibition of voltage-gated calcium channels.

Orexin neurons form direct appositions with MCH neurons (van den Pol et al., 2004) and co-express dynorphin (Chou et al., 2001) and N/OFQ (Maolood and Meister, 2010), suggesting that all of these peptides are likely released onto MCH neurons. It has been shown that orexin and dynorphin have direct excitatory and inhibitory effects on MCH neurons, respectively. Interestingly, the dynorphin effect desensitizes over repeated applications whereas the orexin effect does not (Li and van den Pol, 2006). Therefore, it was of interest to determine whether the inhibitory effect of N/OFQ also desensitizes and whether the effects of dynorphin and N/OFQ share the same pathway. N/OFQ was

applied three times to the same cell for 30 seconds each with a 10-15 minute wash in between applications to allow each response to return to baseline. The amplitude of the outward currents in MCH neurons were attenuated with repeated applications to the same hypothalamic section (Fig. 2.5A, B, n = 4). Furthermore, when dynorphin (40 μM) was tested in the presence of N/OFQ (1 - 10 μM), the dynorphin effect was largely attenuated (Fig. 2.5C-E). These results demonstrate that the N/OFQ effect desensitizes and occludes the responsiveness of MCH neurons to dynorphin.

2.4 Discussion

The present study demonstrates that N/OFQ can directly act as a powerful inhibitor of the MCH system by activating GIRK channels, which may be a cellular mechanism underlying N/OFQ's anti-reward and/or anxiolytic effects. Previously reported cellular effects of N/OFQ in various brain regions include the activation of a barium-sensitive inwardly-rectifying potassium current (Connor et al., 1996b;Vaughan and Christie, 1996;Vaughan et al., 1997;Madamba et al., 1999), suggestive of either GIRK or KATP channels. The NOP receptor can couple to either GIRK (Ikeda et al., 1997) or KATP currents (Armstead, 1999). The present study, by using specific blockers of these two channels, provides definitive evidence for the involvement of GIRK and not KATP channels in N/OFQ's inhibitory effect on MCH neurons. In addition, N/OFQ was found to inhibit voltage-gated calcium currents. Although we cannot rule out an effect on other ionic currents known to be modulated by N/OFQ such as BK and delayed rectifier currents (Chin et al., 2002;Qu et al., 2007a), we conclude that the majority of the

observed inhibition of MCH neurons results from modulation of GIRK and calcium channel activity.

2.4.1 Cotransmission from orexin to MCH neurons

Due to the widespread expression of N/OFQ in the brain (Neal, Jr. et al., 1999b), the source(s) of N/OFQ release onto MCH neurons remains elusive. One likely candidate is from neighbouring orexin neurons because these neurons co-express N/OFQ (Maolood and Meister, 2010) and form direct appositions with MCH neurons (van den Pol et al., 2004). For simplicity, these neurons will be referred to as orexin neurons despite the fact that virtually all of them co-express the endogenous kappa receptor agonist dynorphin (Chou et al., 2001) as well as N/OFQ. Orexin and dynorphin have direct excitatory and inhibitory effects on MCH neurons, respectively, which is a rare case where the postsynaptic cell exhibits opposing responses to two co-transmitted neuropeptides (Li and van den Pol, 2006). Interestingly, the excitatory effect of orexin does not desensitize as fast as the inhibitory effect of dynorphin. When both peptides are applied as a cocktail, the inhibitory effect prevails initially but the cocktail becomes excitatory after repeated applications, reflecting the diminished responsiveness to dynorphin (Li and van den Pol, 2006). In the present study, we demonstrate that N/OFQ also has an inhibitory but desensitizing effect. Therefore, it holds true that the non-desensitizing effect of orexin is likely to dominate following prolonged release of the peptide trio from orexin neurons (Li and van den Pol, 2006). Furthermore, the inhibitory effect of N/OFQ occludes the effect of dynorphin, suggesting that these two opioids activate a common inhibitory pathway in MCH neurons. In all, the present study and that of Li et al (Li and van den Pol, 2006)

demonstrate that the orexin-to-MCH synapse makes for an excellent model to study the complexities of peptide co-transmission.

2.4.2 Functional Considerations

Ample evidence suggests that N/OFQ has a significant inhibitory influence on both anxiety- and addiction-related behaviours. Deletion of the N/OFQ gene increases anxiety-like behaviours (Koster et al., 1999) whereas activation of the NOP receptor has an anxiolytic effect (Gavioli et al., 2002;Griebel et al., 1999;Jenck et al., 1997;Jenck et al., 2000;Varty et al., 2005). N/OFQ also abolishes the conditioned place preference and behavioural sensitization associated with a number of drugs of abuse (Sakoori and Murphy, 2004;Kotlinska et al., 2003;Ciccocioppo et al., 2004). On the other hand, MCH promotes both anxiety and addiction (Borowsky et al., 2002;Chaki et al., 2005;Georgescu et al., 2005;Smith et al., 2006;Roy et al., 2006;Smith et al., 2006;Chung et al., 2009). Therefore, a robust inhibition of MCH neurons by N/OFQ demonstrated by the present data may represent a central mechanism by which N/OFQ attenuates anxiety and addiction.

In contrast, both MCH (Qu et al., 1996;Gomori et al., 2003) and N/OFQ (Polidori et al., 2000;Stratford et al., 1997) have been shown to induce food intake, which appears inconsistent with our finding. However, N/OFQ's orexigenic effect may be site specific, as local injections into the ventromedial (Stratford et al., 1997) or arcuate nuclei of the hypothalamus (Polidori et al., 2000) or the nucleus accumbens shell (Stratford et al., 1997) produces clear hyperphagia whereas no significant effect on feeding is seen following N/OFQ injections into the paraventricular nucleus of the hypothalamus or the

central nucleus of the amygdala (Polidori et al., 2000). As the NOP receptor is commonly coupled to inhibitory $G_{i/o}$ proteins (Hawes et al., 1998), it is likely that the hyperphagic effect of intraventricular N/OFQ (Polidori et al., 2000) is a summed product of its inhibitory actions on many cell groups and systems, which does not necessarily mimic the pattern of endogenous peptide release. As N/OFQ inhibits two major appetite-stimulating neurons of the LH/PFA, namely orexin (Xie et al., 2008) and MCH neurons (this study), this raises a possibility that the local action of N/OFQ within this area may not result in hyperphagia.

2.4.3 Conclusions

The present study demonstrates that N/OFQ is a potent inhibitor of MCH neurons. This inhibition results from a NOP-mediated activation of GIRK channels and inhibition of voltage-gated calcium currents. A N/OFQ-MCH interaction may play a role in the control of anxiety-like behaviours and an individual's vulnerability to addiction.

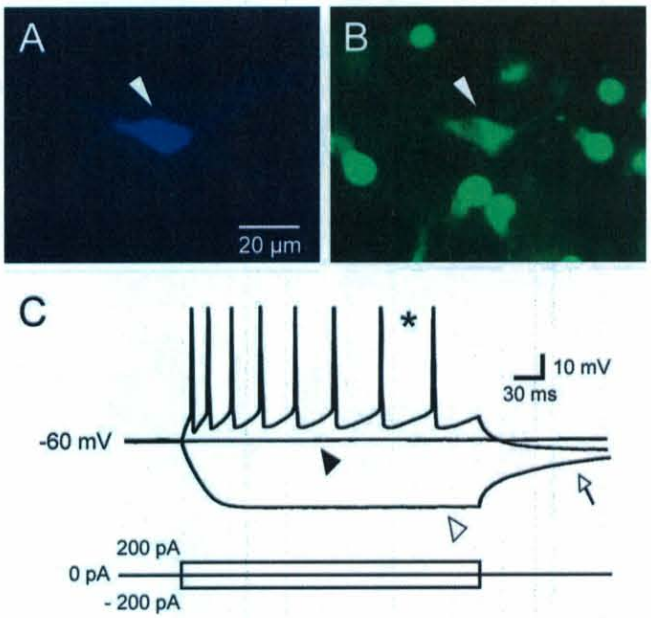


Figure 2.1 Identification of MCH neurons *in vitro*. *A-B*: Post-hoc immunohistochemistry showing co-localization of biocytin (*A*) and MCH (*B*) following whole-cell recording with a pipette solution containing biocytin. Arrowheads point to the recorded cell. *C*: Typical electrophysiological response of an MCH neuron to hyperpolarizing and depolarizing current injections. MCH neurons are characterized by a lack of spontaneous activity (filled arrowhead), h- (hollow arrowhead) and rebound currents (hollow arrow). Upon positive current injection, MCH neurons display spike adaptation (asterisk).

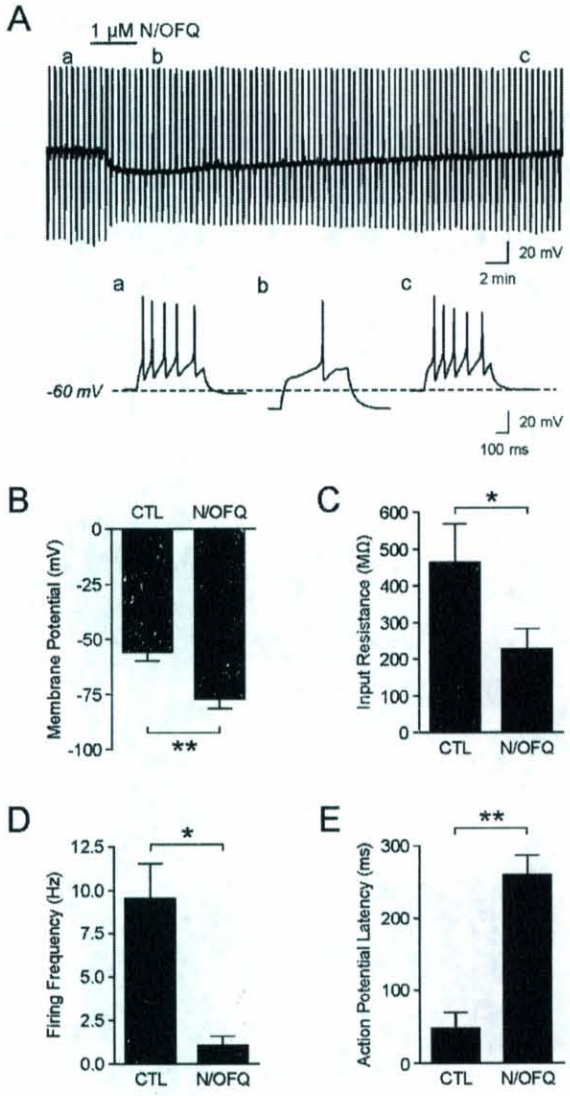

A
1 μM N/OFQ
a
b
c
20 mV
2 min
a
b
c
-60 mV
20 mV
100 ms
B
CTL
N/OFQ
Membrane Potential (mV)
0
-25
-50
-75
-100
**
C
*
Input Resistance (MΩ)
600
500
400
300
200
100
0
CTL
N/OFQ
D
*
Firing Frequency (Hz)
12.5
10.0
7.5
5.0
2.5
0.0
CTL
N/OFQ
E
**
Action Potential Latency (ms)
300
200
100
0
CTL
N/OFQ

Figure 2.2 N/OFQ hyperpolarizes MCH neurons. *A*: Representative current clamp trace from an MCH neuron hyperpolarizing in response to bath application of N/OFQ (1 μM). Vertical lines denote responses to a series of current injections (-200 to +200 pA in 100 pA increments, 300 ms each) applied every 30 seconds. Lower panel: Expanded traces are taken during the +100-pA current injection from the corresponding time points in the upper panel. *B*: Resting membrane potential is significantly hyperpolarized by N/OFQ (n = 4). *C*: Input resistance is significantly reduced by N/OFQ (n = 4). *D-E*: In response to a +100 pA current injection, action potential frequency (*D*) and latency to fire an action potential (*E*) decreases and increases, respectively, during N/OFQ application (n = 4). * $p < 0.05$, ** $p < 0.01$.

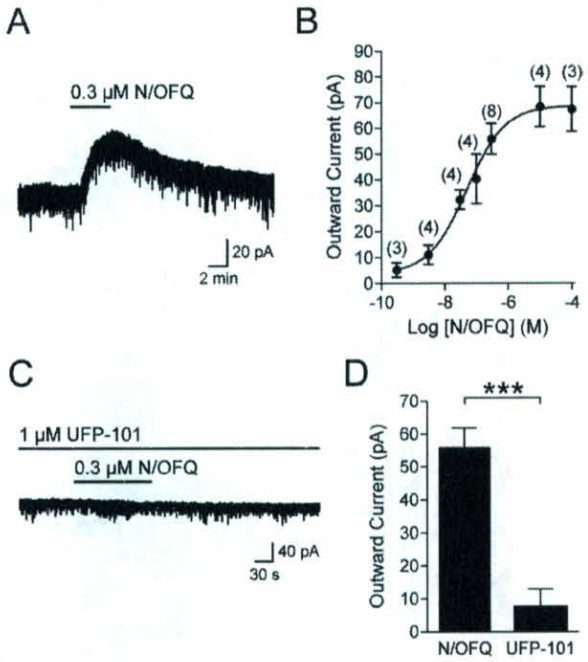


Figure 2.3 N/OFQ induces a NOP receptor-mediated outward current in MCH neurons. *A*: Representative voltage clamp trace of an MCH neuron displaying a TTX-insensitive outward current in response to N/OFQ. *B*: Concentration-response curve of N/OFQ's effect on MCH neurons reveals an EC_{50} of 50.7 nM. Numbers in brackets represent the cell number for each concentration. *C*, D*:* The N/OFQ effect (n = 8) is significantly inhibited by prior exposure to the NOP receptor antagonist UFP-101 (n = 4). *** $p < 0.001$.

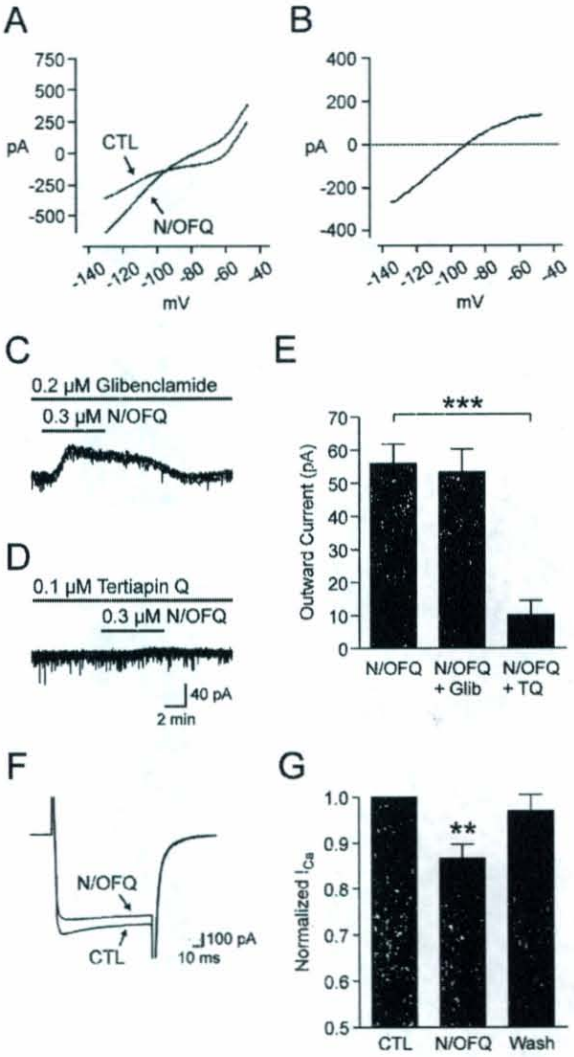

A
750
500
250
pA 0
-250
-500
CTL
N/OFQ
-140 -120 -100 -80 -60 -40
mV
B
400
200
pA 0
-200
-400
-140 -120 -100 -80 -60 -40
mV
C
0.2 µM Glibenclamide
0.3 µM N/OFQ
D
0.1 µM Tertiapin Q
0.3 µM N/OFQ
40 pA
2 min
E
***
Outward Current (pA)
70
60
50
40
30
20
10
0
N/OFQ
N/OFQ + Glib
N/OFQ + TQ
F
N/OFQ
CTL
100 pA
10 ms
G
Normalized I_{Ca}
1.0
0.9
0.8
0.7
0.6
0.5
**
CTL
N/OFQ
Wash

Figure 2.4 N/OFQ activates G-protein dependent inwardly rectifying potassium (GIRK) channels in MCH neurons. *A*: Current responses to voltage ramps before (control; CTL) and during N/OFQ (0.3 μM) application. *B*: Subtraction of the CTL from N/OFQ response in *A* reveals a N/OFQ-induced current with inward rectification that reverses near the equilibrium potential for potassium. *C*: N/OFQ-induced outward currents in MCH neurons persist in the presence of the KATP channel blocker glibenclamide. *D*: The GIRK channel blocker tertiapin Q inhibits N/OFQ-induced outward currents in MCH neurons. Scale bars apply to *C* and *D*. *E*: Grouped data showing a significant attenuation of the N/OFQ effect (n = 8) on MCH neurons in slices exposed to tertiapin Q (TQ, n = 7) but not glibenclamide (Glib, n = 3). *F-G*: N/OFQ (1 μM) inhibits voltage-gated calcium currents in MCH neurons (n = 6). ** $p < 0.01$, *** $p < 0.001$, n.s. non-significant.

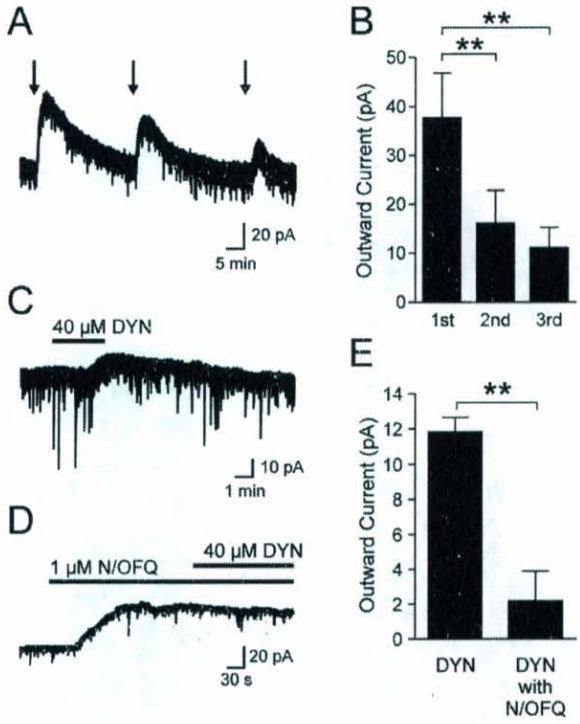


Figure 2.5 N/OFQ effect desensitizes and occludes dynorphin effect. A: Representative experiment showing the response of an MCH neuron to three 30-s applications of N/OFQ (0.3 μM). The start of each application is denoted by an arrow. *B*: Average peak outward currents for the 1st, 2nd and 3rd N/OFQ applications (n = 4). The magnitude of the N/OFQ-induced current decreases with repeated applications. *C-E*: Dynorphin induces an outward current in MCH neurons (n = 4) that is significantly occluded by prior induction of a maximal N/OFQ (1 - 10 μM)-mediated outward current (n = 6). * $p < 0.05$, ** $p < 0.01$.

CHAPTER 3

HYPOPHAGIA AND KATP-DEPENDENT INHIBITION OF OREXIN NEURONS INDUCED BY NOCICEPTIN/ORPHANIN FQ

(Manuscript in preparation)

3.1 Introduction

Nociceptin/orphanin FQ (N/OFQ) is the most recently discovered endogenous opioid (Meunier et al., 1995;Reinscheid et al., 1995). Nociceptin binding to its $G_{i/o}$-coupled nociceptin opioid peptide (NOP) receptor typically results in cellular inhibition via potassium channel activation and/or calcium current inhibition. Structurally, the NOP receptor is closest to the κ opioid receptor, although NOP does not bind to either the μ, δ or κ receptors with any significant affinity and is the only endogenous opioid known that binds to the NOP receptor (Meunier, 1997). N/OFQ actions at the NOP receptor have been implicated in a wide range of physiological and behavioural functions including the control of food intake.

Central N/OFQ injections have been consistently shown to increase food intake, despite the inhibitory nature of the peptide-receptor interaction. Hyperphagia can result from N/OFQ injection into the ventricles (Pomonis et al., 1996), arcuate nucleus (Polidori et al., 2000), ventromedial nucleus or nucleus accumbens shell (Stratford et al., 1997). These experiments have lead to the characterization of N/OFQ as an orexigenic peptide with its effects being attributed to a net inhibitory effect on neurons containing catabolic peptides such as POMC (Polidori et al., 2000). However, NOP receptor knockout animals

have little alterations in their hour-to-hour *ad libitum* food intake (Farhang et al., 2010) suggesting that the role of N/OFQ in feeding regulation may not be so clear.

The lateral hypothalamus/perifornical area (LH/PFA) has long been regarded as a critical brain region involved in motivated behavioural responses, including feeding (Stellar, 1994). The discovery of two peptidergic systems with cell bodies residing more-or-less exclusively within this region, namely the orexins (Sakurai et al., 1998;de Lecea L. et al., 1998) and melanin-concentrating hormone (MCH) (Bittencourt et al., 1992), has initiated a vast amount of research on the roles of these peptides in energy homeostasis and reward. Both orexin (Sakurai et al., 1998) and MCH (Qu et al., 1996;Gomori et al., 2003) stimulate feeding when injected centrally and orexin (Hara et al., 2001) and MCH (Shimada et al., 1998) knockout animals are hypophagic. Despite eating less, orexin knockout animals develop late-onset obesity, demonstrating orexin's additional role in the stimulation of energy expenditure (Sutcliffe and de Lecea, 2000). In contrast, MCH knockout mice are both hypophagic and lean (Shimada et al., 1998). Both orexin and MCH neurons promote reward-related behavioural responses (Harris et al., 2005;Chung et al., 2009) which appear to include palatable food intake (Zheng et al., 2007;Morens et al., 2005). In Chapter 2, we have shown (Parsons and Hirasawa, 2010b) that N/OFQ induces a potent and reversible inhibitory effect on MCH neurons that relies upon the activation of G-protein dependent inwardly rectifying potassium (GIRK) channels while the identity of the effector channel mediating the inhibitory effect on orexin neurons (Xie et al., 2008) has yet to be confirmed. Despite the known inhibitory effect of N/OFQ on these two feeding-stimulatory peptides, the effect of N/OFQ actions within the LH/PFA on food intake remains unknown.

The present study first tested the effect of intra-LH/PFA N/OFQ on food intake in the rat. As the majority of studies testing the effect of N/OFQ on feeding use rats (Pomonis et al., 1996;Polidori et al., 2000;Stratford et al., 1997) we also used rats to replicate the work of Xie et al (2008), which was conducted in mice. We further investigated the underlying mechanism mediating N/OFQ-induced orexin inhibition.

3.2 Materials and Methods

All experiments followed the guidelines set by the Canadian Council on Animal Care and were approved by the Memorial University Institutional Animal Care Committee. Male Sprague Dawley rats were obtained from the breeding colony at Memorial University.

3.2.1 Surgery and behavioural testing

Male-Sprague Dawley rats (250-300g) were anesthetized with isoflurane (4% induction, 2% maintenance). A unilateral stainless-steel guide cannula (22-gauge; .028" OD, .022" ID) was implanted into the right LH/PFA using the following co-ordinates (in mm): -2.9 AP and -1.2 ML with respect to bregma and 2.5 dorsal to the interaural line. Cannulae were fixed in place with skull screws and dental cement. Animals were given 5-7 days to recover from surgery before experimentation. All animals had *ad libitum* access to standard lab chow and water with the exception of testing days. On test days, chow was removed at 9:00 am and animals were given sucrose food pellets without chow (dustless precision pellets, F0021, Bio-Serv) at 1:00 pm. Animals were given an injection of either N/OFQ or saline targeted to the LH/PFA immediately preceding the presentation of the food pellets. Injections (500 nl) took place over 60 seconds via an injection cannula connected to a Hamilton syringe and cannulae were left in place for an additional 30-60 seconds to ensure proper diffusion at the injection site. The amount of pellet intake was measured one and three hours later and animals were then given their regular chow overnight. Overnight chow intake (from 4:00 pm to 9:00 am) was measured. The second test day, which was separated from the first by a non-experimental day where lab chow

and water were available *ad libitum*, was identical to the first except animals received the N/OFQ or saline injection they did not receive on the first test day. The order of injections was alternated from animal to animal.

After experimentation, animals were euthanized with CO_2, brains were removed, frozen and cut at 40 μm on a cryostat. Injection sites were confirmed by a 500 nl injection of blue dye (4% pontamine sky blue), following normal infusion procedures, immediately after CO_2 and prior to removal of the brain. Placements that fell within and outside the LH/PFA were determined blindly and only those animals receiving injections into the LH/PFA were included in the experimental group for analysis.

3.2.2 Electrophysiology

Animals (60-70g) were deeply anesthetized with halothane, decapitated and brains were quickly removed. Coronal hypothalamic slices (250 μm) were sectioned using a vibratome (Leica). Sectioning was performed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 25 $NaHCO_3$, 2 $CaCl_2$, 10 glucose, pH 7.3-7.35. Following dissection, slices were incubated in ACSF at 32-35°C for 30-45 min, then at room temperature until recording. ACSF was continuously bubbled with O_2 (95%)/CO_2 (5%).

Conventional whole-cell patch-clamp recordings were performed on brain slices perfused with ACSF at 1.5-2 ml/min, 26°C, using a Multiclamp 700B amplifier and pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). Different conventional whole cell internal solutions were used in the present study and contained (in mM): 123 K-gluconate (or 123 KCl for high-chloride internal), 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10

HEPES, 4 Na_2-ATP, 0.3 Na-GTP. Another conventional whole cell solution used in the present study consisted of (in mM): 120 K-gluconate, 1 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 3 K_2ATP. For perforated patch recordings, nystatin (0.45 mg/ml) and pluronic acid were dissolved in DMSO and added to an internal solution that contained the following: 120 K-gluconate, 5 MgCl2, 10 EGTA, 40 HEPES. KOH was added to all internal solutions until a pH of 7.3-7.35 was reached. Biocytin (1-1.5 mg/ml) was included daily to label a subset of cells for post-hoc immunohistochemical phenotyping and these sections were processed to visualize biocytin, MCH and orexin-A. Targeted neurons included large cell-bodies neurons (>10 μm diameter) located in the LH/PFA, visualized using an infrared-differential interference contrast microscope. Upon attaining whole cell access, each neuron's electrophysiological characteristics were observed by a series of 300-ms hyperpolarizing (-200 and -100 pA) and depolarizing (100 and 200 pA) current injections in current clamp mode. Cells that did not display voltage responses typical of orexin neurons were not used in the present study. These include a lack of spike adaptation upon positive current injection and the presence of spontaneous action potentials, I_h and rebound currents (Fig. 3.1)(Eggermann et al., 2003;Alberto et al., 2006). All cells displaying the aforementioned electrophysiological criteria were thus included in the present study. All voltage clamp experiments were performed at a holding potential of -70 mV, with the exception of voltage ramps. To determine the effect of N/OFQ on current-voltage relationships, the membrane potential was ramped from -140 to -20 mV (600 ms) in the absence and presence of N/OFQ. Miniature excitatory postsynaptic currents (mEPSCs) were recorded with tetrodotoxin (TTX, 1 μM) and picrotoxin (PTX, 50 μM) added to the ACSF.

3.2.3 Post-Hoc Immunofluorescence

See section 2.2.2 for details. In agreement with previous work from our lab (Alberto et al., 2006;Parsons and Hirasawa, 2010a), we found that we could reliably identify orexin neurons based on electrophysiological characteristics (Fig. 3.1).

3.2.4 Data analysis

Membrane potential and holding current were measured using Clampfit 9.2 (Molecular Devices; Sunnyvale, CA) and miniature excitatory postsynaptic potentials (mEPSCs) were analyzed using MiniAnalysis (Synaptosoft; Decatur, GA). Data are expressed as mean ± S.E.M. The statistical tests used included one-way ANOVA with Dunnett's post test for multiple group comparisons and paired and unpaired Student t-tests for two-group comparisons. Kolmogorov-Smirnov tests were used to generate cumulative distributions and to determine whether N/OFQ had any effect on mEPSC inter-event interval or amplitude in single cells. A value of $p < 0.05$ was considered significant.

3.2.5 Drugs

For electrophysiology experiments, 1000x frozen aliquots of drugs were thawed and diluted with ACSF to their final concentration immediately prior to experimentation. TTX was obtained from Alomone Labs (Jerusalem, Israel) and PTX was obtained from Sigma-Aldrich (St. Louis, MO). N/OFQ (1-13)NH_2, UFP-101, tertiapin Q and glibenclamide were obtained from Tocris Bioscience (Ellisville, MO). N/OFQ (1-13)NH_2

is a bioactive metabolite of N/OFQ and was used in the present study as a NOP receptor agonist. N/OFQ (1-13)NH_2 is referred to as N/OFQ throughout the Results section.

3.3 Results

3.3.1 Effect of intra LH/PFA N/OFQ on palatable food intake

We first investigated the effect of intra-LH/PFA infusions of N/OFQ on food intake. As orexin and MCH neurons are implicated in palatable feeding (Zheng et al., 2007;Morens et al., 2005), we measured the intake of palatable food pellets as well as overnight lab chow intake. Furthermore, as a unilateral lesion of the lateral hypothalamus has been reported to decrease feeding (Grossman and Grossman, 1982;Gold, 1966), our studies were carried out using unilateral injections of N/OFQ in the LH/PFA. N/OFQ was injected at a dose (10 nmol/0.5 µl) shown to increase feeding when injected into the ventromedial nucleus or nucleus accumbens shell (Stratford et al., 1997). We found that unilateral N/OFQ injections into the LH/PFA resulted in a significant reduction in pellet intake over the first hour (Fig. 3.2A, $t = 2.81$, $p < 0.05$, $n = 6$) but not by the third hour (Fig. 3.2A, $t = 2.37$, $p > 0.05$, $n = 6$) and there was no effect on overnight chow intake (Fig. 3.2B, $t = 0.43$, $p > 0.05$, $n = 6$), suggesting that the hypophagic effect of a unilateral intra-LH/PFA N/OFQ injection is temporary. Injections that were determined blindly to fall outside of the LH/PFA did not result in the hypophagia that was observed for correct placements ($t = 1.12$, $p > 0.05$, $n = 4$). Thus, the local action of N/OFQ within the LH/PFA induces temporary hypophagia rather than the hyperphagia typically associated with central N/OFQ injections. As unilateral N/OFQ infusions significantly inhibited food intake, bilateral injections were not investigated.

3.3.2 N/OFQ effect on rat orexin neurons

Two major feeding-stimulatory peptides residing within the LH/PFA include the orexins and MCH. N/OFQ inhibits rat MCH neurons via GIRK channel activation (Parsons and Hirasawa, 2010b) while the inhibitory effect of N/OFQ in orexin neurons was investigated in mice (Xie et al., 2008). As the majority of food intake studies including this one utilize rats, we wanted to determine whether orexin inhibition by N/OFQ extends to rats and if so, the mechanism by which it occurs. Using patch clamp recordings from hypothalamic slices from male Sprague-Dawley rats, we tested N/OFQ at 0.3 μM, a concentration that was shown to elicit a half-maximal response in orexin neurons in transgenic orexin/EGFP C57 mice (Xie et al., 2008). We found that this concentration induced a robust and consistent effect in rat orexin neurons and was used throughout the present study unless otherwise noted. N/OFQ (2-5 minutes) induced a long-lasting hyperpolarization in orexin neurons (15.6 ± 0.7 mV, n = 4, Fig. 3.3A), agreeing with the work of Xie et al (Xie et al., 2008). To investigate the mechanism of inhibition, we examined N/OFQ's effect in orexin neurons that were voltage clamped at -70 mV. N/OFQ (2-5 minutes) induced an outward current in all orexin neurons tested (n = 26, Fig. 3.3B). Outward currents were similar regardless of the conventional whole cell internal solution used ($p > 0.05$) and data were grouped together (Fig. 3.4). The effect of N/OFQ was TTX-insensitive (without TTX, 35.8 ± 3.0 pA, n = 19; with TTX, 36.4 ± 2.2 pA, n = 7) and persistent as currents were never found to return to baseline even hours following agonist washout. This is in contrast to Xie et al (2008) who, despite reporting a long-lasting effect, found that membrane potential and cell excitability in mouse orexin neurons recovered roughly 20 minutes following the washout of N/OFQ. To determine

whether reversible responses could be induced in rat orexin neurons, we briefly applied N/OFQ using a flow-pipette. Reversible responses were observed with short (1-5 second) N/OFQ applications (0.6 μM) and multiple responses could be observed in the same cell. However, applications of 30 seconds or longer were found to induce a persistent outward current (Fig. 3.3C). Thus, the length of N/OFQ exposure to hypothalamic slices determines whether a persistent or reversible inhibitory current is induced in rat orexin neurons. The involvement of the NOP receptor is suggested by a significant attenuation of the outward current in slices that were pre-treated (>5 minutes) with the selective (Calo et al., 2005) competitive NOP receptor antagonist UFP-101 (Fig. 3.3D, F). UFP-101 was applied at the same concentration (1 μM) that was found to significantly attenuate N/OFQ's effect in MCH neurons from the same preparation (Parsons and Hirasawa, 2010b). Currents were also significantly attenuated by postsynaptic loading of the non-hydrolysable GDP analogue GDPβS (Fig. 3.3E, F). The classic opioid receptors were not involved as outward currents persisted in naloxone (Fig. 3.3F). Thus, the orexin neuron response to N/OFQ involves NOP receptor and G-protein activation.

Next, we asked what ion channel(s) mediate the response of orexin neurons to N/OFQ. Results from the study by Xie et al (2008) suggest that activation of a potassium current may contribute to the postsynaptic effect. In our hands, voltage ramps revealed that N/OFQ indeed induced an outward current that reversed near the theoretical potassium equilibrium potential of -104.1 mV. The reversal potential of the response was significantly depolarized when the potassium concentration in the bath was increased four-fold (Fig. 3.5A-B). We then tested the effect of N/OFQ in the presence of potassium channel blockers. N/OFQ responses were inhibited by barium (Fig. 3.5C, F), suggesting

the involvement of a barium-sensitive inwardly-rectifying potassium channel. These include G-protein dependent inwardly rectifying potassium (GIRK) and ATP-sensitive potassium (KATP) channels which can be blocked using tertiapin Q and glibenclamide, respectively. Outward currents still persisted in the presence (>5 minutes) of tertiapin Q (Fig. 3.5, F) at a concentration shown to attenuate N/OFQ-induced responses in MCH neurons in the same slice preparation (Chapter 2). Rather, we found that blocking KATP channels with glibenclamide abolished the N/OFQ response in orexin neurons (Fig. 3.5E, F), implicating KATP, and not GIRK, channels as the effector channel. To further test a KATP channel involvement in the response, we bath applied diazoxide (200-500 μM) to open KATP channels. As expected (Parsons and Hirasawa, 2010a), this resulted in an outward current in orexin neurons (data not shown). The effect of diazoxide significantly occluded that of N/OFQ (Fig. 3.5G). As KATP channels are regulated by the level of intracellular ATP (Nichols, 2006), we also confirmed the presence of an N/OFQ-induced outward current in orexin neurons using nystatin-based perforated patch recordings to maintain intrinsic intracellular ATP levels (n=4). Despite the KATP-dependent nature of the outward current, the persistent N/OFQ effect could not be reversed by additional glucose from 10 to 15 mM (n = 3; Fig. 3.5H). Thus, N/OFQ induces a persistent activation of KATP channels in orexin neurons that cannot be reversed by increasing the amount of extracellular glucose. This is in contrast to the transient GIRK-dependent inhibition that N/OFQ was found to exert on neighbouring MCH neurons (chapter 2) (Parsons and Hirasawa 2010b).

The NOP receptor is coupled to $G_{i/o}$ proteins and is typically linked to adenylyl cyclase inhibition (Meunier et al., 1995;Reinscheid et al., 1995), suggesting that PKA-

induced activation of KATP channels (Beguin et al., 1999) is not a likely mechanism of N/OFQ's effect in orexin neurons. On the other hand, N/OFQ has previously been shown to activate PKC (Lou et al., 1997), and PKC can activate KATP channels through the phosphorylation of a conserved threonine residue in their pore-forming subunits (Light et al., 2000). Thus, we examined the role of PKC in the present response by using the PKC inhibitor calphostin C. In calphostin C-treated slices (100 nM, >60 minutes), N/OFQ induced a significantly attenuated outward current (Fig. 3.5I-J) suggesting at least a partial role of PKC. Together, these data suggest that N/OFQ inhibits orexin neurons through a PKC-dependent activation of KATP channels.

The work by Xie et al (2008) also demonstrated a synaptic effect of N/OFQ on mouse orexin neurons. Thus, we conducted experiments in the rat to determine whether N/OFQ also has an inhibitory effect on excitatory synaptic transmission in the rat. We found no significant overall effect of N/OFQ on mEPSC inter-event interval or amplitude in orexin neurons (Fig. 3.6). However, using Kolmogorov–Smirnov statistics, we found that 2 of the 8 cells tested (25%) did show a significant and reversible decrease in mEPSC inter-event interval but not amplitude. Thus, the presynaptic modulation of orexin neurons by N/OFQ appears to be more pronounced in the mouse compared to the rat.

3.4 Discussion

Different brain regions vary in their sensitivity to the hyperphagic effect of N/OFQ. The present study demonstrates the first region, to the best of our knowledge, in which N/OFQ acts to induce a decrease in feeding. In agreement with Xie et al (2008), N/OFQ also had a potent inhibitory effect on rat orexin neurons. We further showed that the mechanism of inhibition is due to a NOP- and PKC-dependent activation of KATP channels.

3.4.1 N/OFQ and food intake

N/OFQ is typically associated with hyperphagia when injected centrally. A number of different N/OFQ injection sites, including the ventricles, the ventromedial and arcuate hypothalamic nuclei and the nucleus accumbens shell have been shown to elicit feeding (Pomonis et al., 1996;Polidori et al., 2000;Stratford et al., 1997). In contrast, we found that the unilateral injection of N/OFQ into the LH/PFA, where the feeding-stimulatory orexin and MCH neurons exist, decreased the intake of palatable food pellets within the hour following N/OFQ injection. We can speculate that both orexin and MCH neuron inhibition are involved in the observed hypophagia, although the effect of N/OFQ on orexin neurons *in vitro* is long-lasting whereas the hypophagia was not. It is noteworthy to point out that the present study only used unilateral injections into the right LH/PFA and that orexin and MCH neurons in the left hemisphere were presumably never affected by the drug treatment. Thus, a greater and more sustained response may be observed following bilateral injections. Supporting this possibility is the report that

unilateral lesions of the lateral hypothalamus only transiently decreases food intake (Grossman and Grossman, 1982;Gold, 1966).

In light of the present findings, N/OFQ appears not to be a simple orexigenic peptide. We have identified the LH/PFA as a target region where N/OFQ can exert a hypophagic response. Thus, N/OFQ's role in food intake appears to be site-specific in that it can induce hyperphagia or hypophagia depending upon its site(s) of action. Although NOP receptor knockout animals were found to have attenuated deprivation-induced hyperphagia, suggesting an orexigenic role of the endogenous N/OFQ system, 24-hour *ad libitum* feeding was unaffected and detailed analysis of hour-to-hour *ad libitum* feeding revealed a small increase in nocturnal feeding and a small decrease in daytime feeding (Farhang et al., 2010). Together, these data suggest that N/OFQ can exert a bi-directional effect on feeding behaviour in a brain area- and context-dependent manner.

The palatable food pellets used in the present study provided us with a means to drive sufficient, measureable daytime food intake during the post-injection intervals at which food intake was measured. Furthermore, as both orexin and MCH neurons are implicated in hedonic feeding (Zheng et al., 2007;Morens et al., 2005;Borowsky et al., 2002), it was of interest to determine whether N/OFQ actions in the LH/PFA could affect palatable food intake. Although overnight chow intake was measured, animals were not presented with the chow until 3 hours post-injection, a time at which the effect on palatable food intake was no longer evident. Thus, the finding that N/OFQ did not affect overnight chow intake does not necessarily suggest that N/OFQ in the LH/PFA solely modulates rewarding food intake. As the antagonism of orexin and MCH signaling has

been shown to inhibit both palatable food and regular lab chow intake (Haynes et al., 2000;Zheng et al., 2007;Shearman et al., 2003;Morens et al., 2005;Borowsky et al., 2002), we would hypothesize that the effect of N/OFQ in the LH/PFA is a general one on food intake and not just specific for the palatable sugar pellets used in the present study.

3.4.2 N/OFQ-induced inhibition of orexin neurons

N/OFQ is an inhibitory peptide that has been shown to activate a potassium current in neurons from multiple brain areas (Connor et al., 1996a;Vaughan and Christie, 1996;Vaughan et al., 1997;Eriksson et al., 2000). Typically, these currents are confirmed GIRK currents (Chapter 2)(Parsons and Hirasawa, 2010b;Farhang et al., 2010) or are presumed to be GIRK-mediated based on their inward-rectification and barium-sensitivity. However, the effect of N/OFQ in orexin neurons persisted in the presence of a GIRK channel blocker and is instead KATP-dependent. The effect in orexin neurons also sustains long after agonist washout. In fact, inhibition was observed in one cell that lasted over 4 hours following a 5-minute exposure to 300 nM N/OFQ. This is in contrast to a GIRK-dependent effect of N/OFQ on MCH neurons which is readily reversible (chapter 2) (Parsons and Hirasawa, 2010b). Although reversible effects could be observed in orexin neurons with brief flow-pipette applications (1 or 5 seconds), persistent outward currents were observed with the same N/OFQ concentration and application length that induced a reversible effect in MCH neurons. In fact, readily reversible outward currents induced by N/OFQ have been observed for neurons in a number of regions including the arcuate nucleus (Farhang et al., 2010), ventromedial hypothalamus (Lee et al., 1997), periaqueductal grey (Vaughan et al., 2003), ventral tegmental area (Zheng et al., 2002),

substantia gelatinosa (Luo et al., 2001), ventromedial medulla (Vaughan et al., 2001), hippocampus (Amano et al., 2000), locus coeruleus (Connor et al., 1996a) and dorsal raphe (Vaughan and Christie, 1996). The presently observed response behaves more like the response seen in the thalamic reticular nucleus in which the outward current does not readily return to baseline following N/OFQ washout (Meis et al., 2002). Interestingly, it has been suggested that NOP receptor splice variants exist which may represent high- and low-affinity binding sites (Mathis et al., 1997;Mathis et al., 1999;Peluso et al., 1998;Liao et al., 2010). In such a case, NOP receptor splice variants may be expressed differently in orexin versus MCH neurons as well as in thalamic reticular neurons versus the aforementioned regions in which there was a reversible N/OFQ effect. Alternatively, a prolonged postsynaptic effect may be a frequent feature of KATP channel activation by ligand-induced intracellular signaling as the inhibitory effects of insulin and leptin in the ventromedial and arcuate hypothalamic nuclei are PI3 kinase and KATP-dependent and long-lasting (Spanswick et al., 2000;Spanswick et al., 1997). It would be of interest to determine whether the persistent effect of N/OFQ in thalamic reticular neurons is KATP-dependent as well.

3.4.3 PKC-dependent activation of KATP channels by N/OFQ

The NOP receptor is coupled to $G_{i/o}$ proteins and therefore NOP activation is typically associated with an inhibition of adenylyl cyclase activity (Meunier et al., 1995;Reinscheid et al., 1995). Additionally, N/OFQ acting at the NOP receptor has been shown to activate PKC in a PLC- and Ca^{++}-dependent manner (Lou et al., 1997). PKC, in turn, can phosphorylate a conserved threonine residue in the pore-forming subunit of

KATP channels, leading to their activation (Light et al., 2000). Furthermore, the maintenance phase of long-term potentiation involves the persistent activity of PKC, in particular the atypical PKC isoform PKMζ (Sacktor, 2008), and thus it is possible that a similar mechanism may account for the persistence of N/OFQ's effect in the present study. However, much of the data supporting a PKC role in KATP activation comes from studies of Kir6.2-containing KATP channels while we have previously shown that KATP channels in orexin neurons are composed of Kir6.1 and SUR1 subunits (Chapter 4)(Parsons and Hirasawa, 2010a). In vascular smooth muscle, in which KATP channels are composed of Kir6.1 and SUR2B subunits, PKC has actually been shown to promote channel closure by Kir6.1 phosphorylation (Ko et al., 2008;Shi et al., 2008). Precisely how PKC modulates Kir6.1/SUR1-containing KATP channels, which are found in orexin neurons, is unclear. Another possibility for PKC regulation of KATP channels is by the production of free radicals. N/OFQ has been shown to increase O_2^- by a PKC-dependent mechanism (Armstead, 2002) and it is recognized that free radicals, including O_2^-, can activate KATP channels (Krippeit-Drews et al., 1999;Tokube et al., 1998;Avshalumov and Rice, 2003). It is interesting to note that oxidative stress can also result in prolonged PKC activity (Palumbo et al., 1992) which offers another potential mechanism underlying the sustained outward current observed in the present study.

3.4.5 Conclusions

Despite N/OFQ being known to stimulate food intake when injected centrally, we demonstrate that local N/OFQ actions within the LH/PFA reduces palatable food intake. Thus, the effect of endogenous N/OFQ on feeding is likely complex. The finding that

N/OFQ's inhibitory effect on orexin neurons is dependent on KATP channels is novel and contrasts with the mechanism of N/OFQ-induced inhibition of MCH neurons (Chapter 2)(Parsons and Hirasawa, 2010b). Overall, the present study demonstrates a long-lasting KATP-dependent orexin neuron inhibition which may contribute to N/OFQ-induced hypophagia. As both orexin and MCH neurons are implicated in obesity, anxiety, addiction and nociception, among others, there are likely a number of behavioural and physiological consequences of N/OFQ action within the LH/PFA. In fact, similar injections have been observed to block stress-induced analgesia (Gerashchenko et al., 2010). Our results suggest that N/OFQ plays a more complex role in feeding regulation than previously thought and is worth more detailed investigation in future studies. The finding that orexin and MCH neuron inhibition by N/OFQ relies on different mechanisms provides a pharmacological means by which one can investigate the relative contribution of one system over the other on N/OFQ-mediated effects.

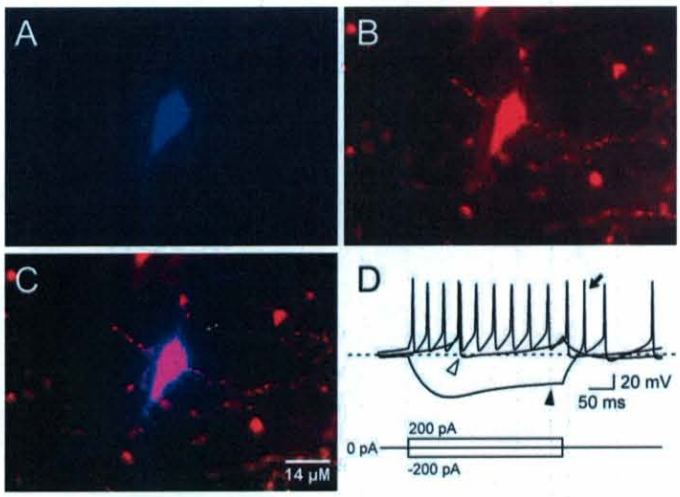


Figure 3.1 Identification of orexin neurons *in vitro*. *A-C*: Post-hoc immunohistochemistry showing co-localization of biocytin (*A*) and orexin-A (*B*) following whole-cell recording with a pipette solution containing biocytin. Merged image is shown in *C*. *D*: Typical electrophysiological response of an orexin neuron to hyperpolarizing and depolarizing current injections. Orexin neurons are characterized by the presence of spontaneous activity (hollow arrowhead), h- (filled arrowhead) and rebound currents that may or may not initiate action potential firing (arrow). Upon positive current injection, orexin neurons do not display significant spike adaptation.

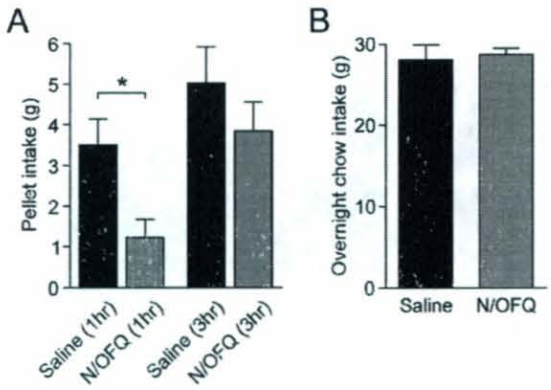


Figure 3.2 Intra-LH/PFA N/OFQ decreases palatable food intake. *A*: N/OFQ injected into the LH/PFA (n = 6) significantly reduced sugar pellet intake during the following hour but not by 3 hours post-injection. Cumulative food intake is presented for the 3 hour measure and thus includes the first hour of intake. *B*. N/OFQ injection at 1:00 pm had no effect on subsequent overnight chow intake (n = 6). ** $p < 0.01$.

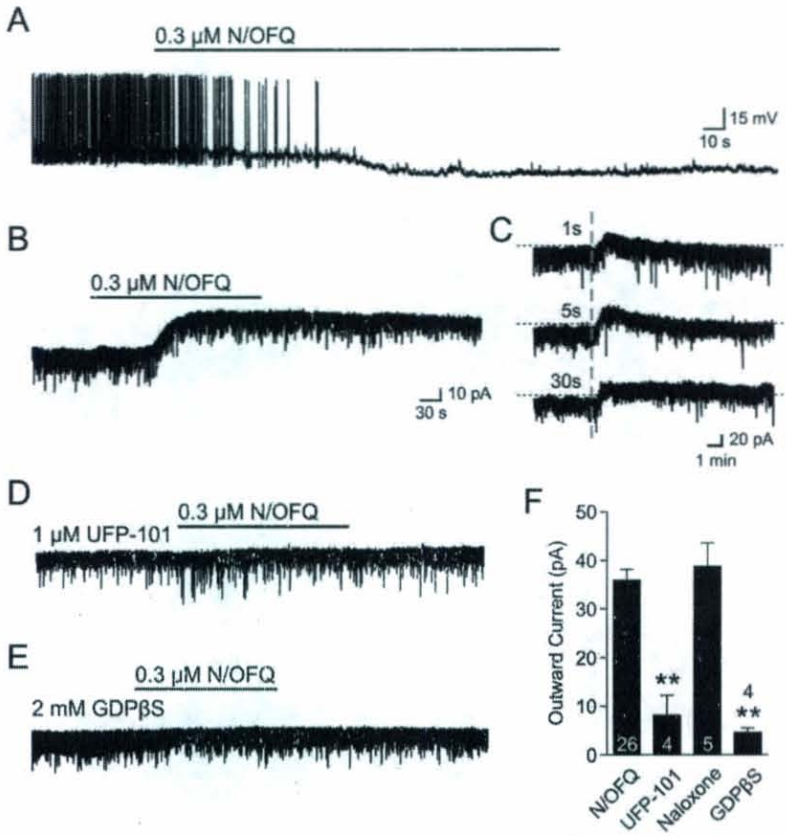

A
0.3 μM N/OFQ
15 mV
10 s
B
0.3 μM N/OFQ
10 pA
30 s
C
1s
5s
30s
20 pA
1 min
D
1 μM UFP-101
0.3 μM N/OFQ
E
0.3 μM N/OFQ
2 mM GDPβS
F
Outward Current (pA)
50
40
30
20
10
0
**
**
4
26
4
5
N/OFQ
UFP-101
Naloxone
GDPβS

Figure. 3.3. N/OFQ inhibits orexin neurons via NOP receptor activation. *A*: Bath application of N/OFQ induces a persistent inhibition of orexin neurons. B: Representative voltage clamp trace showing a TTX-insensitive, persistent outward current in an orexin neuron induced by N/OFQ bath application. *C*: Responses to N/OFQ are reversible for short (1, 5s) but not longer (30s) applications. Applications of 30s or shorter were applied via flow pipette. Vertical dotted grey line represents the beginning of the N/OFQ application for each trace. Horizontal dotted black lines are included to reference the baseline for each trace. Note the sustained effect in the bottom trace. *D-F*: N/OFQ responses are significantly attenuated by UFP-101 (*D*) and GDPβS (*E*). Grouped data is shown in *F*. Numbers in bars represent the number of cells examined in each group. ** $p < 0.05$.

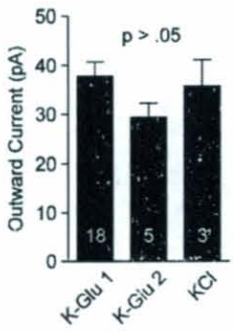


Figure 3.4 Magnitude of N/OFQ-induced outward currents as measured using different internal solutions. Outward currents induced by N/OFQ were similar regardless of the conventional whole-cell internal solution used. K-Glu1 refers to a solution containing (in mM): 123 K-gluconate, 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10 HEPES, 4 Na_2-ATP, 0.3 Na-GTP. K-Glu2 refers to a solution containing (in mM): 120 K-gluconate, 1 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 3 K_2ATP. KCl refers to the high-chloride internal which differs from K-Glu1 only in that K-gluconate is replaced by equimolar KCl. Numbers in bars represent the number of cells examined in each group.

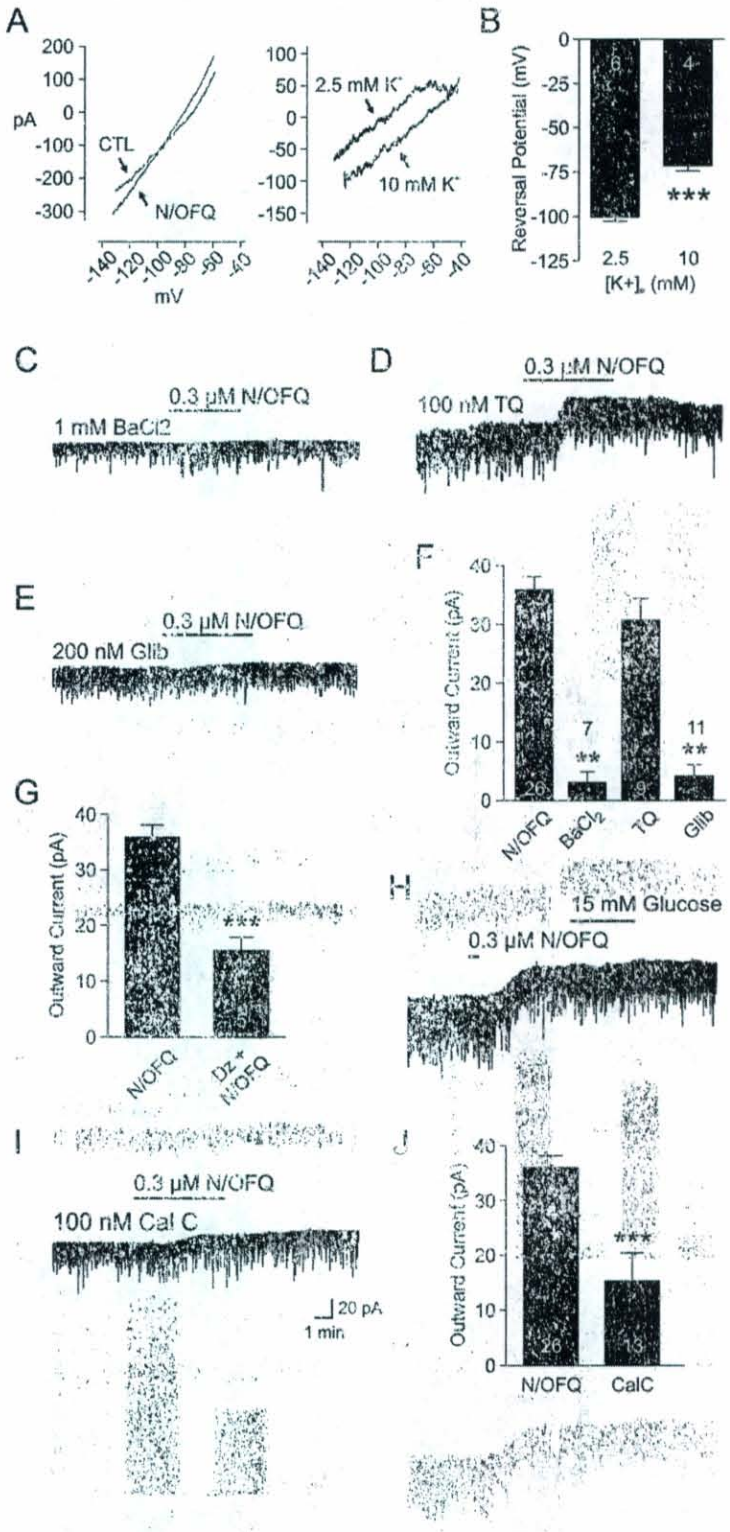

A
200
100
0
-100
-200
-300
pA
CTL
N/OFQ
-140 -120 -100 -80 -60 -40
mV
100
50
0
-50
-100
-150
2.5 mM K+
10 mM K+
-140 -120 -100 -80 -60 -40
B
0
-25
-50
-75
-100
-125
Reversal Potential (mV)
6
4
***
2.5
10
[K+]o (mM)
C
0.3 µM N/OFQ
1 mM BaCl2
D
0.3 µM N/OFQ
100 nM TQ
E
0.3 µM N/OFQ
200 nM Glib
F
40
30
20
10
0
Outward Current (pA)
26
7
**
9
11
**
N/OFQ
BaCl2
TQ
Glib
G
40
30
20
10
0
Outward Current (pA)
***
5
N/OFQ
Dz+ N/OFQ
H
15 mM Glucose
0.3 µM N/OFQ
I
0.3 µM N/OFQ
100 nM Cal C
20 pA
1 min
J
40
30
20
10
0
Outward Current (pA)
***
26
13
N/OFQ
CalC

Figure 3.5 N/OFQ-induced orexin inhibition is dependent on PKC and KATP channel activation. *A*: Voltage-ramps before (control; CTL) and during N/OFQ application generate a visibly different current response (right). Subtraction (N/OFQ - CTL) reveals a current-voltage relationship that is near the potassium equilibrium (left). Increasing the extracellular potassium concentration 4-fold (from 2.5 to 10 mM) shifted the current-voltage relationship of the N/OFQ-induced response rightward. *B*. Grouped data showing that the reversal potential of the N/OFQ-induced response is dependent on the amount of extracellular potassium. *C-F*: N/OFQ responses are attenuated by BaCl2 (*C*) and glibenclamide (*E*) but not tertiapin Q (*D*). Grouped data are shown in *F*. *G*: Prior application of diazoxide (Dz, 200-500 μM) attenuates the N/OFQ effect. *H*: Outward currents induced by N/OFQ are not reversed by increasing the bath concentration of glucose from 10 to 15 mM. *I-J*: The PKC inhibitor calphostin C significantly attenuates the magnitude of the N/OFQ-induced current. Numbers in bars represent the number of cells examined in each group. ** $p < 0.01$, *** $p < 0.005$.

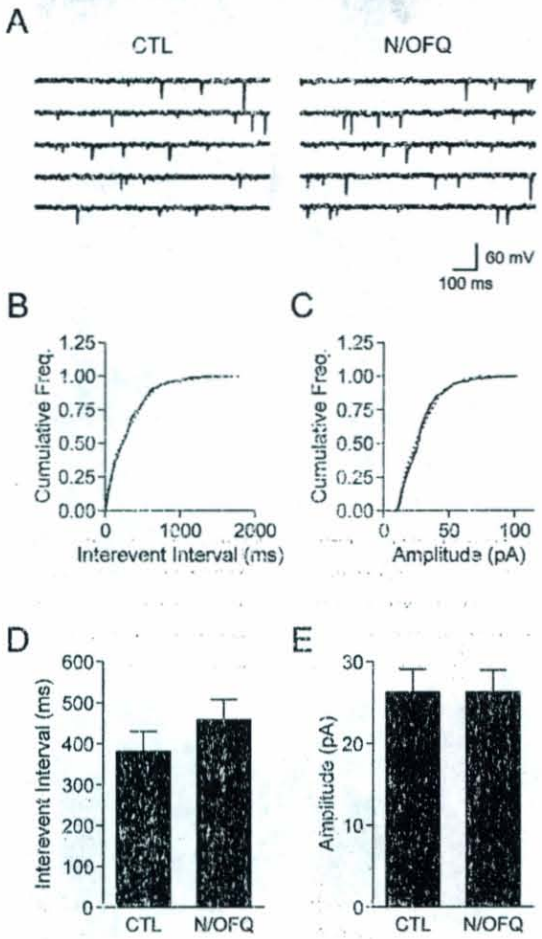


Figure 3.6 N/OFQ has no effect on excitatory synaptic transmission onto orexin neurons. *A*: mEPSC traces from a representative orexin neuron before and during N/OFQ (300 nM, 5 min) application. *B-C*: Cumulative frequency (freq.) graphs for mEPSC inter-event interval (*C*) and amplitude (*D*) for the orexin neuron shown in *A*. *D-E*: Grouped data (n = 8) of N/OFQ's effect on mEPSC inter-event interval (*D*) and amplitude (*E*) in orexin neurons.

CHAPTER 4

ATP-SENSITIVE POTASSIUM CHANNEL-MEDIATED LACTATE EFFECT ON OREXIN NEURONS: IMPLICATIONS FOR BRAIN ENERGETICS DURING AROUSAL

(Published in the Journal of Neuroscience)

4.1 Introduction

The brain, although comprising only about 2% of body weight, is responsible for 25% of total body glucose utilization necessitating a continuous supply from the periphery (Magistretti et al., 1995). This is thought to be ensured by the glucose homeostatic mechanism involving 'glucosensors' that exist in the hypothalamus and brainstem (Marty et al., 2007;Levin et al., 2004). Glucosensing neurons are excited (glucose-excited; GE) or inhibited (glucose-inhibited; GI) by extracellular glucose and induce appropriate counterregulatory responses to restore glucose homeostasis (Levin, 2001). Glucosensing in certain GE and GI neurons depends on glucokinase, a critical enzyme that catalyzes glycolysis (Balfour et al., 2006;Dunn-Meynell et al., 2002;Kang et al., 2004;Lynch et al., 2000), suggesting that glucose metabolism is directly involved in their electrophysiological response to glucose.

While some neurons clearly utilize glucose, astrocytes seem to be the main cell type that metabolizes glucose in the active brain, providing neurons with lactate as an energy substrate (Pellerin and Magistretti, 1994;Pellerin et al., 2007). Due to the heavy lactate dependence, it is plausible that the brain has mechanisms to monitor lactate levels

and control energy substrate levels. Mediobasal hypothalamic GE neurons not only respond to glucose but also to lactate, suggesting that they may also act as lactate sensors (Ainscow et al., 2002;Yang et al., 1999). However, as glucose and lactate do not always fluctuate in tandem within the brain, it is important to determine whether neuronal sensors exist that have selective sensitivity to lactate.

Orexin neurons induce food intake (Sakurai et al., 1998) and regulate autonomic functions (Ferguson and Samson, 2003) including stimulation of hepatic glucose production (Yi et al., 2009). Furthermore, these neurons play an important role in wakefulness (Hagan et al., 1999;Sakurai, 2007) through widespread projections to major arousal related cell groups (Date et al., 1999). Therefore, orexin neurons are well-suited to correlate brain activity and energy supply. Previous studies show that orexin neurons are GI; inhibited by increases and excited by decreases in extracellular glucose in a metabolism-independent manner (Burdakov et al., 2005b;Yamanaka et al., 2003;Burdakov et al., 2006). This glucose effect is acute and mostly short-lived, as the majority of orexin neurons adapt to the new glucose level within several minutes (Williams et al., 2008). This suggests that orexin neurons sense rapid changes rather than the absolute concentration of glucose. However, the time course of glucose fluctuations is not rapid in the lateral hypothalamus where orexin neurons are located (Voigt et al., 2004), and it remains uncertain how orexin neurons respond to slower and longer changes in glucose levels. Furthermore, orexin neurons lack detectable levels of glucokinase (Dunn-Meynell et al., 2002) and are excited by lactate (Gonzalez et al., 2008), indicating that these neurons may rely on lactate as a main energy source. Here we demonstrate that orexin neurons do not directly sense glucose, but instead detect astrocyte-derived lactate

which is translated into varying levels of neuronal activity. Our results provide insight into how orexin neurons may integrate the energy status of the brain as lactate sensors and orchestrate coordinated physiological responses.

4.2 Materials and methods

All experiments were performed following the guidelines set out by the Canadian Council on Animal Care and approved by the Memorial University Institutional Animal Care Committee. Male Sprague Dawley rats (60-70 g) were obtained from the breeding colony at Memorial University and C57BL/6NCrl mice (3-4 wk) from Charles River Laboratories (Quebec, Canada).

4.2.1 Electrophysiology

Animals were deeply anesthetized with halothane, decapitated and brains were quickly removed. Coronal hypothalamic slices (250 μm) were sectioned using a vibratome (Leica). Sectioning was performed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 25 $NaHCO_3$, 2 $CaCl_2$, 2.5 glucose, pH 7.3-7.35. Following dissection, slices were incubated in ACSF at 32-35°C for 30-45 min, then at room temperature until recording. ACSF was continuously bubbled with O_2 (95%)/CO_2 (5%).

Patch-clamp recordings, visually assisted with an infrared differential-interference contrast microscope (Leica), were performed on brain slices perfused with ACSF at 1.5-2 ml/min, 26°C, using a Multiclamp 700B amplifier and pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). The glucose concentration used for recording was 2.5 mM

unless otherwise noted. The internal solution for conventional whole-cell recordings contained (mM): 123 K gluconate, 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10 HEPES, 4 Na_2-ATP, 0.3 Na-GTP, pH 7.3. Na_2-ATP was omitted for the ATP-free internal solution. In a subset of experiments, the composition of ACSF and internal solution were as previously described (Burdakov et al., 2006;Gonzalez et al., 2008). For these experiments, the ACSF consisted of (mM): 125 NaCl, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 21 $NaHCO_3$, 10 HEPES, 1.0 glucose, pH = 7.3. The intracellular solution consisted of (mM): 120 K gluconate, 10 KCl, 0.1 EGTA, 10 HEPES, 4 K_2ATP, 1 Na_2ATP, 2 $MgCl_2$, pH = 7.3. For perforated patch clamp recordings, nystatin (0.45 mg/ml final) and pluronic acid were dissolved in DMSO and added to an internal solution that contained (mM): 120 K gluconate, 5 $MgCl_2$, 10 EGTA and 40 HEPES, pH 7.3. Biocytin (1-1.5 mg/ml) was also included in the conventional whole-cell and nystatin internal solutions for post-hoc immunohistochemical phenotyping. To aid biocytin entry into nystatin-perforated cells, a positive current was applied for 2-5 min following experimentation (Hirasawa et al., 2003). Cell-attached patch experiments used a 150 mM NaCl pipette solution. Following cell-attached experiments, cells were re-patched in whole-cell mode for electrophysiological and immunohistochemical characterization.

Orexin neurons displayed spontaneous firing as well as I_h, low threshold spike and minimal spike adaptation in response to 300-ms negative and positive current steps (-200 to +200 pA in 100 pA increments). These characteristics were clearly distinguishable from those of melanin-concentrating hormone (MCH) neurons that were also localized in the same region (Eggermann et al., 2003;Alberto et al., 2006). As subpopulations of orexin neurons (D versus H-type; Williams et al., 2008) have been shown to display

different rates of desensitization to the inhibitory glucose effect, it was of interest to distinguish these two subpopulations in the present study. This was accomplished based on observing either the presence (D-type) or absence (H-type) of action potential firing immediately following the offset of a hyperpolarizing current step. 148 of the recorded cells that had typical electrophysiological characteristics of orexin neurons were successfully filled with biocytin and of these, 134 cells (90.5%) were orexin-A immunopositive. Because of the high success rate of deducing the neurochemical phenotype from the electrophysiological criteria, these criteria were used for identifying orexin cells for further analysis. Voltage clamp experiments were performed at a holding potential of -70 mV. In a subset of cells, voltage ramps (-140 to 0 mV in 1.4 s or -140 to -20 in 0.6 s) were applied to determine current-voltage relationships. To examine the effect of positive current injection on the firing activity, a current ramp from 0 to 100 pA was applied over 5 s in current clamp mode.

4.2.2 Immunofluorescence

See section 2.2.2 for the immunohistochemical protocol used for detecting biocytin, orexin and MCH in sections used for electrophysiology. For immunohistochemical analysis of Kir subunits, fresh-frozen sections (10-15 μm) of rat brains (separate from the rats used for electrophysiology) were fixed with 4% paraformaldehyde for 5 min, washed (3 X 10-15 minutes in PBS) and then heated (80-95 °C) for 30 minutes in sodium citrate buffer containing 3M NaCl and 0.01M tri-sodium citrate dihydrate. After being cooled in the sodium citrate buffer for another 30 min, sections were washed (3 X 10-15 minutes in PBS) and incubated in a blocking solution consisting of 5% normal donkey serum and 0.3% triton

X-100 in 0.1 M PBS. Sections were then treated sequentially with antibodies in the following order: rabbit anti-Kir6.1 (1:100; Alomone Labs, Jerusalem, Israel) or rabbit anti-Kir6.2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), 2 days at 4 °C; Cy2-donkey anti-rabbit (1:500), 5 hr at room temperature; goat anti-orexin-A (1:1,000), overnight at 4 °C; Cy3-donkey anti-goat (1:500), 3 hr at room temperature. Antibodies were diluted in blocking solution. Washing in PBS (3 X 10-15 minutes) occurred between each step with the exception of after the blocking solution. Sections were coverslipped and immunofluorescence was visualized using a confocal microscope (FV300 scan head, BX50WI upright microscope; Olympus). Cy2 and Cy3 signals were scanned sequentially through a 3-section stack in z-step increments of 1 μm. The three images were stacked together using Fluoview software (Olympus), saved as separate Cy2 and Cy3 images which were combined in Corel Paint Shop Pro Photo XI. Minor adjustments were made in Paint Shop to optimize brightness and contrast.

4.2.3 Data analysis

Action potential frequency, membrane potential and holding current were measured using Mini Analysis 6.0 (Synaptosoft; Decatur, GA) and Clampfit 9.2 (Molecular Devices; Sunnyvale, CA). Data are expressed as mean ± S.E.M. Instantaneous action potential frequency was calculated as the inverse of each action potential interval. A 15% or greater change in firing frequency was considered significant for individual cells. Statistical tests used included one-way ANOVAs for multiple group comparisons and paired and unpaired Student t-tests for two-group comparisons. Tukey's multiple comparison tests were carried out when ANOVA found significance. $P < 0.05$ was

considered significant. Calculation of the EC_{50} was performed by fitting the experimental data with the sigmoidal dose-response equation (variable slope) using Prism 4 (GraphPad).

4.2.4 Drugs

1000x frozen aliquots of drugs were thawed and diluted with ACSF to the final concentration immediately prior to each experiment. All drugs were obtained from Sigma-Aldrich (St Louis, MO), except glibenclamide and pinacidil (Tocris Bioscience, Ellisville, MO) and tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel). Tolbutamide was dissolved in DMSO with a final DMSO concentration of 0.1%.

4.3 Results

4.3.1 Orexin neurons selectively utilize lactate to fuel spontaneous activity

To determine whether lactate is preferentially utilized by orexin neurons, we used alpha-cyano-4-hydroxycinnamate (4-CIN), a specific inhibitor of monocarboxylate transporters (MCTs) which are responsible for lactate transport across the plasma membrane. In cell-attached mode, the spontaneous firing of orexin neurons was significantly inhibited by 0.5 mM 4-CIN (control, CTL: 2.6 ± 0.4 Hz vs. 4-CIN: 0.7 ± 0.9 Hz, $p < 0.05$, $n = 6$, Fig. 4.1A).

To further establish the role of lactate, we tested whether or not exogenous lactate is sufficient to support the firing activity of orexin neurons in the absence of glucose. First, extracellular glucose was removed for 20 min to determine the behaviour of orexin neurons in glucose-free conditions. Cell-attached recordings revealed a complete but reversible cessation of firing activity in 9 of 10 cells (Fig. 4.1B), indicating that glucose is necessary for spontaneous activity. Results were similar regardless of the baseline glucose being 2.5 or 10 mM, thus data were combined. Inhibition induced by glucose-deprivation had a relatively long latency (13.6 ± 1.2 min) that did not differ from that of the 4-CIN effect (14.6 ± 2.1 min, $p > 0.05$). In perforated patch whole cell recordings, hyperpolarization was induced by 0 mM glucose in the presence of 1 μM TTX ($n = 2$, Fig. 4.1C), suggesting a direct postsynaptic effect. In an additional four cells that were silenced by the lack of glucose, lactate (5 mM) completely restored their firing activity (119.8 ± 38.4% of baseline firing frequency, Fig. 4.1D). Because lactate can be used as a fuel by both neurons and astrocytes (Tabernero et al., 1996;Ainscow et al., 2002), it is

possible that the lactate effect on neuronal firing activity was mediated by neighbouring astrocytes taking up lactate, which in turn stimulated neurons. To test this possibility, we utilized acetate which is taken up and metabolized by astrocytes but not neurons (Waniewski and Martin, 1998). Acetate should mimic lactate if astrocytes are responsible for metabolizing and mediating the lactate effect. However, bath application of acetate (5 mM) was unable to reverse the silencing effect of glucose deprivation on orexin neurons (n = 3, Fig. 4.1E), indicating that astrocytes did not mediate the excitatory effect of lactate on orexin neurons.

Astrocytes are also known as the major cell type that metabolizes glucose and releases lactate into the extracellular space (Pellerin and Magistretti, 1994). Therefore, we hypothesized that orexin neurons depend on lactate released endogenously by astrocytes. To test this hypothesis, hypothalamic slices were pretreated with the glial toxin fluoroacetate (FAC; 5 mM, 50-80 min). During the final 20 min, glucose was removed to deplete any existing energy substrates. Then, either glucose (1-2.5 mM, n = 11) or equicaloric lactate (2-5 mM, n = 9) was applied in the continuing presence of FAC, at which time the firing rate was examined using conventional whole-cell patch clamp. The control group only received glucose deprivation for 20 min followed by glucose restoration (n = 7). As shown in Fig. 4.2, we found that FAC prevented glucose from reversing orexin neuron's firing activity to the control level. In contrast, exogenous lactate following glucose deprivation completely restored the firing rate in FAC-treated cells. Taken together, these data suggest that within the slice preparation, lactate is necessary and sufficient to maintain the spontaneous activity of orexin neurons and that glucose is converted to lactate endogenously by astrocytes.

4.3.2 KATP channels mediate lactate sensing

ATP-sensitive potassium (KATP) channels are prime candidates to mediate the lactate effect on firing frequency and membrane potential (Ashford et al., 1990;Song et al., 2001). Using perforated patch clamp recordings, we found that the hyperpolarization induced by glucose deprivation (from -46.7 ± 1.0 mV to -61.1 ± 4.6 mV, $n = 4$, $p < 0.05$, Fig. 4.3A) was blocked by glibenclamide, suggesting that KATP channels mediate the hyperpolarization. In fact, in every cell tested, glibenclamide unmasked a depolarization (from -44.9 ± 1.3 mV to -33.6 ± 2.3 mV in glucose-free condition, $n = 5$, $p < 0.001$, Fig. 4.3B) which lead to irreversible cell damage that could not be rescued even with 10 mM glucose up to 60 min. The fact that glibenclamide-treated cells appeared unable to survive a 20-min glucose deprivation period suggests a neuroprotective role of KATP channels in orexin neurons during severe energy depletion. To confirm the role of lactate in the glucose-deprivation effect, we tested the KATP channel blocker tolbutamide (1 mM) on the 4-CIN-induced inhibition of orexin neurons as seen in Fig. 4.1A. We found that tolbutamide significantly attenuated the effect of 0.5 mM 4-CIN ($n = 4$, Fig. 4.3C).

Interestingly, we found that the activity of arcuate neurons of an unknown phenotype was independent of lactate uptake. A 20-min application of 4-CIN (0.5 mM) had no effect on the firing frequency of arcuate neurons recorded using perforated patch clamp ($n = 4$, Fig. 4.3C, D), whereas 20-min glucose deprivation induced a depolarization similar to that seen in orexin neurons when KATP channels are blocked (Fig. 4.3B, D). Thus, lactate availability is monitored by KATP channels in orexin neurons and lactate preference is at least somewhat unique to this particular phenotype.

4.3.3 Kir6.1 and SUR1 subunits comprise KATP channels in orexin neurons

KATP channels are composed of 4 identical pore-forming subunits (Kir6.1 or 6.2) and 4 identical modulatory sulfonylurea subunits (SUR1, 2A or 2B). Double immunofluorescence labelling was conducted to determine the subunit composition of KATP channels in orexin neurons, which revealed the expression of Kir6.1 but not Kir6.2 (Fig. 4.4A-F). Furthermore, using conventional whole-cell recordings, we found that 0.2 mM diazoxide, the SUR1/2B-containing KATP channel opener, induced an outward current (n = 11, Fig. 4.4G, I). The diazoxide-induced current was sensitive to the KATP channel blocker glibenclamide (n = 5, Fig. 4.4I), insensitive to TTX (n = 3, Fig. 4.4G) and reversed near the potassium equilibrium potential (-87.6 ± 1.4 mV, n = 4, Fig.4.4H). In contrast, the SUR2A/2B-containing KATP channel opener pinacidil (0.25-0.75 mM) had no effect (n = 8, Fig. 4.4I). Thus, KATP channels in orexin neurons are composed of Kir6.1 and SUR1 subunits.

To confirm the metabolism-dependent modulation of KATP channels in orexin neurons, we manipulated intracellular ATP levels. Conventional whole-cell recordings with an ATP-free pipette solution were used to dilute cytosolic ATP. This induced a glibenclamide-sensitive outward current in 5-15 min after achieving whole-cell access (n = 5, Fig. 4.4J). Further, in order to inhibit metabolism, the mitochondrial uncoupler CCCP (2 μM) was bath applied, which resulted in a sustained outward current in both conventional and perforated whole-cell configurations (n = 12). The effect of CCCP was also blocked by glibenclamide (n = 5, Fig. 4.4J). These results suggest that KATP channels expressed in orexin neurons are sensitive to the metabolic state of the cell.

4.3.4 Orexin neurons are lactate sensors

Lactate sensors need to be capable of detecting differences in lactate concentrations within a certain range and converting these differences into specific output signals. To determine whether orexin neurons have the characteristics of lactate sensors, conventional whole cell recordings were performed on orexin neurons in hypothalamic slices incubated (> 20 min) with various concentrations of lactate in the absence of glucose (n = 4 for each concentration). The effect of lactate on the firing rate was found to be concentration dependent (EC_{50} = 2.1 mM, Fig 4.5A), indicating that these neurons can act as lactate sensors.

Next we asked whether physiologically-relevant glucose concentrations were converted to distinct endogenous levels of lactate. Glucose concentrations were changed from 2.5 to 1 mM in 0.5 mM increments, a range that corresponds to meal-to-meal fluctuations in the brain (Routh, 2002). Slices were allowed to adjust to each concentration for 30-60 min while the spontaneous firing frequency of orexin neurons was analyzed using conventional whole cell mode. Using this paradigm, we found that on average, the firing activity was independent of glucose concentration (n = 9-12, Fig. 4.5B). Nonetheless, the firing frequency varied among different orexin neurons, leaving a possibility that some neurons increased or decreased their firing in response to glucose fluctuations. To test this possibility, we repeatedly monitored the activity of individual orexin neurons in cell-attached mode while gradually decreasing the extracellular glucose concentration from 2.5 to 1 mM (n = 3) or increasing from 1 to 2.5 mM (n = 2) in 0.5 mM increments. Again, there was no difference in the activity within this range (Fig. 4.5C, D).

We then sought to determine whether the lack of changes in firing activity between 1 and 2.5 mM glucose was because the endogenous lactate level had reached a plateau at 1 mM glucose or because the orexin neuron's ability to convert lactate availability into firing frequency was saturated. Since 4-CIN is a competitive blocker that competes against lactate for MCTs, utilizing a lower concentration of this compound allowed us to monitor relative levels of endogenous lactate (Broer et al., 1999). Brain slices were incubated in ACSF with 1 or 2.5 mM glucose, in combination with a lower concentration of 4-CIN for 30-60 min. As shown in Fig. 4.5E, conventional whole cell recordings revealed that 4-CIN (0.1 mM) had no significant effect at 2.5 mM glucose (n = 19 for 2.5 mM glucose alone, n = 13 for 2.5 mM glucose/0.1 mM 4-CIN), whereas the same concentration of 4-CIN in combination with 1 mM glucose significantly decreased the firing rate of orexin neurons (n = 21 for 1 mM glucose, n = 11 for 1 mM glucose/0.1 mM 4-CIN). Cells incubated with 1 mM glucose and 0.1 mM 4-CIN were depolarized by tolbutamide, suggesting that the low or lack of activity in this condition is due to the activation of KATP channels (n = 2, Fig. 4.5F). These findings suggest that 2.5 mM extracellular glucose results in more endogenous lactate release in comparison to 1 mM glucose. However, a steady firing frequency within this glucose range in the absence of 4-CIN (Fig. 4.5B-D) indicates that the metabolism-sensing mechanism involving KATP channels is saturated at >1 mM glucose in our preparation. In support of this, only one out of 8 cells responded with an excitation when lactate (5 mM, 4-5 min) was applied in addition to 2.5 mM glucose (data not shown).

4.3.5 Orexin neurons are less excitable in low extracellular glucose

Brain glucose levels are known to drop as low as 0.2 (Silver and Erecinska, 1994) and 0.7 mM (de Vries et al., 2003) during insulin-induced hypoglycemia and overnight fasting, respectively. Thus, we also tested the effect of glucose concentrations lower than 1 mM for 20 min with cell-attached patch and found a reduction in action potential frequency (Fig. 4.6A, B). The effect was concentration-dependent (EC_{50} = 0.59 mM; Fig. 4.6C), which was mainly due to a greater proportion of cells responding as the ambient glucose concentration became lower: 9 of 10 cells to 0 mM, 4 of 5 to 0.5 mM, 3 of 7 to 0.75 mM and 0 of 5 cells to 1 mM (Fig. 4.6D). Increasing glucose from 2.5 to 10 mM for 20 min had no effect on firing frequency (n = 5, Fig. 4.6C, D). For those that responded, the latency to inhibition was independent of glucose concentration (Fig. 4.6E). Thus, KATP channels in orexin neurons can be activated by the low range of physiologically relevant glucose concentrations.

An increase in membrane resistance due to lactate-induced closure of potassium channels can be expected to make orexin neurons more sensitive to subsequent stimulations. To test this idea, the response of orexin neurons to positive current injections was recorded in the absence or presence of lactate (5 mM). Hypothalamic slices were initially incubated in 0.5 mM glucose for at least 20 min to activate KATP channels. In this condition, an incremental current injection through the conventional whole-cell recording pipette (from 0 to 100 pA over 5 s) induced a gradual increase in firing frequency in every cell tested. When lactate was applied, both the baseline activity (without lactate: 0.2 ± 0.1 Hz, with lactate: 2.3 ± 0.7 Hz, n = 8, $p < 0.05$) and the rate of increase in firing frequency during current injection (without lactate: 0.10 ± 0.025 Hz/pA,

with lactate: 0.13 ± 0.020 Hz/pA, n = 8, $p < 0.05$) became significantly higher (Fig. 4.7). These data demonstrate that low availability of energy substrate not only decreases the basal firing rate of orexin neurons but also blunts their excitability to subsequent inputs.

4.3.6 Short-term glucose effect

The inhibitory effect demonstrated above had relatively long latencies (> 10 min). In contrast, it has been shown that an increase or decrease in extracellular glucose induces a robust inhibition or excitation, respectively, within several minutes in the majority of orexin neurons examined (Burdakov et al., 2005b;Yamanaka et al., 2003). Thus, the short-term effect (2-5 min) of glucose recorded with cell-attached patch was also analyzed. Surprisingly, no robust or consistent short-term effects were seen in our hands (Fig. 4.8, Fig. 4.9A), even with the same combinations of glucose concentrations, ACSF and/or internal solution as previously reported (Burdakov et al., 2006;Gonzalez et al., 2008) (Fig. 4.9B). We also tried dissolving glucose immediately prior to experiments to increase the proportion of α– over β–anomer, since glucose powder contains predominantly α-D-(+)-glucose, which converts to β-D-(+)-glucose over a few hours in solution, reaching equilibrium of α:β=36:64. However, this made no difference. As previous reports utilized mice (C57BL/6 background) (Yamanaka et al., 2003;Burdakov et al., 2005b;Guyon et al., 2009), we also examined orexin neurons in 3-5 week old C57BL/6NCrl mice and again observed no robust inhibition in response to glucose increases from 1 to 5 mM (Fig. 4.9B). Nonetheless, switching from 10 to 0 mM induced a modest excitation (Fig. 4.9B, C), providing some support for the GI response in mouse

orexin neurons. The inhibitory effect of long-term 0 mM glucose application was also observed in mice (n = 3, Fig. 4.9C), albeit with a longer latency compared to rats (Fig. 4.8D), suggesting this is a common phenomenon in both species, but more sensitive in rats.

We also considered the functional heterogeneity of orexin neurons. It has been proposed that orexin neurons located in the lateral hypothalamus (LH; lateral to the fornix) are functionally distinct from those in the perifornical area (PFA; dorsal and dorsomedial to the fornix) (Harris and Aston-Jones, 2006). Furthermore, individual orexin neurons have been characterized as either D- or H-type depending on the presence or absence, respectively, of action potentials immediately upon relief from a hyperpolarizing current step. These subpopulations have been shown to display different responses to glucose (Williams et al., 2008). Thus, we investigated whether the cell location or electrophysiological type determined the short-term response to glucose in the present study. Orexin neurons that were non-responsive to glucose were found in both the LH and PFA and consisted of both D- and H-type (Fig. 4.9E). In contrast, all responders (GE and GI) were found in the PFA and all but one (6 of 7) were H-type orexin neurons. Therefore, in our hands, 6 of 12 H-type cells that existed in the PFA responded to short-term glucose changes whereas only 1 of 16 D-type cells in LH or PFA responded. It remains to be seen whether H-type neurons in the LH also respond in the same manner, due to the low total number of this neuron type examined.

DISCUSSION

4.4.1 Orexin neurons prefer lactate over glucose as an energy substrate

The present study demonstrates that orexin neurons *in vitro* depend on astrocyte-derived lactate as their main energy supply. Blockade of MCTs largely attenuates the spontaneous firing of these neurons despite the presence of glucose, suggesting that lactate uptake through MCTs is required. Thus, our study supports the astrocyte-neuron shuttle hypothesis that proposes the importance of astrocyte-derived lactate as a neuronal fuel (Pellerin and Magistretti, 1994). The excitatory effect of lactate on the firing frequency was concentration dependent, suggesting that orexin neurons are lactate sensors capable of detecting differences in extracellular lactate levels. Almost exclusive dependence on lactate indicates that orexin neurons can be influenced by not only the absolute levels of brain glucose but also the efficiency of glucose conversion to lactate, release by astrocytes and uptake by neurons, for example during excitatory transmission (Pellerin and Magistretti, 1994), oxidative stress (Liddell et al., 2009), high fat diet (Pierre et al., 2007) and hypoxia (Vega et al., 2006). Nonetheless, we cannot overlook the possibility that energy substrates additional to lactate may act as a significant fuel source for orexin neurons *in vivo*, which deserves future investigation.

KATP current was found to underlie the reduced excitability. In the absence of glucose and KATP current, orexin neurons develop irreversible depolarization, suggesting that lactate is also necessary for maintaining normal membrane potential and that KATP channels play a neuroprotective role. These results are similar to the effects of hypoxia on substantia nigra neurons, where activation of KATP current induces hyperpolarization in wild type neurons, while depolarization develops in Kir6.2-knockout

neurons (Yamada et al., 2001). Orexin neurons can temporarily maintain activity in the absence of an extracellular energy source, as it takes 10-20 min for glucose deprivation or 4-CIN to take effect. This may be due to astrocytes continuing to release lactate or neurons having their own energy store. The former is unlikely, since disconnecting the lactate shuttle from astrocytes to neurons with 4-CIN does not curtail the latency. Therefore, it appears that orexin neurons have an endogenous supply to support themselves. It has been estimated that neurons contain more than 20 mM of intracellular lactate (Walz and Mukerji, 1988), which may be a provisional energy substrate.

4.4.2 Technical considerations

While perforated and cell-attached patch clamp methods were essential for the present study, conventional whole cell mode was also used. It allowed us to characterize the electrophysiological properties and monitor the basal activity from a number of orexin cells within a limited time frame. Importantly, in our hands, the baseline firing rates in 2.5 mM glucose during the first 1.5 min of whole-cell recordings ($n = 19$) were not significantly different from those in stable (> 5 min) cell-attached recordings ($n = 42$; $p > 0.05$, Fig. 4.10). This suggests that the whole cell configuration did not influence the firing activity at least for the first 1.5 min, consistent with a previous report (Van den Top et al., 2007). To further support our contention that whole cell recordings did not affect our result, we found that ATP-free internal solution took at least 5 min after break-in to have any effect on the holding current. Furthermore, different concentrations of lactate induced distinct firing rates in whole cell mode. Thus, it is unlikely that, during the period

in which our recordings were taken, ATP in the recording pipette would have significantly influenced the membrane potential and firing frequency.

4.4.3 Consideration of discrepancies in the literature

Previous reports have demonstrated an acute inhibitory effect of glucose on a majority of orexin neurons (Burdakov et al., 2005b;Gonzalez et al., 2008;Guyon et al., 2009), while others suggest that orexin neurons are not or not always inhibited by glucose (Liu et al., 2001;Muroya et al., 2001). We did not find a consistent short-term effect despite testing various recording methods and accounting for a number of variables, regardless of the location or electrophysiological properties of orexin neurons. Our results support a previous proposal that hypoglycemia-induced excitation of orexin neurons *in vivo* is due to an indirect mechanism (Cai et al., 2001).

Since studies showing a consistent GI effect utilized mice while others, including ours, have found otherwise using rats, the discrepancy may stem from a species difference. However, upon investigation of mouse neurons, only a modest GI effect was detected if any. Importantly, glucose deprivation also activated KATP channels in mice, albeit with a longer latency than rats. Therefore, the properties of orexin neurons in different species may not be identical. Also, there may be developmental changes in glucose sensitivity. Studies demonstrating the GI response used brain slices from 2-4 week old mice (mostly 2-3 weeks) (Burdakov et al., 2005b;Burdakov et al., 2006;Gonzalez et al., 2008;Guyon et al., 2009), whereas we used 3-4 week old mice and rats.

The inhibitory and excitatory effects of glucose are not necessarily exclusive of each other, because the GI effect is observed at concentrations greater than 1 mM (Burdakov et al., 2005b) whereas the KATP channel-mediated effect is activated below 1 mM. Under certain conditions where both mechanisms are functional, it should result in a parabolic relationship between extracellular glucose concentration and firing frequency of orexin neurons.

4.4.4 KATP channels mediate energy sensing in orexin neurons

Neuronal inhibition in glucose-free conditions has been described as a "ubiquitous" (Mobbs et al., 2001) "run-out-of-fuel" phenomenon, distinct from glucosensing (Gonzalez et al., 2009b). However, we failed to see any hyperpolarizing response to either MCT blockade or prolonged glucose-deprivation in arcuate neurons, suggesting that hyperpolarization is not a universal response to glucose depletion. Additionally, we found that orexin neurons show a concentration-dependent response to low but physiologically-relevant glucose concentrations.

GE neurons in the ventromedial hypothalamus and arcuate nucleus metabolize glucose and produce ATP which results in the modulation of KATP channels that mediate the glucosensitivity (Ashford et al., 1990;Van den Top et al., 2007); (Kang et al., 2006;Miki et al., 2001). These neurons are also excited by lactate, suggesting that they are in fact glucose/lactate sensors (Ainscow et al., 2002;Yang et al., 1999). Despite possessing the same type of ion channels, orexin neurons differ from these typical glucosensors. First, our study shows that orexin neurons do not detect glucose directly, likely due to a lack of glucokinase (Dunn-Meynell et al., 2002). Second, the indirect

glucose sensitivity saturates above 1 mM glucose. It remains to be seen whether this concentration dependence differs in other conditions such as synaptic activation or hypoxia in which lactate production is enhanced (Pellerin and Magistretti, 1994;Vega et al., 2006). Third, glucosensing neurons typically express Kir6.2/SUR1 channels (Miki et al., 2001;Levin et al., 1999) while orexin neurons express Kir6.1/SUR1 channels, similar to glucose-receptive neurons in the ventromedial hypothalamus (Lee et al., 1999). Since Kir6.2 is more sensitive to metabolic state than Kir6.1 (Gribble et al., 1997), a Kir6.1/SUR1 may be less sensitive than Kir6.2/SUR1. Finally, the output of orexin neurons differs from typical anabolic or catabolic neurons. Orexin neurons increase both energy intake and expenditure (Sakurai, 2007) whereas glucose homeostatic neurons inversely regulate energy intake and expenditure. Taken together, despite the presence of functional KATP channels, the influence of energy substrates on orexin neurons is different from previously described glucosensors, and it is unlikely that these neurons are involved in counterregulatory responses for glucose homeostasis.

4.4.5 Physiological Significance

We have demonstrated that lactate disinhibits and primes orexin neurons for excitation, i.e. increases spontaneous firing and the sensitivity to a given excitatory input, respectively. As glutamate stimulates lactate production in astrocytes (Pellerin and Magistretti, 1994), our result indicates that excitatory inputs from other brain areas would have a dual excitatory effect on orexin neurons. Also, since orexin neurons co-express glutamate (Rosin et al., 2003), an activated orexin neuron may trigger a lactate-mediated positive feedback and recruitment of other orexin neurons. Furthermore, MCTs are

proton/monocarboxylate symporters, meaning lactate release accompanies local decline in extracellular pH. Since orexin neurons are excited by low pH (Williams et al., 2007), lactate combined with a drop in pH would be expected to have additive excitatory effects. Together, it appears that excitatory synaptic inputs activate orexin neurons more effectively when accompanied by lactate that signals adequate energy supply. Activated orexin neurons, in turn, will promote or maintain wakefulness, increase food intake and hepatic glucose production. Thus, we propose that orexin neurons play an essential role in gating brain activation in accordance with energy supply and activating physiological responses to meet the energy demands of elevated brain activity (Fig. 4.11). Indeed, extracellular lactate in the brain reaches a higher concentration during the active/awake phase compared to the inactive/sleep phase (Shram et al., 2002), and the discharge of orexin neurons follows a strikingly similar pattern (Lee et al., 2005;Mileykovskiy et al., 2005).

In conclusion, our study highlights lactate as an important regulator of the orexin system. Lactate is not only an energy substrate, but also a paracrine factor that signals the levels of brain activity and fuel availability to orexin neurons. KATP channels play a critical role in this astrocyte-orexin neuron coupling, while also providing neuroprotection. Further investigation of lactate sensors within the central nervous system is essential for a full understanding of the role of lactate in brain energetics.

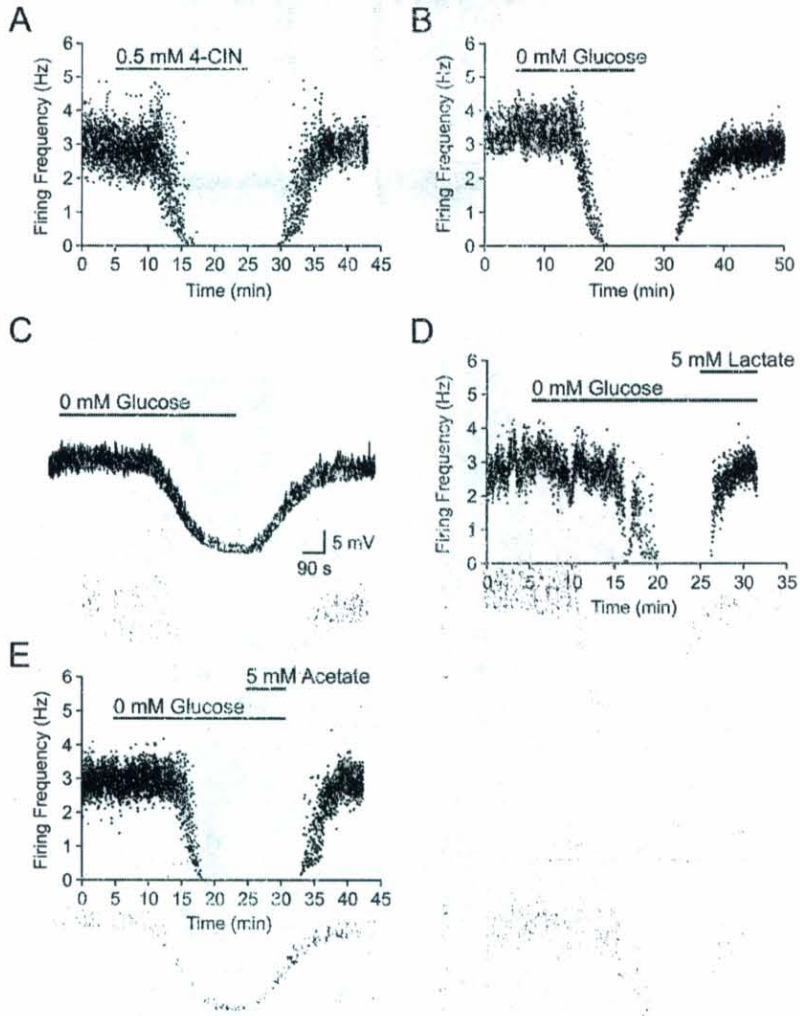


Figure 4.1. Lactate is necessary and sufficient to maintain basal levels of spontaneous activity in orexin neurons. *A,* Representative time-effect plot showing an inhibitory effect of 4-CIN on the firing frequency of an orexin neuron in the presence of 2.5 mM glucose. ***B,*** Representative experiment showing an inhibitory effect of complete glucose deprivation. ***C,*** Glucose deprivation induces a TTX-insensitive reversible hyperpolarization. ***D-E,*** 0 mM glucose effect is completely reversed by lactate ***(D)*** but not by acetate ***(E).***

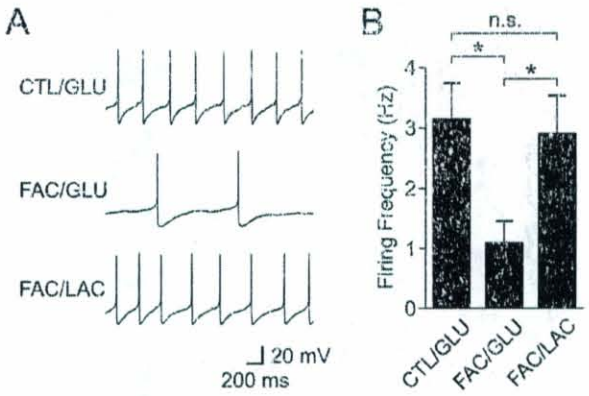


Figure 4.2 Astrocytes supply lactate to support the spontaneous activity of orexin neurons. ***A,*** Sample traces of action potentials from representative orexin neurons in the presence of glucose (GLU) or lactate (LAC) in non-treated (CTL) or fluoroacetate (FAC)-treated slices. Grouped data is shown in ***B.*** * $p < 0.05$. n.s. non-significant.

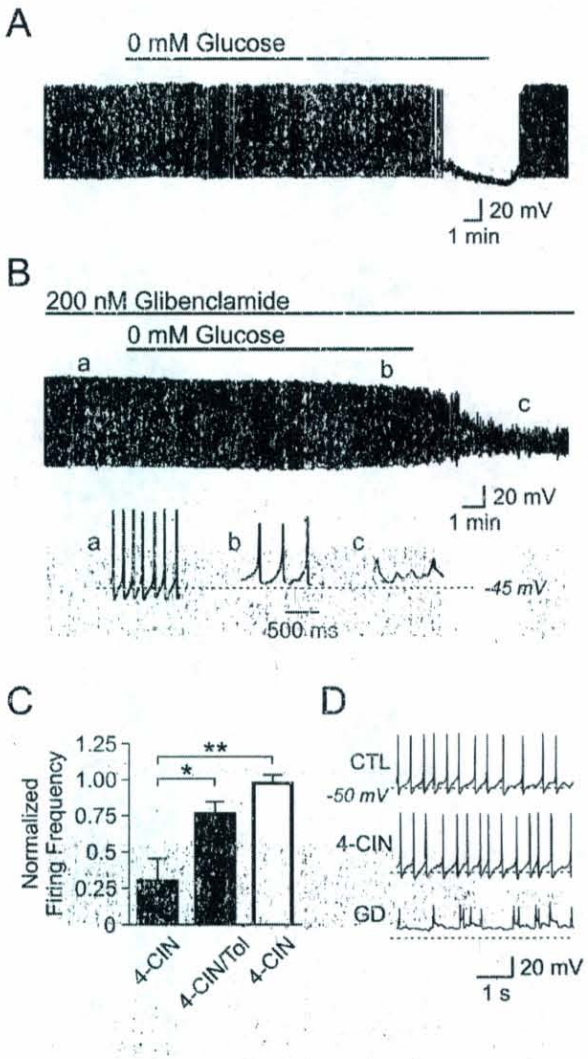

A
0 mM Glucose
20 mV
1 min
B
200 nM Glibenclamide
0 mM Glucose
a
b
c
20 mV
1 min
a
b
c
-45 mV
500 ms
C
Normalized Firing Frequency
1.25
1.00
0.75
0.5
0.25
0
**
*
4-CIN
4-CIN/Tol
4-CIN
D
CTL
-50 mV
4-CIN
GD
20 mV
1 s

Figure 4.3 KATP channels mediate hyperpolarization induced by a lack of energy substrate. ***A***, Representative current-clamp trace showing a spontaneously-active orexin neuron silenced by glucose-free ACSF. ***B***, KATP channel blockade prevents the hyperpolarization induced by 0 mM glucose. Note that glibenclamide unmasks a depolarization. Bottom panels show expanded traces taken at time points denoted by **a, b** and **c**. ***C***, KATP channel blockade with tolbutamide (Tol) attenuates the inhibitory effect of 4-CIN in orexin neurons (filled bars). In contrast, 4-CIN alone has no effect on the firing frequency of neurons in the arcuate nucleus (hollow bar). ***D***, Current-clamp traces from one of the recorded arcuate neurons. These neurons do not respond to 4-CIN (middle panel) but cannot maintain healthy action potentials when faced with 20-min glucose deprivation (GD; bottom panel). * $p < 0.05$, ** $p < 0.01$.

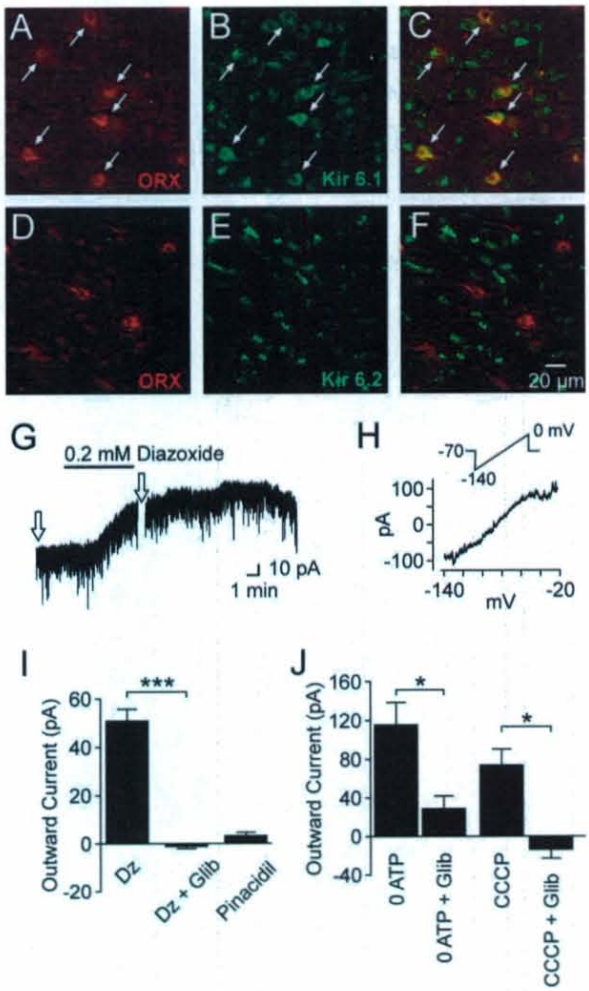

A
B
C
ORX
Kir 6.1
D
E
F
ORX
Kir 6.2
20 µm
G
0.2 mM Diazoxide
10 pA
1 min
H
0 mV
-70
-140
100
0
-100
pA
-140
mV
-20
I
***
Outward Current (pA)
60
40
20
0
-20
Dz
Dz + Glib
Pinacidil
J
*
*
Outward Current (pA)
160
120
80
40
0
-40
0 ATP
0 ATP + Glib
CCCP
CCCP + Glib

Figure 4.4 KATP channel composition in orexin neurons. ***A-C,*** Immunofluorescence labelling of orexin-A (red, ***A***) and Kir6.1 (green, ***B***) displays co-localization (***C***). ***D-F,*** Immunofluorescence labelling of orexin-A (red, ***D***) and Kir6.2 (green, ***E***) displays a lack of co-localization (***F***). ***G,*** Diazoxide induces an outward current in the presence of TTX. Arrows indicate the times of voltage ramp applications. ***H,*** Current-voltage relationship of the diazoxide-induced response, generated by subtracting the current response to voltage ramps in the baseline condition from that in the presence of diazoxide. Inset denotes the voltage ramp protocol used. ***I,*** Grouped data showing diazoxide (Dz) and pinacidil effects on orexin neurons. Diazoxide effect was significantly blocked by glibenclamide (Glib). ***J,*** Outward currents are also induced by postsynaptic dialysis with ATP-free internal solution (0 ATP) and CCCP. These currents are significantly blocked by glibenclamide. * $p < 0.05$, *** $p < 0.005$.

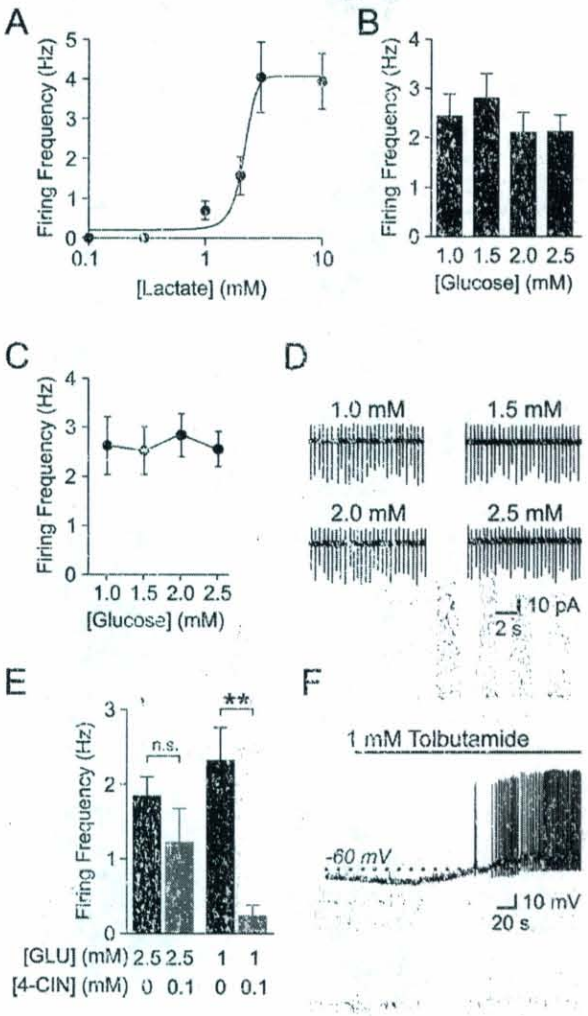

A
Firing Frequency (Hz)
[Lactate] (mM)
B
Firing Frequency (Hz)
[Glucose] (mM)
C
Firing Frequency (Hz)
[Glucose] (mM)
D
1.0 mM
1.5 mM
2.0 mM
2.5 mM
10 pA
2 s
E
Firing Frequency (Hz)
n.s.
**
[GLU] (mM) 2.5 2.5 1 1
[4-CIN] (mM) 0 0.1 0 0.1
F
1 mM Tolbutamide
-60 mV
10 mV
20 s

Figure 4.5 Orexin neurons are lactate sensors. ***A,*** Firing rate is sensitive to the level of extracellular lactate. Glucose-free ACSF was used for these experiments. ***B,*** Firing rate of orexin neurons in various glucose concentrations as indicated. Firing rate remained consistent from 1-2.5 mM glucose. ***C,*** Repeated monitoring of the same neurons also reveals a lack of sensitivity to glucose fluctuation between 1 and 2.5 mM. ***D,*** Sample traces from one of the recorded neurons in ***C***. ***E,*** A low concentration of 4-CIN significantly inhibits orexin neurons in 1 mM glucose (GLU) but not in 2.5 mM. ***F,*** Cells exposed to 0.1 mM 4-CIN and 1 mM glucose are readily depolarized by bath application of tolbutamide. ** $p < 0.01$. n.s. non-significant.

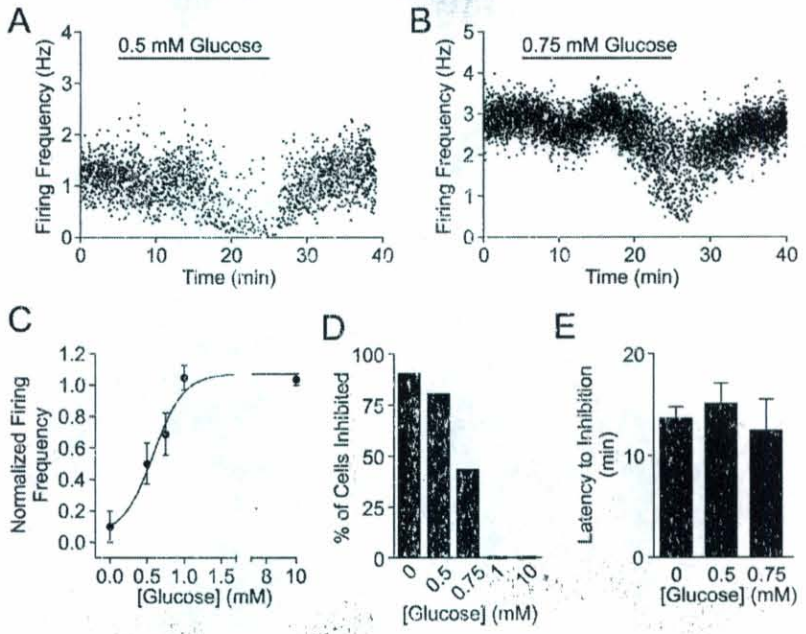


Figure 4.6 Orexin neurons are less active in low glucose concentrations. ***A-B,*** Representative experiments showing that decreasing extracellular glucose from 2.5 to 0.5 ***(A)*** or to 0.75 mM ***(B)*** inhibits spontaneous firing. ***C,*** Concentration-response curve fitted to the average firing frequency of orexin neurons. Baseline frequency in 2.5 mM glucose was used to normalize data. ***D,*** Percentage of orexin neurons inhibited by a 20-min shift in glucose concentration. ***E,*** Latency to inhibition is independent of final glucose concentration.

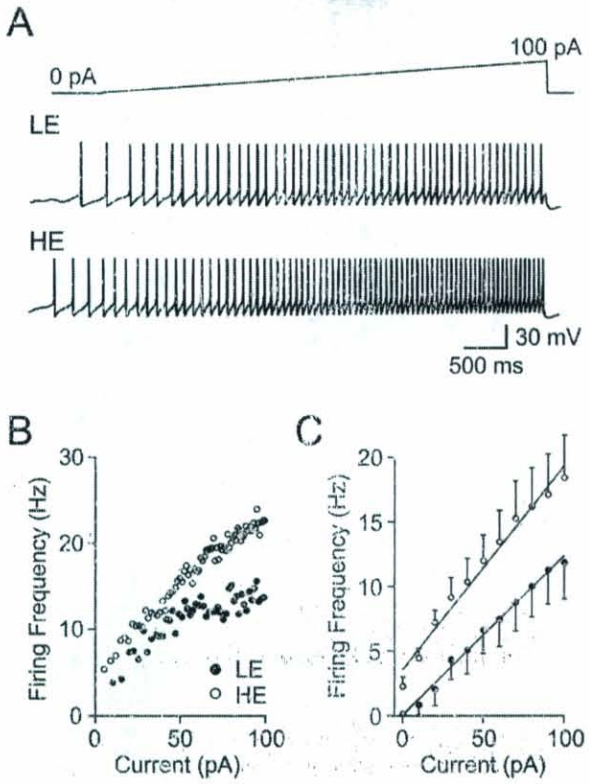


Figure 4.7 Lactate increases the excitability of orexin neurons. ***A,*** Typical responses to positive current ramps from 0 to 100 pA (5 s) in low energy (LE; 0.5 mM glucose) and high energy (HE; 0.5 mM glucose + 5 mM lactate) conditions. ***B,*** Instantaneous firing rate of a representative cell during the incremental current injection for LE (filled circles) or HE (hollow circles) condition. ***C,*** Grouped data showing average firing frequency in 10 pA bins.

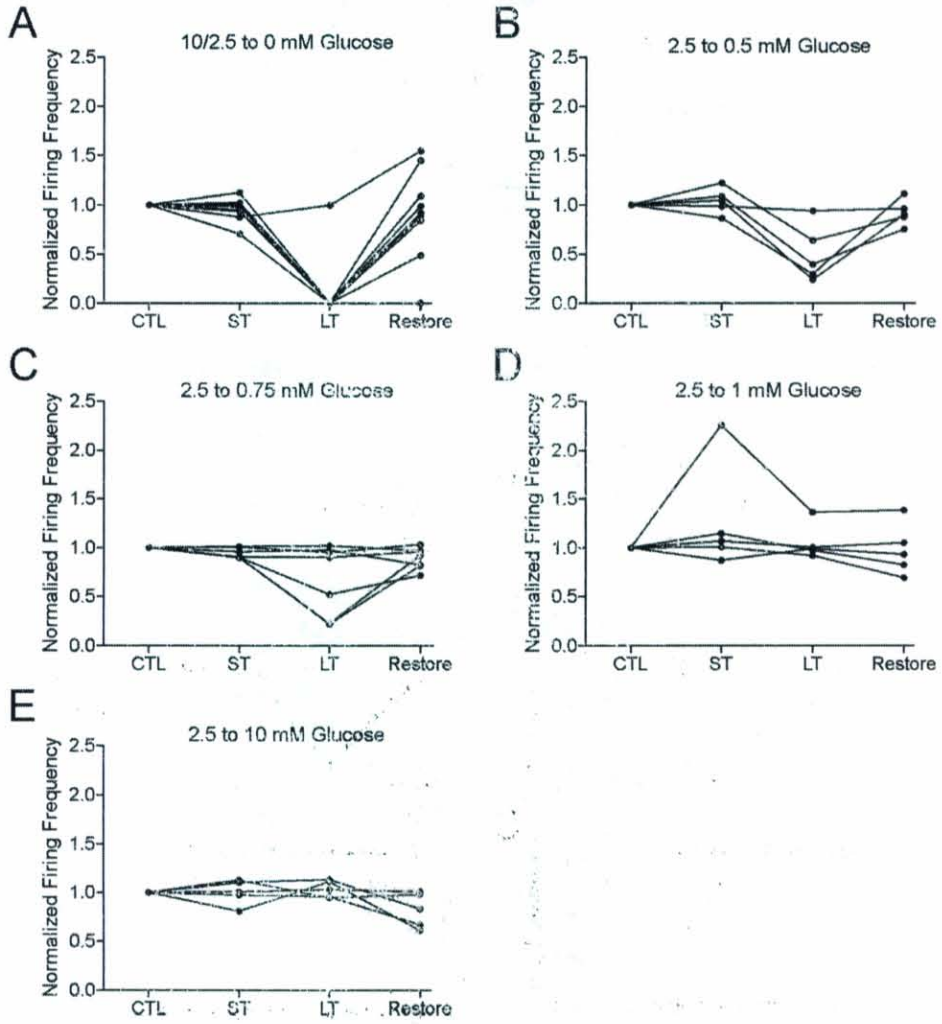


Figure 4.8 Short-term and long-term effects of various glucose concentrations. The normalized firing frequency of individual orexin neurons is shown. ACSF with various glucose concentrations as indicated was applied for 20 min. Short-term (ST; 2-5 min) and long-term (LT; 15-20 min) effects on firing frequency as well as restoration back to baseline concentration (restore) are normalized to control frequency (CTL). There is no clear relationship between the ST and LT effect, regardless of the glucose concentrations tested.

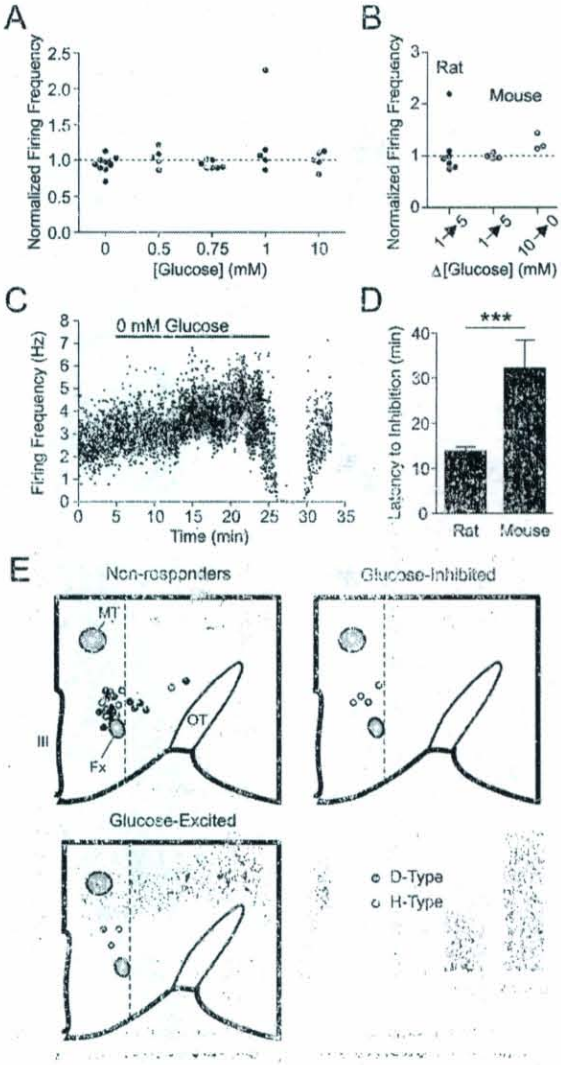

A
Normalized Firing Frequency
2.5
2.0
1.5
1.0
0.5
0.0
0
0.5
0.75
1
10
[Glucose] (mM)
B
Normalized Firing Frequency
Rat
Mouse
3
2
1
0
1→5
1→5
10→0
Δ[Glucose] (mM)
C
0 mM Glucose
Firing Frequency (Hz)
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
Time (min)
D
***
Latency to Inhibition (min)
40
30
20
10
0
Rat
Mouse
E
Non-responders
Glucose-Inhibited
MT
OT
III
Fx
Glucose-Excited
D-Type
H-Type

Figure 4.9 Lack of consistent short-term glucose effects on action potential firing. ***A,*** Normalized firing frequency of rat orexin neurons during the acute phase (2-5 min) of a new glucose concentration as indicated, from a baseline of 2.5 mM. For the 0 mM experiments, a subset of cells was exposed to a baseline of 10 mM glucose. ***B,*** Normalized firing frequency of orexin neurons following glucose changes as indicated in rats (filled circles) and mice (hollow circles). For experiments on rats, ACSF and pipette solutions were adapted from previous studies (Burdakov et al., 2006;Gonzalez et al., 2008). ***C-D,*** Glucose deprivation-induced inhibition is also present in mouse orexin neurons, albeit with a longer latency in comparison to rats ***(D)***. Glucose deprivation exceeding 20 min was tested on some mouse orexin neurons. ***E,*** Schematic of recorded orexin neurons in a hemisected hypothalamus showing the location, electrophysiological type (D vs H) and short-term responsiveness to glucose. Bilateral and anteroposterior dispersal is collapsed into one map to display the distribution pattern relative to the fornix and the 3rd ventricle. Only cells of which the location could be clearly identified by post-hoc immunofluorescence labelling are shown. Fx, fornix; MT, mammillothalamic tract; OT, optic tract; III, 3rd ventricle. Dotted lines denote the lateral border between the perifornical area and lateral hypothalamus. *** $p < 0.001$.

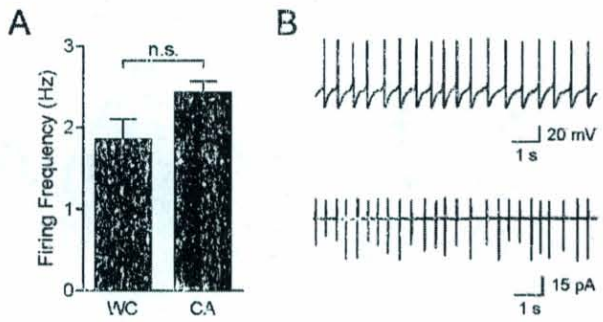


Figure 4.10 Recording methods do not affect the baseline firing rate of orexin neurons. ***A,*** Mean baseline firing frequency of orexin neurons recorded in 2.5 mM glucose using either conventional whole-cell (WC) or cell-attached (CA) recordings. For WC, data were collected within the first 1.5 min after break-in, whereas CA recordings were commonly longer and stable (firing frequency at 5 min from the beginning of recording is shown). No significant difference was found ($p > 0.05$). ***B,*** Representative traces of conventional whole-cell (top) and cell-attached (bottom) recordings. n.s. non-significant.

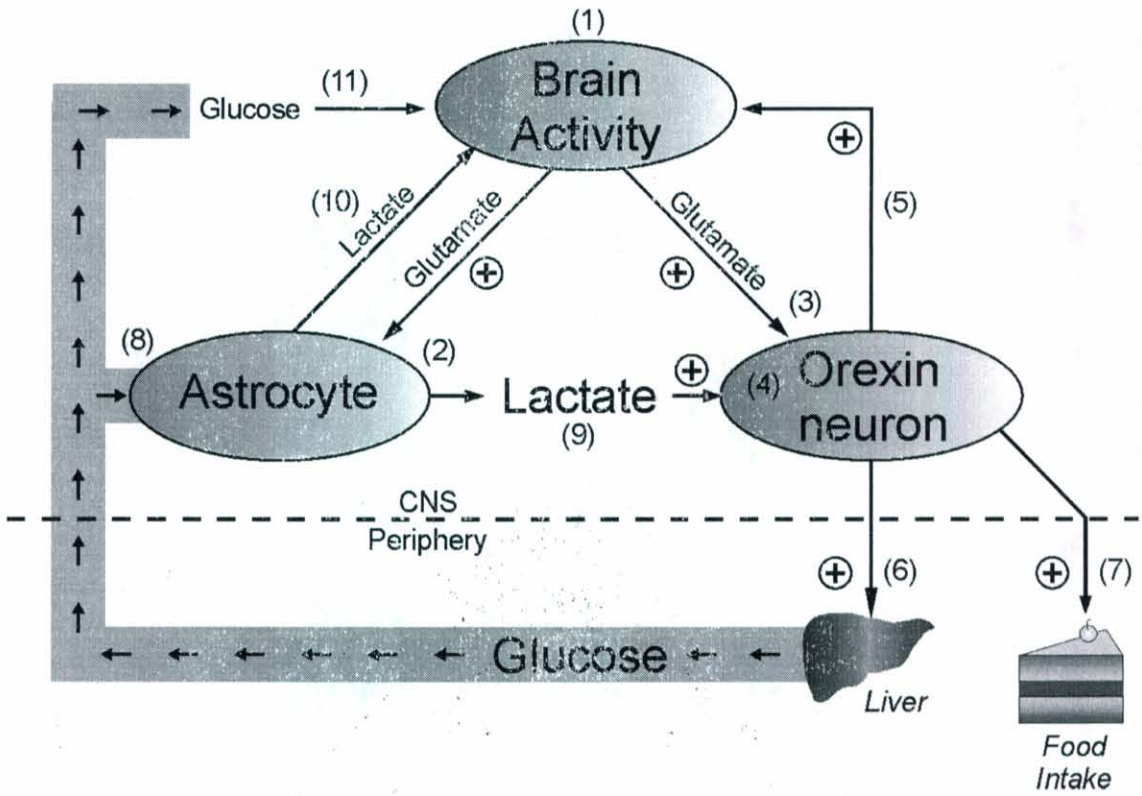

(1)
Brain Activity
(11)
Glucose
(10)
Lactate
Glutamate
Glutamate
(5)
(3)
(8)
Astrocyte
(2)
Lactate
(9)
(4) Orexin neuron
CNS
Periphery
(6)
(7)
Glucose
Liver
Food Intake

Figure 4.11 Orexin neurons as lactate sensors. Schematic drawing depicting a potential mechanism for matching energy substrate supply and brain activity based on the present study and available literature. High levels of brain activity (1) activates orexin neurons via stimulation of astrocytic glucose metabolism and lactate release (2) as well as excitatory transmission (3). Lactate and glutamate signaling are integrated and reflected on the firing rate of orexin neurons (4). Activation of orexin neurons maintains brain activity and arousal (5), increases hepatic glucose production via the sympathetic nervous system (6) and stimulates food intake (7). Increased glucose in the circulation enters the brain where it may be taken up by astrocytes (8) to provide additional lactate as an energy substrate for orexin (9) or other neurons (10). Additionally, glucose may be utilized directly as an energy substrate in other types of neurons (11). Orexin neurons are thus central to the proposed positive feedback mechanism between energy availability, arousal and brain activity.

CHAPTER 5

KATP-DEPENDENT THERMOSENSITIVITY OF OREXIN NEURONS: IMPLICATIONS IN LIPOPOLYSACCHARIDE-INDUCED ANOREXIA

(manuscript in preparation)

5.1 Introduction

The orexin (hypocretin) neurons of the lateral hypothalamus/perifornical area (LH/PFA) are wake-active neurons with well-documented roles in the stimulation of food intake, arousal, motivation and the stabilization of the sleep-wake cycle (Sakurai et al., 1998;de Lecea L. et al., 1998;Hagan et al., 1999;Harris et al., 2005;Chemelli et al., 1999). In contrast, sickness behaviour observed during fever is characterized by hypophagia, behavioural inactivity, anhedonia and fragmented sleep-wake cycles (Dantzer et al., 2008). Suppression of orexin neurons during fever is suggested by recent studies demonstrating that the pyrogen lipopolysaccharide (LPS) suppresses c-Fos induction in orexin neurons (Becskei et al., 2008;Park et al., 2008;Gaykema and Goehler, 2009). Moreover, local warming of the nearby preoptic area was shown to decrease the activity of the majority of wake-active neurons in the LH/PFA (Methippara et al., 2003). It is currently unknown how orexin neurons are inhibited during fever and whether their inhibition contributes to sickness behaviour.

Peripheral infection results in the release of pro-inflammatory cytokines which act within the brain to induce fever (Romanovsky et al., 2005). Cytokines promote the synthesis and release of prostaglandin E_2 (PGE_2) which acts directly on temperature-

sensitive neurons in the preoptic anterior hypothalamus (POAH) to increase body and brain temperatures (Konsman et al., 2002;Boulant, 2000). Fever is accompanied by sickness and although the benefits of acute sickness are understood, prolonged immune system activation, for example in chronic inflammatory diseases, can lead to resource depletion and even contribute to major depression in certain individuals (Dantzer et al., 2008;Pecchi et al., 2009). Despite the well-known PGE_2-dependent central mechanisms of the pyretic response (Boulant, 2000), relatively little is known regarding the precise cell phenotypes and cellular mechanisms underlying sickness behaviours.

Sickness, in particular anorexia and behavioural depression, appears to be largely dependent on the synthesis of PGE_2 (Pecchi et al., 2009). As the pyretic response is dependent upon PGE_2 actions within the brain (Boulant, 2000), it is possible that the resultant temperature increase during inflammation is itself partially responsible for driving some of the neural changes that result in sickness. In the present study, we investigated the cellular mechanism(s) of orexin inhibition during fever and whether this contributes to sickness in the form of anorexia and/or behavioural inactivity. Using patch clamp electrophysiology in acute hypothalamic slices from male Sprague-Dawley rats, we tested the response of orexin neurons to an increase in bath temperature over various ranges. Our results suggest that orexin neurons are inhibited by a direct response to an increase in temperature, not by PGE_2, and that the temperature-induced inhibition of orexin neurons contributes to lipopolysaccharide (LPS)-induced anorexia. We also demonstrate a novel mechanism of orexin neuron thermosensitivity that is reliant on ATP-sensitive potassium (KATP) channels.

5.2 Materials and Methods

All experiments were performed following the guidelines set by the Canadian Council on Animal Care and approved by Memorial University's Institutional Animal Care Committee. Male Sprague Dawley rats were obtained from the breeding colony at Memorial University.

5.2.1 Electrophysiology

Animals (60-70 g) were anesthetized with halothane, decapitated and brains were quickly removed. Coronal hypothalamic slices (250 μm) were sectioned using a vibratome (Leica). Sectioning was performed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 18 $NaHCO_3$, 2.5 glucose, 2 $CaCl_2$, pH 7.3-7.35. Following dissection, slices were incubated in ACSF at 32-35°C for 30-45 min, then at room temperature until recording. ACSF was continuously bubbled with O_2 (95%)/CO_2 (5%).

Patch-clamp recordings, visually assisted by an infrared differential-interference contrast microscope (Leica), were performed on brain slices perfused with ACSF at 1.5-2 ml/min using a Multiclamp 700B amplifier and pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). The conventional whole cell internal solution, adapted from (Balfour et al., 2006), contained (mM): 120 K Gluconate, 1 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 3 K_2ATP, pH 7.3-7.35, 270-280 mOsm. The same solution was used for cell-attached voltage-clamp recordings. For the perforated whole cell internal solution, amphotericin was dissolved in DMSO (60 mg/ml final) and added to (in mM): 110 KCl, 5 $MgCl_2$, 40 HEPES, 10 EGTA, pH 7.3-7.35, 270-280 mOsm. Electrodes had a tip

resistance of 3-8 MΩ. Upon achieving whole-cell access and attaining a series/access resistance of 5-20 MΩ, a series of hyperpolarizing and depolarizing current steps were employed (-200 to +200 pA in 100 pA increments, 300 ms). Orexin and MCH neurons were identified based on their electrophysiological characteristics described elsewhere (Eggermann et al., 2003;Alberto et al., 2006;Parsons and Hirasawa, 2010a;Parsons and Hirasawa, 2010b). The bath temperature was controlled using an in-line heater (TC-324B, Warner Instruments or HW-30, Dagan) with the thermistor placed in close proximity to the hypothalamic section. Temperatures were increased at a rate of approximately 2 °C/min and maintained at the peak test temperature for 1-5 minutes before being returned to the starting temperature. Whole-cell voltage clamp experiments were performed at a holding potential of -50 mV, with the exception of voltage ramps (-140 to -20 mV, 600 ms). As it was sometimes difficult to find spontaneously-active neurons using cell-attached voltage clamp, kainate (0.5-1 μM) was used in some cases to initiate action potential firing. Following all cell-attached experiments, the membrane was ruptured to enter whole-cell mode and the orexin phenotype was confirmed by their electrophysiological characteristics.

5.2.2 Tracing

Male Sprague Dawley rats (PND 19-23) were anesthetized with isoflurane (4% induction, 2% maintenance) and placed in a stereotaxic frame. A small cut was made in the scalp, a hole was drilled into the skull and 300 nl of green retrobeads (LumaFluor Inc.) was injected unilaterally into the locus coeruleus (LC) using a Hamilton syringe. The coordinates used were (mm): -8.9 AP, -1.3 ML, -0.6 DV with respect to the interaural

line. Incisions were sutured and animals were allowed 5-7 days for recovery and to ensure sufficient transport and cellular filling of the retrograde tracer. Following recovery, animals were anesthetized with halothane and hypothalamic slices were obtained as before (section 5.2.1) in ice-cold ACSF. The brainstem was frozen immediately and sectioned at 14 μm on a cryostat (Leica) to determine the injection site. Hypothalamic slices from animals with a successful injection largely confined to the LC were observed under a fluorescent microscope (Leica) and filled cells were targeted for perforated-patch clamp electrophysiological recordings. Experiments were performed only on fluorescent cells that displayed the electrophysiological characteristics of orexin neurons.

5.2.3 Telemetry

Animals (150-200g) were anesthetized with Isoflurane (4% induction, 2% maintenance) and a core telemetry temperature probe (TA10TA-F20, Data Sciences International, St. Paul, MN, USA) was implanted into the peritoneal cavity. During probe implantation, animals were also implanted with unilateral or bilateral cannulae (22-gauge; .028" OD, .022" ID) aimed at the LH/PFA using the following co-ordinates (in mm): AP: -2.3 and ML: ±1.2 with respect to bregma and 2.5 dorsal to the interaural line. Cannulae were fixed in place with skull screws and dental cement. Following surgery, animals were housed individually and allowed 5-7 days to recover before being placed over a telemetry receiver (RPC-1, Data Sciences International). Core body temperature and locomotor activity were automatically recorded every 5 minutes and overnight food intake was measured by the experimenter. Animals initially received an i.p. injection of saline (1 ml/kg body weight) prior to lights-off at 19:00. Two days later, animals were given an i.p.

injection of LPS (100 µg/kg, 1 ml/kg) prior to lights-off, followed three hours later by an intra-LH/PFA injection of 500 nl (over 60 seconds) DMSO or glibenclamide dissolved in DMSO. Intra-LH/PFA injections were performed three hours following LPS treatment as preliminary data demonstrated that a clear body temperature response to LPS could be observed approximately from 3 to 6 hours post-LPS. Injections of glibenclamide/vehicle were carried out using a 2 µl Hamilton syringe and a 25-gauge (.020" OD, .010" ID) injection cannula that extended 1 mm below the implantation cannula. The injection cannula was left in place for an additional 60 seconds to ensure proper diffusion of glibenclamide/vehicle. The following day, animals were euthanized with CO_2 and brains were removed, immediately frozen, and cut at 40 µm on a cryostat to determine cannula placements. A volume of 500 nl of 4% pontamine sky blue was injected immediately following CO_2 and prior to the removal of the brain to aid in the identification of the injection sites. It was determined blindly whether cannula placements fell within or outside of the LH/PFA.

5.2.4 Data Analysis

Action potential frequency, membrane potential, and holding current were measured using Mini Analysis 6.0 (Synaptosoft) and Clampfit 9.2 (Molecular Devices). Data are expressed as mean ± SEM. Instantaneous action potential frequency was calculated as the inverse of each action potential interval. For individual cells, a change in firing frequency of more than 15% was considered significant. In the telemetry experiments, we found the activity counts to be highly variable over 5 minute intervals and thus these data were averaged into 60 minute bins. Statistical tests used included one-

way and two-way ANOVA for multiple-group comparisons and paired and unpaired Student's *t* tests for two-group comparisons. Tukey post-hoc test was used when ANOVA found significance. A value of $p < 0.05$ was considered significant.

5.2.5 Drugs

All compounds were obtained from Sigma-Aldrich (St. Louis, MO) with the exception of PGE_2, glibenclamide (Tocris Bioscience, Ellisville, MO) and TTX (Alomone Labs, Jerusalem, Israel).

5.3 Results

5.3.1 Orexin neurons are temperature-sensitive.

We first used cell-attached voltage-clamp to monitor orexin neuron firing frequency. We found that increasing the bath temperature from 36 to 40 °C resulted in reversible inhibition in 4 out of 5 orexin neurons tested (Fig. 5.1). These data suggest that the majority of orexin neurons are thermosensitive within this range. We then used whole-cell recordings to further investigate the properties of orexin neuron thermosensitivity. In our hands, we found it difficult to maintain stable whole-cell orexin neuron recordings in temperatures above 36 °C. Thus, we asked whether the temperature effect could be initiated at lower sub-physiological temperatures and, if so, whether such an approach can be used to investigate the mechanism of the effect. Using whole-cell recordings, we found that increasing the bath temperature from 26 to 30 °C significantly and reversibly inhibited the spontaneous firing activity of orexin neurons (Fig. 5.2A-C).

As recordings were much more stable at these temperatures, bath temperature changes from 26 to 30 were used to investigate the mechanism of the effect.

To determine if orexin neurons are intrinsically thermosensitive, tetrodotoxin (TTX) was added to the bath to prevent action potential-dependent transmitter release and orexin neurons were voltage-clamped at -50 mV. Under these conditions, orexin neurons responded to a temperature increase with a reversible outward current (Fig. 5.2D, E, n = 5), suggesting intrinsic thermosensitivity of orexin neurons.

We also asked whether orexin neurons are directly sensitive to PGE_2. Bath application of PGE_2 (10 μM, 3-5 minutes) at a concentration ten-fold higher than that previously shown to induce a cellular effect in hypothalamic slices (Ferri et al., 2005) did not influence the basal spontaneous firing frequency of orexin neurons (Fig. 5.3A-B, n = 3). When orexin neurons were voltage clamped at -50 mV, PGE_2 had no effect on the holding current (Fig. 5.3C, n = 3) and voltage ramps applied before and during PGE_2 application revealed no net steady-state current induced by PGE_2 treatment over a range (-140 to -20 mV) of membrane potentials (Fig. 5.3D). Thus, orexin neurons are directly sensitive to temperature changes but not PGE_2.

5.3.2 Increased temperature activates ATP-sensitive potassium channels in orexin neurons

To determine the ion channel(s) involved in the temperature-induced inhibition of orexin neurons, voltage ramps were applied at 26 and again at 30 °C. Current-voltage profiles were visibly different at these temperatures (Fig. 5.4A). Subtracting the current

response at 26 °C from that at 30 °C revealed a temperature-induced current with an inward rectification that reversed near the equilibrium potential for potassium (n = 5, Fig. 5.4B, E). This current was suggestive of KATP channels, which we have recently shown on orexin neurons (Chapter 4)(Parsons and Hirasawa 2010a). To test the involvement of KATP channels in the temperature effect, we treated hypothalamic slices (> 5 min) with the KATP channel blocker tolbutamide (1 mM). Tolbutamide completely blocked the temperature-induced outward currents in orexin neurons (Fig. 5.4C, D, n = 5). Voltage ramps applied before and during the temperature increase in tolbutamide-treated slices (n = 5) did reveal a temperature-induced current that reversed at a significantly more depolarized potential compared to that seen during control (i.e. without tolbutamide) conditions (Fig. 5.4E). When orexin neuron firing frequency was monitored in tolbutamide-treated slices, a rise in temperature was found to induce a slight increase in firing frequency (Fig. 5.4F, G). Thus, temperature appears to activate both inhibitory and excitatory currents in orexin neurons. However, under control conditions the excitatory effect is completely masked by the effect of KATP channel activation. To further confirm a KATP-dependent postsynaptic mechanism, we successfully attenuated the response by postsynaptic loading of 13 mM ATP (Fig. 5.4H, I).

We then asked whether the same mechanism is responsible for the inhibition observed within higher, more physiologically-relevant temperatures. Using cell-attached voltage clamp to monitor orexin neuron firing frequency, we found that tolbutamide not only blocked the inhibition induced by a temperature change from 36 to 40 °C (see Fig. 5.1), but revealed a temperature-induced excitation (Fig. 5.5), in agreement with the data at lower temperatures when KATP channels are blocked. Thus, heat activates multiple

currents in orexin neurons, although the KATP current is dominant, at least in the majority of cases, and results in orexin neuron inhibition.

5.3.3 Temperature-sensitive orexin neurons include those projecting to the locus coeruleus

We also confirmed the temperature response in orexin neurons using perforated patch clamp recordings and demonstrated the effect in smaller, two-degree temperature changes from 28 to 30 °C. Under these conditions, 7 of 9 (78%) orexin neurons were temperature-sensitive (Fig. 5.6A-C). A slight but insignificant (i.e. <15%) increase in firing frequency was observed in the remaining 2 cells.

Although we have seen a temperature-induced inhibition of the majority of orexin neurons in the present study, there were 2 of 9 cells identified with perforated recordings and 1 of 5 cells identified with cell-attached recordings that appeared to be temperature-insensitive. The orexins send a dense excitatory projection to the LC where they act to increase arousal and locomotor activity (Hagan et al., 1999). In an effort to investigate whether increased temperatures could decrease arousal through the inhibition of orexin projections to the LC, we asked whether the orexin neurons projecting to this wake-promoting region are temperature-sensitive or insensitive. We injected a fluorescent retrograde tracer into the LC and recorded from filled cells in the LH/PFA 5-7 days later. When the injection site was largely confined to the locus coeruleus (n = 3, Fig. 5.6D), fluorescent cells were observed (Fig. 5.6D, inset) within the orexin-containing regions of both hemispheres, consistent with the finding that the orexin innervation of the LC is bilateral (Crocker et al., 2005). Few, if any, fluorescent cells were found in the LH/PFA

in animals whose tracer injection landed outside the LC. Using perforated patch clamp recordings, we found that 6 of 6 LC-projecting orexin neurons were inhibited by a bath temperature increase from 28-30 °C (Fig. 5.6E, F). The magnitude of the temperature-induced decrease in firing frequency was similar for LC-projecting orexin neurons (40.8 ± 10.7% of control firing frequency, n = 6) and orexin neurons with unknown projection targets (53.9 ± 5.0% of control firing frequency, n = 7, $p > 0.05$) obtained from perforated patch recordings. Thus, temperature-sensitive orexin neurons include those that project to the wake-promoting LC.

5.3.4 Temperature-induced hyperpolarization in the lateral hypothalamus/perifornical area is specific to orexin neurons

We then asked whether the temperature sensitivity within the LH/PFA is specific to orexin neurons. To determine whether other non-orexin neuronal phenotypes are affected by increased temperatures, non-orexin neurons within the LH/PFA were patched and exposed to a temperature increase from 26 to 30 °C. Electrophysiological responses to current injections revealed three distinct cell types which included orexin neurons (Fig. 5.7A), MCH neurons (Fig. 5.7B) as well as an unknown non-orexin, non-MCH phenotype (Fig. 5.7C). In contrast to orexin neurons, voltage clamp recordings revealed a lack of response to a temperature increase in both MCH neurons (n = 3) and the unknown phenotype (n = 3) (Fig. 5.7D). Thus, KATP-dependent inhibition by heat is at least somewhat unique to orexin neurons within the LH/PFA.

5.3.5 Orexin thermosensitivity is implicated in LPS-induced anorexia

Lastly, we investigated whether orexin inhibition during high temperatures could contribute to either the decrease in locomotor activity or food intake associated with fever. To test this, we induced fever with LPS and prevented the temperature-induced inhibition of orexin neurons by injecting the KATP channel blocker glibenclamide into the LH/PFA, where orexin neurons are concentrated. Using telemetry recordings of body temperature and locomotor activity, we found that LPS (100 µg/kg i.p.) resulted in a significant body temperature increase in comparison to saline (Fig. 5.8A-B, n = 19). LPS injections were followed three hours later by either a control injection (n = 10) or an intra-LH/PFA injection of glibenclamide (n = 9). Animals that received glibenclamide injections outside the LH/PFA did not statistically differ from DMSO animals in terms of temperature, activity or food intake and data from these animals were combined to form the "control injection" group. These included injections that were posterior and/or dorsal to the orexin field.

Glibenclamide had no effect on the LPS-induced increase in body temperature (Fig. 5.8A-B, $F = 0.40$, $p > 0.05$) or suppression of locomotor activity (Fig. 5.8C-D, $F = 0.76$, $p > 0.05$). However, the LPS-induced decrease in overnight food intake was significantly improved in glibenclamide-treated animals (Fig. 5.8E, $t = 1.91$, $p < 0.05$). These data suggest that a KATP channel mediated mechanism, possibly involving orexin neurons, contributes to the hypophagia associated with fever.

5.4 Discussion

Systemic inflammation induces an increase in body temperature which is typically accompanied by a variety of sickness behaviours. The present study reports for the first time the thermosensitive properties of orexin neurons and implicates their temperature-induced inhibition in LPS-induced anorexia. Specifically, we found that KATP channels in orexin neurons are activated by an increase in temperature.

5.4.1 Technical Considerations

KATP channels are modulated by the intracellular level of ATP and a portion of our studies were performed using conventional whole-cell methods in which temperature-induced inhibition was observed despite the presence of "clamping" intracellular ATP at 3 mM via the recording pipette. The ability for KATP channels to respond in such conditions can likely be explained by the fact that cytosol-to-submembrane nucleotide mobility is limited and thus the ATP concentration loaded into the cytosol via the patch pipette is not the amount sensed in KATP channel microdomains (Abraham et al., 2002). Indeed, in the present study, the addition of 10 mM ATP to the pipette was still unable to completely block the temperature-induced response while tolbutamide did. Thus, the ability for KATP channels to open is still preserved at least to a certain degree with 13 mM bulk ATP. Moreover, KATP-dependent hyperpolarization due to glucose deprivation or cyanide/azide-induced metabolic challenge has been observed in conventional whole-cell recordings where exogenous ATP is delivered to the cytosol, as shown in chapter 4 of the present thesis (Parsons and Hirasawa 2010) and by others (Balfour et al., 2006;Ainscow et al., 2002;Parsons and Hirasawa, 2010a), further suggesting that ATP

added to the pipette does not accurately reflect the local domain of ATP sensed by KATP channels. This validates the use of conventional whole-cell recordings for KATP-dependent currents. Nonetheless, we also confirmed the temperature effect with cell-attached and perforated patch recordings, in which intracellular ATP is left intact.

In our whole-cell experiments, we found that an increase from 26 to 30 °C virtually eliminated orexin neuron spontaneous activity. However, although difficult, we were able to find spontaneously-active orexin neurons at 36 °C using cell-attached recordings. This would suggest that that there is at least some degree of desensitization of the temperature response in at least a subpopulation of temperature-sensitive orexin neurons. Although incubating hypothalamic slices in different temperatures from 28 to 40 °C did not significantly alter the mean firing frequency (Fig. 5.9A), the percentage of spontaneously active cells (above 1 Hz) was substantially greater at 28 and 32 °C in comparison to 36 and 40 °C (Fig. 5.9B). Thus, as there appears to be desensitization of the temperature response, these data suggest that many temperature-sensitive orexin neurons do not completely desensitize to increased temperatures.

5.4.2 Orexin neurons as central thermosensors

Thermosensitive neurons are known to exist, most notably, within the POAH (Boulant, 2000). The firing rates of warm-sensitive and cold-sensitive neurons are positively and negatively correlated with temperature, respectively. These neurons not only respond to local temperature changes but also to peripheral changes as they receive projections from the lateral spinothalamic tract (Boulant, 2000). In turn, POAH neurons project to thermoeffector systems in an effort to maintain a stable internal body

temperature. The currently described thermosensitivity of orexin neurons differs from these neurons in terms of the mechanism of thermosensitivity and the effector systems upon which they act.

The most obvious difference between orexin neurons and previously described thermosensitive neurons is that orexin neurons are inhibited whereas the majority of thermosensitive neurons are excited by an increase in temperature. It has been reported that approximately 30% of POAH neurons are warm-sensitive whereas < 5% are cold-sensitive (Boulant, 2000) and this trend appears to hold true for adjacent diencephalic regions (Dean and Boulant, 1989). Much of what is known about the mechanism of neuronal thermosensing involves warm-sensitive neurons, as they are the most abundant thermosensing phenotype. Warming has been shown to exert an excitatory effect in the hypothalamus by a number of mechanisms including a faster inactivation of A-currents (Griffin et al., 1996), modulation of the afterhyperpolarization (Burgoon and Boulant, 2001), activation of a non-selective cation channel (Hori et al., 1999) and activation of TRPV channels (Sharif-Naeini et al., 2008). One of these mechanisms could potentially explain the excitatory effect of temperature in the present study when KATP channels were blocked with tolbutamide. On the other hand, mediators of peripheral cold-sensitivity include TRPM8 and Anktm1 channels, which are activated by temperatures below 25 and 17 °C, respectively (Patapoutian et al., 2003), whereas central cold-sensitivity has been attributed mainly to a synaptic inhibition by neighbouring warm-sensitive neurons (Boulant, 2000). Members of the two-pore domain potassium channels TRAK-1, TRAAK and TREK-2 have been shown to increase their conductivity with

increasing temperatures between 25 and 42 °C (Kang et al., 2005), suggesting a possible role as ionic mediators of cold-sensitivity. However, the orexin neuron inhibition shown here persists in TTX and is blocked by tolbutamide or attenuated by postsynaptic loading of additional ATP. KATP channel activation has been proposed as a plausible mechanism of central thermosensing (Qu et al., 2007b) but, to the best of our knowledge, has not been shown until now. Interestingly, KATP channels are rather ubiquitous in the brain, yet it appears that KATP-dependent thermosensing is somewhat specific to orexin neurons. For example, MCH neurons in mice were recently shown to contain functional KATP channels (Kong et al., 2010) but were shown to be temperature-insensitive in the present study. Moreover, the supraoptic nucleus contains warm-sensitive neurons (Sharif-Naeini et al., 2008) despite the fact that this area possesses a high density of KATP channels (Thomzig et al., 2005). A possible explanation for the discrepancy is that KATP channels in orexin neurons are composed of a rare combination of Kir6.1 and SUR1 subunits (Parsons and Hirasawa, 2010a). The precise mechanism by which temperature modulates these channels remains to be seen.

It is well-established that POAH thermosensitive neurons detect changes in local and peripheral temperatures and direct their outputs to thermoeffector regions in an effort to maintain a stable body temperature. For example, warm sensitive neurons can detect an increase in body and/or environmental temperatures and react in a way which promotes heat loss through autonomic control over brown adipose tissue, skeletal muscle and skin vasculature (Romanovsky, 2007). This appears to be a tightly regulated system whereby temperature changes are detected and countered. In contrast, the role of the orexins in

thermoregulation is less clear. Both an increase (Yoshimichi et al., 2001;Monda et al., 2001) and decrease (Balasko et al., 1999;Jaszberenyi et al., 2002) in body temperature has been observed following i.c.v. injection of orexin-A. Orexin knockout animals do not have a lower core temperature (Mochizuki et al., 2006) although orexin neurons do appear necessary for stress-induced thermogenesis (Zhang et al., 2010). Based on the conflicting data, it remains to be seen whether orexin neurons detect fluctuations in temperature and respond in such a way that contributes to the ongoing maintenance of body temperature homeostasis.

5.4.3 Temperature-induced inhibition of orexin neurons and sickness behaviour

It is well-established that orexin neurons are involved in the regulation of food intake. Central administration of orexin-A increases food intake (Sakurai et al., 1998) and mice lacking orexin neurons were observed to eat roughly 30% less than their wild-type littermates (Hara et al., 2001). The orexin neurons have also been implicated in arousal and physical activity as central orexin administration increases locomotor activity and time spent awake (Hagan et al., 1999;Huang et al., 2001) whereas orexin knockout mice have a difficulty maintaining wakefulness and a narcoleptic phenotype is observed (Chemelli et al., 1999). More recently, they have been shown to play a critical role in promoting reward and motivated behaviours (Borgland et al., 2006;Harris et al., 2005). Together, it is thought that orexin neuron activity coordinates arousal and vigilance with reward-seeking behaviours to increase the chances of finding and consuming food. Thus, many lines of evidence suggest an orexin involvement in the stimulation of food intake, wakefulness and arousal as well as reward and motivated behavioural responding.

On the contrary, fever is associated with decreased appetite, arousal and motivation (Dantzer et al., 2008). Demonstrating an inhibition of orexin neurons by temperature suggests a central mechanism by which this may occur. Although temperature-sensitive orexin neurons project to the wake-promoting LC, orexin neurons appear to be uninvolved in LPS-induced inactivity, at least according to the locomotor activity counts generated in the present telemetry studies. It is quite possible that some aspects of LPS-induced behavioural depression are mediated entirely by an orexin-independent mechanism involving ascending pathways from the brainstem to histaminergic neurons of the tuberomammillary nucleus (Gaykema et al., 2008). Despite an apparent lack of orexin-involvement in LPS-induced locomotor depression, KATP channel blockade after LPS challenge did significantly alleviate hypophagia, suggesting a partial orexin involvement in this aspect of sickness. It is clear that orexin neurons cannot represent the only neural phenotype involved in sickness as LPS or cytokine administration has been shown to activate a number of areas in both the brainstem and hypothalamus (Pecchi et al., 2009) and many thermosensitive and PGE_2-sensitive neurons exist both within and outside the POAH (Dean and Boulant, 1989;Ferri et al., 2005;Chakfe et al., 2006;Matsuda et al., 1992). Therefore, the range of sickness behaviours is likely to result from the coordinated actions of PGE_2 as well as local temperature increases on a variety of neuronal phenotypes. Supporting this view is the finding that different aspects of sickness have a different time course of onset (Dantzer, 2001).

5.4.4 Conclusions

The present data highlights the thermosensitive properties of orexin neurons and introduces the KATP channel as a mechanism of central thermosensing. Upon LPS challenge, blockade of KATP channels within the orexin field alleviated the hypophagia. This is the first report of orexin neuron thermosensitivity and the first to link this neural phenotype to sickness behaviour.

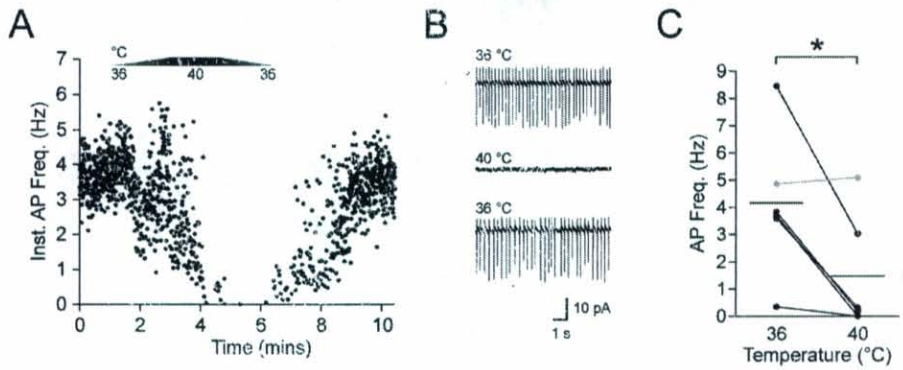


Figure 5.1 Orexin neurons are temperature-sensitive. *A*. Instantaneous action potential frequency (Inst. AP Freq.) plot of a representative orexin neuron recorded using cell-attached voltage clamp. Temperature was ramped from 36 to 40 °C as shown. *B*. Representative cell-attached traces at different temperatures. The cell in *B* is different from that in *A*. C. Grouped data (n = 6) showing a significant inhibition of orexin neurons by temperature. A temperature-insensitive orexin neuron is shown in grey. Horizontal bars indicate the mean values. All cells were included for analysis. * $p < 0.05$.

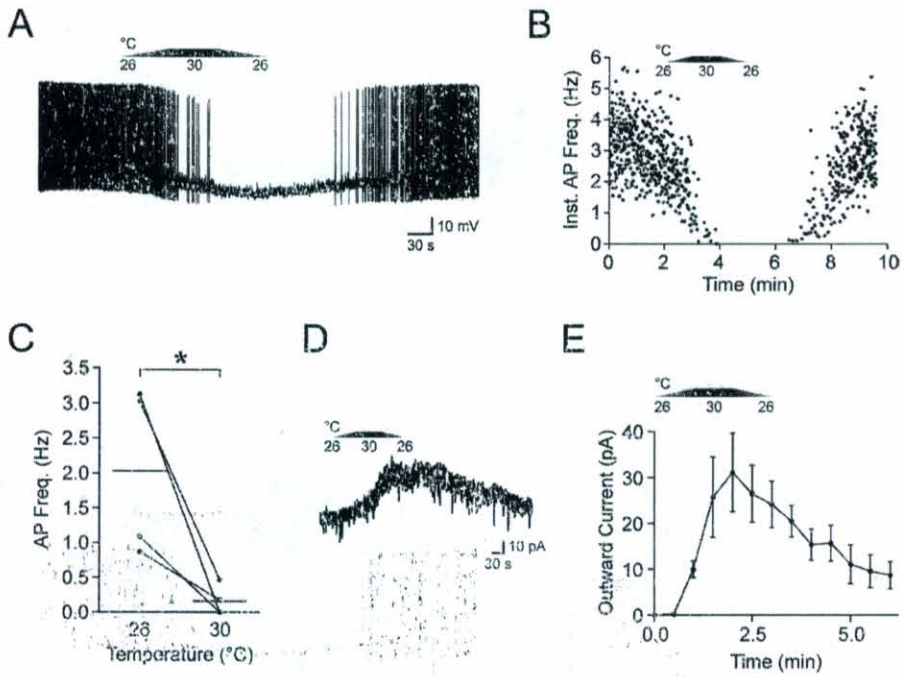


Figure 5.2 Orexin neurons are intrinsically temperature-sensitive. *A-B.* Current clamp trace *(A)* and time plot of instantaneous action potential frequency (Inst. AP Freq; *B*) of a representative orexin neuron exposed to a temperature increase from 26 to 30 °C. Firing frequency inhibition is shown for multiple cells in *C*. *D.* Representative voltage clamp recording of an orexin neuron exposed to TTX. Outward currents were observed upon a temperature increase. Grouped data for voltage-clamped cells in the presence of TTX is shown in *E*. * $p < 0.05$.

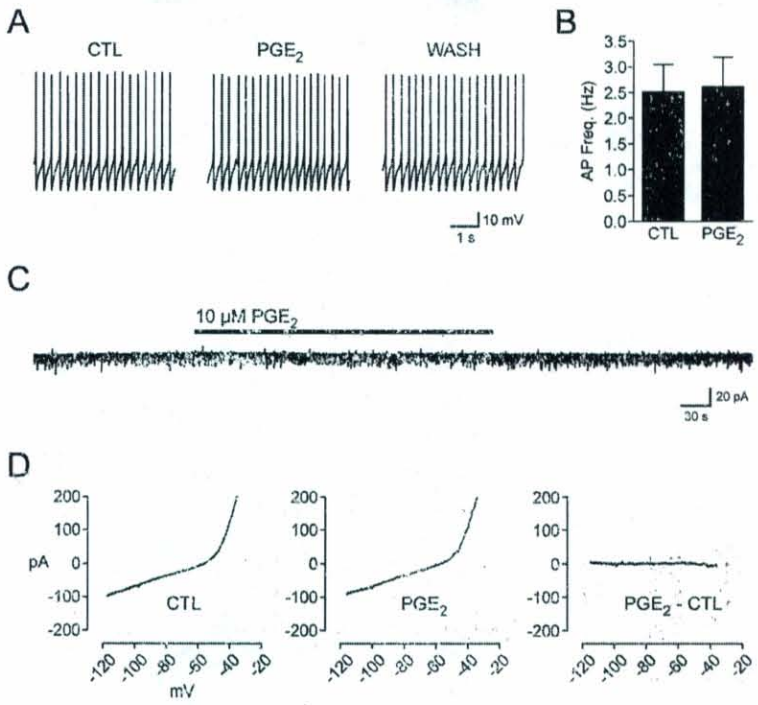


Figure 5.3 PGE_2 does not directly influence orexin neurons. *A-B.* Orexin neuron firing frequency is unaffected by PGE_2 (10 μM) bath application. Grouped data is shown in *B*. *C.* Representative voltage clamp recording showing no current response to PGE_2. *D.* Voltage ramps applied before (left) and during (middle) PGE_2 application reveal no change in current-voltage responses of orexin neurons, as shown in the subtracted trace (right).

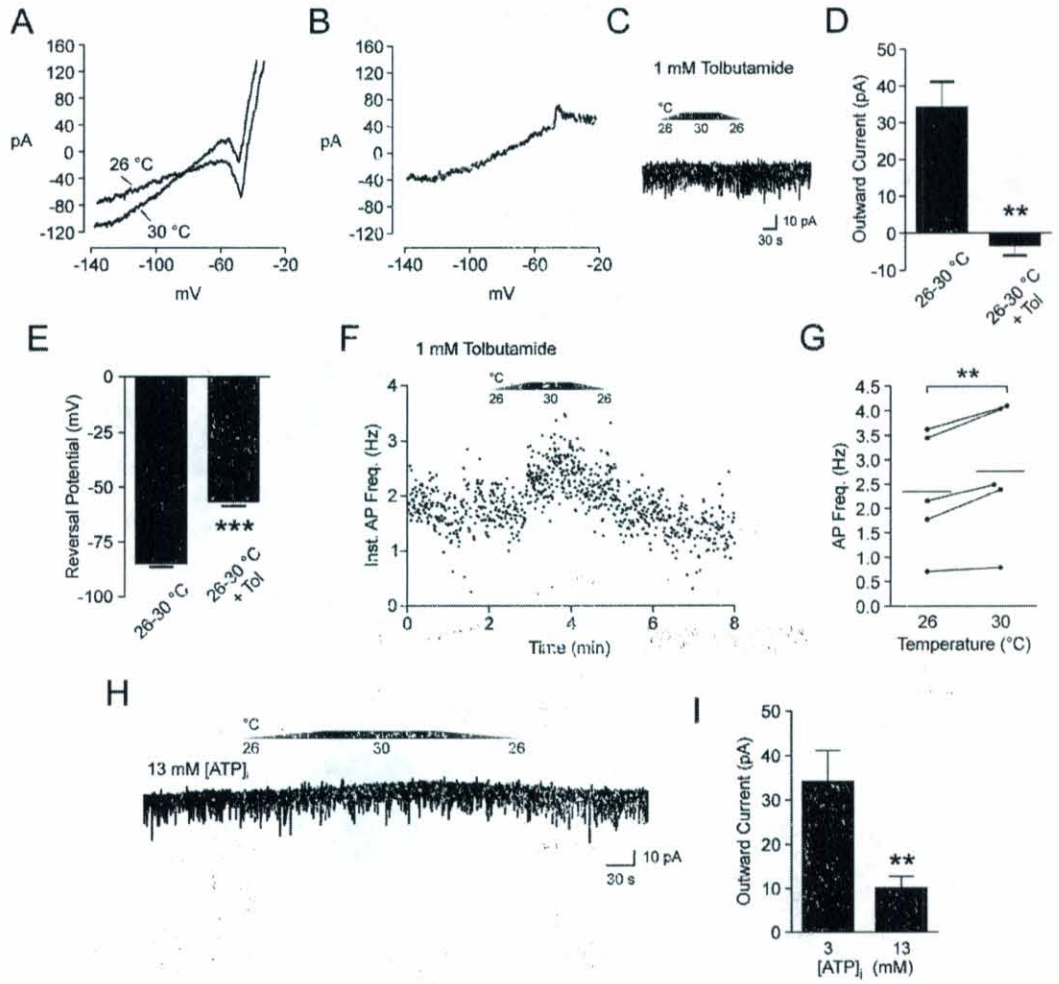

A
B
C
D
pA
mV
26 °C
30 °C
1 mM Tolbutamide
°C
26 30 26
10 pA
30 s
Outward Current (pA)
26-30 °C
26-30 °C + Tol
**
E
F
G
Reversal Potential (mV)
***
Inst. AP Freq. (Hz)
Time (min)
AP Freq. (Hz)
Temperature (°C)
H
I
13 mM [ATP]i
[ATP]i (mM)

Figure 5.4 Temperature activates KATP channels in orexin neurons. *A-B.* Voltage ramps applied before (26 °C) and during (30 °C) the temperature increase reveals a temperature-induced change in the current voltage relationship which is shown in the subtracted trace in *B. C-D.* The KATP channel blocker tolbutamide completely inhibits the temperature-induced outward current. Grouped data is shown in *D. E.* Mean reversal potentials in control conditions and in tolbutamide (tol)-treated slices calculated by subtracting the current-voltage response of orexin neurons in 30 °C from that of 26 °C. *F.* Temperature effect on orexin neuron instantaneous action potential frequency (Inst. AP Freq.) from a slice treated with tolbutamide. Grouped data is shown in *G. H-I.* Adding ATP to the intracellular solution significantly attenuates the temperature response. ** $p < 0.01$, *** $p < 0.005$

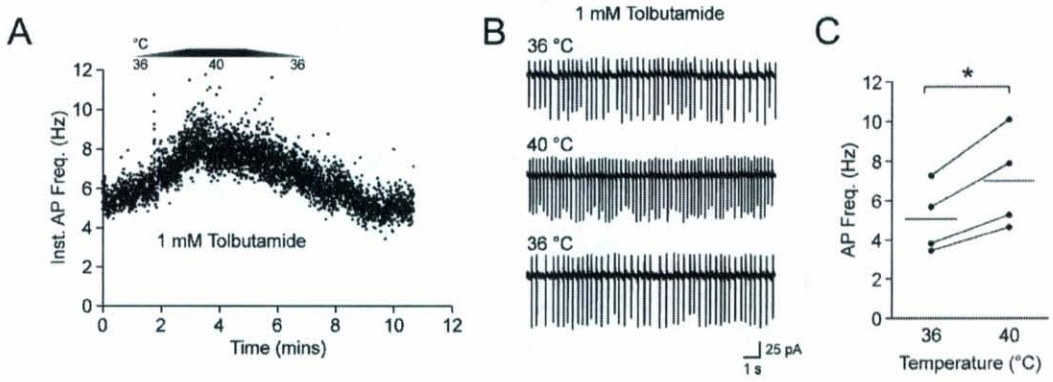


Figure 5.5 KATP channels mediate orexin neuron thermosensitivity over a physiological range. *A*. Instantaneous action potential frequency (Inst. AP Freq.) plot of a representative orexin neuron recorded using cell-attached voltage clamp. Tolbutamide was added to the bath at least 5 minutes prior to the temperature increase. *B*. Representative traces at different temperatures in a tolbutamide-treated cell. The cell in *B* is different from that in *A*. *C*. Grouped data showing a significant excitation of orexin neurons by temperature when KATP channels are blocked with tolbutamide. This is in stark contrast to the inhibition observed when tolbutamide is excluded (see Fig. 5.1). * $p < 0.05$.

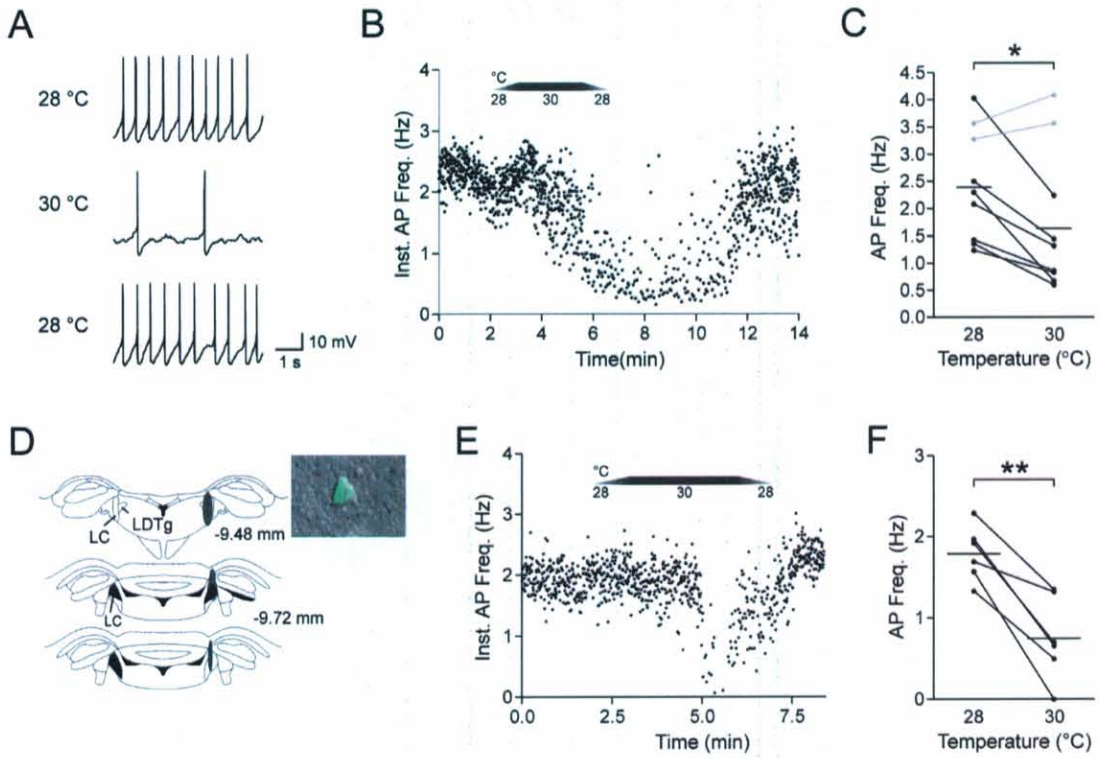


Figure 5.6 Orexin neurons projecting to the locus coeruleus are temperature-sensitive. *A-B.* Representative perforated patch voltage clamp traces at different temperatures *(A)* and a representative time plot of orexin neuron instantaneous action potential frequency (Inst. AP Freq.) when exposed to a temperature increase from 28 to 30 °C. Grouped data is shown in *C*. Note that 2 of the 9 cells tested were not temperature sensitive (grey). *D*. Schematic drawings of the locations of three different successful tracer injections largely confined to the locus coeruleus (LC). *Inset.* Representative DIC image of the LH/PFA with the corresponding fluorescence induced by retrograde tracer injection into the LC. *E-F*. Orexin neurons projecting to the locus coeruleus were temperature-sensitive. Inhibition was seen in 6 of 6 cells tested (*F*). LTDg, laterodorsal tegmental nucleus. * $p < 0.05$, ** $p < 0.01$

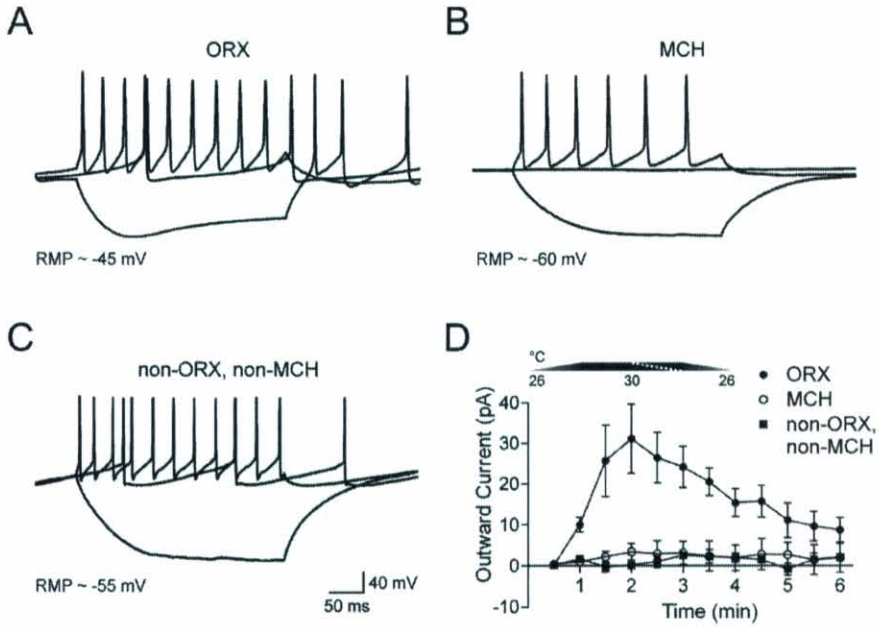


Figure 5.7 Neighbouring MCH and unknown 3rd party neurons do not display orexin-like thermosensitive properties. *A-C.* Electrophysiological responses of three different neuronal phenotypes in the LH/PFA to 300-ms positive and negative current injections. Responses to -100, 0 and +100 pA injections are shown. Orexin neuron electrophysiological characteristics *(A)* are distinct from those of MCH neurons *(B)* which are again distinct from those of an unknown non-orexin, non-MCH phenotype *(C)*. These different phenotypes were each exposed to a temperature increase from 32 to 39/40 °C. *D.* The TTX-insensitive outward current observed in orexin neurons is not evident for MCH neurons or the unknown 3rd cell type. The dotted white line represents the temperature return in the orexin experiments. Bath temperature was maintained between 39 and 40 °C for an additional minute for MCH and 3rd party neurons to ensure a lack of temperature-dependent outward current.

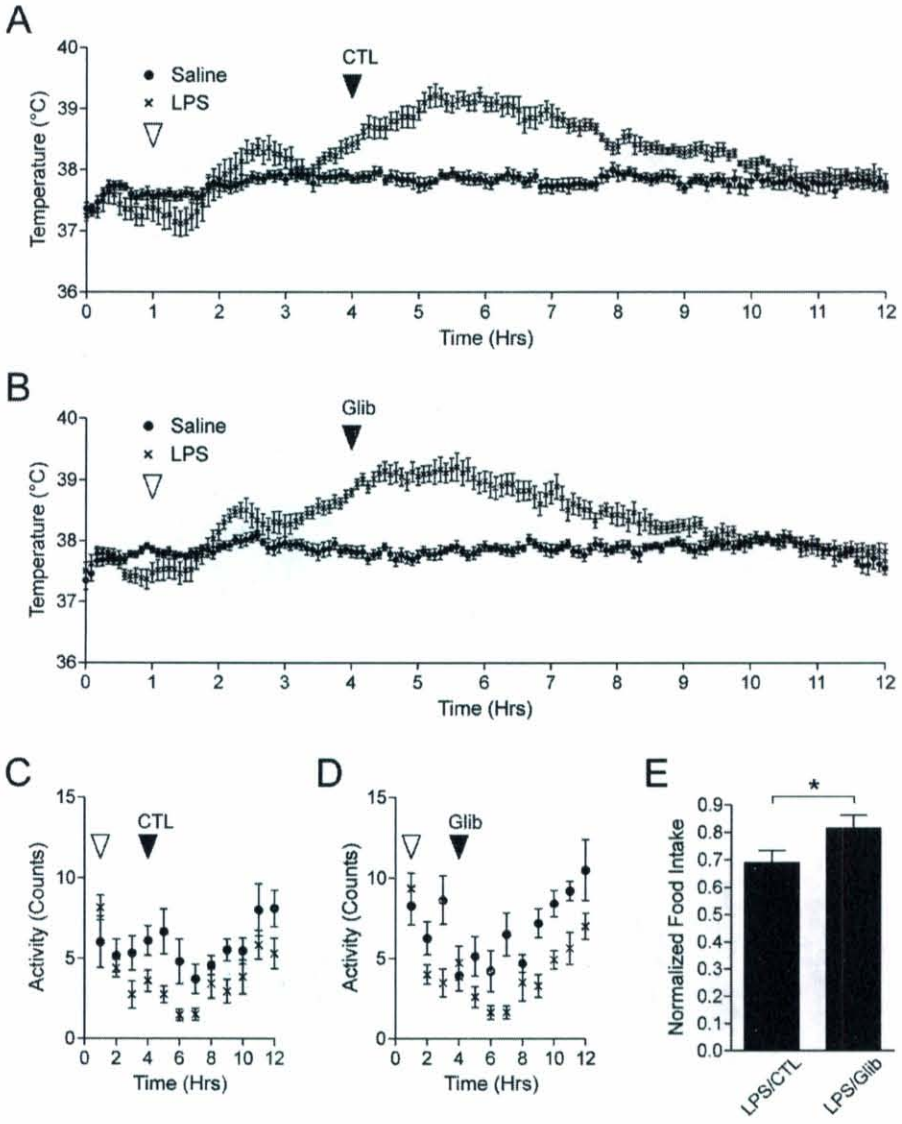

A
Saline
LPS
CTL
Temperature (°C)
Time (Hrs)
B
Saline
LPS
Glib
Temperature (°C)
Time (Hrs)
C
CTL
Activity (Counts)
Time (Hrs)
D
Glib
Activity (Counts)
Time (Hrs)
E
*
Normalized Food Intake
LPS/CTL
LPS/Glib

Figure 5.8 Blockade of temperature-induced orexin neuron inhibition partially alleviates LPS-induced hypophagia. *A-B.* Temperature response (5-min bins) to i.p. injection (hollow arrowhead) of either saline (•) or LPS (x). I.p. injections were followed three hours later by a control (CTL) injection (*A*) or a glibenclamide (glib) injection (*B*) into the LH/PFA (filled arrowhead). Glibenclamide had no effect on the temperature responses induced by LPS. *C-D.* Activity counts (60-min bins) in response to i.p. injection (hollow arrowhead) of either saline (•) or LPS (x). I.p. injections were followed three hours later by a control (CTL) injection (*C*) or a glibenclamide (glib) injection (*D*) into the LH/PFA (filled arrowhead). LPS significantly decreased activity regardless of the intra-LH/PFA injection ($p_{treatment} < 0.05$ for both *C* and *D*). *E.* Overnight food intake after LPS treatment is normalized to that after saline treatment. The LPS-induced decrease in food intake was significantly alleviated by intra-LH/PFA injections of glib. * $p < 0.05$.

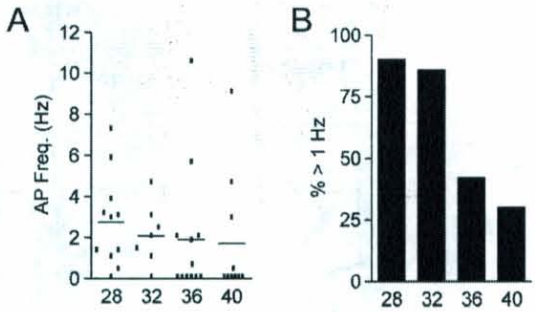


Figure 5.9 Orexin activity in different temperatures. Hypothalamic sections were exposed to a stable temperature (28, 32, 36 or 40 °C) for at least 5 minutes. Orexin neurons were patch clamped using conventional whole-cell methods and their baseline firing rate was recorded. Each dot in (*A*) represents the firing rate of a single orexin neuron. Although the mean firing frequency was not different in the different temperatures (*A*) a high percentage of orexin neurons were active at lower (28 and 32 °C) temperatures compared to higher (36 and 40 °C), more physiologically-relevant temperatures (*B*). *B* represents the percentage of orexin neurons that fired at a spontaneous frequency of more than 1 Hz. The ability to fire action potentials was confirmed in all neurons tested by positive current injections (100 and 200 pA).

Chapter 6

Summary

In the discussion sections of the individual research chapters, I have added "functional considerations" and "technical considerations" sub-sections where I felt they were appropriate. They will not be repeated in this summary section. Rather, I will use this space first to provide a very brief summary of the main findings of the research chapters and then highlight certain inter-manuscript implications that may not have been considered in the individual discussion sections. Rather than ending with a dedicated "future directions" section, I have decided to mix some future ideas in as sub-sections of particular discussion topics.

6.1 Brief summary of the main findings

We found that N/OFQ induced a reversible outward current in MCH neurons that was dependent on the activation of GIRK channels. Similarly, N/OFQ induced an outward current in orexin neurons. However, this current persisted long after agonist washout and was dependent upon PKC and KATP channel activation. As N/OFQ induces a potent inhibition of both the feeding-stimulatory orexin and MCH neurons, we wondered whether the local N/OFQ actions within the LH/PFA would induce hypophagia rather than the typical hyperphagia associated with central N/OFQ injections. We found that intra LH-PFA injections of N/OFQ resulted in the reduced intake of a palatable food.

We also found that orexin neurons are sensitive to cellular metabolism state and that the amount of extracellular energy available, specifically in the form of lactate, determines orexin neuron activity via KATP channel modulation. Furthermore, KATP channels in orexin neurons are also activated by an increase in ambient temperature and we demonstrated a role of orexin neurons in the partial mediation of anorexia which accompanies fever.

6.2 Implications on typical patch clamp experimental protocols

As the majority of experiments in the present thesis were conducted *in vitro*, it is difficult to tell precisely how active KATP channels are in the intact animal. We have demonstrated that glucose concentrations below 1 mM can activate these channels, although these experiments were conducted at sub-physiological temperatures. As we have seen, increasing the temperature also activates KATP channels, suggesting that orexin neurons may be more sensitive to extracellular energy fluctuations at a physiological temperature. These data bring up an interesting argument regarding the glucose and temperature conditions under which patch-clamp electrophysiological experiments should be conducted.

The concentration of glucose within the brain has been estimated to be roughly 10-30% of that within the blood, dependent on the brain region and the state of the animal, giving a range of roughly 1-2.5 mM from meal-to-meal (Burdakov et al., 2005b). A value of as low as 0.7 mM has been measured in the hypothalamus following overnight food deprivation (de Vries et al., 2003). Nonetheless, the typical electrophysiological setup uses 10 mM glucose, an extremely high and physiologically-irrelevant

concentration that is theorized to help cell survival in brain slices. However, by experimenting with different glucose concentrations, I did not notice any obvious difference in cell viability between ACSF glucose concentrations from 1 to 10 mM.

Similarly, for reasons associated with increasing the probability of successful recordings, many electrophysiological experiments are conducted below physiological temperature, somewhere between room temperature and slightly above 30 °C. Heating ACSF to a more physiologically-relevant temperature range of about 36-38 °C can result in oxygen bubbles forming within the recording chamber that can easily hinder the reliability of a recording. This issue is easily countered by heating the ACSF prior to its introduction in the recording chamber. On the other hand, it appears that maintaining a stable recording is generally harder in higher temperatures, forcing recordings to take place at sub-physiological temperatures. Although I realize that the very nature of slice electrophysiology provides an already-artificial situation in that the afferent and efferent connections of a given cell are severed, based on the data presented in this thesis I now firmly believe that every condition that is under user control should be mimicked as close as possible to a physiologically relevant condition while maintaining cell viability.

6.3 N/OFQ-induced inhibition of orexin and MCH neurons

N/OFQ inhibited both orexin and MCH neurons. However, the effect was sustained and KATP-mediated in orexin neurons whereas it was transient and GIRK-mediated in MCH neurons. It is currently unclear whether the prolonged effect in orexin neurons would occur with endogenous release of N/OFQ. Nonetheless, the fact that different effector channels are employed would suggest that orexin and MCH neurons

may behave differently in terms of whether N/OFQ's actions are additive or occlusive to other influencing factors. For example, we demonstrate in chapter 2 that the N/OFQ effect in MCH neurons occludes that of dynorphin as both appear to activate GIRK currents. Dynorphin has also been shown to inhibit orexin neurons, presumably via GIRK channel activation (Li and van den Pol, 2006), and therefore is unlikely to be occluded by N/OFQ. Thus, additional transmitter(s) released with N/OFQ is likely to have different net effects on orexin and MCH neurons.

Regardless of the mechanism and duration of inhibition, N/OFQ injections into the LH/PFA resulted in the reduced intake of palatable sugar pellets. As N/OFQ is inhibitory to both orexin and MCH neurons, it is also unclear whether both or one of these interactions mediate the behavioural effect. There is even a small chance that the hypophagic effect is mediated by an orexin- and MCH-independent mechanism as other potential feeding-related neurons exist in the area, including galanin- and leptin receptor-expressing neurons (Hakansson et al., 1998;Cheung et al., 2001), although the effect of N/OFQ on these cell types in the LH/PFA has yet to be investigated.

6.3.1 Future directions: Investigating whether the sustained effect of N/OFQ on orexin neurons is physiologically-relevant.

N/OFQ bath application induced a long-lasting hyperpolarization of orexin neurons which did not recover after washout for the length of the electrophysiological recordings. Should such a potent and persistent effect be observed physiologically, this would have a tremendous impact on the orexin system and therefore on the associated physiological and behavioural functions of the orexins. It is therefore of interest to

determine whether this duration of inhibition is a product of N/OFQ bath application or whether it can be achieved by N/OFQ release. A way to approach this would be to drive the expression of the light-sensitive channelrhodopsin-2 under the control of the N/OFQ promoter. As N/OFQ cell bodies exist in the LH and surrounding areas (Neal, Jr. et al., 1999b), brain slices generated for the purpose of electrophysiological recordings from orexin neurons would be expected to have plenty of viable N/OFQ neurons as well. Using this setup, one could conceivably record from an orexin neuron while stimulating, at a user-controlled frequency, a group of N/OFQ-expressing neurons. The response in orexin neurons could be monitored and, through the use of NOP receptor inhibitors such as UFP-101, the contribution of endogenous N/OFQ release to any observed effect can be calculated. This can be used to determine whether N/OFQ release can result in the prolonged inhibition of orexin neurons.

A caveat to the above design is that N/OFQ is also found in orexin neurons (Maolood and Meister, 2010) and it therefore may be hard to find a localized stimulation site which evokes N/OFQ release onto the recorded orexin neuron while avoiding stimulation of the recorded neuron itself. Furthermore, as the maximal firing rate of nociceptin neurons *in vivo* is unknown, it will be difficult to draw conclusions about the physiological relevance of the stimulation frequency required to drive a persistent effect, should one be observed. Nonetheless, these experiments should help determine whether endogenous N/OFQ release can induce a long-term inhibition of orexin neurons.

6.3.2 Future directions: Further exploration of N/OFQ-induced hypophagia

We demonstrated in chapter 3 that N/OFQ injection into the LH/PFA region decreases palatable food intake. To fully understand the cellular mediator(s) of N/OFQ's effect, future experiments can be designed to test the effect of similar injections while preventing the effect of N/OFQ on one of the two phenotypes of interest. Repeating the experiments and co-applying either the GIRK blocker tertiapin Q or the KATP blocker glibenclamide with N/OFQ will determine the relative degree of orexin and MCH involvement in the hypophagic response. If neither of these treatments alone blocks the hyperphagic effect then a cocktail of all three agents should be injected. This will establish whether orexin, MCH or orexin and MCH neuron inhibition is required for N/OFQ's effect. Hypophagia persisting in the presence of both blockers would suggest an orexin and MCH-independent action.

It would also be of interest to determine whether N/OFQ's effect within the LH/PFA solely mediated rewarding food intake or whether regular lab chow intake can be altered. To answer this, the experiments in the present thesis could simply be repeated using regular lab chow instead of the sugar pellets. This will aid in our understanding of the role of N/OFQ action within the LH/PFA in the control of hedonic versus homeostatic feeding. As orexin and MCH antagonism has been shown to decrease both homeostatic and hedonic feeding (Haynes et al., 2000;Zheng et al., 2007;Shearman et al., 2003;Morens et al., 2005;Borowsky et al., 2002), I would expect intra-LH/PFA N/OFQ to decrease chow intake as it did sugar pellet intake.

6.3.3 Future directions: Additional effects of intra-LH/PFA N/OFQ

Throughout this thesis I have also highlighted additional roles of the N/OFQ system and how they overlap with known functions of orexin and MCH. As we only tested the effect of intra-LH/PFA N/OFQ on palatable food intake, it would be of interest to determine whether N/OFQ can also act within this region to influence some of the other functions shared by these peptides. Thus, a possible angle to extend the data from chapters 2 and 3 would be to investigate the effect of intra-LH/PFA N/OFQ on tests of anxiety (elevated plus maze, open field, etc) as well as on reward value. With regards to the latter, questions of interest include whether N/OFQ in this area is aversive on its own (as determined using a conditioned place preference paradigm) and if not, whether it decreases the preference associated with a drug of abuse such as cocaine. Performing these experiments with the aforementioned GIRK and KATP blockers can also determine the relative involvement of the orexin versus the MCH system in any observed responses. These experiments will help determine the scope of N/OFQ function in the LH/PFA and may lead to information aiding the design of pharmaceutical treatments for obesity, anxiety and/or addiction.

6.4 KATP channels as critical regulators of orexin neuron function

The threshold of orexin neuron KATP channel activation appears to rely not only on energy substrate availability and temperature but also on the amount of N/OFQ release. As UFP-101 and tolbutamide alone did not appear to influence the holding current and/or firing rate in orexin neurons in my experiments, it is presumed that KATP channels are closed at basal experimental conditions and that the level of N/OFQ release,

if any, is negligible within the slice preparation. To discuss the relevance of orexin neuron KATP channel modulation in the intact animal requires, amongst others, knowledge of endogenous N/OFQ release conditions. Based on studies examining the expression of N/OFQ and NOP receptor mRNA, it is suggested that N/OFQ release in the intact animal may be influenced by a variety of factors including stress (Rodi et al., 2008;Reiss et al., 2007;Green and Devine, 2009), nutritional state (Przydzial et al., 2010;Rodi et al., 2002) and addiction (Kuzmin et al., 2009). Thus, KATP channel activation in orexin neurons can likely occur independently of metabolic challenge and/or high temperature exposure. Interestingly, many lines of research also suggest that N/OFQ is released during infection (Leggett et al., 2009;Miller and Fulford, 2007;Buzas et al., 2002). One study in particular demonstrated an upregulation of N/OFQ in the hypothalamus 4 hours following LPS injection (Leggett et al., 2009), a time which corresponds with the LPS-induced temperature peak observed in the present thesis. This suggests that the simultaneous increase in temperature and N/OFQ release would ensure potent and perhaps prolonged orexin neuron inhibition during infection. As both act on the KATP channel, the glibenclamide injections used to attenuate LPS-induced hypophagia induced in chapter 5 would be expected to block the effects of both temperature and N/OFQ on orexin neurons.

6.4.1 Future directions: Role of KATP channels in orexin neurons *in vivo*

The present thesis in general supports the view that KATP channels play a critical role in orexin neuron regulation. Thus, an important question for future investigation is to determine precisely how important these channels are to the intact animal. This can be

addressed most accurately by developing a genetic mouse line in which Kir6.1 is knocked out specifically in orexin neurons. This can be done using cre-loxP-mediated recombination where mice carrying a lox-flanked Kir6.1 gene are crossed with mice expressing the Cre recombinase under the control of the orexin promoter.

Once these mice (referred to here as CKO mice, for conditional knock-out mice) have been generated, a series of control experiments are initially necessary. These include double-label immunofluorescence for Kir6.1 subunits and the orexin-A peptide as well as electrophysiological experiments testing the effects of N/OFQ, lactate and temperature on slices from the CKO mice. With a successful CKO, we would expect to see a lack of Kir6.1 immunoreactivity in orexin neurons but not a general lack of central Kir6.1. Furthermore, the effects of N/OFQ, lactate and temperature are expected to be blocked.

The importance of KATP channels in orexin neurons can be tested directly by observing the phenotype of the CKO mice versus wild-type mice. Should such mice ever be available, it would be of interest to examine (1) the firing rate of orexin neurons in awake, behaving animals; (2) the properties of their sleep-wake cycle; (3) their feeding behaviour; (4) energy homeostasis and (5) their sickness response to LPS. Based on the data presented in the present thesis, it would be expected that some, if not all, of these properties may be altered. Regardless of the outcome, these experiments directly address the precise importance of KATP channels in orexin neurons to animal physiology and behaviour.

I will elaborate on one of the above suggestions that emphasizes the value of such a CKO model. In chapter 5, *in vivo* data was presented to determine the potential role orexin neuron thermosensing played in the sickness behaviour associated with fever. To

block orexin neuron thermosensing, we injected the KATP channel blocker glibenclamide into the orexin-containing region. However, we cannot be sure that this treatment specifically affected orexin neurons as there are likely other cell types in the area that contain KATP channels, including MCH neurons (Kong et al., 2010) (although we found no evidence of non-orexin thermosensitive neurons in the region). Furthermore, the chances that our glibenclamide injections inhibited KATP channels in all orexin neurons are zero. The orexin field in each hemisphere is simply too large on both medial-lateral and rostral-caudal axes for a single injection to cover and thus a percentage of orexin neurons undoubtedly retained their thermosensing abilities. This experimental design also required a central injection to block KATP channels a few hours following LPS treatment, making it impossible to leave the animals undisturbed during the telemetry recordings of body temperature and locomotor activity. All of these problems are avoided with the aforementioned CKO animals. As we only found a significant attenuation of the hypophagic effect of LPS, the proposed CKO model may uncover additional sickness behaviours contributed to by the orexins.

6.5 Data discrepancy

Discrepancies amongst data are not uncommon in the scientific world. However, it is in our best interest to attempt to discover the reason(s) for a given difference and to reach a general agreement. The major discrepancy from the present thesis is that we were unable to see short-term, metabolism-independent effects of glucose on orexin neuron activity. It is commonly accepted that orexin neurons are inhibited by glucose and this mechanism has even received media attention as a reason why we may feel tired after a

big meal (for example, see http://www.newscientist.com/article/dn9272-why-we-need-a-siesta-after-dinner.html). We have made an extensive effort to replicate this data (Burdakov et al., 2005a) and could not. Our inability to see a short-term effect cannot be attributed to recording conditions or species difference. We suggested in chapter 4 that the short-term effect has generally been shown in animals of a younger age (< 2 weeks old) in comparison to our animals (3-5 weeks old). Although this is a small time window, there may exist a rapid developmental change in the sensitivity of orexin neurons to glucose changes above 1 mM. Should the age difference not be the case, details such as housing conditions, diet, light/dark cycle and time of slice preparation may have an influence on the proposed surface recognition of glucose (Burdakov et al., 2006). Nonetheless, until the discrepancy can be resolved, the precise way in which orexin neurons are influenced by extracellular energy fluctuations (as a net result of metabolism-independent glucosensing and metabolism-dependent lactate-sensing) remains ambiguous.

6.6 Concluding remarks

The data presented in this thesis shed light on the role of N/OFQ in feeding regulation and extends the existing literature regarding orexin and MCH neuron regulation. I hope that these data will change the view of N/OFQ as a strict orexigenic peptide as well as the view that orexin neurons are metabolically-insensitive, glucose-inhibited cells. The orexin involvement in sickness that I show in chapter 5 is a novel and exciting finding that will hopefully spark interest in the thermosensitivity and/or PGE_2-sensitivity of other feeding, arousal and reward-related neural phenotypes to gain a more

complete understanding of the cellular mechanisms of sickness behaviour. Furthermore, by investigating the endogenous regulators of the orexin system, the present thesis has defined a novel role for these neuropeptides in balancing brain activity with energy supply (see Fig. 4.11). However, during metabolic challenge or during infection, orexin inhibition by low energy substrate, high temperatures or N/OFQ may represent a mechanism which promotes an energy-conserving state designed to aid survival.

References

Abraham MR, Selivanov VA, Hodgson DM, Pucar D, Zingman LV, Wieringa B, Dzeja PP, Alekseev AE, Terzic A (2002) Coupling of cell energetics with membrane metabolic sensing. Integrative signaling through creatine kinase phosphotransfer disrupted by M-CK gene knock-out. J Biol Chem 277:24427-24434.

Adamantidis A, de LL (2009) A role for Melanin-Concentrating Hormone in learning and memory. Peptides 30:2066-2070.

Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de LL (2007) Neural substrates of awakening probed with optogenetic control of hypocretin neurons. Nature 450:420-424.

Ainscow EK, Mirshamsi S, Tang T, Ashford ML, Rutter GA (2002) Dynamic imaging of free cytosolic ATP concentration during fuel sensing by rat hypothalamic neurones: evidence for ATP-independent control of ATP-sensitive K(+) channels. J Physiol 544:429-445.

Akiyama M, Yuasa T, Hayasaka N, Horikawa K, Sakurai T, Shibata S (2004) Reduced food anticipatory activity in genetically orexin (hypocretin) neuron-ablated mice. Eur J Neurosci 20:3054-3062.

Alberto CO, Trask RB, Quinlan ME, Hirasawa M (2006) Bidirectional dopaminergic modulation of excitatory synaptic transmission in orexin neurons. J Neurosci 26:10043-10050.

Amano T, Matsubayashi H, Tamura Y, Takahashi T (2000) Orphanin FQ-induced outward current in rat hippocampus. Brain Res 853:269-274.

Anand BK, Brobeck JR (1951) Localization of a "feeding center" in the hypothalamus of the rat. Proc Soc Exp Biol Med 77:323-324.

Armstead W (2002) NOC/oFQ activates PKC and generates superoxide to impair hypotensive cerebrovasodilation after hypoxia/ischemia. Med Sci Monit 8:BR8-BR14.

Armstead WM (1999) Nociceptin/orphanin FQ dilates pial arteries by K(ATP) and K(ca) channel activation. Brain Res 835:315-323.

Ashford ML, Boden PR, Treherne JM (1990) Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K+ channels. Pflugers Arch 415:479-483.

Avshalumov MV, Rice ME (2003) Activation of ATP-sensitive K+ (K(ATP)) channels by H2O2 underlies glutamate-dependent inhibition of striatal dopamine release. Proc Natl Acad Sci U S A 100:11729-11734.

Balasko M, Szelenyi Z, Szekely M (1999) Central thermoregulatory effects of neuropeptide Y and orexin A in rats. Acta Physiol Hung 86:219-222.

Balfour RH, Hansen AM, Trapp S (2006) Neuronal responses to transient hypoglycaemia in the dorsal vagal complex of the rat brainstem. J Physiol 570:469-484.

Becskei C, Riediger T, Hernadfalvy N, Arsenijevic D, Lutz TA, Langhans W (2008) Inhibitory effects of lipopolysaccharide on hypothalamic nuclei implicated in the control of food intake. Brain Behav Immun 22:56-64.

Beguin P, Nagashima K, Nishimura M, Gonoi T, Seino S (1999) PKA-mediated phosphorylation of the human K(ATP) channel: separate roles of Kir6.2 and SUR1 subunit phosphorylation. EMBO J 18:4722-4732.

Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, Vale W, Sawchenko PE (1992) The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. J Comp Neurol 319:218-245.

Borgland SL, Taha SA, Sarti F, Fields HL, Bonci A (2006) Orexin A in the VTA is critical for the induction of synaptic plasticity and Behavioral Sensitization to cocaine. Neuron 49:589-601.

Borowsky B, Durkin MM, Ogozalek K, Marzabadi MR, DeLeon J, Lagu B, Heurich R, Lichtblau H, Shaposhnik Z, Daniewska I, Blackburn TP, Branchek TA, Gerald C, Vaysse PJ, Forray C (2002) Antidepressant, anxiolytic and anorectic effects of a melanin-concentrating hormone-1 receptor antagonist. Nat Med 8:825-830.

Boulant JA (2000) Role of the preoptic-anterior hypothalamus in thermoregulation and fever. Clin Infect Dis 31 Suppl 5:S157-S161.

Boutrel B, Cannella N, de LL (2010) The role of hypocretin in driving arousal and goal-oriented behaviors. Brain Res 1314:103-111.

Broer S, Broer A, Schneider HP, Stegen C, Halestrap AP, Deitmer JW (1999) Characterization of the high-affinity monocarboxylate transporter MCT2 in Xenopus laevis oocytes. Biochem J 341:529-535.

Burdakov D, Gerasimenko O, Verkhratsky A (2005a) Physiological changes in glucose differentially modulate the excitability of hypothalamic melanin-concentrating hormone and orexin neurons in situ. J Neurosci 25:2429-2433.

Burdakov D, Jensen LT, Alexopoulos H, Williams RH, Fearon IM, O'Kelly I, Gerasimenko O, Fugger L, Verkhratsky A (2006) Tandem-pore K+ channels mediate inhibition of orexin neurons by glucose. Neuron 50:711-722.

Burdakov D, Luckman SM, Verkhratsky A (2005b) Glucose-sensing neurons of the hypothalamus. Philos Trans R Soc Lond B Biol Sci 360:2227-2235.

Burgoon PW, Boulant JA (2001) Temperature-sensitive properties of rat suprachiasmatic nucleus neurons. Am J Physiol Regul Integr Comp Physiol 281:R706-R715.

Buzas B, Rosenberger J, Kim KW, Cox BM (2002) Inflammatory mediators increase the expression of nociceptin/orphanin FQ in rat astrocytes in culture. Glia 39:237-246.

Cai XJ, Evans ML, Lister CA, Leslie RA, Arch JR, Wilson S, Williams G (2001) Hypoglycemia activates orexin neurons and selectively increases hypothalamic orexin-B levels: responses inhibited by feeding and possibly mediated by the nucleus of the solitary tract. Diabetes 50:105-112.

Calo G, Guerrini R, Rizzi A, Salvadori S, Burmeister M, Kapusta DR, Lambert DG, Regoli D (2005) UFP-101, a peptide antagonist selective for the nociceptin/orphanin FQ receptor. CNS Drug Rev 11:97-112.

Chakfe Y, Zhang Z, Bourque CW (2006) IL-1beta directly excites isolated rat supraoptic neurons via upregulation of the osmosensory cation current. Am J Physiol Regul Integr Comp Physiol 290:R1183-R1190.

Chaki S, Funakoshi T, Hirota-Okuno S, Nishiguchi M, Shimazaki T, Iijima M, Grottick AJ, Kanuma K, Omodera K, Sekiguchi Y, Okuyama S, Tran TA, Semple G, Thomsen W (2005) Anxiolytic- and antidepressant-like profile of ATC0065 and ATC0175: nonpeptidic and orally active melanin-concentrating hormone receptor 1 antagonists. J Pharmacol Exp Ther 313:831-839.

Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98:437-451.

Cheung CC, Hohmann JG, Clifton DK, Steiner RA (2001) Distribution of galanin messenger RNA-expressing cells in murine brain and their regulation by leptin in regions of the hypothalamus. Neuroscience 103:423-432.

Chin JH, Harris K, MacTavish D, Jhamandas JH (2002) Nociceptin/orphanin FQ modulation of ionic conductances in rat basal forebrain neurons. J Pharmacol Exp Ther 303:188-195.

Chou TC, Lee CE, Lu J, Elmquist JK, Hara J, Willie JT, Beuckmann CT, Chemelli RM, Sakurai T, Yanagisawa M, Saper CB, Scammell TE (2001) Orexin (hypocretin) neurons contain dynorphin. J Neurosci 21:RC168.

Chung S, Hopf FW, Nagasaki H, Li CY, Belluzzi JD, Bonci A, Civelli O (2009) The melanin-concentrating hormone system modulates cocaine reward. Proc Natl Acad Sci U S A 106:6772-6777.

Ciccocioppo R, Angeletti S, Sanna PP, Weiss F, Massi M (2000) Effect of nociceptin/orphanin FQ on the rewarding properties of morphine. Eur J Pharmacol 404:153-159.

Ciccocioppo R, Economidou D, Fedeli A, Angeletti S, Weiss F, Heilig M, Massi M (2004) Attenuation of ethanol self-administration and of conditioned reinstatement of alcohol-seeking behaviour by the antiopioid peptide nociceptin/orphanin FQ in alcohol-preferring rats. Psychopharmacology (Berl) 172:170-178.

Ciccocioppo R, Panocka I, Polidori C, Regoli D, Massi M (1999) Effect of nociceptin on alcohol intake in alcohol-preferring rats. Psychopharmacology (Berl) 141:220-224.

Civelli O (2008) The orphanin FQ/nociceptin (OFQ/N) system. Results Probl Cell Differ 46:1-25.

Connor M, Vaughan CW, Chieng B, Christie MJ (1996a) Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurones in vitro. Br J Pharmacol 119:1614-1618.

Connor M, Yeo A, Henderson G (1996b) The effect of nociceptin on Ca2+ channel current and intracellular Ca2+ in the SH-SY5Y human neuroblastoma cell line. Br J Pharmacol 118:205-207.

Crocker A, Espana RA, Papadopoulou M, Saper CB, Faraco J, Sakurai T, Honda M, Mignot E, Scammell TE (2005) Concomitant loss of dynorphin, NARP, and orexin in narcolepsy. Neurology 65:1184-1188.

Dantzer R (2001) Cytokine-induced sickness behavior: mechanisms and implications. Ann N Y Acad Sci 933:222-234.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9:46-56.

Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M (1999) Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci U S A 96:748-753.

de Lecea L., Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, Frankel WN, van den Pol AN, Bloom FE,

Gautvik KM, Sutcliffe JG (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A 95:322-327.

de Vries MG, Arseneau LM, Lawson ME, Beverly JL (2003) Extracellular glucose in rat ventromedial hypothalamus during acute and recurrent hypoglycemia. Diabetes 52:2767-2773.

Dean JB, Boulant JA (1989) In vitro localization of thermosensitive neurons in the rat diencephalon. Am J Physiol 257:R57-R64.

Dube MG, Kalra SP, Kalra PS (1999) Food intake elicited by central administration of orexins/hypocretins: identification of hypothalamic sites of action. Brain Res 842:473-477.

Duncan EA, Rider TR, Jandacek RJ, Clegg DJ, Benoit SC, Tso P, Woods SC (2006) The regulation of alcohol intake by melanin-concentrating hormone in rats. Pharmacol Biochem Behav 85:728-735.

Dunn-Meynell AA, Routh VH, Kang L, Gaspers L, Levin BE (2002) Glucokinase is the likely mediator of glucosensing in both glucose-excited and glucose-inhibited central neurons. Diabetes 51:2056-2065.

Eggermann E, Bayer L, Serafin M, Saint-Mleux B, Bernheim L, Machard D, Jones BE, Muhlethaler M (2003) The wake-promoting hypocretin-orexin neurons are in an intrinsic state of membrane depolarization. J Neurosci 23:1557-1562.

Eggermann E, Serafin M, Bayer L, Machard D, Saint-Mleux B, Jones BE, Muhlethaler M (2001) Orexins/hypocretins excite basal forebrain cholinergic neurones. Neuroscience 108:177-181.

Eriksson KS, Stevens DR, Haas HL (2000) Opposite modulation of histaminergic neurons by nociceptin and morphine. Neuropharmacology 39:2492-2498.

Estabrooke IV, McCarthy MT, Ko E, Chou TC, Chemelli RM, Yanagisawa M, Saper CB, Scammell TE (2001) Fos expression in orexin neurons varies with behavioral state. J Neurosci 21:1656-1662.

Farhang B, Pietruszewski L, Lutfy K, Wagner EJ (2010) The role of the NOP receptor in regulating food intake, meal pattern, and the excitability of proopiomelanocortin neurons. Neuropharmacology 59:190-200.

Ferguson AV, Samson WK (2003) The orexin/hypocretin system: a critical regulator of neuroendocrine and autonomic function. Front Neuroendocrinol 24:141-150.

Ferri CC, Yuill EA, Ferguson AV (2005) Interleukin-1beta depolarizes magnocellular neurons in the paraventricular nucleus of the hypothalamus through prostaglandin-mediated activation of a non selective cationic conductance. Regul Pept 129:63-71.

Flier JS, Maratos-Flier E (1998) Obesity and the hypothalamus: novel peptides for new pathways. Cell 92:437-440.

Gavioli EC, Rae GA, Calo' G, Guerrini R, De Lima TC (2002) Central injections of nocistatin or its C-terminal hexapeptide exert anxiogenic-like effect on behaviour of mice in the plus-maze test. Br J Pharmacol 136:764-772.

Gaykema RP, Goehler LE (2009) Lipopolysaccharide challenge-induced suppression of Fos in hypothalamic orexin neurons: their potential role in sickness behavior. Brain Behav Immun 23:926-930.

Gaykema RP, Park SM, McKibbin CR, Goehler LE (2008) Lipopolysaccharide suppresses activation of the tuberomammillary histaminergic system concomitant with behavior: a novel target of immune-sensory pathways. Neuroscience 152:273-287.

Georgescu D, Sears RM, Hommel JD, Barrot M, Bolanos CA, Marsh DJ, Bednarek MA, Bibb JA, Maratos-Flier E, Nestler EJ, DiLeone RJ (2005) The hypothalamic neuropeptide melanin-concentrating hormone acts in the nucleus accumbens to modulate feeding behavior and forced-swim performance. J Neurosci 25:2933-2940.

Georgescu D, Zachariou V, Barrot M, Mieda M, Willie JT, Eisch AJ, Yanagisawa M, Nestler EJ, DiLeone RJ (2003) Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. J Neurosci 23:3106-3111.

Gerashchenko D, Horvath TL, Xie XS (2010) Direct inhibition of hypocretin/orexin neurons in the lateral hypothalamus by nociceptin/orphanin FQ blocks stress-induced analgesia in rats. Neuropharmacology.

Gervasoni D, Darracq L, Fort P, Souliere F, Chouvet G, Luppi PH (1998) Electrophysiological evidence that noradrenergic neurons of the rat locus coeruleus are tonically inhibited by GABA during sleep. Eur J Neurosci 10:964-970.

Gervasoni D, Peyron C, Rampon C, Barbagli B, Chouvet G, Urbain N, Fort P, Luppi PH (2000) Role and origin of the GABAergic innervation of dorsal raphe serotonergic neurons. J Neurosci 20:4217-4225.

Gold RM (1966) Aphagia and adipsia produced by unilateral hypothalamic lesions in rats. Am J Physiol 211:1274-1276.

Gomori A, Ishihara A, Ito M, Mashiko S, Matsushita H, Yumoto M, Ito M, Tanaka T, Tokita S, Moriya M, Iwaasa H, Kanatani A (2003) Chronic intracerebroventricular infusion of MCH causes obesity in mice. Melanin-concentrating hormone. Am J Physiol Endocrinol Metab 284:E583-E588.

Gompf HS, Moldavan MG, Irwin RP, Allen CN (2005) Nociceptin/orphanin FQ (N/OFQ) inhibits excitatory and inhibitory synaptic signaling in the suprachiasmatic nucleus (SCN). Neuroscience 132:955-965.

Gonzalez JA, Horjales-Araujo E, Fugger L, Broberger C, Burdakov D (2009a) Stimulation of orexin/hypocretin neurones by thyrotropin-releasing hormone. J Physiol 587:1179-1186.

Gonzalez JA, Jensen LT, Fugger L, Burdakov D (2008) Metabolism-independent sugar sensing in central orexin neurons. Diabetes 57:2569-2576.

Gonzalez JA, Reimann F, Burdakov D (2009b) Dissociation between sensing and metabolism of glucose in sugar sensing neurones. J Physiol 587:41-48.

Green MK, Devine DP (2009) Nociceptin/orphanin FQ and NOP receptor gene regulation after acute or repeated social defeat stress. Neuropeptides 43:507-514.

Gribble FM, Ashfield R, Ammala C, Ashcroft FM (1997) Properties of cloned ATP-sensitive K+ currents expressed in Xenopus oocytes. J Physiol 498 (Pt 1):87-98.

Griebel G, Perrault G, Sanger DJ (1999) Orphanin FQ, a novel neuropeptide with anti-stress-like activity. Brain Res 836:221-224.

Griffin JD, Kaple ML, Chow AR, Boulant JA (1996) Cellular mechanisms for neuronal thermosensitivity in the rat hypothalamus. J Physiol 492 (Pt 1):231-242.

Grossman SP, Grossman L (1982) Iontophoretic injections of kainic acid into the rat lateral hypothalamus: effects on ingestive behavior. Physiol Behav 29:553-559.

Guilleminault C, Carskadon M, Dement WC (1974) On the treatment of rapid eye movement narcolepsy. Arch Neurol 30:90-93.

Gulia KK, Mallick HN, Kumar VM (2003) Orexin A (hypocretin-1) application at the medial preoptic area potentiates male sexual behavior in rats. Neuroscience 116:921-923.

Guyon A, Tardy MP, Rovere C, Nahon JL, Barhanin J, Lesage F (2009) Glucose inhibition persists in hypothalamic neurons lacking tandem-pore K+ channels. J Neurosci 29:2528-2533.

Hagan JJ, et al. (1999) Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc Natl Acad Sci U S A 96:10911-10916.

Hahn JD (2010) Comparison of melanin-concentrating hormone and hypocretin/orexin peptide expression patterns in a current parceling scheme of the lateral hypothalamic zone. Neurosci Lett 468:12-17.

Hakansson ML, Brown H, Ghilardi N, Skoda RC, Meister B (1998) Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neurosci 18:559-572.

Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T (2001) Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron 30:345-354.

Hara J, Gerashchenko D, Wisor JP, Sakurai T, Xie X, Kilduff TS (2009) Thyrotropin-releasing hormone increases behavioral arousal through modulation of hypocretin/orexin neurons. J Neurosci 29:3705-3714.

Harris GC, Aston-Jones G (2006) Arousal and reward: a dichotomy in orexin function. Trends in Neurosciences 29:571-577.

Harris GC, Wimmer M, Aston-Jones G (2005) A role for lateral hypothalamic orexin neurons in reward seeking. Nature 437:556-559.

Hassani OK, Lee MG, Jones BE (2009) Melanin-concentrating hormone neurons discharge in a reciprocal manner to orexin neurons across the sleep-wake cycle. Proc Natl Acad Sci U S A 106:2418-2422.

Hawes BE, Fried S, Yao X, Weig B, Graziano MP (1998) Nociceptin (ORL-1) and mu-opioid receptors mediate mitogen-activated protein kinase activation in CHO cells through a Gi-coupled signaling pathway: evidence for distinct mechanisms of agonist-mediated desensitization. J Neurochem 71:1024-1033.

Haynes AC, Jackson B, Chapman H, Tadayyon M, Johns A, Porter RA, Arch JR (2000) A selective orexin-1 receptor antagonist reduces food consumption in male and female rats. Regul Pept 96:45-51.

Hervieu GJ, Cluderay JE, Harrison D, Meakin J, Maycox P, Nasir S, Leslie RA (2000) The distribution of the mRNA and protein products of the melanin-concentrating hormone (MCH) receptor gene, slc-1, in the central nervous system of the rat. Eur J Neurosci 12:1194-1216.

Hetherington AW, Ranson SW (1940) Hypothalamic lesions and adiposity in the rat. Anat Rec 78:149-172.

Hirasawa M, Mouginot D, Kozoriz MG, Kombian SB, Pittman QJ (2003) Vasopressin differentially modulates non-NMDA receptors in vasopressin and oxytocin neurons in the supraoptic nucleus. J Neurosci 23:4270-4277.

Hori A, Minato K, Kobayashi S (1999) Warming-activated channels of warm-sensitive neurons in rat hypothalamic slices. Neurosci Lett 275:93-96.

Horvath TL, Peyron C, Diano S, Ivanov A, Aston-Jones G, Kilduff TS, van den Pol AN (1999) Hypocretin (orexin) activation and synaptic innervation of the locus coeruleus noradrenergic system. J Comp Neurol 415:145-159.

Huang H, Acuna-Goycolea C, Li Y, Cheng HM, Obrietan K, van den Pol AN (2007) Cannabinoids excite hypothalamic melanin-concentrating hormone but inhibit hypocretin/orexin neurons: implications for cannabinoid actions on food intake and cognitive arousal. J Neurosci 27:4870-4881.

Huang ZL, Qu WM, Li WD, Mochizuki T, Eguchi N, Watanabe T, Urade Y, Hayaishi O (2001) Arousal effect of orexin A depends on activation of the histaminergic system. Proc Natl Acad Sci U S A 98:9965-9970.

Ikeda K, Kobayashi K, Kobayashi T, Ichikawa T, Kumanishi T, Kishida H, Yano R, Manabe T (1997) Functional coupling of the nociceptin/orphanin FQ receptor with the G-protein-activated K+ (GIRK) channel. Brain Res Mol Brain Res 45:117-126.

Jain MR, Horvath TL, Kalra PS, Kalra SP (2000) Evidence that NPY Y1 receptors are involved in stimulation of feeding by orexins (hypocretins) in sated rats. Regul Pept 87:19-24.

Jaszberenyi M, Bujdoso E, Kiss E, Pataki I, Telegdy G (2002) The role of NPY in the mediation of orexin-induced hypothermia. Regul Pept 104:55-59.

Jenck F, Moreau JL, Martin JR, Kilpatrick GJ, Reinscheid RK, Monsma FJ, Jr., Nothacker HP, Civelli O (1997) Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress. Proc Natl Acad Sci U S A 94:14854-14858.

Jenck F, Wichmann J, Dautzenberg FM, Moreau JL, Ouagazzal AM, Martin JR, Lundstrom K, Cesura AM, Poli SM, Roever S, Kolczewski S, Adam G, Kilpatrick G (2000) A synthetic agonist at the orphanin FQ/nociceptin receptor ORL1: anxiolytic profile in the rat. Proc Natl Acad Sci U S A 97:4938-4943.

Johnson PL, Truitt W, Fitz SD, Minick PE, Dietrich A, Sanghani S, Traskman-Bendz L, Goddard AW, Brundin L, Shekhar A (2010) A key role for orexin in panic anxiety. Nat Med 16:111-115.

Kang D, Choe C, Kim D (2005) Thermosensitivity of the two-pore domain K+ channels TREK-2 and TRAAK. J Physiol 564:103-116.

Kang L, Dunn-Meynell AA, Routh VH, Gaspers LD, Nagata Y, Nishimura T, Eiki J, Zhang BB, Levin BE (2006) Glucokinase is a critical regulator of ventromedial hypothalamic neuronal glucosensing. Diabetes 55:412-420.

Kang L, Routh VH, Kuzhikandathil EV, Gaspers LD, Levin BE (2004) Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons. Diabetes 53:549-559.

Karschin C, Ecke C, Ashcroft FM, Karschin A (1997) Overlapping distribution of K(ATP) channel-forming Kir6.2 subunit and the sulfonylurea receptor SUR1 in rodent brain. FEBS Lett 401:59-64.

Kauer JA, Malenka RC (2007) Synaptic plasticity and addiction. Nat Rev Neurosci 8:844-858.

Kawauchi H, Kawazoe I, Tsubokawa M, Kishida M, Baker BI (1983) Characterization of melanin-concentrating hormone in chum salmon pituitaries. Nature 305:321-323.

Kayaba Y, Nakamura A, Kasuya Y, Ohuchi T, Yanagisawa M, Komuro I, Fukuda Y, Kuwaki T (2003) Attenuated defense response and low basal blood pressure in orexin knockout mice. Am J Physiol Regul Integr Comp Physiol 285:R581-R593.

Kennedy AR, Todd JF, Dhillo WS, Seal LJ, Ghatei MA, O'Toole CP, Jones M, Witty D, Winborne K, Riley G, Hervieu G, Wilson S, Bloom SR (2003) Effect of direct injection of melanin-concentrating hormone into the paraventricular nucleus: further evidence for a stimulatory role in the adrenal axis via SLC-1. J Neuroendocrinol 15:268-272.

Ko EA, Han J, Jung ID, Park WS (2008) Physiological roles of K+ channels in vascular smooth muscle cells. J Smooth Muscle Res 44:65-81.

Kokkotou E, Jeon JY, Wang X, Marino FE, Carlson M, Trombly DJ, Maratos-Flier E (2005) Mice with MCH ablation resist diet-induced obesity through strain-specific mechanisms. Am J Physiol Regul Integr Comp Physiol 289:R117-R124.

Kong D, Vong L, Parton LE, Ye C, Tong Q, Hu X, Choi B, Bruning JC, Lowell BB (2010) Glucose stimulation of hypothalamic MCH neurons involves K(ATP) channels, is modulated by UCP2, and regulates peripheral glucose homeostasis. Cell Metab 12:545-552.

Konsman JP, Parnet P, Dantzer R (2002) Cytokine-induced sickness behaviour: mechanisms and implications. Trends Neurosci 25:154-159.

Korotkova TM, Sergeeva OA, Eriksson KS, Haas HL, Brown RE (2003) Excitation of ventral tegmental area dopaminergic and nondopaminergic neurons by orexins/hypocretins. Journal of Neuroscience 23:7-11.

Koster A, Montkowski A, Schulz S, Stube EM, Knaudt K, Jenck F, Moreau JL, Nothacker HP, Civelli O, Reinscheid RK (1999) Targeted disruption of the orphanin FQ/nociceptin gene increases stress susceptibility and impairs stress adaptation in mice. Proc Natl Acad Sci U S A 96:10444-10449.

Kotlinska J, Rafalski P, Biala G, Dylag T, Rolka K, Silberring J (2003) Nociceptin inhibits acquisition of amphetamine-induced place preference and sensitization to stereotypy in rats. Eur J Pharmacol 474:233-239.

Krippeit-Drews P, Kramer C, Welker S, Lang F, Ammon HP, Drews G (1999) Interference of H2O2 with stimulus-secretion coupling in mouse pancreatic beta-cells. J Physiol 514 (Pt 2):471-481.

Kuru M, Ueta Y, Serino R, Nakazato M, Yamamoto Y, Shibuya I, Yamashita H (2000) Centrally administered orexin/hypocretin activates HPA axis in rats. Neuroreport 11:1977.

Kuzmin A, Bazov I, Sheedy D, Garrick T, Harper C, Bakalkin G (2009) Expression of pronociceptin and its receptor is downregulated in the brain of human alcoholics. Brain Res 1305 Suppl:S80-S85.

Lammers GJ, Pijl H, Iestra J, Langius JA, Buunk G, Meinders AE (1996) Spontaneous food choice in narcolepsy. Sleep 19:75-76.

Lee K, Dixon AK, Richardson PJ, Pinnock RD (1999) Glucose-receptive neurones in the rat ventromedial hypothalamus express KATP channels composed of Kir6.1 and SUR1 subunits. J Physiol 515 (Pt 2):439-452.

Lee K, Nicholson JR, McKnight AT (1997) Nociceptin hyperpolarises neurones in the rat ventromedial hypothalamus. Neuroscience letters 239:37-40.

Lee MG, Hassani OK, Jones BE (2005) Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. J Neurosci 25:6716-6720.

Leggett JD, Dawe KL, Jessop DS, Fulford AJ (2009) Endogenous nociceptin / orphanin FQ system involvement in hypothalamic-pituitary-adrenal axis responses: relevance to models of inflammation. J Neuroendocrinol 21:888-897.

Levin BE (2001) Glucosensing neurons do more than just sense glucose. Int J Obes Relat Metab Disord 25 Suppl 5:S68-S72.

Levin BE, Dunn-Meynell AA, Routh VH (1999) Brain glucose sensing and body energy homeostasis: role in obesity and diabetes. Am J Physiol 276:R1223-R1231.

Levin BE, Routh VH, Kang L, Sanders NM, Dunn-Meynell AA (2004) Neuronal glucosensing: what do we know after 50 years? Diabetes 53:2521-2528.

Li Y, Gao XB, Sakurai T, van den Pol AN (2002) Hypocretin/Orexin excites hypocretin neurons via a local glutamate neuron-A potential mechanism for orchestrating the hypothalamic arousal system. Neuron 36:1169-1181.

Li Y, Li S, Wei C, Wang H, Sui N, Kirouac GJ (2010) Orexins in the paraventricular nucleus of the thalamus mediate anxiety-like responses in rats. Psychopharmacology (Berl) 212:251-265.

Li Y, van den Pol AN (2005) Direct and indirect inhibition by catecholamines of hypocretin/orexin neurons. J Neurosci 25:173-183.

Li Y, van den Pol AN (2006) Differential target-dependent actions of coexpressed inhibitory dynorphin and excitatory hypocretin/orexin neuropeptides. J Neurosci 26:13037-13047.

Li Y, van den Pol AN (2008) Mu-opioid receptor-mediated depression of the hypothalamic hypocretin/orexin arousal system. J Neurosci 28:2814-2819.

Liao YY, Teng SF, Lin LC, Kolczewski S, Prinssen EP, Lee LJ, Ho IK, Chiou LC (2010) Functional heterogeneity of nociceptin/orphanin FQ receptors revealed by (+)-5a Compound and Ro 64-6198 in rat periaqueductal grey slices. Int J Neuropsychopharmacol1-13.

Liddell JR, Zwingmann C, Schmidt MM, Thiessen A, Leibfritz D, Robinson SR, Dringen R (2009) Sustained hydrogen peroxide stress decreases lactate production by cultured astrocytes. J Neurosci Res 87:2696-2708.

Light PE, Bladen C, Winkfein RJ, Walsh MP, French RJ (2000) Molecular basis of protein kinase C-induced activation of ATP-sensitive potassium channels. Proc Natl Acad Sci U S A 97:9058-9063.

Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, Qiu X, de Jong PJ, Nishino S, Mignot E (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98:365-376.

Liss B, Bruns R, Roeper J (1999) Alternative sulfonylurea receptor expression defines metabolic sensitivity of K-ATP channels in dopaminergic midbrain neurons. EMBO J 18:833-846.

Liu RJ, van den Pol AN, Aghajanian GK (2002) Hypocretins (orexins) regulate serotonin neurons in the dorsal raphe nucleus by excitatory direct and inhibitory indirect actions. J Neurosci 22:9453-9464.

Liu XH, Morris R, Spiller D, White M, Williams G (2001) Orexin a preferentially excites glucose-sensitive neurons in the lateral hypothalamus of the rat in vitro. Diabetes 50:2431-2437.

Lou LG, Ma L, Pei G (1997) Nociceptin/orphanin FQ activates protein kinase C, and this effect is mediated through phospholipase C/Ca2+ pathway. Biochem Biophys Res Commun 240:304-308.

Ludwig DS, Tritos NA, Mastaitis JW, Kulkarni R, Kokkotou E, Elmquist J, Lowell B, Flier JS, Maratos-Flier E (2001) Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance. J Clin Invest 107:379-386.

Luo C, Kumamoto E, Furue H, Yoshimura M (2001) Nociceptin-induced outward current in substantia gelatinosa neurones of the adult rat spinal cord. Neuroscience 108:323-330.

Lynch RM, Tompkins LS, Brooks HL, Dunn-Meynell AA, Levin BE (2000) Localization of glucokinase gene expression in the rat brain. Diabetes 49:693-700.

Madamba SG, Schweitzer P, Siggins GR (1999) Nociceptin augments K(+) currents in hippocampal CA1 neurons by both ORL-1 and opiate receptor mechanisms. J Neurophysiol 82:1776-1785.

Magistretti PJ, Pellerin L, Martin JL (1995) Brain energy metabolism: an integrated cellular perspective. In: Neuropsychopharmacology: The Fourth Generation of Progress (Bloom F, Kupfer DJ, eds), pp 657-670. New York: Lippincott Williams & Wilkins.

Maidment NT, Chen Y, Tan AM, Murphy NP, Leslie FM (2002) Rat ventral midbrain dopamine neurons express the orphanin FQ/nociceptin receptor ORL-1. Neuroreport 13:1137-1140.

Maolood N, Meister B (2010) Nociceptin/orphanin FQ peptide in hypothalamic neurones associated with the control of feeding behaviour. J Neuroendocrinol 22:75-82.

Margules DL, Olds J (1962) Identical "feeding" and "rewarding" systems in the lateral hypothalamus of rats. Science 135:374-375.

Marty N, Dallaporta M, Thorens B (2007) Brain glucose sensing, counterregulation, and energy homeostasis. Physiology 22:241-251.

Mathis JP, Goldberg IE, Letchworth SR, Ryan-Moro JP, Pasternak GW (1999) Identification of a high-affinity orphanin FQ/nociceptin(1-11) binding site in mouse brain. Synapse 34:181-186.

Mathis JP, Ryan-Moro J, Chang A, Hom JS, Scheinberg DA, Pasternak GW (1997) Biochemical evidence for orphanin FQ/nociceptin receptor heterogeneity in mouse brain. Biochem Biophys Res Commun 230:462-465.

Matsuda T, Hori T, Nakashima T (1992) Thermal and PGE2 sensitivity of the organum vasculosum lamina terminalis region and preoptic area in rat brain slices. J Physiol 454:197-212.

Meis S (2003) Nociceptin/orphanin FQ: actions within the brain. Neuroscientist 9:158-168.

Meis S, Munsch T, Pape HC (2002) Antioscillatory effects of nociceptin/orphanin FQ in synaptic networks of the rat thalamus. J Neurosci 22:718-727.

Methippara MM, Alam MN, Szymusiak R, McGinty D (2003) Preoptic area warming inhibits wake-active neurons in the perifornical lateral hypothalamus. Brain Res 960:165-173.

Meunier JC (1997) Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. Eur J Pharmacol 340:1-15.

Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377:532-535.

Miki T, Liss B, Minami K, Shiuchi T, Saraya A, Kashima Y, Horiuchi M, Ashcroft F, Minokoshi Y, Roeper J, Seino S (2001) ATP-sensitive K+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. Nat Neurosci 4:507-512.

Miki T, Nagashima K, Tashiro F, Kotake K, Yoshitomi H, Tamamoto A, Gonoi T, Iwanaga T, Miyazaki J, Seino S (1998) Defective insulin secretion and enhanced insulin action in KATP channel-deficient mice. Proc Natl Acad Sci U S A 95:10402-10406.

Miki T, Seino S (2005) Roles of KATP channels as metabolic sensors in acute metabolic changes. J Mol Cell Cardiol 38:917-925.

Mileykovskiy BY, Kiyashchenko LI, Siegel JM (2005) Behavioral correlates of activity in identified hypocretin/orexin neurons. Neuron 46:787-798.

Miller TR, Fulford AJ (2007) Regulation of nociceptin/orphaninFQ secretion by immune cells and functional modulation of interleukin-2. Peptides 28:2243-2252.

Mobbs CV, Kow LM, Yang XJ (2001) Brain glucose-sensing mechanisms: ubiquitous silencing by aglycemia vs. hypothalamic neuroendocrine responses. Am J Physiol Endocrinol Metab 281:E649-E654.

Mochizuki T, Klerman EB, Sakurai T, Scammell TE (2006) Elevated body temperature during sleep in orexin knockout mice. Am J Physiol Regul Integr Comp Physiol 291:R533-R540.

Mogil JS, Grisel JE, Reinscheid RK, Civelli O, Belknap JK, Grandy DK (1996) Orphanin FQ is a functional anti-opioid peptide. Neuroscience 75:333-337.

Monda M, Viggiano A, Mondola P, De L, V (2001) Inhibition of prostaglandin synthesis reduces hyperthermic reactions induced by hypocretin-1/orexin A. Brain Res 909:68-74.

Morens C, Norregaard P, Receveur JM, van DG, Scheurink AJ (2005) Effects of MCH and a MCH1-receptor antagonist on (palatable) food and water intake. Brain Res 1062:32-38.

Muroya S, Uramura K, Sakurai T, Takigawa M, Yada T (2001) Lowering glucose concentrations increases cytosolic Ca2+ in orexin neurons of the rat lateral hypothalamus. Neurosci Lett 309:165-168.

Murphy NP, Maidment NT (1999) Orphanin FQ/nociceptin modulation of mesolimbic dopamine transmission determined by microdialysis. J Neurochem 73:179-186.

Nakamura T, Uramura K, Nambu T, Yada T, Goto K, Yanagisawa M, Sakurai T (2000) Orexin-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system. Brain research 873:181-187.

Neal CR, Jr., Mansour A, Reinscheid R, Nothacker HP, Civelli O, Akil H, Watson SJ, Jr. (1999a) Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding. J Comp Neurol 412:563-605.

Neal CR, Jr., Mansour A, Reinscheid R, Nothacker HP, Civelli O, Watson SJ, Jr. (1999b) Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. J Comp Neurol 406:503-547.

Nichols CG (2006) K-ATP channels as molecular sensors of cellular metabolism. Nature 440:470-476.

Nishi M, Houtani T, Noda Y, Mamiya T, Sato K, Doi T, Kuno J, Takeshima H, Nukada T, Nabeshima T, Yamashita T, Noda T, Sugimoto T (1997) Unrestrained nociceptive

response and disregulation of hearing ability in mice lacking the nociceptin/orphaninFQ receptor. EMBO J 16:1858-1864.

Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E (2000) Hypocretin (orexin) deficiency in human narcolepsy. Lancet 355:39-40.

Pachoud B, Adamantidis A, Ravassard P, Luppi PH, Grisar T, Lakaye B, Salin PA (2010) Major impairments of glutamatergic transmission and long-term synaptic plasticity in the hippocampus of mice lacking the melanin-concentrating hormone receptor-1. J Neurophysiol 104:1417-1425.

Palumbo EJ, Sweatt JD, Chen SJ, Klann E (1992) Oxidation-induced persistent activation of protein kinase C in hippocampal homogenates. Biochem Biophys Res Commun 187:1439-1445.

Park ES, Yi SJ, Kim JS, Lee HS, Lee IS, Seong JK, Jin HK, Yoon YS (2004) Changes in orexin-A and neuropeptide Y expression in the hypothalamus of the fasted and high-fat diet fed rats. J Vet Sci 5:295-302.

Park SM, Gaykema RP, Goehler LE (2008) How does immune challenge inhibit ingestion of palatable food? Evidence that systemic lipopolysaccharide treatment modulates key nodal points of feeding neurocircuitry. Brain Behav Immun 22:1160-1172.

Parsons MP, Hirasawa M (2010a) ATP-sensitive potassium channel-mediated lactate effect on orexin neurons: implications for brain energetics during arousal. J Neurosci 30:8061-8070.

Parsons MP, Hirasawa M (2010b) GIRK channel-mediated inhibition of melanin-concentrating hormone neurons by nociceptin/orphanin FQ. J Neurophysiol.

Parton LE, Ye CP, Coppari R, Enriori PJ, Choi B, Zhang CY, Xu C, Vianna CR, Balthasar N, Lee CE, Elmquist JK, Cowley MA, Lowell BB (2007) Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. Nature 449:228-232.

Patapoutian A, Peier AM, Story GM, Viswanath V (2003) ThermoTRP channels and beyond: mechanisms of temperature sensation. Nat Rev Neurosci 4:529-539.

Pecchi E, Dallaporta M, Jean A, Thirion S, Troadec JD (2009) Prostaglandins and sickness behavior: old story, new insights. Physiol Behav 97:279-292.

Pellerin L, Bouzier-Sore AK, Aubert A, Serres S, Merle M, Costalat R, Magistretti PJ (2007) Activity-dependent regulation of energy metabolism by astrocytes: an update. Glia 55:1251-1262.

Pellerin L, Magistretti PJ (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci 91:10625-10629.

Peluso J, LaForge KS, Matthes HW, Kreek MJ, Kieffer BL, Gaveriaux-Ruff C (1998) Distribution of nociceptin/orphanin FQ receptor transcript in human central nervous system and immune cells. J Neuroimmunol 81:184-192.

Peyron C, Sapin E, Leger L, Luppi PH, Fort P (2009) Role of the melanin-concentrating hormone neuropeptide in sleep regulation. Peptides 30:2052-2059.

Peyron C, Tighe DK, van den Pol AN, de LL, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18:9996-10015.

Pierre K, Parent A, Jayet PY, Halestrap AP, Scherrer U, Pellerin L (2007) Enhanced expression of three monocarboxylate transporter isoforms in the brain of obese mice. J Physiol 583:469-486.

Plaza-Zabala A, Martin-Garcia E, de LL, Maldonado R, Berrendero F (2010) Hypocretins regulate the anxiogenic-like effects of nicotine and induce reinstatement of nicotine-seeking behavior. J Neurosci 30:2300-2310.

Polidori C, de Caro G, Massi M (2000) The hyperphagic effect of nociceptin/orphanin FQ in rats. Peptides 21:1051-1062.

Pomonis JD, Billington CJ, Levine AS (1996) Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats. Neuroreport 8:369-371.

Przydzial MJ, Garfield AS, Lam DD, Moore SP, Evans ML, Heisler LK (2010) Nutritional state influences Nociceptin/Orphanin FQ peptide receptor expression in the dorsal raphe nucleus. Behav Brain Res 206:313-317.

Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes WF, Przypek R, Kanarek R, Maratos-Flier E (1996) A role for melanin-concentrating hormone in the central regulation of feeding behaviour. Nature 380:243-247.

Qu L, Li Y, Tian H, Wang Z, Cui L, Jin H, Wang W, Yang L (2007a) Effects of PKC on inhibition of delayed rectifier potassium currents by N/OFQ. Biochem Biophys Res Commun 356:582-586.

Qu L, Liu X, Wu C, Leung LS (2007b) Hyperthermia decreases GABAergic synaptic transmission in hippocampal neurons of immature rats. Neurobiol Dis 27:320-327.

Reinscheid RK, Civelli O (2002) The orphanin FQ/nociceptin knockout mouse: a behavioral model for stress responses. Neuropeptides 36:72-76.

Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma Jr FJ, Civelli O (1995) Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science 270:792.

Reiss D, Wolter-Sutter A, Krezel W, Ouagazzal AM (2007) Effects of social crowding on emotionality and expression of hippocampal nociceptin/orphanin FQ system transcripts in mice. Behav Brain Res 184:167-173.

Rodi D, Polidori C, Bregola G, Zucchini S, Simonato M, Massi M (2002) Pro-nociceptin/orphanin FQ and NOP receptor mRNA levels in the forebrain of food deprived rats. Brain research 957:354-361.

Rodi D, Zucchini S, Simonato M, Cifani C, Massi M, Polidori C (2008) Functional antagonism between nociceptin/orphanin FQ (N/OFQ) and corticotropin-releasing factor (CRF) in the rat brain: evidence for involvement of the bed nucleus of the stria terminalis. Psychopharmacology (Berl) 196:523-531.

Romanovsky AA (2007) Thermoregulation: some concepts have changed. Functional architecture of the thermoregulatory system. Am J Physiol Regul Integr Comp Physiol 292:R37-R46.

Romanovsky AA, Almeida MC, Aronoff DM, Ivanov AI, Konsman JP, Steiner AA, Turek VF (2005) Fever and hypothermia in systemic inflammation: recent discoveries and revisions. Front Biosci 10:2193-2216.

Rosin DL, Weston MC, Sevigny CP, Stornetta RL, Guyenet PG (2003) Hypothalamic orexin (hypocretin) neurons express vesicular glutamate transporters VGLUT1 or VGLUT2. J Comp Neurol 465:593-603.

Routh VH (2002) Glucose-sensing neurons-Are they physiologically relevant? Physiol Behav 76:403-414.

Roy M, David NK, Danao JV, Baribault H, Tian H, Giorgetti M (2006) Genetic inactivation of melanin-concentrating hormone receptor subtype 1 (MCHR1) in mice exerts anxiolytic-like behavioral effects. Neuropsychopharmacology 31:112-120.

Sacktor TC (2008) PKMzeta, LTP maintenance, and the dynamic molecular biology of memory storage. Prog Brain Res 169:27-40.

Saito Y, Cheng M, Leslie FM, Civelli O (2001) Expression of the melanin-concentrating hormone (MCH) receptor mRNA in the rat brain. J Comp Neurol 435:26-40.

Sakamaki R, Uemoto M, Inui A, Asakawa A, Ueno N, Ishibashi C, Hirono S, Yukioka H, Kato A, Shinfuku N, Kasuga M, Katsuura G (2005) Melanin-concentrating hormone enhances sucrose intake. Int J Mol Med 15:1033-1039.

Sakoori K, Murphy NP (2004) Central administration of nociceptin/orphanin FQ blocks the acquisition of conditioned place preference to morphine and cocaine, but not conditioned place aversion to naloxone in mice. Psychopharmacology (Berl) 172:129-136.

Sakurai T (2007) The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. Nat Rev Neurosci 8:171-181.

Sakurai T, et al. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92:573-585.

Sakurai T, Nagata R, Yamanaka A, Kawamura H, Tsujino N, Muraki Y, Kageyama H, Kunita S, Takahashi S, Goto K, Koyama Y, Shioda S, Yanagisawa M (2005) Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice. Neuron 46:297-308.

Saper CB, Scammell TE, Lu J (2005) Hypothalamic regulation of sleep and circadian rhythms. Nature 437:1257-1263.

Schuld A, Hebebrand J, Geller F, Pollmacher T (2000) Increased body-mass index in patients with narcolepsy. Lancet 355:1274-1275.

Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. Nature 404:661-671.

Sears RM, Liu RJ, Narayanan NS, Sharf R, Yeckel MF, Laubach M, Aghajanian GK, DiLeone RJ (2010) Regulation of nucleus accumbens activity by the hypothalamic neuropeptide melanin-concentrating hormone. J Neurosci 30:8263-8273.

Sharif-Naeini R, Ciura S, Bourque CW (2008) TRPV1 gene required for thermosensory transduction and anticipatory secretion from vasopressin neurons during hyperthermia. Neuron 58:179-185.

Shearman LP, Camacho RE, Sloan SD, Zhou D, Bednarek MA, Hreniuk DL, Feighner SD, Tan CP, Howard AD, Van der Ploeg LH, MacIntyre DE, Hickey GJ, Strack AM (2003) Chronic MCH-1 receptor modulation alters appetite, body weight and adiposity in rats. Eur J Pharmacol 475:37-47.

Shi Y, Cui N, Shi W, Jiang C (2008) A short motif in Kir6.1 consisting of four phosphorylation repeats underlies the vascular KATP channel inhibition by protein kinase C. J Biol Chem 283:2488-2494.

Shimada M, Tritos NA, Lowell BB, Flier JS, Maratos-Flier E (1998) Mice lacking melanin-concentrating hormone are hypophagic and lean. Nature 396:670-674.

Shirasaka T, Miyahara S, Takasaki M, Kannan H (2001) Nociceptin/orphanin FQ and [Phe(1)psi(CH2-NH)Gly2]nociceptin(1-13)NH2 modulates the activity of hypothalamic paraventricular nucleus neurons in vitro. Brain Res 890:147-153.

Shram N, Netchiporouk L, Cespuglio R (2002) Lactate in the brain of the freely moving rat: voltammetric monitoring of the changes related to the sleep-wake states. Eur J Neurosci 16:461-466.

Silva JP, von MF, Howell J, Thorens B, Wolfrum C, Stoffel M (2009) Regulation of adaptive behaviour during fasting by hypothalamic Foxa2. Nature 462:646-650.

Silver IA, Erecinska M (1994) Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals. J Neurosci 14:5068-5076.

Smith DG, Davis RJ, Rorick-Kehn L, Morin M, Witkin JM, McKinzie DL, Nomikos GG, Gehlert DR (2006) Melanin-concentrating hormone-1 receptor modulates neuroendocrine, behavioral, and corticolimbic neurochemical stress responses in mice. Neuropsychopharmacology 31:1135-1145.

Song Z, Levin BE, McArdle JJ, Bakhos N, Routh VH (2001) Convergence of pre- and postsynaptic influences on glucosensing neurons in the ventromedial hypothalamic nucleus. Diabetes 50:2673-2681.

Spanswick D, Smith MA, Groppi VE, Logan SD, Ashford MLJ (1997) Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. Nature 390:521-525.

Spanswick D, Smith MA, Mirshamsi S, Routh VH, Ashford ML (2000) Insulin activates ATP-sensitive K+ channels in hypothalamic neurons of lean, but not obese rats. Nat Neurosci 3:757-758.

Stellar E (1994) The physiology of motivation. 1954. Psychol Rev 101:301-311.

Stratford TR, Holahan MR, Kelley AE (1997) Injections of nociceptin into nucleus accumbens shell or ventromedial hypothalamic nucleus increase food intake. Neuroreport 8:423-426.

Sutcliffe JG, de Lecea L (2000) The hypocretins: Excitatory neuromodulatory peptides for multiple homeostatic systems, including sleep and feeding. Journal of Neuroscience Research 62:161-168.

Sweet DC, Levine AS, Billington CJ, Kotz CM (1999) Feeding response to central orexins. Brain Res 821:535-538.

Tabernero A, Vicario C, Medina JM (1996) Lactate spares glucose as a metabolic fuel in neurons and astrocytes from primary culture. Neurosci Res 26:369-376.

Takahashi K, Lin JS, Sakai K (2006) Neuronal activity of histaminergic tuberomammillary neurons during wake-sleep states in the mouse. J Neurosci 26:10292-10298.

Takano M, Xie LH, Otani H, Horie M (1998) Cytoplasmic terminus domains of Kir6.x confer different nucleotide-dependent gating on the ATP-sensitive K+ channel. J Physiol 512 (Pt 2):395-406.

Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM (2000) Reduced number of hypocretin neurons in human narcolepsy. Neuron 27:469-474.

Thomzig A, Laube G, Pruss H, Veh RW (2005) Pore-forming subunits of K-ATP channels, Kir6.1 and Kir6.2, display prominent differences in regional and cellular distribution in the rat brain. Journal of Comparative Neurology 484:313-330.

Thomzig A, Wenzel M, Karschin C, Eaton MJ, Skatchkov SN, Karschin A, Veh RW (2001) Kir6.1 is the principal pore-forming subunit of astrocyte but not neuronal plasma membrane K-ATP channels. Mol Cell Neurosci 18:671-690.

Thorpe AJ, Kotz CM (2005) Orexin A in the nucleus accumbens stimulates feeding and locomotor activity. Brain Res 1050:156-162.

Tokube K, Kiyosue T, Arita M (1998) Effects of hydroxyl radicals on KATP channels in guinea-pig ventricular myocytes. Pflugers Arch 437:155-157.

Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH, Guan XM (1998) Distribution of orexin receptor mRNA in the rat brain. FEBS Lett 438:71-75.

Tsunematsu T, Fu LY, Yamanaka A, Ichiki K, Tanoue A, Sakurai T, van den Pol AN (2008) Vasopressin increases locomotion through a V1a receptor in orexin/hypocretin neurons: implications for water homeostasis. J Neurosci 28:228-238.

Tucker SJ, Gribble FM, Zhao C, Trapp S, Ashcroft FM (1997) Truncation of Kir6.2 produces ATP-sensitive K+ channels in the absence of the sulphonylurea receptor. Nature 387:179-183.

van den Pol AN, Acuna-Goycolea C, Clark KR, Ghosh PK (2004) Physiological properties of hypothalamic MCH neurons identified with selective expression of reporter gene after recombinant virus infection. Neuron 42:635-652.

Van den Top M, Lyons DJ, Lee K, Coderre E, Renaud LP, Spanswick D (2007) Pharmacological and molecular characterization of ATP-sensitive K+ conductances in CART and NPY/AgRP expressing neurons of the hypothalamic arcuate nucleus. Neuroscience 144:815-824.

Varty GB, Hyde LA, Hodgson RA, Lu SX, McCool MF, Kazdoba TM, Del Vecchio RA, Guthrie DH, Pond AJ, Grzelak ME, Xu X, Korfmacher WA, Tulshian D, Parker EM, Higgins GA (2005) Characterization of the nociceptin receptor (ORL-1) agonist, Ro64-6198, in tests of anxiety across multiple species. Psychopharmacology (Berl) 182:132-143.

Vaughan CW, Bagley EE, Drew GM, Schuller A, Pintar JE, Hack SP, Christie MJ (2003) Cellular actions of opioids on periaqueductal grey neurons from C57B16/J mice and mutant mice lacking MOR-1. Br J Pharmacol 139:362-367.

Vaughan CW, Christie MJ (1996) Increase by the ORL1 receptor (opioid receptor-like1) ligand, nociceptin, of inwardly rectifying K conductance in dorsal raphe nucleus neurones. Br J Pharmacol 117:1609-1611.

Vaughan CW, Connor M, Jennings EA, Marinelli S, Allen RG, Christie MJ (2001) Actions of nociceptin/orphanin FQ and other prepronociceptin products on rat rostral ventromedial medulla neurons in vitro. J Physiol 534:849-859.

Vaughan CW, Ingram SL, Christie MJ (1997) Actions of the ORL1 receptor ligand nociceptin on membrane properties of rat periaqueductal gray neurons in vitro. J Neurosci 17:996-1003.

Vega C, Sachleben R, Gozal D, Gozal E (2006) Differential metabolic adaptation to acute and long-term hypoxia in rat primary cortical astrocytes. J Neurochem 97:872-883.

Voigt JP, Nwaiser B, Rex A, Mayer C, Fink H (2004) Effect of 5-HT1A receptor activation on hypothalamic glucose. Pharmacol Res 50:359-365.

Walz W, Mukerji S (1988) Lactate release from cultured astrocytes and neurons: a comparison. Glia 1:366-370.

Waniewski RA, Martin DL (1998) Preferential utilization of acetate by astrocytes is attributable to transport. J Neurosci 18:5225-5233.

Williams RH, Alexopoulos H, Jensen LT, Fugger L, Burdakov D (2008) Adaptive sugar sensors in hypothalamic feeding circuits. Proc Natl Acad Sci U S A 105:11975-11980.

Williams RH, Jensen LT, Verkhratsky A, Fugger L, Burdakov D (2007) Control of hypothalamic orexin neurons by acid and CO2. Proc Natl Acad Sci U S A 104:10685-10690.

Willie JT, Sinton CM, Maratos-Flier E, Yanagisawa M (2008) Abnormal response of melanin-concentrating hormone deficient mice to fasting: hyperactivity and rapid eye movement sleep suppression. Neuroscience 156:819-829.

Winsky-Sommerer R, Yamanaka A, Diano S, Borok E, Roberts AJ, Sakurai T, Kilduff TS, Horvath TL, de LL (2004) Interaction between the corticotropin-releasing factor system and hypocretins (orexins): a novel circuit mediating stress response. J Neurosci 24:11439-11448.

Xie X, Wisor JP, Hara J, Crowder TL, LeWinter R, Khroyan TV, Yamanaka A, Diano S, Horvath TL, Sakurai T, Toll L, Kilduff TS (2008) Hypocretin/orexin and nociceptin/orphanin FQ coordinately regulate analgesia in a mouse model of stress-induced analgesia. J Clin Invest 118:2471-2481.

Yamada K, Ji JJ, Yuan H, Miki T, Sato S, Horimoto N, Shimizu T, Seino S, Inagaki N (2001) Protective role of ATP-sensitive potassium channels in hypoxia-induced generalized seizure. Science 292:1543-1546.

Yamamoto T, Nozaki-Taguchi N, Chiba T (2002) Analgesic effect of intrathecally administered orexin-A in the rat formalin test in the rat hot plate test. British Journal of Pharmacology 137:170-176.

Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, Tominaga M, Yagami K, Sugiyama F, Goto K, Yanagisawa M, Sakurai T (2003) Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron 38:701-713.

Yamanaka A, Tsujino N, Funahashi H, Honda K, Guan JL, Wang QP, Tominaga M, Goto K, Shioda S, Sakurai T (2002) Orexins activate histaminergic neurons via the orexin 2 receptor. Biochem Biophys Res Commun 290:1237-1245.

Yang XJ, Kow LM, Funabashi T, Mobbs CV (1999) Hypothalamic glucose sensor: similarities to and differences from pancreatic beta-cell mechanisms. Diabetes 48:1763-1772.

Yi CX, Serlie MJ, Ackermans MT, Foppen E, Buijs RM, Sauerwein HP, Fliers E, Kalsbeek A (2009) A major role for perifornical orexin neurons in the control of glucose metabolism in rats. Diabetes 58:1998-2005.

Yoshimichi G, Yoshimatsu H, Masaki T, Sakata T (2001) Orexin-A regulates body temperature in coordination with arousal status. Exp Biol Med (Maywood) 226:468-476.

Zhang S, Zeitzer JM, Yoshida Y, Wisor JP, Nishino S, Edgar DM, Mignot E (2004) Lesions of the suprachiasmatic nucleus eliminate the daily rhythm of hypocretin-1 release. Sleep 27:619-627.

Zhang W, Sunanaga J, Takahashi Y, Mori T, Sakurai T, Kanmura Y, Kuwaki T (2010) Orexin neurons are indispensable for stress-induced thermogenesis in mice. J Physiol 588:4117-4129.

Zheng F, Grandy DK, Johnson SW (2002) Actions of orphanin FQ/nociceptin on rat ventral tegmental area neurons in vitro. Br J Pharmacol 136:1065-1071.

Zheng H, Patterson LM, Berthoud HR (2007) Orexin signaling in the ventral tegmental area is required for high-fat appetite induced by opioid stimulation of the nucleus accumbens. J Neurosci 27:11075-11082.

Zhu Y, Miwa Y, Yamanaka A, Yada T, Shibahara M, Abe Y, Sakurai T, Goto K (2003) Orexin receptor type-1 couples exclusively to pertussis toxin-insensitive G-proteins, while orexin receptor type-2 couples to both pertussis toxin-sensitive and -insensitive G-proteins. J Pharmacol Sci 92:259-266.