CELLULAR MECHANISMS OF THERMOSENSING IN OREXIN NEURONS

By

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Abstract

Hypothalamic orexin neurons promote energy intake and expenditure. Thus, satiety and behavioural inactivity that accompany post-prandial rises in body temperature may, in part, be explained by the inhibition of orexin neurons. Using whole-cell patch clamp on rat brain slices, I investigated cellular mechanisms of orexin thermosensing. Orexin neurons are inhibited by elevated temperatures *in-vitro* but neighboring melanin-concentrating hormone neurons are not temperature-sensitive. Orexin neuronal inhibition is mediated by ATP-sensitive potassium channels, which are modulated by the UCP2 inhibitor genipin. Warming additionally revealed an increase in glutamatergic transmission (mEPSCs). This effect was attenuated by the transient receptor potential vanilloid-1 (TRPV1) channel inhibitor AMG9810, suggesting mediation by pre- and post-synaptic TRPV1 channels. Orexin neurons in rats overfed with high-fat western-diet had impaired thermosensing mechanisms. In conclusion, this study suggests that post-prandial thermogenesis inhibits orexin neurons to promote satiety and lethargic behaviours and additionally implicates a role for orexin thermosensing in diet-induced overconsumption.

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List of Abbreviations

ACSF, artificial cerebrospinal fluid

AgRP, agouti related peptide

AMG9810, (2*E*)-*N*-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-
propenamide

Arc, arcuate nucleus of the hypothalamus

BDNF, brain-derived neurotrophic factor

BMI, body mass index

CART, cocaine and amphetamine regulated transcript

cGMP, cyclic guanosine monophosphate

GABA, gamma-aminobutyric acid

KATP, ATP-sensitive potassium

LC, locus coeruleus

LH, lateral hypothalamus

L-NAME, L-NG-Nitroarginine methyl ester

LPS, lipopolysaccharide

MCH, melanin concentrating hormone

MCHR1, melanin concentrating hormone receptor 1

mEPSC, miniature excitatory postsynaptic current

mIPSC, miniature inhibitory postsynaptic current

NAcc, nucleus accumbens

NO, nitric oxide

NOS, nitric oxide synthase

NPY, neuropeptide Y

OX1, orexin receptor 1

OX2, orexin receptor 2

PFA, perifornical area

PI3K, phosphoinositide 3-kinase

PKA, protein kinase A

PKC, protein kinase C

PKG, protein kinase G

PO/AH, preoptic anterior hypothalamus

POMC, pro-opiomelanocortin

PVN, paraventricular nucleus

Ras, rat sarcoma

sEPSC, spontaneous excitatory postsynaptic current

SUR, sulphonylurea receptor

TRPV, transient receptor potential vanilloid

TRPV1, transient receptor potential vanilloid-1

TTX, tetrodotoxin

UCP, uncoupling protein

UCP2, uncoupling protein-2

VMH, ventromedial hypothalamus

VTA, ventral tegmental area

α-MSH, α-melanocyte stimulating hormone

Chapter 1: Introduction

1.1   The hypothalamus is critical for homeostatic regulation

Homeostasis is the maintenance of the body's internal milieu at a relatively constant condition. The maintenance of a stable internal environment is a remarkably large job for a relatively small brain structure: the hypothalamus. The hypothalamus integrates peripheral signals and communicates with numerous brain regions and peripheral targets using a variety of neurohormones and synaptic connections to carry out its functions. The hypothalamus regulates body temperature, food intake, energy expenditure, the sleep-wake cycle, motivated behaviours, thirst and circadian rhythm among other physiological functions. The hypothalamus essentially functions as a biological thermostat: it operates using a physiological set-point. If internal or external factors result in a deviation from this set-point, the hypothalamus works to correct this divergence. Good examples of the maintenance of a hypothalamic set-point are the defense of a particular body weight by balancing energy intake and expenditure (Keesey and Corbett, 1984), or the strict maintenance of a stable internal body temperature despite changes in ambient temperature (Kurz, 2008).

1.2   Hypothalamic nuclei and homeostatic control

The hypothalamus is divided up into various distinct nuclei that carry out specific functions. Several of these nuclei will be discussed in detail as they pertain to hypothalamic

regulation of energy balance and body temperature, as these topics will become the focus of the present thesis.

1.2.1   Hypothalamic control of energy balance

1.2.1.1 Lateral hypothalamus and perifornical area in the control of energy balance

The lateral hypothalamus and perifornical area (LH/PFA) have been long-known to play an important role in energy homeostasis. Early studies have demonstrated that LH/PFA lesions reduce food intake (Anand and Brobeck, 1951b;Grossman et al., 1978;Stricker et al., 1978) often to the point of starvation (Anand and Brobeck, 1951a). In addition to anorexia, LH/PFA lesions increase energy expenditure which is associated with elevated motor activity (Morrison, 1968). What is more, electrical stimulation of the LH/PFA in cats was demonstrated to induce hyperphagia (Delgado and Anand, 1953). Together, these and similar studies implicated the LH/PFA as the "feeding centre" of the brain (Elmquist et al., 1999). Two neuronal populations exist within the LH/PFA that regulate energy balance: orexin and melanin-concentrating hormone (MCH) neurons.

1.2.1.1.1   Orexin neurons

Orexin neurons produce the orexin/hypocretin neuropeptides, orexin-A and orexin-B (or hypocretin-1 and hypocretin-2, respectively) which are derived from the same precursor peptide. The orexin neuropeptides were first described by two independent research groups in 1998. One group called these newly discovered peptides hypocretins, a name derived from two words:

hypothalamus, due to the peptide's localization, and incretin, since its amino acid sequence is similar to that of the incretin family of hormones (de Lecea et al., 1998). Shortly thereafter, a second group coined the term orexin which comes from the Greek word for appetite, 'orexis', since fasted animals showed an upregulation of the orexin mRNA (Sakurai et al., 1998).

Although neurons containing the orexin neuropeptides are localized exclusively to the LH/PFA, they have widespread projections within the hypothalamus and throughout the brain (Peyron et al., 1998;Chen et al., 1999;Date et al., 1999;Nambu et al., 1999) and spinal cord (van den Pol, 1999). The densest extra-hypothalamic projection is to the noradrenergic locus coeruleus (LC) for promoting arousal, while other connections include the septal nuclei of the basal forebrain, thalamic nuclei, zona incerta, subthalamic nucleus, central gray, substantia nigra, raphe nuclei, parabrachial nuclei, medullary reticular formation and the nucleus tractus solitarius, among others (Peyron et al., 1998). This widespread distribution suggests that the orexins may play a neuromodulatory role in many brain regions (de Lecea et al., 1998).

The orexins are primarily excitatory neurotransmitters (de Lecea, 1998;Sutcliffe and de Lecea, 2000). Whole cell recordings of hypothalamic slices revealed that both inhibitory and excitatory miniature postsynaptic currents are facilitated by orexin (van den Pol et al., 1998). Two types of orexin receptors exist (OX1 and OX2), and are both responsive to the two orexin peptides. Orexin receptors can be found throughout the central nervous system, including the hippocampus, amygdala, hypothalamus, medulla, nucleus accumbens, pons, striatum, substantia nigra, thalamus, pituitary and spinal cord, among others (Hervieu et al., 2001;Cluderay et al., 2002). Orexin's effects are dose-dependent and operate via G-protein-mediated activation of plasma membrane Ca^{2+} channels which facilitate Ca^{2+} influx and cell depolarization (van den Pol et al., 1998). Intracerebroventricular (ICV) administration of orexin-A increases food intake

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(Sakurai et al., 1998;Haynes et al., 1999;Haynes et al., 2000;Rodgers et al., 2002) whereas the effects of orexin-B on food intake are inconsistent (Sakurai et al., 1998;Sweet et al., 1999;Edwards et al., 1999;Lubkin and Stricker-Krongrad, 1998;Haynes et al., 1999). In addition, both orexin-A and orexin-B increase motor activity, although the effect of orexin-B is less potent and the behavioural responses they elicit differ (Jones et al., 2001). Together, these studies suggest differential roles for orexin-A and orexin-B in energy homeostasis.

The expression of the orexin neuropeptides and their receptors are altered in different feeding states. For instance, fasting is associated with an increase in prepro-orexin mRNA (Sakurai et al. 1998), and orexin receptor OX1 and OX2 mRNA (Lu et al., 2000). Furthermore, genetic overexpression of orexin under conditions of high-fat diet can increase energy expenditure and protect against weight gain (Funato et al., 2009). Indeed, obesity-resistant rats have increased basal and orexin-A-induced physical activity and increased OX1 and OX2 mRNA expression (Teske et al., 2006).

1.2.1.1.2 MCH neurons

MCH-expressing neurons are localized in the LH/PFA as well as the zona-incerta. Hypothalamic neurons producing the MCH peptide were first described by Bittencourt and colleagues in 1992 (Bittencourt et al., 1992). Despite being densely intermingled with orexin-producing neurons in the LH/PFA, MCH neurons are an anatomically distinct neuronal population (Broberger et al., 1998;Elias et al., 1998;Peyron et al., 1998). MCH neurons, however, share many synaptic contacts with orexin neurons within the LH/PFA (Burt et al., 2011).

MCH neurons also play a role in regulating energy balance. Like the orexin-A, ICV infusion of MCH stimulates feeding (Ito et al., 2003;Rossi et al., 1999), and MCH expression levels are reversibly increased during calorie restriction (Qu et al., 1996). Deletion of the prepro-MCH gene results in reduced food intake and body weight, coupled with elevated locomotor activity (Shimada et al., 1998). In addition, disruption of the MCH receptor MCHR1 leads to hyperphagic, hyperactive mice that are resistant to diet-induced obesity (Chen et al., 2002;Marsh et al., 2002). It is also known that MCH expression is altered in different metabolic states. For instance, MCH is upregulated in leptin-deficient, genetically obese *ob/ob* mice (Qu et al., 1996), and ablation of MCH in ob/ob mice leads to a lean phenotype attributable to increased energy expenditure and core body temperature (Segal-Lieberman et al., 2003). Together, these findings demonstrate an important role for the MCH neuropeptide in regulating energy intake and expenditure.

1.2.1.2 Other hypothalamic areas involved in the control of energy balance

In addition to the LH/PFA, other hypothalamic nuclei have also been shown to play a critical role in the regulation of energy homeostasis, including but not limited to, the arcuate nucleus of the hypothalamus (ARC), the ventromedial hypothalamic nucleus (VMH), and the paraventricular nucleus (PVN). These nuclei are discussed in more detail in sections 1.2.1.2.1, 1.2.1.2.2, and 1.2.1.2.3, respectively.

1.2.1.2.1 Arcuate nucleus of the hypothalamus

Two populations of neurons in the ARC modulate food intake. One population is neuropeptide Y (NPY) and agouti-related peptide (AgRP)-producing neurons, while the other population expresses α-melanocyte-stimulating hormone (α-MSH), derived from the precursor peptide pro-opiomelanocortin (POMC), in addition to cocaine-and amphetamine-related transcript (CART) (Smith and Ferguson, 2008). The ARC is in close proximity to the median eminence, a circumventricular organ that lacks a proper blood-brain barrier. Thus, neurons in this region are thought to receive various peripheral signals conveying the energy status of the organism (Sainsbury et al., 2002).

NPY/AgRP neurons of the ARC promote positive energy balance. In fact, NPY is a potent orexigenic peptide that stimulates feeding when injected centrally (Clark et al., 1984;Levine and Morley, 1984). However, its activity is not required for feeding behaviour as mice deficient in NPY have normal food intake and body weight (Erickson et al., 1996). Furthermore, AgRP injected centrally also triggers food intake (Rossi et al., 1998). NPY/AgRP neurons project to other hypothalamic nuclei, including PVN, dorsomedial hypothalamic nucleus, and the LH/PFA, through which they regulate feeding behaviour and energy expenditure (Morton and Schwartz, 2001).

Neurons expressing α-MSH/CART within the ARC promote negative energy balance. Indeed, ICV administration of these neuropeptides reduces food intake (Tsujii and Bray, 1989;Edwards et al., 2000). Deletion of the α-MSH MC4 receptor results in hyperphagia and obesity (Huszar et al., 1997), as does deletion of the POMC gene (Yaswen et al., 1999). Anorexic α-MSH/CART neurons have been shown to be directly inhibited by neighbouring

orexigenic NPY/AgRP neurons (Sainsbury et al., 2002). Indeed, AgRP is the endogenous antagonist to MC4 receptors (Ellacott and Cone, 2004).

The two populations of neurons within the ARC are differentially regulated in obesity. For instance, the expression of orexigenic NPY and AgRP are upregulated in the hypothalamus of leptin deficient *ob/ob* mice while the expression of anorexigenic α-MSH and CART are downregulated (Morton and Schwartz, 2001).

1.2.1.2.2   Ventromedial hypothalamus

Originally referred to as the "satiety centre", the VMH was shown to stimulate satiety, and thus, was thought to functionally oppose the activity of the LH/PFA as the "feeding centre", supporting a "dual-center" hypothesis for energy homeostasis (King, 2006). Indeed, in contrast to LH/PFA lesions, VMH lesions result in hyperphagia and weight gain (Sclafani, 1971;Debons et al., 1982). Additionally, electrical stimulation of the VMH impairs food intake in fasted animals (Wyrwicka and Dobrzecka, 1960;Oomura et al., 1967). More recent work has demonstrated that POMC neurons of the ARC activate neurons of the VMH to decrease food intake (Wisse and Schwartz, 2003;Xu et al., 2003). Overall, it is now thought that damage to VMH BDNF neurons or POMC projections to the VMH underly abnormal fat metabolism and feeding behaviours associated with VMH lesions (King, 2006).

1.2.1.2.3 Paraventricular nucleus of the hypothalamus

The PVN is primarily involved in regulation of the neuroendocrine system (Hill, 2012). The PVN contains magnocellular neurons, which release vasopressin and oxytocin from the posterior pituitary, as well as medial parvocellular neurons, which regulate the production of various pituitary hormones via the release of signalling peptides (Hill, 2012). However, early lesion studies implicated the PVN in the initiation of feeding (Sainsbury et al., 2002). The PVN is thought to integrate information about energy homeostasis from NPY/AgRP and α-MSH/CART neurons of the ARC as well as the POMC neurons of the nucleus of the solitary tract (Ellacott and Cone, 2004). Release of α-MSH from POMC neurons of the ARC directly activates MC4s in the PVN to promote satiety (Hill, 2012). Meanwhile, orexigenic NPY neurons inhibit PVN activity through direct innervations of parvocellular neurons (Hill, 2012). Thus, the PVN provides an interface where neuropeptides regulating energy balance can act on the neuroendocrine system.

1.2.2 Hypothalamic regulation of body temperature

The maintenance of a stable core body temperature (CBT) is vital for survival in homeotherms (Nakamura, 2011). CBT in homeotherms is optimized to facilitate metabolic processes and bodily function. The hypothalamus is a critical brain region in controlling the thermogenic set-point of the body; large deviations from this set-point are likely to result in organ failure and death. Indeed, the hypothalamus integrates body temperature information with control of sympathetic activity to preserve a relatively constant body temperature despite environmental thermal challenges. In addition to maintaining CBT, the hypothalamus also

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mediates the rise in body temperature associated with the febrile response, in order to slow the growth of pathogens and facilitate host immune activity for rapid recovery from infection (Nakamura, 2011). The preoptic and anterior hypothalamus (PO/AH) and the LH/PFA, as discussed in sections 1.2.2.1 and 1.2.2.2, respectively, both play a role in the central homeostatic regulation of CBT.

1.2.2.1 Preoptic and anterior hypothalamus in thermoregulation

The PO/AH has been suggested to be the "thermoregulatory center" of the body, where information from peripheral and central thermosensors converge (Nakamura, 2011). Indeed, the PO/AH receives thermal information from peripheral sensory neurons of the dorsal horn via the lateral parabrachial nucleus (Nakamura, 2011). In addition, local temperature changes are also detected by PO/AH neurons. For instance, local cooling of the PO/AH results in activation of thermogenic brown adipose tissue (BAT) (Imai-Matsumura et al., 1984) as well as vasoconstriction and shivering thermogenesis (Hammel et al., 1960). In contrast, local warming of the PO/AH facilitates heat loss via cutaneous vasodilation (Freeman and Davis, 1959) and salivary secretion (Kanosue et al., 1990) in rodents. Although the PO/AH is considered to be the chief regulator of thermal homeostasis, the LH/PFA also plays a role in thermoregulation.

1.2.2.2 Lateral hypothalamus and perifornical area in thermoregulation

The LH/PFA has been implicated in the regulation of CBT. Indeed, disinhibition of LH/PFA neurons by $GABA_A$ receptor antagonist bicuculline increases BAT metabolism and

sympathetic nerve activity (Cerri and Morrison, 2005). Retrograde transport of pseudorabies virus from inoculated BAT tissue has confirmed that both orexin and MCH neurons send projections transynaptically to BAT (Oldfield et al., 2002). Indeed, orexin-A administration is associated with sympathetic activity, increased BAT temperature, and increased abdominal temperature (Monda et al., 2003). Moreover, ICV administration of orexin-1 receptor antagonist SB-334867-A increases thermogenic uncoupling protein-1 (UCP1) mRNA expression in BAT of genetically obese *ob/ob* mice (Haynes et al., 2002), suggesting that orexin-A is an important regulator of the sympathetic nervous system. In contrast, ICV infusion of MCH in mice decreases UCP1 mRNA expression in BAT and reduces CBT (Ito et al., 2003). Thus, LH/PFA neuropeptides orexin and MCH have been associated with both the activation and inhibition of sympathetic BAT thermogenesis, respectively.

It has been suggested that food intake and sympathetic activity are reciprocally-related, such that activation of the sympathetic nervous system acts as a satiety mechanism to inhibit feeding activity (Bray, 2000). Furthermore, since orexin and MCH neurons project directly to hypothalamic feeding centres as well as transynaptically to thermogenic BAT, it has been proposed that these neuronal populations act as 'command neurons' (Oldfield et al., 2007), whose role is organizing coordinated changes in food intake and energy expenditure. The relationship between thermoregulation and food intake is discussed in the following section.

1.3   Thermoregulatory control of food intake

The rise in body temperature that occurs during and following food intake is known as post-prandial thermogenesis. There are two components of eating that result in rises in body

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temperature. First, the thermic effect of the food itself, due to its breakdown, and second, the associated diet-induced thermogenesis as a result of sympathetic activity (Himms-Hagen, 1984). The theory on the thermoregulatory control of food intake proposes that post-prandial thermogenesis acts as a satiety signal to prevent overeating and hyperthermia (Brobeck, 1948). Indeed, when ambient temperatures are high, food intake is reduced, and when ambient temperatures are low, food intake is elevated (Brobeck, 1948). Other studies have confirmed an increase in food intake in the cold (Sanes et al., 1975;Fregly, 1954), and meal size has been negatively correlated with environmental temperature (Davies, 1977). Finally, increased ambient temperature is associated with lower body mass index (BMI) in humans, possibly via a reduction in appetite (Daly, 2013).

In further support of the thermoregulatory control of food intake, studies have confirmed rises in brain temperature associated with feeding (Abrams and Hammel, 1964;Rampone and Shirasu, 1964). For instance, food intake resulted in a mean rise in PO/AH temperature greater than $1°C$ in rats (Abrams and Hammel, 1964). A second study detected a mean feeding-induced rise in extra-hypothalamic intercranial temperature exceeding $0.5°C$ in fasted rats (Rampone and Shirasu, 1964). Moreover, local heating of the PO/AH reduces food intake (Andersson and Larsson, 1961;Spector et al., 1968). Together, these studies suggest that local changes in brain temperature can modify ingestive behaviour.

Changes in peripheral body temperature have also been observed with food intake. Indeed, De Vries and colleagues found the consistent termination of meals when the rat liver had a precise temperature of $39.3°C$, regardless of meal size (De Vries et al., 1993). Although similar measurements cannot be performed in humans, skin temperature increases of $0.8-1.5°C$ (proximal to the liver) during and after meal consumption have been documented in man,

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remaining high 60-90 minutes after the meal (Westerterp-Plantenga et al., 1990). Thus, both central and peripheral body temperature changes can be observed with food intake and may contribute to the thermoregulatory control of food intake. However, the mechanism by which rises in body temperature during and after a meal promote satiety, or how these mechanisms respond to changes in energy status, are not fully understood.

1.3.1   Effect of energy status on thermoregulation

The metabolic status of an organism has a significant impact on thermoregulatory processes. For instance, the genetically obese *ob/ob* mouse has a reduced CBT, thus requiring less energy to maintain its body temperature and facilitating storage of excess energy as fat (James and Trayhurn, 1981). Moreover, diet-induced thermogenesis in BAT is defective in three types of obese animals, the genetically obese *fa/fa* Zucker rat and rats with obesity induced by hypothalamic gold thioglucose lesions or surgical hypothalamic lesions (Himms-Hagen, 1984). In addition to impaired thermogenic responses, obese *fa/fa* Zucker rats also show an impaired febrile response to LPS (Rosenthal et al., 1996). These effects may be explained by the fact that *ob/ob* obese mice have reduced sympathetic activity in BAT that leads to functional atrophy, coupled with a reduction in mRNA for uncoupling protein 1 in BAT, a protein that mediates the thermogenic effect of BAT (Reichling et al., 1988). Overall, if rises in body temperature are a signal for satiety, impaired diet-induced thermogenesis in obesity could predispose animals to overeat. Whether fundamental changes in thermoregulatory mechanisms occur during short-term exposure to high-fat foods, preceding obesity, remain to be determined.

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In a state of negative energy balance, obese and normal-weight human subjects showed a reduced thermogenic response to food intake (Westerterp-Plantenga et al., 1990). Furthermore, fasted rats showed a reduced thermogenic response to LPS compared to fed controls (Inoue and Luheshi, 2010). This lack of thermogenic feedback during fasting could be a mechanism to promote food intake when energy stores are depleted. Overall, it has been suggested that the regulation of BAT thermogenesis is part of an energy buffering mechanism (Himms-Hagen, 1984), which can dissipate excess energy as heat and when defective, can result in an impaired ability to regulate energy balance.

1.3.2 Thermosensitive neurons as a mediator of thermoregulatory control of food intake

It is thought that the mechanism for the thermoregulatory control of food intake is a central mediator that is temperature-sensitive. Thermosensitive neurons are a good candidate for mediating the thermoregulatory control of food intake since they respond to central and peripheral thermal signals and are present in hypothalamic areas important for the regulation of food intake and energy expenditure.

1.4 Thermosensitive neurons are necessary for homeostatic control

In order to maintain homeostatic control of body temperature, it is vital for the brain regions responsible for thermoregulation to be able to detect temperature changes. Indeed, it is through the actions of temperature-sensitive, or thermosensitive, neurons that the PO/AH is able to integrate local and peripheral thermal information via neural connections to elicit the

appropriate thermoregulatory responses (Boulant, 2000). In addition to the homeostatic regulation of CBT, thermosensitive neurons of the PO/AH also play a role in homeostatic control of the cardiovascular system, central and peripheral osmolality, motor behaviours, emotional state, arousal, and reward (Hori, 1991). Thus, thermosensitive hypothalamic neurons may be important for coordinating both thermal and non-thermal homeostatic functions (Hori, 1991). Importantly, thermosensitive neurons may also be responsible for the homeostatic regulation of energy balance. For instance, glucose-responsive neurons of the LH/PFA involved in feeding behaviour are responsive to peripheral (Imai-Matsumura et al., 1984) and central thermal stimulation (Imai-Matsumura and Nakayama, 1983). However, the significance of thermosensitive neurons in the regulation of energy homeostasis remains to be elucidated.

1.5   Thermosensitive neurons

Various neuronal populations that have been implicated in thermoregulation respond to changes in temperature by adjusting their firing activity. There are two types of thermosensitive neurons: warm-sensitive neurons increase their firing in response to rises in brain temperature whereas cold-sensitive neurons increase their firing in response to decreases in brain temperature (Nakayama et al., 1978;Hori et al., 1980). The most widely-known thermosensitive neurons exist in the PO/AH, where they are known to play an important role in maintaining CBT. Early *in vivo* (Nakayama et al., 1961;Hardy et al., 1964) and *in vitro* (Hori et al., 1980;Kelso et al., 1982) studies confirmed that neurons in the PO/AH are temperature-sensitive. The majority of PO/AH neurons are temperature insensitive, however, approximately 30% are warm-sensitive and 10% are cold-sensitive (Kelso et al., 1982;Kobayashi, 1986).

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In addition to the PO/AH, thermosensitive neurons have been found in numerous other brain regions, including the LH/PFA (Imai-Matsumura and Nakayama, 1983;Imai-Matsumura et al., 1984;Parsons et al., 2012), PVN (Inenaga et al., 1987), VMH (Imai-Matsumura et al., 1988), medulla oblongata (Kobayashi and Murakami, 1982), hippocampus (de la Pena et al., 2005), red nucleus of the midbrain (Asami et al., 1988), dorsal root ganglia (Babes et al., 2004), and superior cervical ganglion (Smith et al., 2004). Although some populations of hypothalamic thermosensitive neurons appear to be involved in the regulation of energy balance, the functional significance of extra-hypothalamic thermosensing neurons needs to be further explored.

1.5.1   Known neuronal mechanisms for thermosensing

There have been various neuronal thermosensing mechanisms described including activation of non-selective cation channels (Hori et al., 1999), ionic currents (Zhao and Boulant, 2005), inactivation of A-currents (I_A) (Griffin et al., 1996), modulation of hyperpolarization-induced current (I_h) (Cai et al., 2012), activation of tetrodotoxin (TTX)-sensitive Na^+ channels (Kiyohara et al., 1990), as well as activation of members of the TRPV family of channels, including TRPV1 (Sharif-Naeini et al., 2008), TRPV2 (Caterina et al., 1999), TRPV3 (Xu et al., 2002), TRPV4 (Guler et al., 2002), and TRPM8 (Peier et al., 2002;McKemy et al., 2002). Moreover, our previous study has confirmed orexin neurons are thermosensitive via KATP channel activation, which is a novel thermosensing mechanism (Parsons et al., 2012).

1.5.1.1 TRPV1 channels are thermosensitive

Increasing the bath temperature in the presence of KATP channel antagonists revealed an excitatory synaptic response in orexin neurons (Parsons et al., 2012). However, the channels mediating the synaptic response to warming in orexin neurons remain unknown. Thus, in the present thesis I have investigated the role of TRPV1 channels in the excitatory thermosensory response of orexin neurons.

TRPV1 channels are the most widely known member of the TRPV family of thermosensitive channels. TRPV1 receptors are multi-state cation channels that can be activated by the vanilloid capsaicin (found in 'hot' chili peppers), heat and low pH (Caterina et al., 1997), voltage (Voets et al., 2004;Gunthorpe et al., 2000;Piper et al., 1999) and lipids (Szallasi and Blumberg, 1999;Julius and Basbaum, 2001;Caterina and Julius, 2001;Clapham, 2003;Prescott and Julius, 2003;Cortright and Szallasi, 2004;Jung et al., 2004). TRPV1 channels are additionally regulated by kinases including PKA, PKC and CaMK11 via multiple phosphorylation sites (Rosenbaum and Simon, 2007).

Generally, TRPV1 channels are expressed in various cortical and subcortical brain regions (Toth et al., 2005). Specifically, TRPV1 mRNA and protein (Mezey et al., 2000) and TRPV1-sensitive glutamatergic terminals (Sasamura et al., 1998) have been localized to hypothalamic nuclei. Although TRPV1 channels are traditionally associated with the detection of noxious stimuli in the periphery, TRPV1 presence in the brain suggests that the receptor may play a different role. Indeed, hypothalamic TRPV1 channels have been shown to participate in synaptic transmission as their activation facilitates glutamatergic signaling (Karlsson et al., 2005;Yokoyama et al., 2009).

1.5.1.2 Orexin neurons are thermosensitive via ATP-sensitive potassium channel activation

ATP-sensitive potassium (KATP) channels are inward rectifier channels that are inhibited by ATP and activated by Mg^{2+}-bound nucleotides (Nichols, 2006). These channels have been proposed to act as metabolic sensors due to their unique ability to electrically respond to changes in the cell's metabolic state (Nichols, 2006). Indeed, when metabolism is low, KATP channels are active resulting in cell hyperpolarization and inactivity (McTaggart et al., 2010). On the other hand, when metabolism is high, cells are depolarized by KATP channel closing, leading to cellular activity (McTaggart et al., 2010). KATP channels are most widely-known for their role in regulating glucose homeostasis in the pancreatic β-cell. Indeed, KATP channels are activated and promote insulin secretion from the pancreas when plasma glucose levels are decreased (McTaggart et al., 2010).

Orexin neurons possess KATP channels with four identical pore-forming Kir6.1 subunits and four identical modulatory sulfonylurea SUR1 subunits (Parsons and Hirasawa, 2010). Although Kir6.2/SUR1 subunits for KATP channels are more common in neurons (Karschin et al., 1998;Thomzig et al., 2005), the Kir6.1/SUR1 subunit combination has been reported in cholinergic interneurons (Thomzig et al., 2003) as well as pyramidal neurons and interneurons of the CA1 (Zawar et al., 1999). Our previously reported mechanism of KATP-dependent thermosensing appears to be unique to orexin neurons (Parsons et al., 2012), as no other studies, to the best of our knowledge, have reported KATP channels to be a mediator of neuronal thermosensing.

Orexin neurons respond to elevated temperatures with an inhibitory, hyperpolarizing current (Parsons et al., 2012). Other populations of neurons inhibited by rises in temperature,

including those of the PO/AH, are thought to be interneurons that receive inhibitory synaptic input from local warm-sensitive neurons (Kelso et al., 1982). On the other hand, orexin neurons are intrinsically thermosensitive, as application of TTX to block action potential-dependent activity does not abolish their ability to respond to changes in temperature (Parsons et al., 2012).

Given that orexin neuron thermosensitivity is mediated by KATP channels, it is possible that the ability of orexin neurons to detect temperature changes is modulated by factors that regulate intracellular ATP production by mitochondria. However, whether KATP channel-dependent thermosensing in orexin neurons is modulated by endogenous regulators of ATP synthesis is unknown.

1.6   Cellular metabolism and role of uncoupling proteins

Mitochondria are the metabolic energy-producing organelles of the cell. They are composed of two membranes, an inner and an outer mitochondrial membrane, separated by the intermembrane space. The innermost compartment of a mitochondrion is referred to as the matrix (see Fig. 1). During oxidative phosphorylation, hydrogen pumps in the inner membrane actively pump hydrogen ions out of the inner mitochondrial matrix and into the intermembrane space, creating a proton gradient. This gradient is used to drive the synthesis of ATP as hydrogen ions re-enter the matrix via the protein ATP synthase. Uncoupling proteins (UCPs) are mitochondrial proteins that uncouple oxidative phosphorylation from ATP synthesis (Fig. 1). UCPs provide an alternative pathway for displaced hydrogen ions to re-enter the inner mitochondrial matrix and diminish the proton gradient across the inner mitochondrial membrane.

The activity of UCPs is therefore associated with reduced ATP synthesis and reactive oxygen species (ROS) production, while releasing stored energy in the form of heat (Fig. 2).

There are several homologues of the uncoupling protein (UCP) family, including UCP1, UCP2 and UCP3. UCP1 is the most widely known UCP which is expressed in BAT and facilitates thermogenesis through its uncoupling activity. UCP3 is expressed in large quantities in muscle and BAT and its activity is known to be altered by thermogenic stimuli (Gong et al., 1997). UCP2 is the only uncoupling protein found in the brain (Fleury et al., 1997).

1.6.1   Uncoupling protein 2 modulates the activity of ATP-sensitive potassium channels

UCP2 is intensely expressed in the hypothalamus, the ventral septal region, the medulla, the ventricular system and the cerebellum (Sakurai et al., 1998). In addition to being expressed in the brain, UCP2 mRNA can also be found in BAT, white adipose tissue (WAT), skeletal muscle, gastrointestinal tract, macrophages, bone marrow, and organs including the thymus, heart, lung, kidney and spleen (Erlanson-Albertsson, 2003). Upon its initial discovery in 1997, it was proposed that UCP2 likely plays a role in energy homeostasis, including body weight, inflammation and thermoregulation (Fleury et al., 1997). UCP2 upregulation has since been associated with metabolic disorders, including genetic models of obesity such as ob/ob and db/db mice (Gimeno et al., 1997). Importantly, research in humans suggests that UCP2 mRNA expression in adipose tissue is positively correlated with BMI (Millet et al., 1997).

UCP2 appears to be significant for whole body thermoregulation. Indeed, UCP2 expression can be found in brain regions important for body temperature regulation, including the hypothalamus (Ricquier and Bouillaud, 2000), which provides sympathetic input to BAT to

promote thermogenesis. Interestingly, orexin neuron-specific UCP2 overexpression in mice results in a reduced CBT and increased lifespan (Conti et al., 2006). Although these mice show no difference in food intake when compared with control animals, they reveal an increase in energy efficiency and hypothalamic temperature. This finding suggests that the uncoupling activity of UCP2 in orexin neurons is playing an important role in thermoregulation and longevity, likely by producing heat and reducing ROS production, respectively (Conti et al., 2006). Indeed, UCP2-expressing brain regions have been shown to have an increased temperature relative to UCP2-absent brain regions (Horvath et al., 1999).

1.7 Thermosensitive orexin neurons and the thermoregulatory control of food intake

Orexin neurons are inhibited by physiological rises (37-39°C) in bath temperature *in vitro* (Parsons et al., 2012). Consequently, the inhibition of orexin neurons *in vivo* could result from physiological processes that elevate body temperatures, including fever, food intake, exercise, or by environmental temperature fluctuations. Indeed, the inhibition of orexin neurons has been demonstrated during fever (Becskei et al., 2008;Park et al., 2008;Gaykema and Goehler, 2009) when body temperatures are elevated. Additionally, rises in ambient temperature are known to reduce food intake (Brobeck, 1948).

The ability for orexin neurons to respond to temperature changes within the physiological range, coupled with the role of orexin neurons in the control of energy balance, suggests a functional role for orexin thermosensing in the regulation of food intake. Since orexin neurons promote food-seeking behaviours, the inhibition of these neurons by elevated temperatures likely attenuates food intake and locomotor activity. Indeed, the inhibition of orexin neurons during fever has been proposed to be the source of sickness behaviours associated with infection

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(Becskei et al., 2008;Gaykema and Goehler, 2009), including lethargic behaviours (reduced arousal or motivation) and anorexia (Konsman et al., 2002;De La Garza, 2005;Dantzer et al., 2008). Given that orexin neurons integrate control of food intake and sympathetic activity with thermogenic signals, these neurons could be the means by which increases in brain temperature attenuate food intake.

This hypothesis is supported by studies reporting an interaction between orexin-mediated food intake and body temperature. For instance, one study reports that under normal conditions, orexin-A administration (1.5nmol, ICV) induces hyperphagia when food is readily available, however, the same dose of orexin-A administered 6-hours prior to food presentation reduces food intake (Monda et al., 2003). The authors suggest that this effect is attributable to an orexin-A induced rise in body temperature (Monda et al., 2003). Indeed, orexin-A infusion into the third ventricle of the brain was previously shown to increase CBT in a dose-dependent manner (Yoshimichi et al., 2001). Thus, a rise in body temperature may attenuate orexin-induced eating behaviour.

In all, orexin neurons may be an important mediator of attenuated food intake during rises in body temperature, since they coordinate changes in energy balance by regulating thermogenesis and food intake. Furthermore, since orexin neurons respond to changes in energy balance, the regulation of thermosensing in orexin neurons could be altered in conditions of fasting or high-fat diet.

1.8   Rationale and objectives

Two key neuronal populations exist nearly exclusively within the LH/PFA, namely orexin neurons and MCH neurons. Both of these neuronal systems have been shown to be critical for the maintenance of energy homeostasis and play a role in thermoregulation. This thesis will focus primarily on examining the cellular mechanisms of thermosensing in orexin neurons.

1.8.1   Objective 1- Investigate potential modulators of KATP channel-dependent thermosensing in orexin neurons

Our previous work has demonstrated that warming causes the reversible inhibition of orexin neurons in a KATP-channel dependent manner. However, the mechanisms by which warming facilitates KATP current are unknown. Since UCP2 activity can modulate intracellular ATP and ultimately KATP channel activity, I explored whether this protein modulates KATP channel activation in orexin neurons during warming. Additionally, the role of nitric oxide synthesis in the thermosensing response of orexin neurons was also investigated.

1.8.2   Objective 2- Characterize the underlying mechanism for the synaptic effect of warming in orexin neurons

In addition to the inhibitory current response to warming in orexin neurons, I observed an additional excitatory synaptic effect of warming. In this thesis I describe the underlying synaptic mechanisms for an increase in excitatory transmission during warming, with an emphasis on the role of TRPV1 channels.

1.8.3 Objective 3- Explore the effects of high-fat diet on orexin thermosensing mechanisms

Orexin neurons are known to be sensitive to changes in diet. Thus, I tested the hypothesis that the thermosensing mechanisms of orexin neurons are altered by short-term exposure to high-fat, palatable diet. Potential modulators for the altered thermosensing mechanisms are discussed.

Chapter 2: Materials and Methods

2.1   Animal Model

Male Sprague-Dawley rats were obtained from a breeding colony at Memorial University of Newfoundland at 3 weeks of age and were housed in an Animal Care facility until sacrifice. Rats were fed *ad libitum* chow (LabDiet Autoclavable Rodent Diet 5010) or western-style diet (WD; TestDiet AIN-76A Western Diet, cat#1810060). Diet composition is as follows: 3.08kcal/g; 12.7% fat, 28.7% protein and 58.5% carbohydrates by calories for chow diet and 4.55kcal/g; 40.0% fat (from milk and corn oil), 15.8% protein and 44.2% carbohydrates by calories for WD. Food intake and body weight measurements were obtained just prior to sacrifice, as necessary. All manipulations were performed following guidelines set by the Canadian Council on Animal Care and were approved Memorial University of Newfoundland's Institutional Animal Care Committee.

2.2   Electrophysiology

Whole cell patch clamp recordings were performed on acute brain slices obtained from 3-4 week old male Sprague-Dawley rats. Rats were anesthetized with 4% isoflurane, sacrificed by decapitation, and brains were quickly removed and placed into 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. Coronal hypothalamic slices 250µm thick were obtained using a vibratome (VT-1000, Leica Microsystems). Following dissection, slices were incubated

in ACSF at 32-35°C for 30-45 minutes, then at room temperature prior to recording. ACSF was continuously bubbled with O_2 (95%)/CO_2 (5%).

Patch-clamp recordings were performed with an infrared-differential interference contrast optics microscope (DM-LFSA, Leica Microsystems) using a Multiclamp 700B amplifier and pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). Slices were perfused with ACSF bubbled with O_2 (95%)/CO_2 (5%) at a rate of 2.5-3.0ml/min. Cells were chosen based on their location and morphology; orexin and MCH neurons are localized to the LH/PFA, proximal and dorsal to the fornix, and are typically large in size (20-30μm) and round or oval in shape.

Recordings were conducted using conventional whole cell internal solution containing (in mM): 120 K-gluconate, 1 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 5 K_2ATP, pH 7.29-7.30 with KOH. Biocytin (1-1.5 mg/ml) was added to the internal pipette solution for post-hoc immunohistochemical phenotyping. Glass pipette electrodes had a tip resistance 2-5MΩ when filled with internal solution.

A temperature probe placed in close proximity to the slice and an in-line heater (TC-324B, Warner Instruments or HW-30, Dagan) were used to regulate and control the bath temperature. Experimental protocol involved increasing the bath temperature at a rate of approximately 3°C/min which was maintained at the peak for several minutes before returning to baseline temperature. For comparison purposes, control cells were always tested with the same temperature range as the treatment group.

When whole-cell access with a series/access resistance of 5-20MΩ was achieved, a series of depolarizing and hyperpolarizing current steps were applied (-200 to +200 pA in 100 pA increments, 300-600 ms). Orexin and MCH neurons have distinct electrophysiological

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characteristics, which in our experience are highly effective in differentiating phenotype (approximately 80-90% of cells selected in this manner are orexin or MCH-immunopositive) (Parsons and Hirasawa, 2010;Alberto and Hirasawa, 2010) (Fig. 3).

Whole-cell voltage clamp recordings were conducted at a holding potential of -50mV. Miniature excitatory postsynaptic currents (mEPSCs) were recorded with TTX (1μM) in the bath. Throughout the recording, -20mV square pulses were applied for 100ms to monitor series resistance. Experiments in which the series resistance changed by approximately >20% were excluded from analysis. At least two different animals were used for each experiment. Typically, two to four hypothalamic slices were obtained from a rat and 1 to 3 cells were recorded from each slice.

2.3   Post-hoc Immunohistochemistry

Immediately following experimentation, slices were fixed in 10% formalin and kept at 4°C for at least 16 hours. Slices were washed with PBS and treated with primary antibodies of goat anti-orexin-A (1:1000–3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-MCH (1:1000–4000; Phoenix Pharmaceuticals, Belmont, CA, USA) at 4°C for 3 days. Slices were washed and treated with Cy3-conjugated donkey anti-goat, Cy2-conjugated donkey anti-rabbit and streptavidin-conjugated AMCA (1:500; Jackson ImmunoResearch, West Grove, PA, USA) and remained at room temperature in the dark for 3 hours before wash and mounting. Slices were mounted on glass slides using Fluorescent Mounting Medium (Dako, Glostrup, Denmark) and stored at 4°C until viewing. A fluorescence microscope was used to visualize cells

stained with orexin-A (Cy3), MCH (Cy2) or biocytin (AMCA). Double-labelled cells confirmed cellular identity.

2.4   Chemical Compounds

Drugs were applied in the bath using a perfusion system. TTX was obtained from Alomone Labs (Jerusalem, Israel). Glibenclamide was obtained from Tocris Bioscience (Ellisville, MO, USA). Tolbutamide and N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Genipin was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). AMG9810 was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Antagonists and inhibitors were aliquoted and frozen until use. Drugs were applied in the bath for a minimum of 5-10 minutes before manipulating the bath temperature.

2.5   Statistical Analysis

Miniature postsynaptic currents, membrane potential and holding current were measured using Mini Analysis 6.0 (Synaptosoft) and Clampfit 9.2 (Molecular Devices). Paired and unpaired Student t-tests (one- or two-tailed) were used for two-group comparisons, while one-way ANOVA with Tukey post-test was used for multiple group comparisons. The Kolmogorov-Smirnov test was used for analysis of mEPSCs. Criteria for cell exclusion from mEPSC analysis include a frequency less than 100 events per minute or a change in mEPSC amplitude of more than 25% over the course of an experiment. Data points that were >2 S.D. away from the mean

were excluded from analysis. All data are expressed as mean $\pm$ S.E.M. A value of $p < 0.05$ was considered significant.

Chapter 3: Results

3. 1   CURRENT RESPONSE TO WARMING IN OREXIN NEURONS

3.1.1   Orexin neurons are inhibited by increases in environmental temperature

We have previously shown that orexin neurons respond to both physiological and subphysiological increases in temperature with an inhibitory current (Parsons et al., 2012). I was able to replicate these findings using whole cell voltage clamp recordings, which revealed a reversible outward current with a bath temperature increase from 32 to 37°C in the presence of TTX (1μM; n=7, p<0.005; Fig. 4A, C). This suggests that the current response to warming is intrinsic to orexin neurons and is not mediated by synaptic inputs. As can be observed in the representative trace (Fig. 4A), the KATP current closely follows the bath temperature, suggesting that even subtle temperature changes elicit a response. Furthermore, as previously demonstrated by Parsons et al. (2012), KATP current can easily be visualized with a 2°C change in temperature (37 to 39°C). Parsons et al., (2012) also show that this current is KATP channel-mediated, as KATP channel-blocker tolbutamide (100μM) abolished the warming-induced current in orexin neurons (n=5, p<0.005; Fig. 4A, B). The inhibitory current response was observed with various subphysiological and physiological temperature changes, and the magnitude of the response did not differ between groups (Fig. 4C).

3.1.2 MCH neuronal thermosensitivity is temperature-specific

In order to determine whether thermosensing was specific to orexin neurons in the LH/PFA, local MCH neurons were also tested for thermosensitivity. Like orexin neurons, MCH neurons also showed a reversible inhibitory current by increases in bath temperature from 30-36°C (p<0.05, relative to baseline change; Fig. 5A). This effect persists in the presence of TTX, suggesting that this response is intrinsic to MCH neurons (p<0.05, relative to baseline change; Fig. 5C). However, when tested with a physiological temperature increase from 37 to 40°C, MCH neurons no longer displayed an inhibitory current response (Fig. 5B). Indeed, the response was significantly attenuated relative to the subphysiological range of 30 to 36°C (Fig. 5C). Thus, unlike orexin neurons, MCH neurons are not intrinsically thermosensitive within the physiological temperature range.

3.1.3 UCP2 and NO synthesis modulates the KATP current response to warming in orexin neurons

Since the activity of UCP2 is associated with a decrease in ATP production and is expressed in the LH/PFA (Richard et al., 1999), I hypothesized that this protein may modulate the KATP current response to warming in orexin neurons. Thus, the current response to warming was tested in the presence of UCP2 inhibitor genipin (50μM). Genipin significantly reduced the peak inhibitory current response to a bath temperature increase from 32°C to 37°C in the presence of TTX (control n=7, genipin n=5, p<0.05; Fig. 6). This finding suggests that UCP2 activation in the postsynaptic neuron contributes to warming-induced KATP current.

In addition to reducing ATP production within the cell, UCP2 activity is associated with a decrease in reactive oxygen species (ROS) production (Fig. 1, Fig. 2) (Mailloux and Harper, 2011). ROS production, in turn, attenuates nitric oxide (NO) synthesis by disinhibiting enzyme NO synthase (NOS) (Kumar et al., 2009). Thus, UCP2 activity is associated with an increase in NO synthesis, which can subsequently activate KATP channels directly (Kawano et al., 2009) or via downstream pathways including Ras/PI3K (Lin et al., 2004;Mirshamsi et al., 2004) and cGMP/PKG (Chai and Lin, 2010;Negrete et al., 2011). Therefore, UCP2 could activate KATP channels by facilitating NOS activity in the postsynaptic neuron. Application of NOS inhibitor L-NAME (500μM) reduced the KATP current response to a bath temperature increase from 30-36°C in orexin neurons (control n=5, L-NAME n=6, p<0.05; Fig. 7), indicating that NO synthesis is important for modulating the postsynaptic response to warming in orexin neurons. This suggests that UCP2 could activate KATP current either directly by decreasing intracellular ATP and/or by facilitating NO synthesis.

3.2   SYNAPTIC RESPONSE TO WARMING IN OREXIN NEURONS

3.2.1   Warming facilitates excitatory transmission in orexin neurons

Although warming in orexin neurons results in an inhibitory KATP current response, the synaptic response to warming in orexin neurons has not yet been explored. Therefore, the synaptic effect of warming was investigated using voltage clamp recordings. Recordings were conducted with a holding potential of -50mV in the presence of TTX to block action potential-dependent transmitter release via membrane-bound voltage-gated sodium channels. Warming in the physiological range (37-40°C) revealed an increase in miniature excitatory postsynaptic

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currents mEPSCs (Fig. 8A). Furthermore, warming in the subphysiological range (32-37°C) was also associated with a significant and reversible increase in the frequency of mEPSCs (Fig. 8B). A cumulative distribution of mEPSC frequency at 32°C and 37°C revealed significant differences between the two conditions in each cell tested (p<0.0001, Kolmogorov-Smirnov (K-S) test; Fig 8C shows one representative cell). Analysis of a group of cells revealed a significant and reversible increase in mEPSC frequency with a bath temperature increase from 32°C to 37°C (n=6, p<0.005; Fig. 8C, inset), suggesting an increase in presynaptic excitatory neurotransmitter release. In addition, differences between mEPSC amplitude distributions are also significant in each cell tested (p<0.0001, K-S test; Fig 8D shows one representative cell). Quantification of data from a group of cells revealed that mEPSC amplitude was reversibly elevated by warming (n=7, p<0.005; Fig. 8D, inset), implying that during warming orexin neurons have enhanced sensitivity to presynaptically-released excitatory neurotransmitter. The excitatory currents that were elevated during warming in my recording conditions are likely glutamatergic. Indeed, at the holding potential of -50mV, mEPSCs were inward currents and mIPSCs were outward currents since the theoretical reversal potentials for $GABA_A$ and glutamate receptors are approximately -65mV and -10mV, respectively. Consequently, I investigated the mechanism by which warming could facilitate glutamatergic transmission in orexin neurons.

3.2.2   TRVP1 channels mediate the synaptic effect of warming

One mechanism by which heat could facilitate excitation at the orexin synapse is via the activation of transient receptor potential vanilloid-1 (TRPV1) channels. TRPV1 channels are activated by warming, and are present in the hypothalamus (Mezey et al., 2000;Sasamura et al.,

1998) where orexin neurons are located. TRPV1 channel activation is associated with calcium influx and cell depolarization (Rosenbaum and Simon, 2007). Thus, I hypothesized that TRPV1 channels could mediate the increase in mEPSC frequency or amplitude observed with warming. To investigate this possibility, the synaptic effects of warming were measured in the presence of TRPV1 channel antagonist AMG9810 (500nM). When normalized to the 32°C AMG9810 baseline, the percent increase in mEPSC frequency by warming was significantly attenuated in the presence of AMG9810 (control n=6, AMG9810 n=8, $p < 0.05$, Fig. 9A, B), suggesting that the presynaptic increase in glutamate release is mediated by TRPV1 channel activation. In addition, the percent increase in mEPSC amplitude by warming was abolished by AMG9810 (AMG9810 n=7, $p < 0.05$; Fig. 9C) relative to control cells (n=7). These results suggest that pre- and post-synaptic TRPV1 channels mediate the increase in excitatory transmission during elevated temperatures in orexin neurons. It is important to note that AMG9810 does not completely abolish the increase in synaptic activity during warming, and thus another mechanism may also be involved.

3.2.3   Synaptic response to warming is independent of KATP channel activation

Since warming also activates KATP channels in orexin neurons, I explored whether warming-induced KATP channel activity could also account for the synaptic effects of warming. Tolbutamide had no effect on the warming-induced increase in mEPSC frequency with a bath temperature change from 32 to 37°C (control n=6, tolbutamide n=5, $p > 0.05$; Fig. 10A, B). Furthermore, the KATP current response to warming was not correlated with the percent increase in the frequency of spontaneous EPSC (sEPSC) ($R^2 = 0.1525$, $p > 0.05$; Fig. 10C) or

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mEPSCs (R^2=0.2035, p>0.05; Fig. 10D), suggesting that the increase in presynaptic glutamate release by warming is independent of the inhibitory postsynaptic KATP current. Furthermore, the postsynaptic increase in mEPSC amplitude with warming was not affected by tolbutamide application (p>0.05; not shown). In addition, a linear regression analysis revealed that the percent change in mEPSC amplitude with warming was not correlated with the simultaneous KATP current response (R^2=0.009682, p>0.05; Fig. 10E). Moreover, bath application of AMG9810 in the presence of TTX had no effect on the outward current response to warming (control n=7, AMG9810 n=6, p>0.05; Fig. 11A, B). These findings suggests that during warming TRPV1 channels and KATP channels are activated simultaneously and independently of one another, with differential effects on orexin neuron excitability.

3.2.4 UCP2 does not modulate synaptic response to warming in orexin neurons

Uncoupling protein 2 (UCP2), which produces heat at the orexin synapse (Horvath et al., 1999), could be a modulator of excitatory transmission. In order to determine the role of UCP2-mediated heat production on the synaptic effects of warming, UCP2 inhibitor genipin (50μM) was applied and thermosensitivity tested. Warming in the presence of genipin did not appear to abolish the excitatory response (Fig. 12A). Indeed, orexin neurons exposed to genipin showed no change in mEPSC frequency (control n=6, genipin n=8, p>0.05, Fig. 12B) or amplitude at 37°C (control n=7, genipin n=8, p>0.05, Fig. 12C). This finding suggests that UCP2 modulates the inhibitory KATP current response but not the synaptic response to warming.

3.3 EFFECTS OF DIET ON THERMOSENSING IN OREXIN NEURONS

3.3.1 Short-term exposure to western diet (WD) induces overfeeding

Orexin neurons integrate central and peripheral signals about energy status to control food intake and energy expenditure. Palatability of food and associated environmental cues can induce food intake via orexin signaling, leading to caloric overconsumption (Choi et al., 2010). Thus, if orexin thermosensing mechanisms are important for the regulation of food intake, it is possible that these mechanisms might be altered in conditions of high-fat, palatable diet in ways to promote overeating. Therefore, I hypothesized that orexin neuron thermosensitivity is impaired following exposure to western-style diet (WD). To test this hypothesis, rats were fed WD *ad libitum* for 7-11 days prior to sampling for *in vitro* experimentation. WD rats consumed significantly more calories than their chow counterparts over a 7 day span when fed *ad libitum* (chow n=5, WD n=18, $p<0.0001$; Fig. 13A). These changes in consumption preclude any change in body weight over the 7 day period ($p>0.05$; Fig. 13B). Body weight prior to experimentation was not statistically different between groups ($p>0.05$, not shown).

3.3.2 Thermosensing mechanisms in orexin neurons are susceptible to changes in diet

The thermosensing mechanisms of orexin neurons were evaluated in WD-fed rats. Orexin neurons from rats fed WD showed a reduced peak inhibitory current response (n=5) to warming compared with those from chow-fed controls (n=7, $p<0.05$; Fig. 14A, B). The reduced response was blocked by KATP channel inhibitors tolbutamide (100μM) or glibenclamide (200μM) (WD n=5, WD + KATP inhib. n=5, $p<0.01$; Fig. 14A, B). suggesting that the attenuated response is due to reduced KATP channel activation.

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The synaptic effect of warming in orexin neurons was also investigated in WD-exposed rats (Fig. 15A). WD had no effect on mEPSC frequency or amplitude at 37°C (chow n=6, WD n=7, p>0.05; Fig. 15B, D, respectively). However, WD blocked the increase in mEPSC frequency (chow n=6, WD n=5) and amplitude (chow n=7, WD n=5) observed with warming from 32 to 37°C with warming (frequency p<0.005, amplitude p<0.05, Fig. 15C, E, respectively). Overall, these results suggest that the thermosensing mechanisms of orexin neurons are plastic and susceptible to short-term changes in diet that may contribute to overeating.

Chapter 4: Discussion

4   Cellular mechanisms of thermosensing in orexin neurons

The aim of this thesis was to examine the cellular mechanisms of thermosensing in orexin neurons. Our lab has previously shown that orexin neurons respond to rises in temperature with an inhibitory current mediated by KATP channel activation. In this thesis, I explored possible modulators of the warming-induced KATP current in orexin neurons. Additionally, I provide evidence that orexin neurons respond to warming with an increase in excitatory synaptic transmission, which is mediated by the activation of TRPV1 channels. Furthermore, I demonstrate that these thermosensing mechanisms are altered by short-term exposure to high-fat, palatable, western-diet, leading to food overconsumption.

4.1   Inhibition of orexin neurons by rises in body temperature

Our previous study has shown that orexin neurons are thermosensitive within the physiological temperature range (Parsons et al., 2012). Importantly, electrophysiological recordings from orexin neurons revealed a novel thermosensing mechanism: the reversible activation of inhibitory KATP current with an increase in bath temperature (Parsons et al., 2012). This finding indicates that orexin neurons are cold-sensitive. Whereas PO/AH cold-sensitive neurons are thought be indirect cold-sensors that receive inhibitory synaptic input from local warm-sensitive neurons (Kelso et al., 1982), orexin neurons are intrinsically thermosensitive, as

application of TTX to block action potential-dependent activity does not abolish the response (Parsons et al., 2012).

I examined the underlying thermosensing mechanisms of orexin neurons using whole cell patch clamping technique with a bath temperature increase from 32 to 37°C and from 37 to 40°C. Warming within a physiological and subphysiological range produced an inhibitory current response comparable with that of our previous study (Parsons et al., 2012). Thermosensing appears to be specific to orexin neurons in the LH/PFA, as I demonstrate that local MCH neurons are not temperature-sensitive within the physiological range. This finding is similar to that of our previous study which found that LH/PFA neurons of unknown phenotype (not orexin- or MCH-immunopositive) were also unresponsive to physiological temperature fluctuations (Parsons et al., 2012).

Together, these findings suggest that orexin neurons may be inhibited by increases in body and brain temperature within the physiological range, which occurs during fever (Dantzer, 2001), food intake (Abrams and Hammel, 1964;Rampone and Shirasu, 1964) and exercise-induced hyperthermia (Nybo, 2012). Since orexin neurons promote food intake, energy expenditure and arousal, one would expect their inhibition to reduce food intake and promote behavioural inactivity. In support of this possibility, orexin neurons show a reduction in c-Fos immunoreactivity (marker for active neurons) following treatment with the fever-inducing pyrogen LPS (Becskei et al., 2008;Park et al., 2008;Gaykema and Goehler, 2009), which is not mediated by inflammatory cytokines (Grossberg et al., 2011). What is more, LPS-induced fever is accompanied by sickness behaviours including reduced food intake (Langhans et al., 1989) and lethargy (Grossberg et al., 2011;Dantzer, 2001), behaviours which can be attributed to the inhibition of orexin neurons. Importantly, although we have observed the direct inhibition of

orexin neurons by rises in temperature *in vitro*, further studies are required to confirm the functional significance of these data *in vivo*.

4.2   Modulation of KATP channel-dependent thermosensing in orexin neurons by UCP2

KATP channel activity is known to be modulated by the intracellular ATP/ADP ratio. Thus, the KATP current response to warming in orexin neurons may be mediated by a reduction in intracellular ATP which disinhibits these channels. Indeed, increasing intracellular ATP to 13mM abolished the current response to warming in orexin neurons (Parsons et al., 2012). Consequently, endogenous modulators of intracellular ATP could alter the current response to warming in orexin neurons.

One potential endogenous modulator of intracellular ATP is the uncoupling protein (UCP2). UCP2 reduces mitochondrial ATP synthesis by uncoupling oxidative phosphorylation from ATP synthesis. Consequently, one would expect the inhibition of UCP2 to cause a respective increase in mitochondrial ATP production, promoting subsequent KATP channel closure. UCP2 has been shown to modulate KATP channels in hypothalamic neuronal populations including POMC neurons of the arcuate nucleus (Parton et al., 2007) and MCH neurons of the LH/PFA (Kong et al., 2010). Furthermore, UCP2 is known to regulate insulin secretion in pancreatic β-cells by a KATP channel-dependent mechanism (Zhang et al., 2001).

Since UCP2 mRNA (Sakurai et al., 1998) and protein (Horvath et al., 1999) are expressed in the LH/PFA, I hypothesized that UCP2 may modulate the KATP current response to warming in orexin neurons. Using whole cell patch clamp technique, I found that UCP2 inhibitor genipin reduced the KATP current response to elevated bath temperatures in orexin

neurons, implicating UCP2 as a modulator of warming-induced KATP activation in orexin neurons.

In addition to diminishing ATP production, UCP2 activity reduces the production of reactive oxygen species (ROS), which in turn inhibits nitric oxide (NO) synthesis. Since NO can activate KATP channels both directly (Kawano et al., 2009) and indirectly (Lin et al., 2004;Mirshamsi et al., 2004;Chai and Lin, 2010;Negrete et al., 2011), UCP2 may modulate KATP current by increasing NO synthesis. Indeed, application of NO synthesis inhibitor L-NAME reduced the KATP current response to warming, suggesting that NO is important for the warming-induced inhibitory current in orexin neurons. In all, it appears that UCP2 modulates KATP channel-mediated thermosensing in orexin neurons by reducing ATP production and/or increasing NO synthesis.

4.3   Synaptic effect of warming in orexin neurons is mediated by TRPV1 channels

In addition to the KATP current response, I demonstrated an excitatory synaptic effect of warming in orexin neurons that was mediated by the activation of pre- and post-synaptic TRPV1 channels. These findings are in agreement with other reports demonstrating TRPV1 channel modulation of glutamatergic EPSCs in the nucleus of the solitary tract (Doyle et al., 2002;Shoudai et al., 2010;Peters et al., 2010), dorsolateral periaqueductal gray (Xing and Li, 2007), dorsal horn of the spinal cord (Nakatsuka et al., 2002;Baccei et al., 2003;Premkumar et al., 2005;Sikand and Premkumar, 2007), spinothalamic tract (Kim et al., 2009), striatum (Musella et al., 2009), locus coeruleus (Marinelli et al., 2002), substantia nigra (Marinelli et al., 2003), as well as the paraventricular hypothalamus (Li et al., 2004;Boychuk et al., 2013).

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Since TRPV1 channel activation can induce NO synthesis (Okere et al.,
2000;Zschenderlein et al., 2011), this was a potential mechanism by which warming-induced
TRPV1 channel activity could induce KATP channel opening. Thus, I investigated whether the
KATP current and the synaptic responses are correlated. I found that application of TRPV1
antagonist AMG9810 did not abrogate the KATP current response to warming. Additionally,
KATP channel blockers tolbutamide and glibenclamide failed to attenuate the synaptic response
to warming, suggesting that the two responses are independent of one another. Together, these
findings suggest that orexin neurons respond to warming with both inhibition and excitation,
mediated by two independent mechanisms (Fig. 16). However, in our recording conditions,
KATP channel activation usually predominates (Parsons et al., 2012) and the neuron is
hyperpolarized despite depolarizing TRPV1 channel-mediated cation influx.

4.4   Technical Considerations

The present study conducted conventional whole cell recordings with a pipette filled with
internal solution containing 5mM of ATP. This concentration reflects the estimated
endogenously-produced ATP concentration in orexin neurons (Liu et al., 2011). The "clamping"
of intracellular ATP in whole cell recordings may be problematic when measuring ATP-sensitive
KATP current. Indeed, the KATP current responses observed in the present study were slightly
smaller than those of our previous study (Parsons et al., 2012), likely a consequence of using a
higher concentration of intracellular ATP in the recording pipette (5mM vs. 3mM). Indeed,
increasing the concentration of ATP in the internal solution to 13mM abolished the warming-
induced activation of KATP channels in orexin neurons (Parsons et al., 2012).

However, previous studies have observed KATP-mediated current using whole cell recording with an ATP-containing internal solution (Balfour et al., 2006;Ainscow et al., 2002;Parsons and Hirasawa, 2010;Parsons et al., 2012). This may be attributable to the idea that ATP levels in microdomains proximal to KATP channels are relatively unaffected by "clamping" of the intracellular ATP during whole cell recording (Abraham et al., 2002), suggesting that ATP introduced via the recording pipette may not significantly impair channel activity. Indeed, in the present study, KATP current was significantly increased during warming despite the use of 5mM ATP in the internal solution. Thus, although orexin KATP channels are sensitive to exogenous ATP introduced during whole cell recording, the use of endogenous levels of ATP in the internal solution does not block the activity of KATP channels. We confirmed that UCP2 modulates the KATP current response to warming in orexin neurons by demonstrating that a UCP2 inhibitor abolishes the warming-induced current. The UCP2 inhibitor genipin used in this study is a natural cross-linking reagent (Elzoghby et al., 2011). As a result, there has been some speculation as to whether the cross-linking activity of genipin could be mediating its effects on UCP2. To the contrary, genipin's inhibitory effect of action on UCP2 appears to be independent of its protein cross-linking activity, as a synthetic derivative of genipin that lacks cross-linking capability still inhibits UCP2 activity (Zhang et al., 2006). Instead, genipin is thought to act on UCP2 via direct protein-protein interaction. Thus, the inhibition of the KATP current response by genipin in the present study is likely attributable to the direct inhibition of UCP2 activity and not the cross-linking activity of the protein.

As discussed above, I observed an increase in synaptic activity with elevated bath temperatures in orexin neurons. It is possible that this increase in activity with warmer temperatures could simply be due to an increase in protein activity at higher temperatures,

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facilitating vesicle transport and release. However, this would not explain the effect of AMG9810, which attenuated the synaptic response to warming in my recording conditions. Indeed, a warming-induced increase in EPSCs was reported in neurons of the nucleus of the solitary tract, which was specific to those expressing TRPV1 (Shoudai et al., 2010;Peters et al., 2010), arguing that this phenomenon is TRPV1-mediated and not simply a warming effect. The low frequency of mEPSCs observed in MCH neurons (less than 100 mEPSCs per minute in most cases) in my recordings has prevented analysis of these events for comparison purposes. Finally, although TRPV1 channel-specific binding has been demonstrated in the lateral hypothalamus (Roberts et al., 2004), future immunohistological study needs to confirm the presence of TRPV1 receptor expression in orexin neurons to substantiate these findings.

Interestingly, although relatively few orexin neurons display inhibitory postsynaptic currents (IPSCs) in my recording conditions, those that did appeared to display an increase in IPSCs in addition to EPSC's during elevated temperatures (not shown). For purposes of time, this finding was not explored in detail. However, it is a noteworthy observation given that an increase in IPSCs coupled with the inhibitory KATP current likely supports a prevailing inhibitory effect of warming, despite an increase in excitatory transmission. The overall inhibitory effect of warming was revealed in the majority of orexin neurons in our previous study using cell-attached recordings (Parsons et al., 2012).

4.5 Western diet alters the thermosensing mechanisms of orexin neurons

4.5.1 Western diet attenuates KATP current in response to warming in orexin neurons

This study was the first to determine that the thermosensing mechanisms of orexin neurons are altered by changes in diet. Short-term exposure to WD (7-11 days) significantly attenuated the warming-induced KATP current. The current was tolbutamide-sensitive, indicating that the impaired response was due to a reduction in warming-induced channel activity. However, the underlying mechanism by which WD reduces the warming-induced KATP current in orexin neurons has not yet been elucidated.

It is possible that the attenuation of the KATP current response in condition of WD is due to direct modification of KATP channel function or sensitivity. For instance, KATP channel sensitivity can be altered by exposure to lipids (Baukrowitz and Fakler, 2000). Since dietary fatty acids alter plasma free fatty acid (FFA) composition (Raatz et al., 2001), and a high-fat, high-sucrose diet is associated with elevated circulating FFAs in mice (Yang et al., 2012), it is possible that WD, which is high in saturated fatty acids, alters the thermosensing mechanism of orexin neurons by increasing circulating FFAs, directly affecting KATP channel sensitivity. Alternatively, a WD-induced change in intracellular ATP concentrations could also directly affect KATP current.

The modulation of the intracellular ATP/ADP ratio has also been shown to be important for regulating KATP channel activity (Dunne and Petersen, 1986;Misler et al., 1986). However, it has been suggested that the absolute levels of ATP and ADP, not their ratio, are important for determining channel activity, since ADP can activate KATP channels in β-cells in the absence of ATP (Hopkins et al., 1992). Indeed, ATP inhibits KATP channels by acting on the Kir6.2

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subunit. whereas ADP activates these channels by binding with the SUR1 subunit (Schulze et al., 2007). Importantly, intracellular ATP and ADP levels in β-cells are susceptible to changes in glucose concentrations (Kakei et al., 1986;Cook et al., 1988;Ghosh et al., 1991;Detimary et al., 1996), which are likely elevated in WD-exposed rats. Thus, the results obtained in my WD-fed animals could be due to a diet-induced modification of the intracellular levels of the adenosine nucleotides (ATP or ADP) or their ratio, reducing KATP channel activation.

In addition to diet directly altering intracellular ATP levels, it is possible that the sensitivity of KATP channels to intracellular ATP could also be affected in a diet-dependent manner. Indeed, chronic (two month) exposure to a high-sucrose diet increased the sensitivity of pancreatic β-cell KATP channels to intracellular ATP, making the channels less likely to open in the presence of lower intracellular ATP concentrations (Velasco et al., 2012), an effect which was observed prior to the onset of type-2 diabetes. If short-term exposure to a high sucrose diet (one week) has a similar effect on orexin KATP channels, this may explain the reduced KATP current response to warming I observed in rats fed WD, which is also high in sucrose (34%). Further experiments will be required to determine if there is a similar mechanism at play in short-term WD-fed animals.

As mentioned previously, it has been suggested that changes in ATP and ADP concentrations fluctuate in the microdomains of KATP channels rather than global cytosolic changes. There have been several hypotheses for the potential candidates that could regulate ATP and ADP at the level of individual channels. For instance, creatine kinase (Krippeit-Drews et al., 2003) and adenylate kinase (Schulze et al., 2007) have been shown to modulate KATP channel activity. In addition, direct interaction with membrane anion phospholipids (Fan and Makielski, 1997), specifically, phosphatidylinositol 4,5-bisphosphate (PIP_2) (Hilgemann and

Ball, 1996), as well as long-chain acyl-coenzyme A esters (Larsson et al., 1996) can modulate

KATP channel activity. Thus, an alteration in KATP channel sensitivity by interaction with the

nucleotides, lipids or proteins at the level of the microdomains could explain the altered channel

thermosensitivity observed in WD-fed animals.

4.5.1.1 Western diet may attenuate orexin KATP current by a UCP2-dependent mechanism

The mechanism by which WD attenuates KATP-mediated current in orexin neurons

remains unknown. However, it is known that the expression of UCP2 is susceptible to diet. For

instance, UCP2 is upregulated in WAT after 7 days (Fleury et al., 1997) and 4 weeks (Matsuda et

al., 1998) of high-fat diet feeding, suggesting that UCP2 may be upregulated in the WD-fed

animals in this study. Exposure to a high-fat diet additionally increases circulating FFAs, which

are known to activate UCP2 (Mattiasson and Sullivan, 2006). Thus, upregulation of UCP2 by

high-fat diet or FFAs likely causes a respective decrease in ATP synthesis, facilitating KATP

current. On the contrary, I observed a reduced KATP current response to warming in WD-fed

animals. Admittedly, it is possible that an increase in circulating FFAs could increase ATP

production in the cell due to increased energy availability, thereby decreasing KATP current in

response to warming. Alternatively, since UCP2 also facilitates mitochondrial proliferation, it

has been suggested that the intracellular ATP concentration is actually elevated by UCP2 activity

in a tissue-specific manner (Diano et al., 2003). Thus, instead of reducing ATP production,

upregulation of UCP2 by exposure to WD could increase intracellular ATP concentrations by

promoting mitochondrial proliferation. In order to determine whether UCP2 mediates the

reduced KATP current in WD-fed rats, it will be necessary to determine if UCP2 is functionally

altered in orexin neurons under conditions of WD, and if so, what effect this has on intracellular ATP concentrations, and ultimately, KATP channel activation by warming.

4.5.2 Western diet reduces the synaptic effect of warming in orexin neurons

The synaptic effect of warming in this study was also attenuated by short-term exposure to WD. Since TRPV1 channels mediate the synaptic effect of warming, it is possible that TRPV1 channel sensitivity was altered under conditions of WD. There are a number of factors which can alter TRPV1 channel sensitivity. In particular, it has been demonstrated that the TRPV1 temperature threshold for activation is reduced at low pH and in the presence of agonists. For instance, TRPV1 receptors can sensitize to a lower threshold of heat activation in the presence of capsaicin or extracellular protons by altering the free energy, biasing them towards an open state (Rosenbaum and Simon, 2007). Furthermore, various molecules and proteins can lower the threshold for activation of these channels, including ATP (Tominaga et al., 2004), tyrosine kinases and G-protein coupled receptors (Vellani et al., 2001;Tominaga et al., 2001). Inflammatory mediators, including prostaglandins, can also sensitize TRPV1 channels (Rosenbaum and Simon, 2007), suggesting that these receptors may be important in inflammatory disease states, including obesity. However, sensitization of the TRPV1 receptor does not explain the reduced channel activity observed in this study under conditions of WD. Indeed, if TRPV1 channels are mediating the effect of WD on the synaptic response to warming, it is likely through a desensitization of TPRV1 channels. One possibility that could explain these observations is that TRPV1 channels possess a self-regulatory process by which they desensitize over time. It is thought that the rise in intracellular calcium that results from channel activation in

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turn acts to reduce channel sensitivity (Rosenbaum and Simon, 2007). Thus it is tempting to speculate that a reduction in TRPV1 channel activity during *ad libitum* exposure to WD could be due to a desensitization of the receptor. This possibility would need to be demonstrated by a reduction in receptor activity in the presence of TRPV1 agonists or by immunohistochemical staining revealing a reduction in agonist binding. If so, TRPV1 channel desensitization could occur in WD when high levels of ATP are being sustained, such that TRPV1 channels become less responsive to rises in body temperature that would normally activate them.

4.6   Physiological significance of warming-induced inhibition of orexin neurons

4.6.1   Thermoregulatory control of food intake

The theory on the thermoregulatory control of food intake proposes that central heat-sensors detect post-prandial rises in body temperature to inhibit food intake and prevent overeating and hyperthermia. In fact, factors that increase body temperature, including fever (Dantzer, 2001) and elevated environmental temperatures (Brobeck, 1948) are associated with reduced food intake.. Since orexin neurons integrate temperature-sensing capabilities and promote food intake, the thermosensing mechanisms of orexin neurons may represent a central mechanism by which rises in body temperature promote satiety. In support of this hypothesis, the inhibition of orexin neurons during elevated body temperatures results in anorexia (Langhans et al., 1989). An important caveat is that the regulation of feeding is a complex behaviour that involves more than just the thermosensing system. Indeed, many factors impact feeding, including learned associations, opportunity, social factors, time of day, gut peptides such as

cholecystokinin and glucagon as well as endogenous hormones including leptin and insulin, among others (Woods, 1998).

4.6.2 Western diet-induced impairment of thermosensing may contribute to caloric overconsumption

Short-term exposure to WD attenuates the response to warming in orexin neurons. Since orexin activity promotes food intake and is influenced by food palatability and associated cues (Choi et al., 2010), the reduced temperature response following WD likely results in a disinhibition of food intake. Accordingly, compared to chow-fed animals, WD-fed animals are likely to overeat due to impairment in the ability to sense rises in body temperature that normally act as a satiety signal. Indeed, I found that animals exposed to WD over a 7 day period consumed more calories than their chow-fed littermates. It is of future interest to measure energy expenditure and locomotor activity, to see if these behaviours are also altered in early exposure to WD in an orexin-dependent manner. Overall, the disinhibition of food intake in orexin neurons upon exposure to a high-fat, palatable diet may be a contributing factor in high-fat diet-induced overeating which often leads to weight gain and obesity.

4.6.3 Thermoregulatory control of energy expenditure

As described above, orexin neurons also promote energy expenditure, thus the inhibition of these neurons could also explain lethargic behaviour associated with fever (Grossberg et al., 2011;Dantzer, 2001). Thus, I propose that the inhibition of orexin activity during elevated body

temperatures could be a mechanism to not only reduce food intake, but also physical activity. In fact, the sickness behaviours during fever would be advantageous for reducing energy expenditure to promote recovery from infection.

It is important to discuss that orexin neurons have many other roles aside from regulating thermogenesis and energy balance, which are likely affected by warming-induced neuronal inhibition. For instance, orexin neurons also play a role in reward, motivation, arousal, and neuroendocrine function.

4.6.4 Orexin neurons and the reward system

Orexin activity has been associated with consumption of palatable food in particular, suggesting that orexin is important for the rewarding aspects of food. For instance, reward-based feeding is thought to be mediated by ventral tegmental area connections with LH orexin neurons (Zheng et al., 2007). What is more, palatability of food and associated environmental cues can induce food intake via orexin signaling, leading to overconsumption and obesity (Choi et al., 2010). Moreover, orexin-A administration into the third ventricle induces consumption of high-fat diet (Clegg et al., 2002), which is known to be rewarding (Perello et al., 2010). Furthermore, glucose-sensitive neurons of the LH/PFA , which includes orexin neurons (Parsons and Hirasawa, 2010;Burdakov et al., 2006), are thought to participate in the regulation of motivated feeding (Oomura, 1988). Together, these data suggest that the inhibition of orexin neurons during rises in body temperature likely reduces the rewarding value of food. In support of this possibility, animals with LPS-induced fever demonstrate reduced motivation to obtain food rewards (Kent et al., 1992;Bret-Dibat et al., 1995;Bret-Dibat et al., 1997;Bret-Dibat and Dantzer,

2000;De La Garza et al., 2004), an effect which may be mediated by hypothalamic nuclei (Kent et al., 1996).

4.6.5   Orexin neurons in arousal

In addition to their role in promoting feeding behaviour, orexin neurons promote arousal and wakefulness. First, knockout of the orexin gene (Chemelli et al., 1999) and mutation of the orexin-2 receptor (Lin et al., 1999) were shown to produce the chronic sleep disorder narcolepsy. Second, orexin neurons are most active during waking and arousal (Lee et al., 2005;Takahashi et al., 2008), with maximal activity observed during exploratory behaviour in freely-moving animals (Mileykovskiy et al., 2005), and virtually inactive during sleep (Lee et al., 2005;Takahashi et al., 2008). Third, orexin neurons project to many brain regions involved in the maintenance of wakefulness, including the ventral tegmental area, nucleus accumbens, amygdala and prefrontal cortex (Alexandre et al., 2013), although the densest projection is to noradrenergic cells of the locus coeruleus (LC) (Peyron et al., 1998). Indeed, orexin A-induced activation of LC neurons was shown to promote arousal and locomotor activity in rats (Hagan et al., 1999). Likewise, fever, which is accompanied with a rise in body temperature, promotes lethargic behaviours, implicating orexin neurons as a potential modulator of this behavioural response.

4.6.6   Other functions of orexin neurons

In addition to their primary functions in energy balance, arousal and reward systems, orexin neurons also play a role in regulating neuroendocrine (van den Pol et al., 1998) and

autonomic systems (Nixon et al., 2012). An early immunohistochemical report confirmed that orexin neurons project to many brain regions responsible for regulating physiological functions such as blood pressure, body temperature, and neuroendocrine function (Peyron et al., 1998). Indeed, it has been demonstrated that ICV injection of orexin-A *in vivo* increases heart rate (Shirasaka et al., 1999;Zhang et al., 2005), respiration (Zhang et al., 2005), and blood pressure (Samson et al., 1999;Shirasaka et al., 1999;Zhang et al., 2005) while suppressing the neurohormone prolactin (Russell et al., 2000). Furthermore, orexin-A stimulated the release of various neurohormones from hypothalamic explants in *vitro*, including neurotensin, vasoactive intestinal polypeptide, somatostatin, corticotropin releasing factor, and luteinizing hormone releasing hormone (Russell et al., 2000). Thus, the inhibition of orexin neurons by rises in body temperature could have widespread implications, including the modification of autonomic and neuroendocrine activity. In all, the inhibition of orexin neurons by rises in body temperature associated with fever, food intake, and exercise likely results in an altered behavioural state including reduced food intake, arousal, motivation and locomotion. These behaviours are characteristic of fever-induced sickness behaviours, for example, that promote recovery from infection.

4.7   Conclusions

In this thesis I have demonstrated that warming-induced orexin neuronal inhibition by KATP channel activation is modulated by the uncoupling protein UCP2. Warming additionally activates TRPV1 channels at the orexin synapse to facilitate excitatory transmission at both pre- and post-synaptic membranes. Since the inhibitory current response usually predominates over

the excitatory response to warming, orexin activity is abolished by rises in temperature. Since orexin neurons promote food intake and energy expenditure and also detect physiological changes in temperature, it is possible that these neurons can utilize body temperature information to prevent caloric overconsumption. Thus, I propose that orexin neuronal inhibition may be an important physiological mechanism by which rises in body temperature promote hypophagia and behavioural inactivity. What is more, short-term exposure to western diet, which is high in fat and palatable, attenuates the ability of orexin neurons to detect changes in temperature. These findings may help to explain overeating that is associated with exposure to high-fat foods.
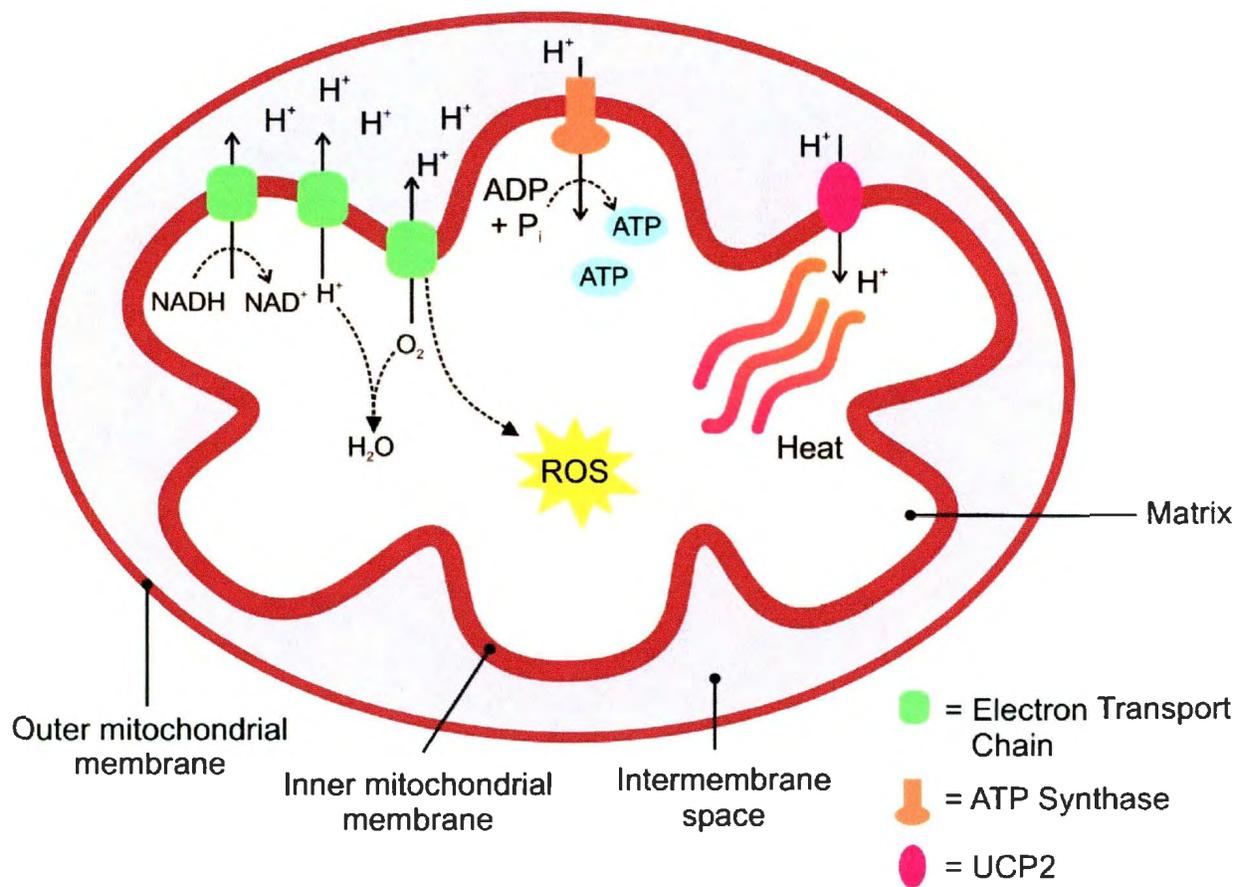
Figure 1. Overview of mitochondrial metabolism and uncoupling proteins.

Mitochondria are responsible for producing ATP, the cell's useable form of energy. During a process known as oxidative phosphorylation, electrons from donors (such as NADH) move down the electron transport chain and react with electron acceptors (such as oxygen) via redox reactions. Energy released by the electron transport chain is used to pump H^+ ions from the matrix into the intermembrane space, creating a proton gradient across the membrane. The resultant proton gradient is used to transport H^+ ions back into the matrix via the protein ATP synthase, which actively phosphorylates ADP into ATP. Reactive oxygen species (ROS) are a natural by-product of oxidative phosphorylation that can result in cell damage.

Uncoupling proteins (UCPs) such as UCP2 uncouple the process of oxidative phosphorylation from ATP synthesis by providing an alternative pathway for H^+ ions to re-enter

the matrix. UCP2 therefore diminishes the protein gradient across the inner mitochondrial membrane, releasing energy in the form of heat. Thus, the activity of UCP2 is associated with a reduction in ATP synthesis and ROS production and an increase in heat dissipation.
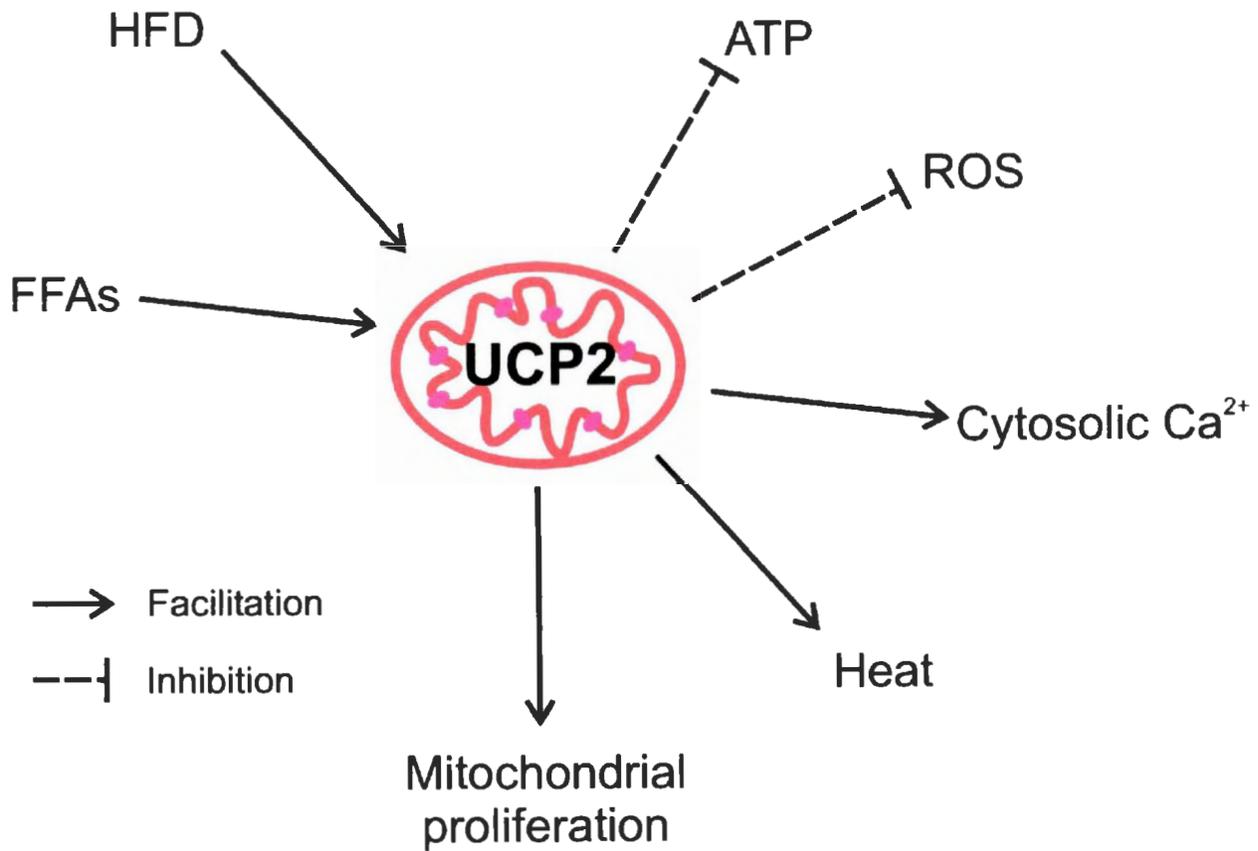
Figure 2. Overview of regulators and functions of UCP2.

UCP2 is activated by high-fat diet (HFD) and free fatty acids (FFAs). In turn, UCP2 activity

inhibits ATP synthesis and ROS production by uncoupling oxidative phosphorylation from ATP

synthesis, diminishing the proton gradient across the inner mitochondrial membrane and

releasing energy in the form of heat. Additionally, UCP2 increases cytosolic Ca^{2+} concentrations

by reducing Ca^{2+} uptake by mitochondria. Finally, UCP2 has been shown to increase

mitochondrial proliferation.

A.

Biocytin　　　Orexin-A　　　Overlay

B.

Biocytin　　　MCH　　　Overlay
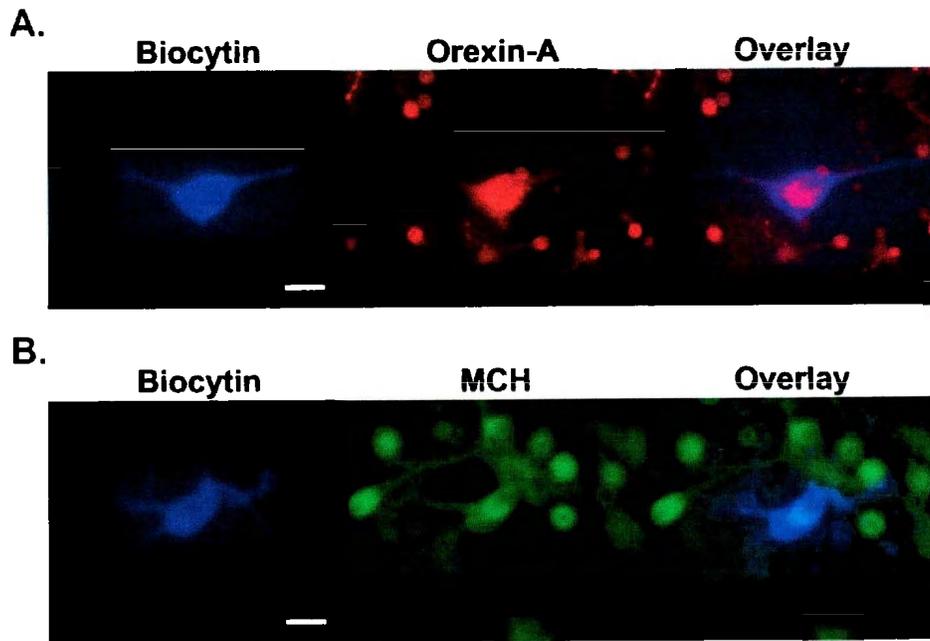
Figure 3. Immunohistochemical phenotyping of orexin and MCH neurons.

A. Post-hoc immunohistochemistry shows co-localization of biocytin (blue) and orexin-A (red) following whole cell recording with an internal pipette solution containing biocytin. *B*. Post-hoc immunohistochemistry reveals co-localization of biocytin (blue) and MCH (green) following whole cell recording. Scale bar is 20µm.
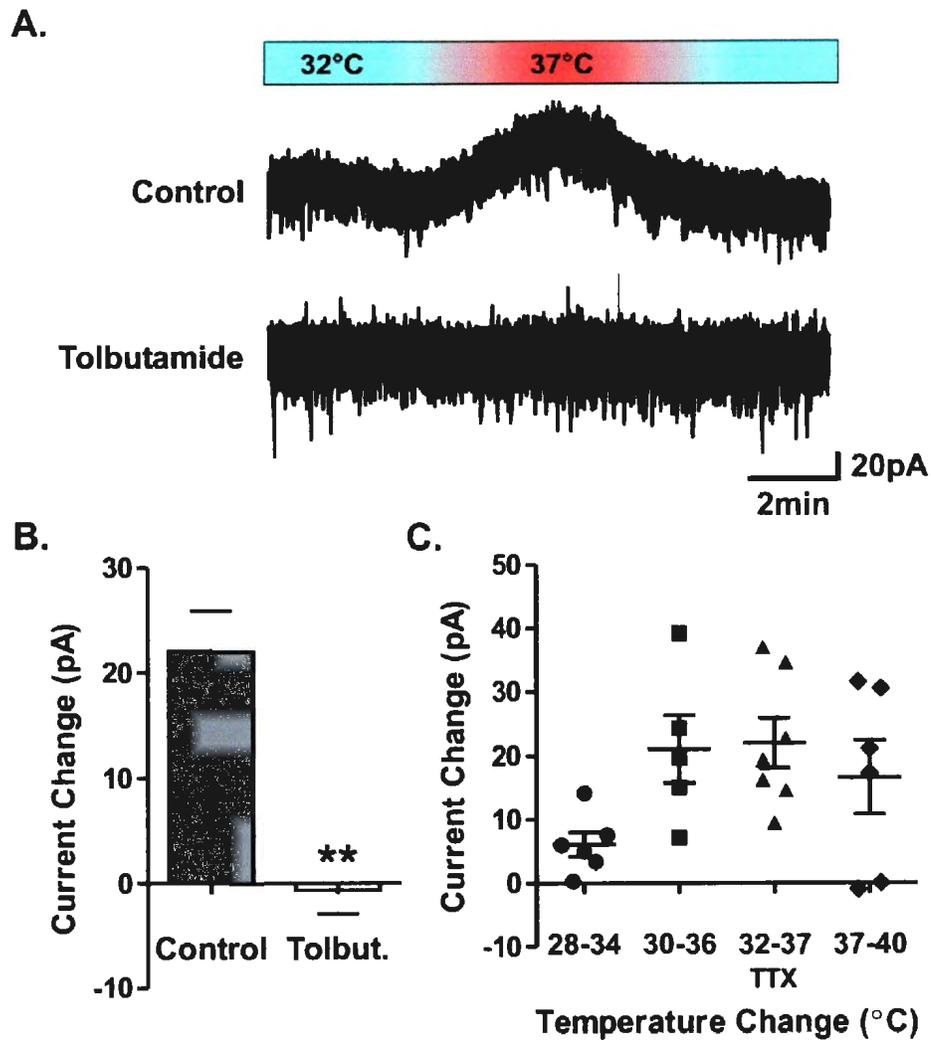
Figure 4. Warming inhibits orexin neurons in a KATP channel-dependent manner.
Representative whole cell voltage clamp recording (*A.*) and summary graph (*B.*) showing that
increasing the bath temperature from 32-37°C causes a reversible outward (inhibitory) current in
orexin neurons in the presence of 1μM TTX (control, n=7). The warming-induced current is
mediated by KATP channel activation as bath application of KATP channel inhibitor
tolbutamide (100μM) blocks the current response (n=5). *C.* The current response is observed
over various subphysiological and physiological temperature ranges. Each data point represents
the response of an individual cell. Cells were obtained from different animals. The current

responses do not differ between the physiological range and the subphysiological ranges. The

current was unaffected by TTX. ** p<0.005.
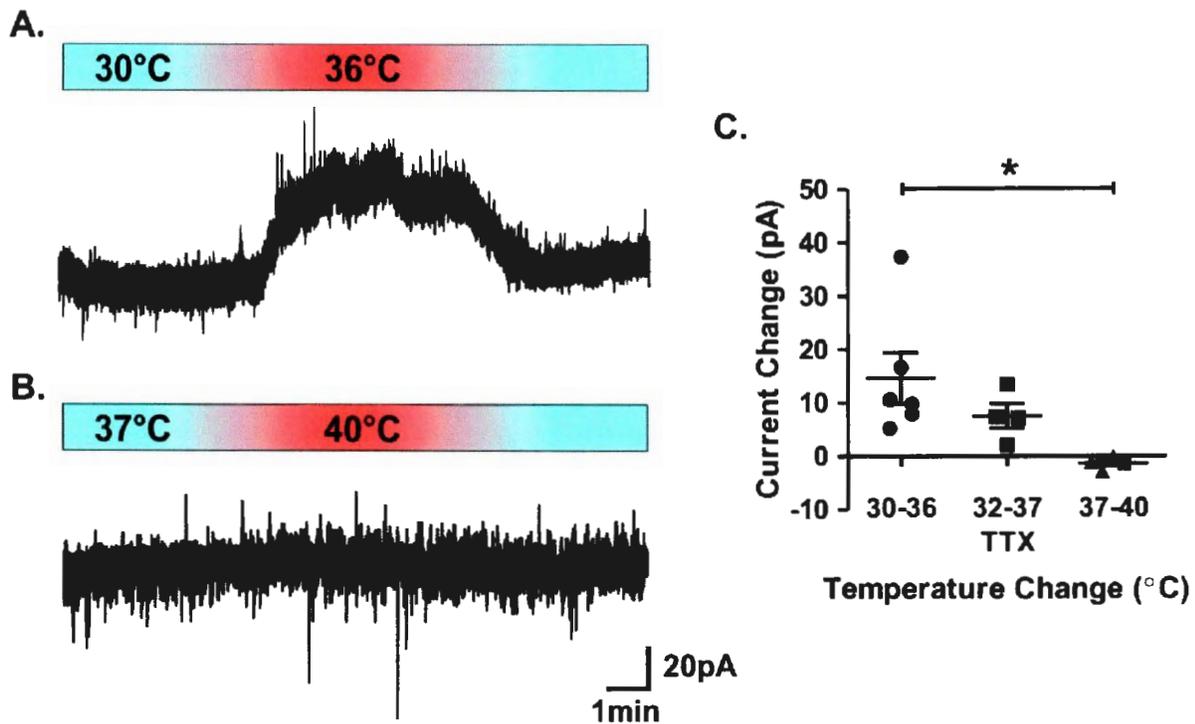
Figure 5. MCH neuron thermosensitivity is temperature-dependent.

A. Representative voltage clamp recording reveals that MCH neurons are temperature-sensitive within the subphysiological range (30-36°C). *B.* Voltage clamp recordings within the physiological range (37-40°C) reveals that MCH neurons are not thermosensitive within the physiological range. *C.* While the warming-induced current is apparent with warming at subphysiological temperatures (30-36°C), it gradually decreases as the temperature rises (32-37°C) until it is abolished at physiological temperatures (37-40°C). * $p < 0.05$.
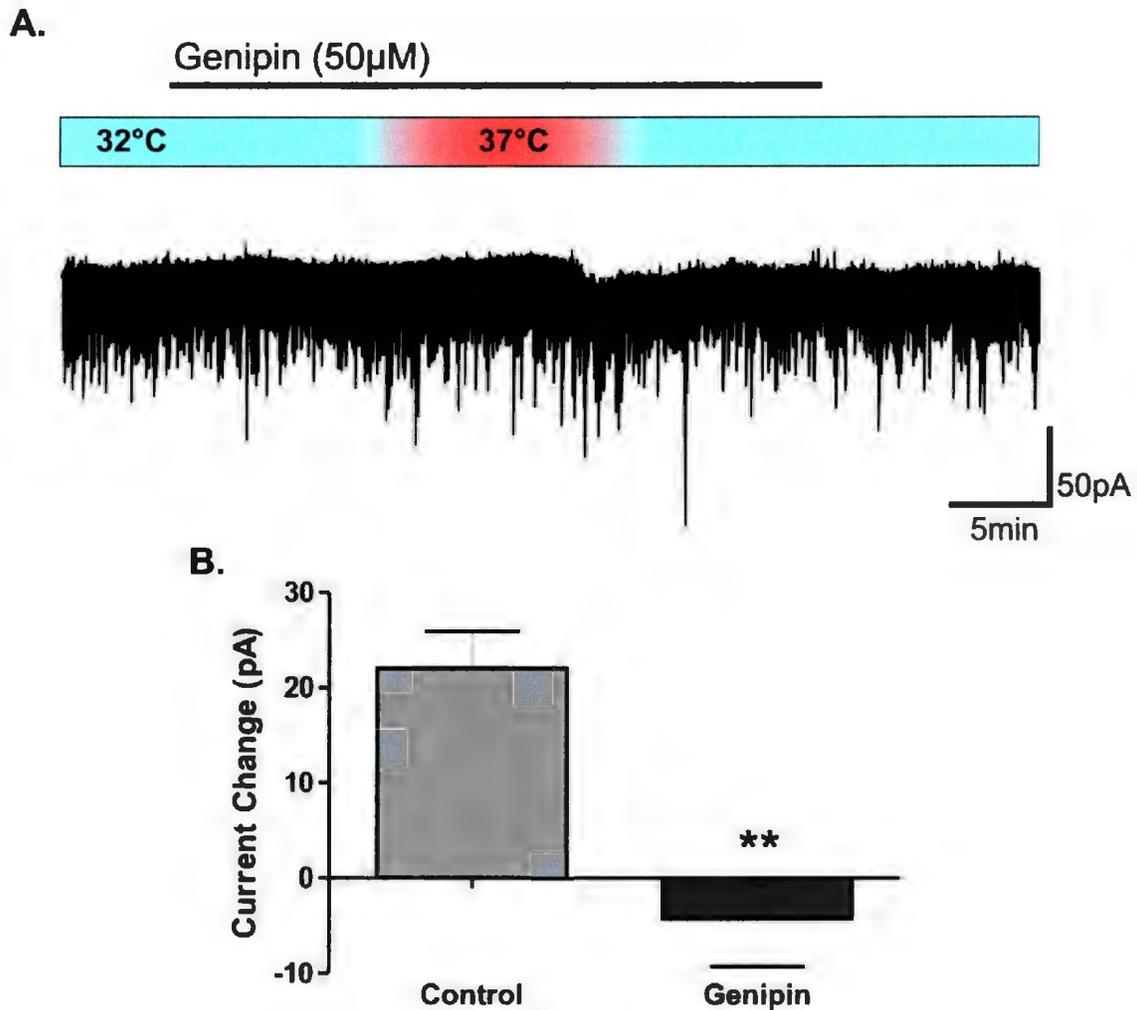
Figure 6. UCP2 modulates the KATP channel-dependent thermosensing in orexin neurons.
A. Representative voltage clamp recording of an orexin neuron reveals that application of UCP2 inhibitor genipin (50μM) abolishes the KATP current in response to warming in the presence of TTX. *B.* The magnitude of the current response to warming from 32-37°C is significantly attenuated by application of genipin (n=5), relative to the response elicited in control cells (n=7). ** p<0.05.
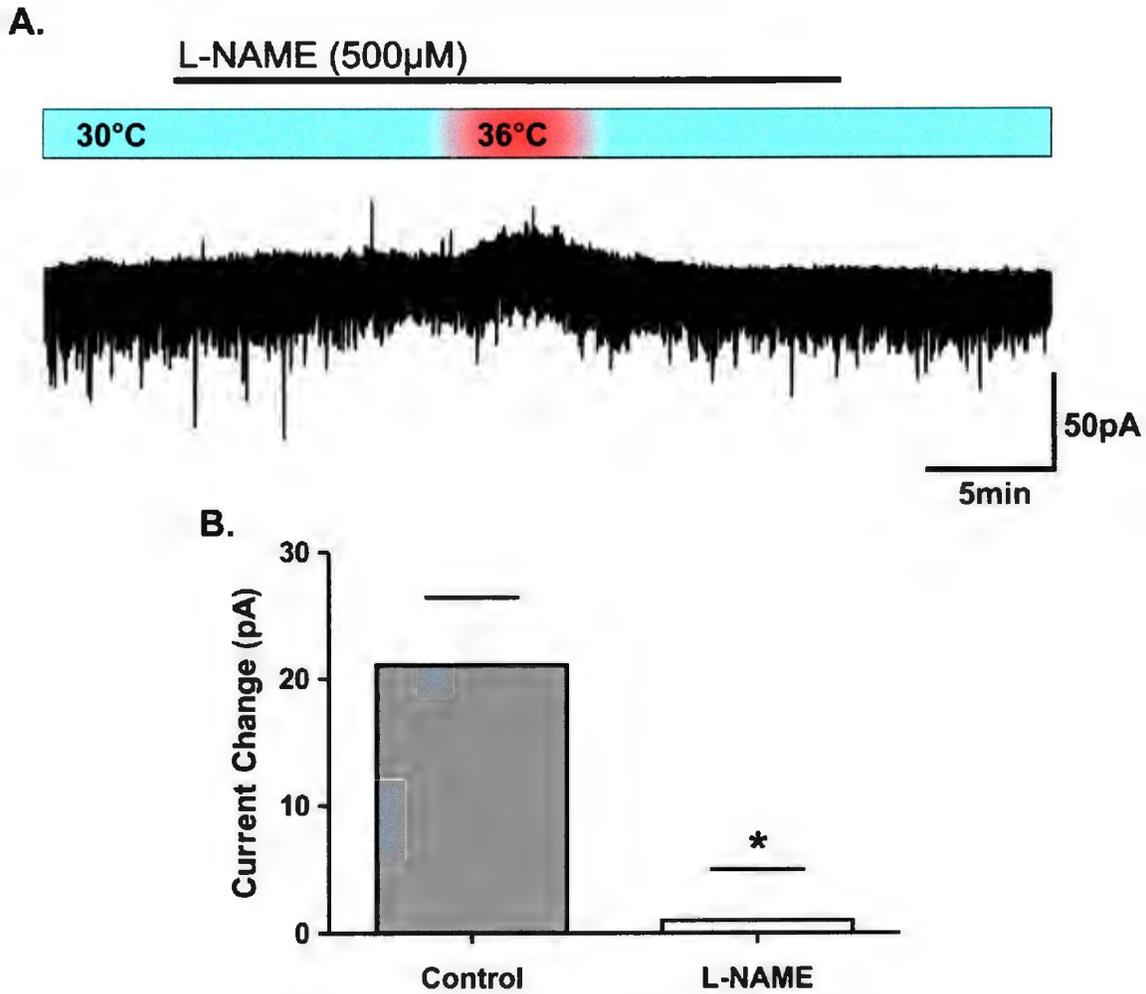
Figure 7. NO synthesis modulates the KATP channel-dependent thermosensing in orexin neurons.

A. Representative voltage clamp recording of an orexin neuron reveals that NO synthesis inhibitor L-NAME (500μM) attenuates the KATP current response to warming from 30-36°C. *B.* The magnitude of the current response was significantly reduced in cells exposed to L-NAME (n=6) relative to controls (n=5). * $p < 0.05$.
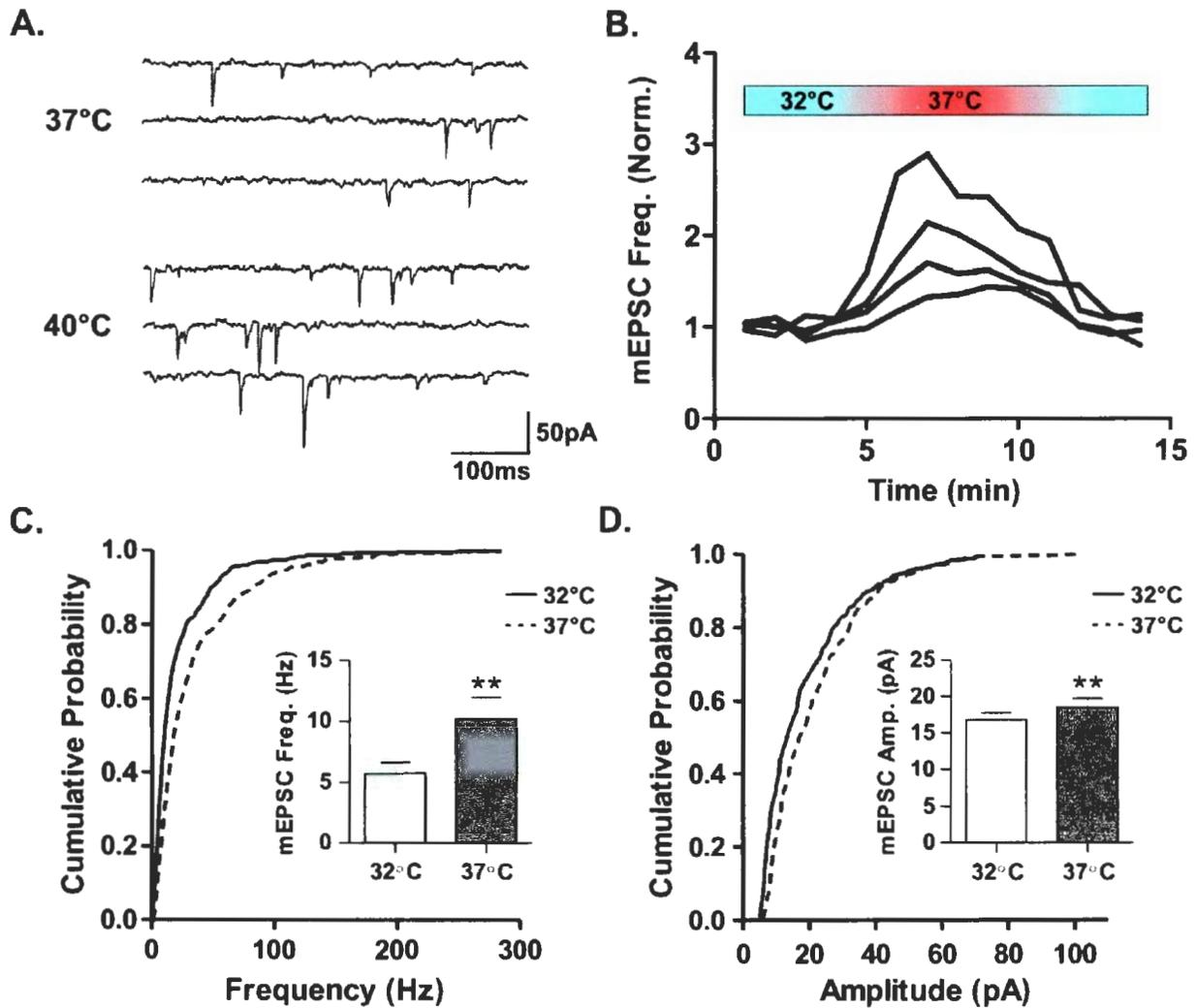
Figure 8. Warming facilitates excitatory transmission in orexin neurons.

A. In addition to the KATP current response, TTX application reveals an increase in excitatory transmission during warming in the physiological range (37-40°C). *B.* Time-course analysis reveals that mEPSC frequency is directly correlated with the bath temperature. Each line represents the response of an individual cell. *C.* Representative cumulative distribution for one cell reveals a significant increase in mEPSC frequency with a bath temperature increase from 32-37°C, which was significant in each cell tested (p<0.0001). *Inset:* There is a significant increase in mEPSC frequency with warming between 32-37°C (n=6). *D.* Representative cumulative

distribution for one cell reveals a significant increase in mEPSC amplitude with a bath

temperature increase from 32-37°C, which was significant in each cell tested (p<0.0001). *Inset:*

Increasing the bath temperature from 32-37°C was associated with a significant increase in

mEPSC amplitude (n=7). ** p<0.005.
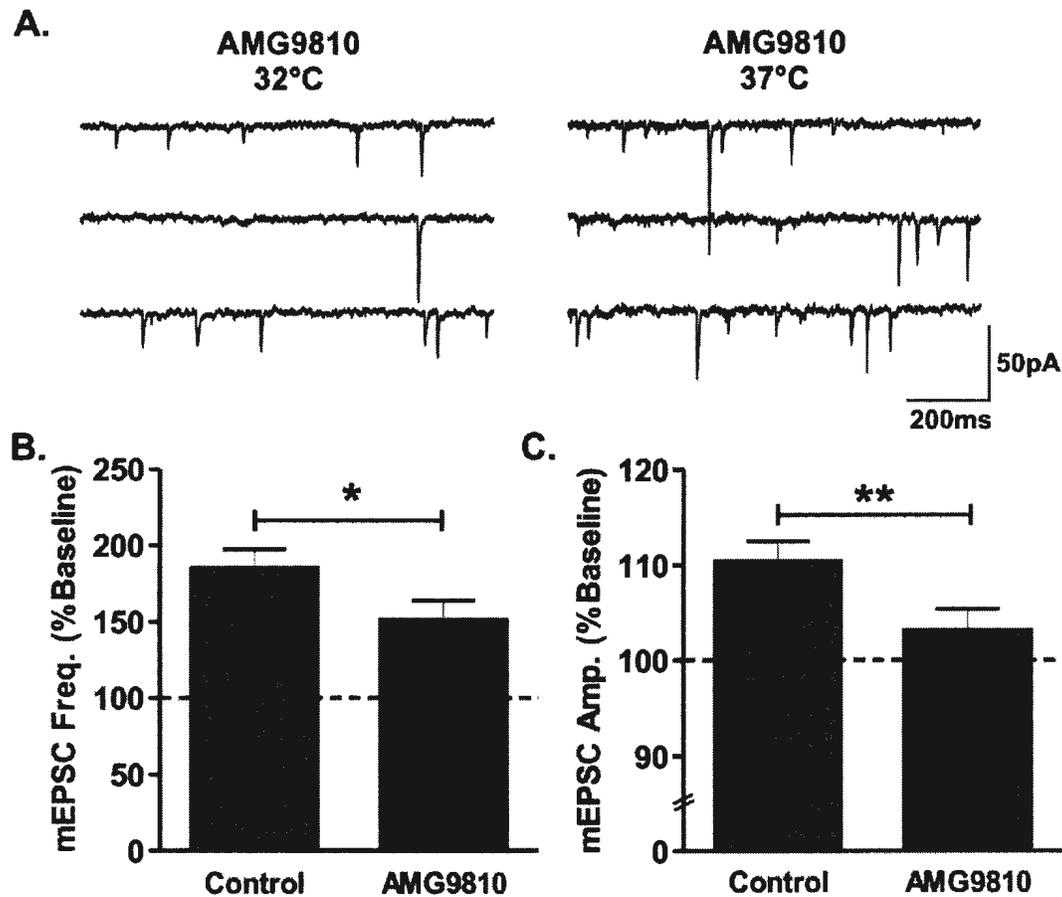
Figure 9. TRPV1 channels mediate the synaptic effect of warming in orexin neurons.

A. Representative traces reveal that TRPV1 channel antagonist AMG9810 attenuates the warming-induced increase in excitatory transmission in orexin neurons. *B.* Data presented represent groups of cells that are normalized to their own 32°C TTX baseline (dashed line). AMG9810 significantly reduces the increase in mEPSC frequency associated with warming from 32-37°C (n=8), relative to control cells (n=6). *C.* AMG9810 abolishes the postsynaptic increase in mEPSC amplitude with a bath temperature increase from 32-37°C (n=7), relative to control cells (n=7). * $p<0.05$, ** $p<0.01$.
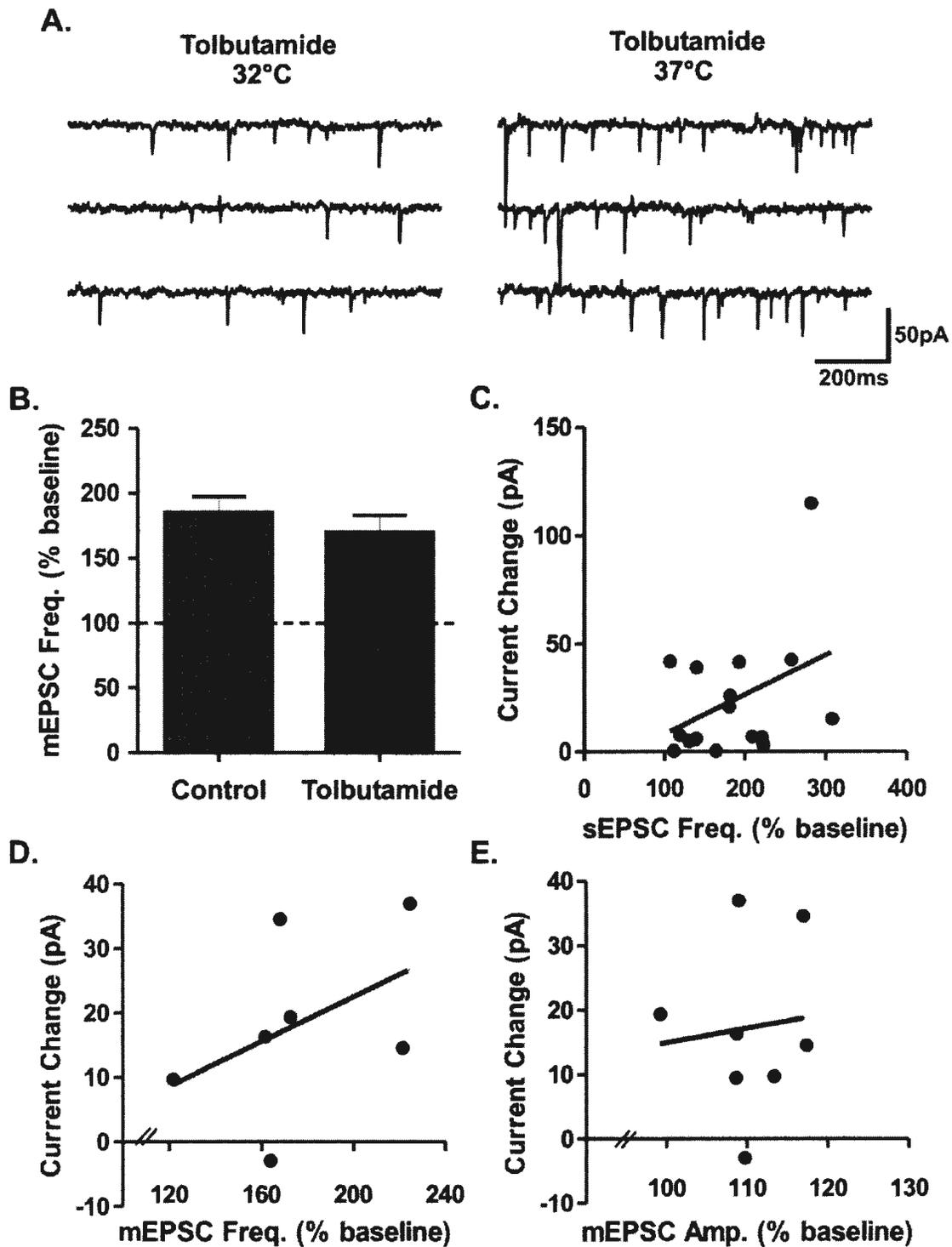
Figure 10. Synaptic response to warming is independent of KATP channel activation in orexin neurons.

A. Representative voltage clamp traces reveal that KATP channel inhibitor tolbutamide does not

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block the synaptic effect of warming. *B.* Tolbutamide (100μM) application has no effect on the percent increase in mEPSC frequency associated with warming (grouped data are normalized to own 32°C baseline control n=6, tolbutamide n=5). Furthermore, the KATP current response shows no correlation with the increase in sEPSC frequency *(C., R^2 = 0.1525)*, or mEPSC frequency *(D., R^2 = 0.2035)* with warming (each data point represents the responses of one cell). *E.* The KATP current response is not correlated with the increase in mEPSC amplitude with warming *(R^2 = 0.009682)*.
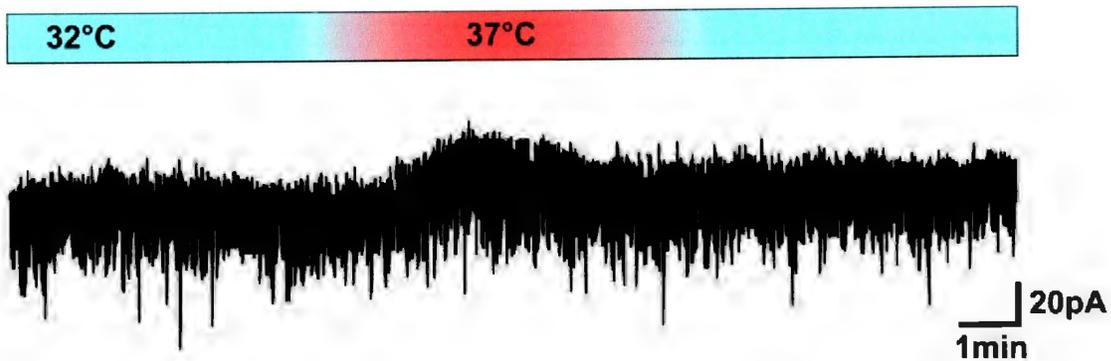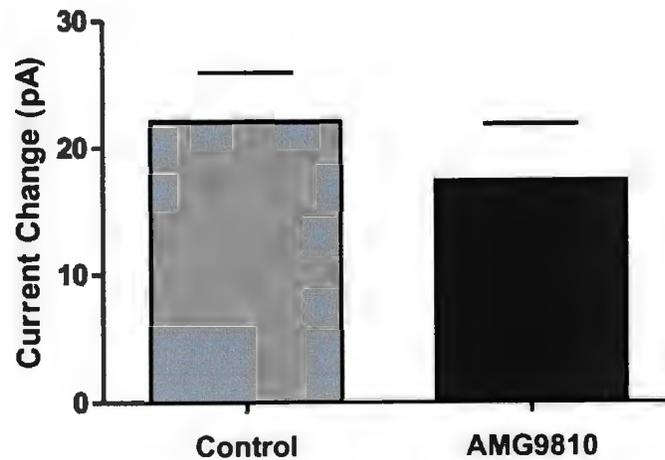
Figure 11. KATP current response to warming in orexin neurons is independent of TRPV1 channels.

A. Representative trace showing that application of TRPV1 channel inhibitor AMG9810 (500nM) does not abolish the current response to warming from 32-37°C in orexin neurons. *B.* Compared to control cells (n=7), the magnitude of the warming-induced KATP current is not affected by application of AMG9810 (n=6).
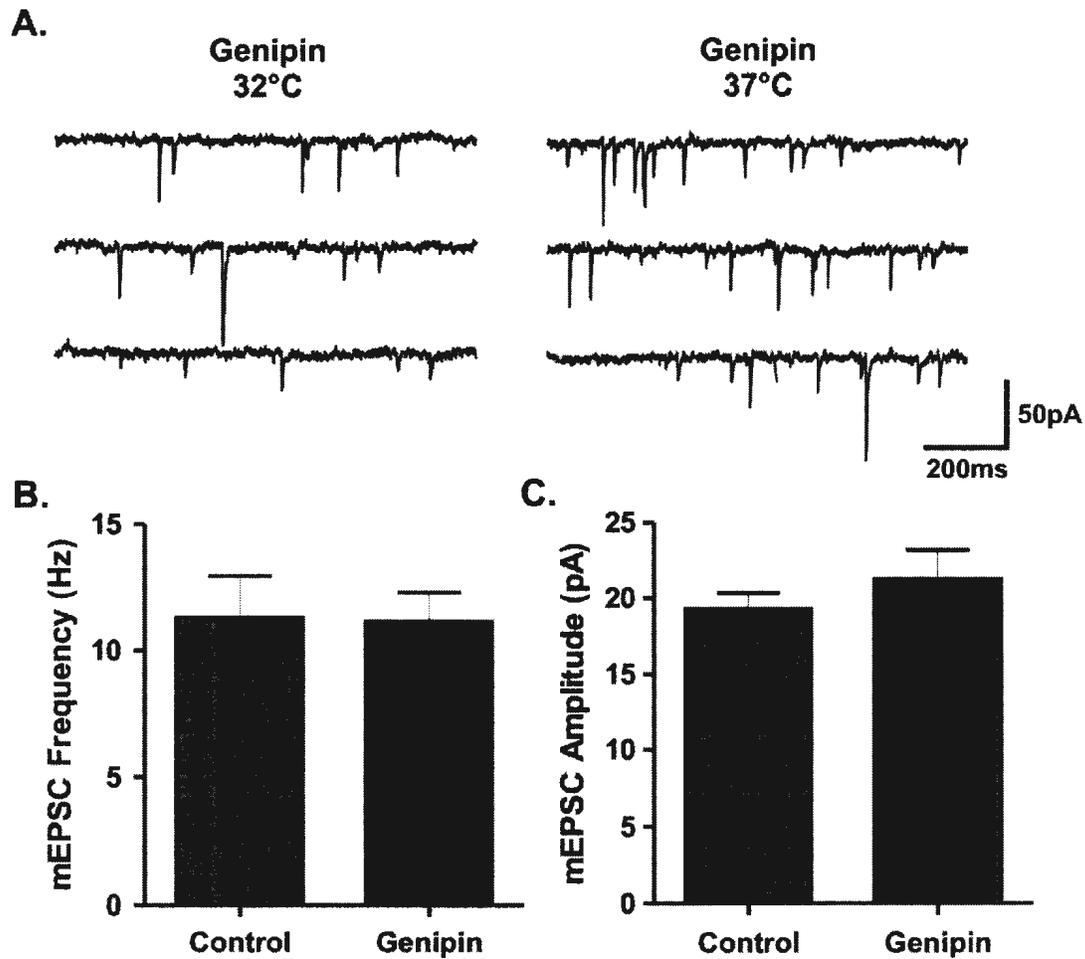
Figure 12. Synaptic effect of warming is independent of UCP2 activity in orexin neurons. *A.* Representative traces show that application of UCP2 inhibitor genipin has no effect on excitatory transmission in orexin neurons with a bath temperature increase between 32-37°C. *B.* Quantification of mEPSC frequency reveals no significant differences between mEPSC frequency of control (n=6) and genipin-treated (n=8) cells at 37°C. *C.* Genipin fails to significantly alter mEPSC amplitude at 37°C (n=8) relative to control cells (n=7).
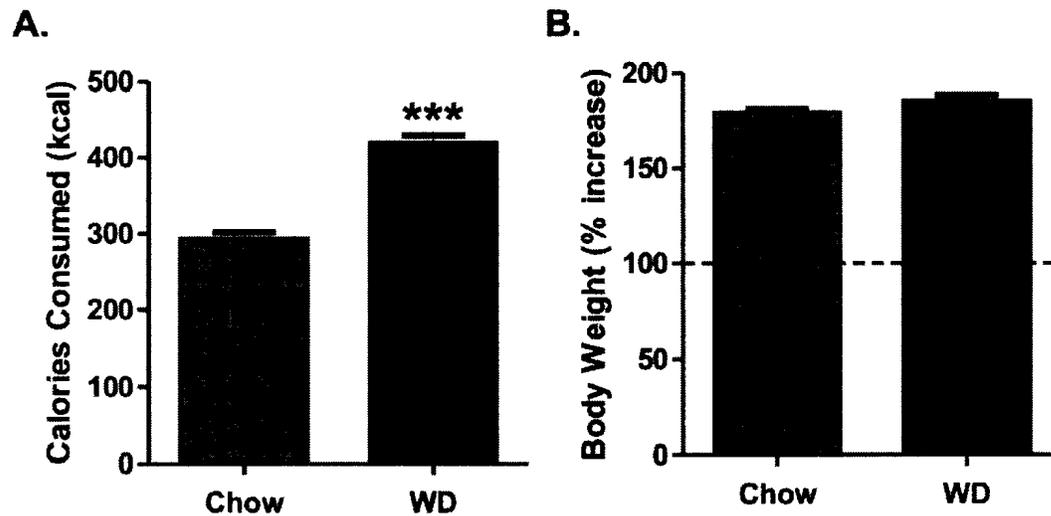
Figure 13. Short-term exposure to western-diet induces overfeeding.

A. Juvenile rats (3 weeks old) exposed to WD (n=18) for 7 days *ad libitum* consumed significantly more calories than rats fed chow *ad libitum* for the same length of time (n=5). *B.* Despite caloric overconsumption, rats fed WD for 7 days did not gain more weight than their chow fed counterparts over 7 days. *** p<0.0001.

A.

Control

WD

WD +
Tolbut.

32°C   37°C

20 pA

2 min

B.

Current Change (pA)

Control   WD   WD + KATP Inhib.
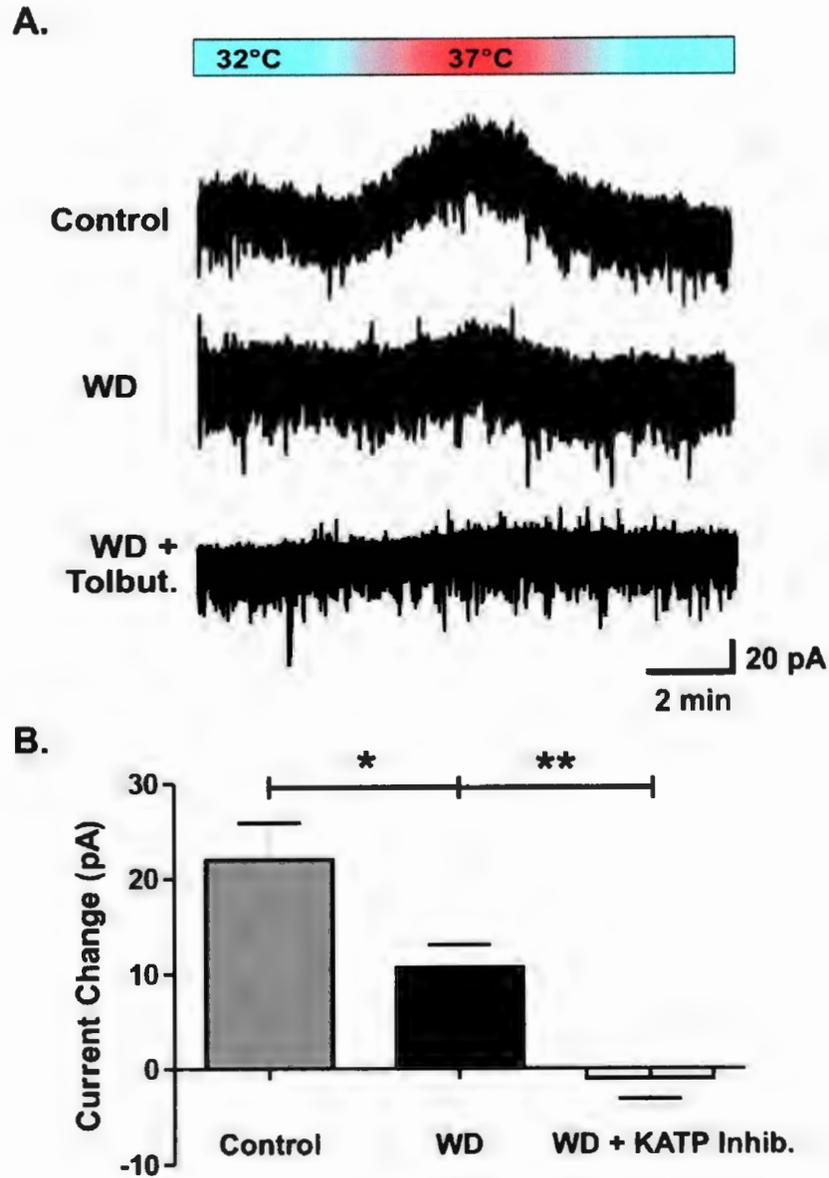
Figure 14. KATP current response to warming in orexin neurons is attenuated by short-term exposure to WD.

Representative voltage clamp traces (*A.*) and summary graph (*B.*) reveal that orexin neurons from rats fed WD for 7-11 days showed a reduced KATP current response to warming (n=5) relative to chow-fed controls (n=7). The reduced response in WD- was mediated by reduced

KATP current activation, as KATP channel inhibitors tolbutamide (100μM) or glibenclamide (200μM) abolished the warming response (n=5). * p<0.05, ** p<0.01.
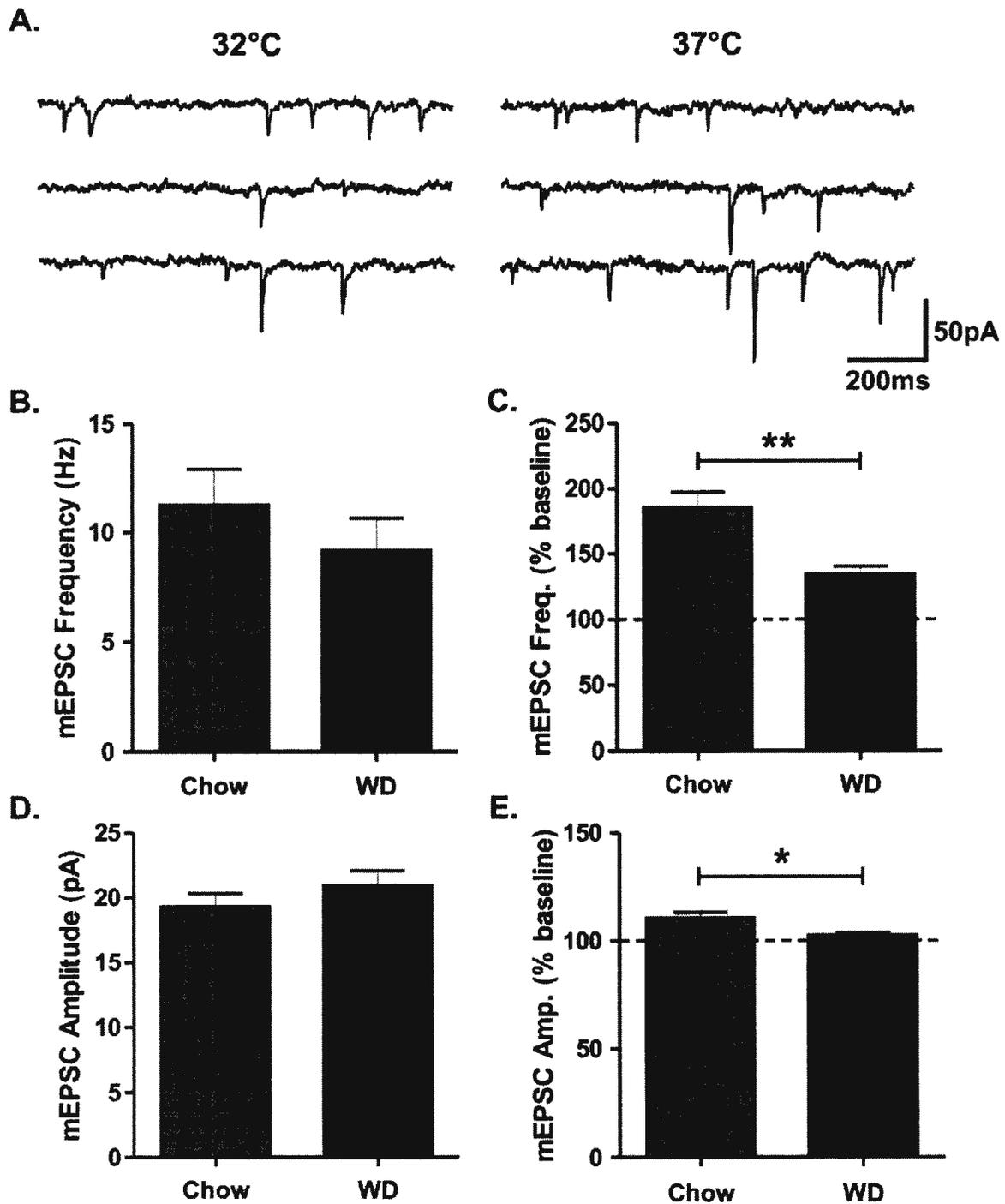
Figure 15. WD reduces the synaptic effect of warming in orexin neurons.

A. Representative voltage clamp traces from an orexin neuron obtained from a WD-fed rat show

an attenuated synaptic response to warming. *B.* Quantification of mEPSCs reveals no effect of

diet on mEPSC frequency at 37°C. (chow n=6, WD n=7). However, the increase in mEPSC frequency with warming from 32-37°C was significantly reduced *(C.)* in WD-fed animals (n=7) relative to chow-fed controls (n=6). *D.* WD did not significantly alter mEPSC amplitude at 37°C (n=5) relative to chow controls (n=7). *E.* WD significantly impaired the increase in mEPSC amplitude associated with warming from 32-37°C (n=5) compared to chow controls (n=7). * p<0.05, ** p<0.005
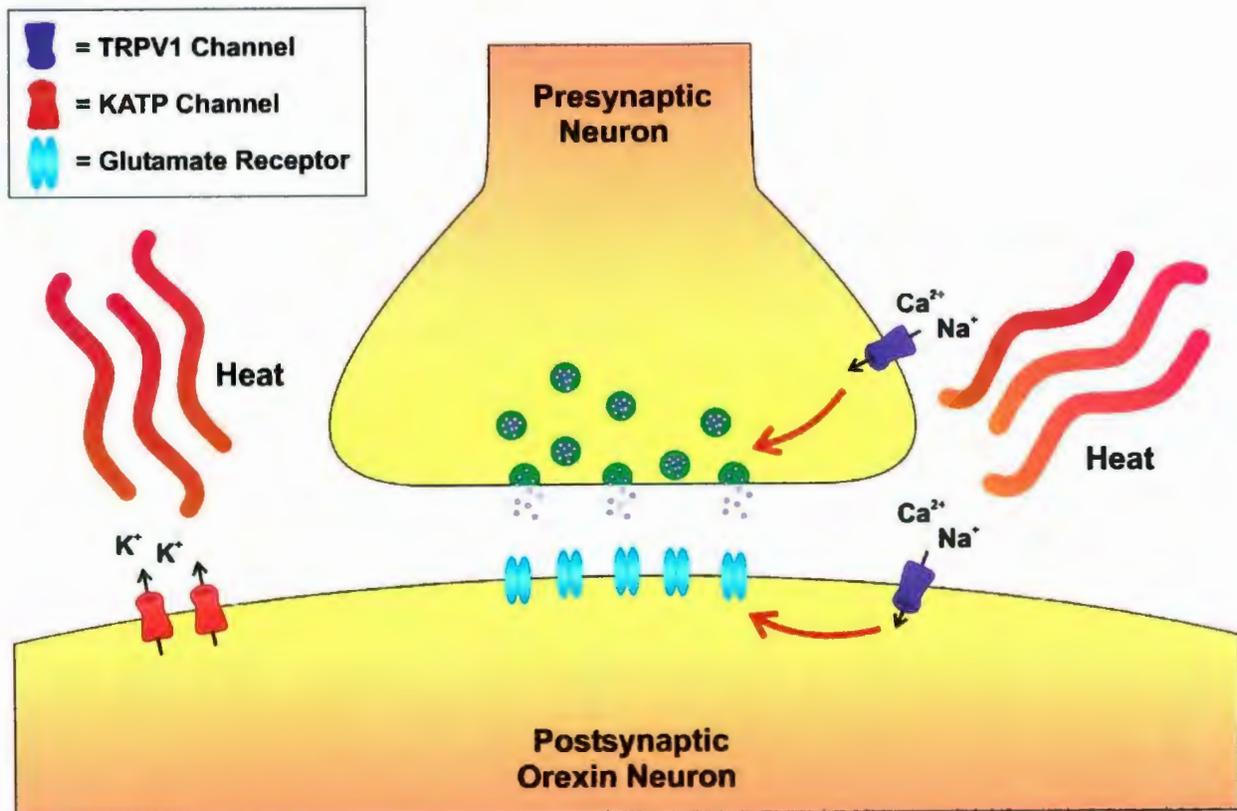
Figure 16. Postulated cellular mechanisms of warming in orexin neurons.

Warming activates KATP channels in the postsynaptic membrane, leading to cell

hyperpolarization. Simultaneous activation of heat-sensitive pre- and post-synaptic TRPV1

channels facilitates glutamate release and augments glutamate receptor sensitivity, respectively.

Overall, elevated temperatures activate inhibitory KATP channel-mediated current in orexin

neurons in addition to facilitating excitatory glutamatergic transmission onto orexin neurons by

activating TRPV1 channels.

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