EFFECT OF HIGH-FAT DIET ON HYPOTHALAMIC FUNCTIONS

by © Victoria Linehan

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ABSTRACT

The increased prevalence of high-fat food in modern society has led to an epidemic of overeating and obesity. High-fat diets are known to modulate homeostatic and reward pathways in the brain that regulate feeding, which may underlie caloric overconsumption. Such modulation may involve functional plasticity of feeding-related neurons including orexin and melanin-concentrating hormone (MCH) neurons of the lateral hypothalamus. However, how these neurons respond to high-fat diet at a cellular level and whether their responses precede obesity is incompletely understood. We used *in vitro* electrophysiology on acute brain slices from rats fed a high-fat, Western Diet (WD) or a standard chow to assess WD-induced plasticity of orexin and MCH neurons.

We found that orexin neurons display dynamic responses to WD. Excitatory transmission to orexin neurons is potentiated as early as 1 day and 1 week of WD feeding. At the same time, we found that a novel activity-dependent presynaptic long-term depression occurs only in the WD condition in orexin neurons, which is mediated by mGluR5 and retrograde cannabinoid signaling. This LTD may be a homeostatic mechanism to limit overactivation of the orexin system. With longer feeding, these effects on excitatory transmission become attenuated while inhibitory transmission is increased, which would limit the excitability of orexin neurons.

Contrastingly, WD induced a delayed activation of MCH neurons by 4 weeks of feeding through increased excitatory synaptic input and direct membrane depolarization due to an inhibition of the Na^+/K^+-ATPase. The latter depends on the activity of

cyclooxygenase and is mimicked by prostaglandin E_2, an inflammatory mediator. These changes are persistent and further exacerbated by prolonged feeding. Furthermore, this activation of MCH neurons underlies increased WD intake and weight gain, which likely contributes to diet-induced obesity. This is the first study to link excitation of feeding-related neurons with brain inflammation as a mechanism to explain high fat diet-induced weight gain.

In summary, we have found that complex, time-dependent plasticity occurs in the lateral hypothalamus over the course of WD feeding. This plasticity likely affects food intake and weight gain, as well as other physiological functions of orexin and MCH neurons. Therefore, our study may provide valuable insights into developing treatments for obesity.

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1dWD, one day of WD feeding;

1wWD, one week of WD feeding;

4wWD, four weeks of WD feeding;

11wWD, eleven weeks of WD feeding;

ACSF, artificial cerebrospinal fluid;

AgRP, agouti-related peptide;

AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide;

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;

ANOVA (RM), analysis of variance (repeated measures);

APAP, acetaminophen;

cAMP, cyclic adenosine monophosphate;

BDNF, brain-derived neurotrophic factor;

COX, cyclooxygenase;

CB1R, type 1 cannabinoid receptor;

Ctrl, control;

CPPG, (RS)-α-cyclopropyl-4-phosphonophenylglycine;

DAP5, D-2-amino-5-phosophonovalerate;

CV, coefficient of variation;

DAP5, D-2-amino-5-phosphonovalerate;

DHK, dihydrokainic acid;

DHPG, (S)-3,5-dihydroxyphenylglycine;

DMSO, dimethyl sulfoxide;

DNQX, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione;

EPSC, excitatory postsynaptic current;

γDGG, γ-D-glutamylglycine;

GABA, γ-aminobutyric acid;

GDPβS, guanosine 5′-β-thiodiphosphate trilithium salt;

GLAST, glutamate-aspartate transporter;

GLT-1, glutamate transporter-1;

GnRH, gonadotropin-releasing hormone;

GW 803430, 6-(4-chloro-phenyl)-3-[3-methoxy-4-(2-pyrrolidin-1-yl-ethoxy)-phenyl]-3H-thieno[3,2-D]pyrimidin-4-one;

HFD, high-fat diet;

HFS, high-frequency stimulation;

IgG, immunoglobulin G;

IL-1β, interleukin-1β;

IL-6, interleukin 6;

I.P., intraperitoneal;

IPSC, inhibitory postsynaptic current;

LTD, long-term depression;

LTP, long-term potentiation;

MCH, melanin-concentrating hormone;

MCHR1/MCHR2, MCH receptor 1/2;

mEPSC, miniature excitatory postsynaptic current;

mGluR, metabotropic glutamate receptor;

mIPSC, miniature inhibitory postsynaptic current;

MPEP, 6-methyl-2-(phenylethynyl)pyridine;

NAcc, nucleus accumbens;

NKA, Na^+/K^+-ATPase;

NMDA, N-methyl-D-aspartate;

NPY, neuropeptide Y;

NSNA, non-stationary noise analysis;

OX1R/OX2R, orexin receptor 1/2;

PBS, phosphate buffered saline;

PGE_2, prostaglandin $E_{2;}$

PKC, protein kinase C;

POMC, pro-opiomelanocortin;

PPR, paired pulse ratio;

RMP, resting membrane potential;

SD, standard deviation;

SEM, standard error of the mean;

TNFα, tumor necrosis factor α;

TTX, tetrodotoxin;

VTA, ventral tegmental area;

WD, western diet

CHAPTER 1

INTRODUCTION AND OVERVIEW

1.1 Regulation of food intake and body weight

Body weight is regulated by the brain and determined by the balance between energy taken in as food and energy used for metabolic processes. Normally, food intake is regulated in a homeostatic manner; however, hedonic or reward-based feeding also occurs in the absence of metabolic need. Therefore, the interaction and activities of these two feeding mechanisms determine overall energy balance and body weight regulation (Lutter & Nestler, 2009).

Although often presented as two separate mechanisms, homeostatic and reward-based feeding are interrelated. As such, homeostatic circuits can recruit the reward circuitry to drive food intake. For instance, the rewarding value of food increases when energy stores are low, while satiety limits reward-based feeding pathways (Morton, Cummings, Baskin, Barsh, & Schwartz, 2006). Moreover, since the reward system can lead to overconsumption of energy-dense food, this system could have been evolutionarily advantageous for long-term energy balance to increase energy stores when food is abundant for future periods when it may be scarce (Bellisari, 2008). However, in modern society where high-fat foods are readily available, caloric overconsumption driven by the reward system is not countered by subsequent food scarcity, which can lead to weight gain (Volkow, Wang, & Baler, 2011). Accordingly, in the past few decades, there has been a steady rise in obesity rates, which has been tied to reward-based overconsumption of high-fat foods (Lutter & Nestler, 2009). Therefore, understanding how high-fat diets affect both homeostatic and reward pathways underlying food intake is critical to develop strategies to prevent and treat diet-induced obesity.

1.1.1 Homeostatic feeding mechanisms and circuitry

Homeostatic feeding mechanisms ensure sufficient energy is ingested and stored in the body to meet current and future energy demands for physiological functions. Conversely, when energy reserves are in excess, homeostatic mechanisms limit feeding behaviours. Homeostatic centers can also regulate energy balance by modulating energy expenditure through metabolism, thermogenesis, sympathetic activity, and physical activity (Lenard & Berthoud, 2008).

The hypothalamus is the main brain region that controls energy homeostasis. First order neurons in the mediobasal hypothalamus integrate information on the body's energy state from humoural signals and vagal afferents and then project to second order neurons to adjust food intake and energy expenditure accordingly. These first order neurons include two main populations in the arcuate nucleus: appetite-promoting Agouti-related peptide/Neuropeptide Y (AgRP/NPY)-expressing neurons and appetite suppressing pro-opiomelanocortin (POMC)-expressing neurons (Schwartz, Woods, Porte, Seeley, & Baskin, 2000). Since these neurons are located near the median eminence, which has a semi-permeable blood-brain barrier (Morita & Miyata, 2013), they are able to directly sense circulating hormones and macronutrients. These peripheral factors include triglycerides, amino acids, and glucose, as well as the satiety hormones leptin and insulin, and the hunger hormone ghrelin. When energy stores are abundant, satiety signals such as leptin, insulin, glucose, and triglycerides are elevated, which activate POMC neurons to attenuate feeding. Contrastingly, when energy stores are low, hunger signals such as ghrelin activate AgRP/NPY neurons (Fig. 1.1; Belgardt, Okamura, & Brüning, 2009).

AgRP/NPY neurons promote food intake by directly inhibiting POMC neurons (Cowley et al., 2001) and by their other efferent pathways via second order neurons. These second order neurons include neurons in the paraventricular nucleus, ventromedial nucleus, and the lateral hypothalamus, which project widely throughout the central nervous system to coordinate relevant physiological responses to the body's energy state (Schwartz et al., 2000).

1.1.2 Hedonic and reward feeding mechanisms and circuitry

While food intake occurs in response to metabolic need, it can also occur independent of need due to the rewarding properties of food. There are two main components underlying reward-based feeding: "liking" and "wanting." "Liking" is the hedonic aspect of feeding representing the pleasure experienced during reward seeking and is mediated through hedonic hotspots including the nucleus accumbens (NAcc) and cortical structures, while the "wanting" aspect of reward-based feeding encompasses the motivational aspects of feeding and is thought to be mediated mainly by the mesolimbic dopamine system (Berridge, 2009).

This reward system involves projections between the ventral tegmental area (VTA) and NAcc, along with connections to executive centers such as the prefrontal cortex and limbic structures (Fig. 1.1; Volkow et al., 2011). A key neurotransmitter for motivational salience is dopamine, which is produced by VTA neurons and released into the NAcc (Hernandez & Hoebel, 1988; Salamone, Cousins, McCullough, Carriero, & Berkowitz, 1994). Dopamine release in the NAcc is associated with post-ingestive

pleasure ratings in humans (D. M. Small, Jones-Gotman, & Dagher, 2003) and can lead to excessive intake of palatable food (Berridge, Ho, Richard, & DiFeliceantonio, 2010). The rewarding nature and motivation underlying palatable food intake is salient. Accordingly, mice will work to obtain food rewards (Guegan et al., 2013), show a strong preference for palatable, high-fat diets (Geiger et al., 2009; Rockwood & Bhathena, 1990), and continue to consume palatable food in the presence of aversive stimuli (Teegarden & Bale, 2007). Similarly, humans also show a strong preference for palatable, calorie-dense food (Drewnowski & Greenwood, 1983).

This motivational control of food intake is physiologically significant as it is necessary to reinforce food seeking, particularly energy-dense food, which is an essential behaviour for survival. For example, dopamine signaling through the mesolimbic pathway is thought to coordinate arousal and locomotor activation for general feeding behaviour (Szczypka et al., 1999) and for learning food associations (Schultz, 2010), highlighting the role of reward circuitry in energy intake.

1.2 High-fat diet effects on the brain

High-fat diets influence the activity of neurons both within the homeostatic and reward circuitry to affect further high-fat diet intake. Moreover, the effects of high-fat diet extend beyond feeding circuits and can affect cognition, anxiety, and sleep.

1.2.1 Effect on homeostatic circuitry

The hypothalamus undergoes a variety of changes during high-fat diet feeding, such as inflammation and resistance to satiety signals. Collectively, these changes reduce the ability of homeostatic circuits to maintain a lean body weight, and therefore weight gain and obesity commonly result. High-fat diet feeding induces an inflammatory response in the hypothalamus (Thaler et al., 2012) that is thought to cause several disruptions to the homeostatic circuitry. First, it is thought to underlie leptin and insulin resistance, where these hormones are unable to initiate their respective signaling cascades in target cells (El-Haschimi, Pierroz, Hileman, Bjørbaek, & Flier, 2000; Posey et al., 2009). Accordingly, while leptin can reduce NPY and increase POMC mRNA expression in chow-fed controls, it has no effect on animals fed a high-fat diet (van den Heuvel et al., 2014). As a result, neither leptin nor insulin can reduce food intake in high-fat diet-fed animals at the same concentrations as chow controls. Therefore, with prolonged high-fat diet feeding, the amount of leptin and insulin required to suppress feeding becomes greater, leading to higher plasma leptin and insulin levels (Lin, Thomas, Storlien, & Huang, 2000; Posey et al., 2009), indicative of increased energy stores and weight gain.

Other changes linked to inflammation include endoplasmic reticulum stress and associated apoptosis (Moraes et al., 2009), reactive gliosis (Horvath et al., 2010), and decreased neurogenesis (McNay, Briançon, Kokoeva, Maratos-Flier, & Flier, 2012) in the arcuate nucleus and other hypothalamic regions. These changes may underlie some of the perturbations of neural activity and synaptic remodeling of the arcuate nucleus after high-fat diet feeding (Horvath et al., 2010). Such changes include increased excitatory

transmission and decreased inhibitory transmission to POMC neurons within three days of high-fat diet feeding (Benani et al., 2012), indicating a homeostatic response that would limit food intake. Moreover, diet-induced obese mice have increased POMC and decreased NPY mRNA expression, along with a reduction in inhibitory synapses to POMC neurons and a reduction in excitatory synapses to NPY neurons (Horvath et al., 2010). Similarly, the percentage of electrically silent AgRP neurons is increased by diet-induced obesity (Dietrich, Liu, & Horvath, 2013).

In summary, while compensatory changes in synaptic remodeling may occur in the homeostatic circuitry, overall the effect of leptin and insulin resistance appear to predominate. This leads to fat accrual, metabolic disturbances, and weight gain with prolonged high-fat diet consumption.

1.2.2 Effect on reward circuitry

As described in the previous section (1.1), activation of the reward circuitry is critical for the promotion of palatable food intake, specifically of high-fat diets. Short-term high-fat diet exposures lead to a long-lasting increase in glutamatergic transmission on dopaminergic VTA neurons, which is thought to prime future high-fat diet intake, as it is associated with increased food approach behaviours (Liu et al., 2016). However, the VTA is also sensitive to homeostatic factors such as insulin, ghrelin, and leptin (Abizaid et al., 2006; Liu et al., 2016; Thompson & Borgland, 2013). Accordingly, direct administration of insulin or leptin into the VTA reduces food intake (Hommel et al., 2006; Liu et al., 2016), while ghrelin administration into the VTA triggers feeding

(Abizaid et al., 2006). These data illustrate that the interaction between the reward and homeostatic circuitry determines high-fat diet intake and the potential resultant weight gain. Nevertheless, obesity rates worldwide have been steadily increasing over the past few decades (Ng et al., 2014), suggesting that the hedonic and reward system may often outweigh homeostatic factors.

Interestingly, prolonged activation of the reward pathway is thought to lead to compensatory changes that limit motivation and pleasure, known as reward hypofunction (Guo, Simmons, Herscovitch, Martin, & Hall, 2014; Johnson & Kenny, 2010; Vucetic, Kimmel, & Reyes, 2011). Reward hypofunction is evident in high-fat diet-fed animals as they show a decrease in preference for food rewards such as sucrose, saccharin, and high-fat diet (Carlin, Hill-Smith, Lucki, & Reyes, 2013; Carlin et al., 2016; Rabasa et al., 2016; Sharma, Fernandes, & Fulton, 2013). This is thought to be mediated by plasticity of the reward circuitry, such as decreased dopamine release, sensitivity, and receptor expression (Geiger et al., 2009; Sharma et al., 2013). Importantly, reward hypofunction also leads to withdrawal-like symptoms when high-fat diet is not available, such as an increased preference for rewarding foods and increased anxiety and compulsive-like behaviour that drives further high-fat diet intake (Carlin et al., 2016; Johnson & Kenny, 2010; Sharma et al., 2013). For example, during obesity, there is decreased dopamine receptor 2 expression and dopamine release in the NAcc, which is linked to compulsive eating in rats (Johnson & Kenny, 2010). Therefore, these adaptive changes may represent a dependence on continued high-fat diet intake and contribute to the difficulty in dieting and losing weight.

On the other hand, overeating in obesity has also been attributed to hyper-responsivity of reward-related brain regions that encode cues for palatable food. This is supported by evidence that visual food cues lead to greater activation of reward centers such as the dorsal striatum in obese people compared to lean counterparts (Rothemund et al., 2007). Burger and Slice have suggested that both hypo-responsivity in reward pathways associated with food intake and hyper-responsivity in reward pathways that process food cues contribute to obesity in a "dynamic vulnerability model" (Burger & Stice, 2011). Further research is required to fully understand how high-fat diet affects individual components of the reward circuitry and may provide further insight on the pathogenesis of obesity.

1.2.3 Effects on other brain regions and functions

In addition to acting on feeding circuits, high-fat diet consumption affects other brain functions including sleep, mood, cognition and memory. Namely, high-fat diet can disrupt circadian rhythms (Kohsaka et al., 2007), increase time asleep, and accordingly, decrease arousal (Tanno, Terao, Okamatsu-Ogura, & Kimura, 2013). Moreover, high-fat diet leads to deficits in spatial and procedural memory as well as in general intellectual function. These deficits are attributed to increased saturated fatty acids (Greenwood & Winocur, 1996) that lead to brain inflammation (Pistell et al., 2010) and impaired neurogenesis in the hippocampus by lipid peroxidation (Lindqvist et al., 2006; Park et al., 2010). Additionally, alterations in insulin resistance and glucose regulation may be involved (McNay et al., 2010), as insulin resistance affects synaptic plasticity in the

hippocampus (Stranahan et al., 2008), while glucose treatment can ameliorate cognitive issues (Greenwood & Winocur, 2001).

Finally, in humans, there is an association between high-fat diet intake and depression and anxiety (Jacka et al., 2010). Similarly in rats, high-fat diet can aggravate depressive behaviours in a genetic model of depression (Abildgaard et al., 2011). Interestingly, this may be dependent on the length of high-fat diet feeding as brief, 1 week exposures can reduce anxiety behaviours (Prasad & Prasad, 1996) and stress induced by early life trauma (Maniam & Morris, 2010). Contrastingly, longer high-fat diet feeding is reported to increase anxiety-like behaviour (Del Rosario, McDermott, & Panee, 2012; Souza et al., 2007). Therefore, high-fat diet leads to a variety of time-dependent pathological changes in energy homeostasis, reward, cognition, emotion, and sleep.

1.3 Lateral hypothalamus

Of particular interest for high-fat diet intake are two neuronal populations in the lateral hypothalamus: orexin and melanin-concentrating hormone (MCH) neurons. These neurons are connected to homeostatic centers as well as the reward circuitry and may act as an interface between these two components of feeding (Fig. 1.1). Moreover, while both neurons can promote food intake, they have a greater ability to increase the consumption of palatable, high-fat food compared to low-fat chow, which suggests a role in diet-induced obesity. Finally, orexin and MCH neurons can also mediate other brain functions that are perturbed by high-fat diet. Therefore, these neurons may mediate many of the effects of high-fat diet on the brain.

1.3.1 Orexin neurons

Orexin neurons are localized to the lateral hypothalamus and perifornical area (Peyron et al., 1998). These neurons selectively express the orexin peptides A and B (also known as hypocretin 1 and 2), which are cleaved from the same precursor, prepro-orexin (de Lecea et al., 1998; Sakurai et al., 1998). Orexins can act on two orexin receptors OX1R and OX2R; however, OX1R has a greater affinity for orexin A, while OX2R has equal affinities for both peptides (Sakurai et al., 1998). Orexin neurons receive diverse afferents from different brain areas (Burdakov, Karnani, & Gonzalez, 2013; González, Iordanidou, Strom, Adamantidis, & Burdakov, 2016; Sakurai et al., 2005; Yoshida, McCormack, España, Crocker, & Scammell, 2006), which allow them to integrate various metabolic and environmental cues. In turn, they also project widely to allow a coordinated physiological response that generally promotes arousal and motivated behaviours (Tsujino & Sakurai, 2013).

1.3.1.1 Role of orexin neurons in energy homeostasis

Orexin peptides play a significant role in food intake as both orexin A and B dose-dependently increase (Sakurai et al., 1998), while antagonists decrease (Haynes et al., 2000), food intake in freely fed rats. However, since orexins do not change total daily food intake (Blais et al., 2017) and orexin neurons are rapidly inactivated at the onset of food intake (González, Jensen, et al., 2016), they may be more involved in the initiation of food intake rather than increasing the amount of food consumed. Therefore, orexin neurons may have less of a role in the homeostatic regulation of feeding and instead may coordinate the arousal and motivation necessary for food seeking. This is supported by

evidence of increased orexin neuron activity during fasting that drives locomotion and food seeking behaviour (Diano, Horvath, Urbanski, Sotonyi, & Horvath, 2003; Haynes et al., 2000; Sakurai et al., 1998; Yamada, Okumura, Motomura, Kobayashi, & Kohgo, 2000; Yamanaka et al., 2003). Furthermore, lack of orexin signaling reduces food anticipatory activity during food restriction (Akiyama et al., 2004), entrainable feeding schedules (Mieda et al., 2004), and binge eating (Alcaraz-Iborra, Carvajal, Lerma-Cabrera, Valor, & Cubero, 2014).

The role of orexin neurons in anticipatory and motivated food intake is most likely related to reward processing (Borgland et al., 2009; Perello et al., 2010) as they promote consumption of non-caloric rewards, such as saccharin (Cason & Aston-Jones, 2013). In line with this, orexin neurons are also involved in substance abuse, as they increase ethanol, cocaine, and heroin intake (Borgland, Taha, Sarti, Fields, & Bonci, 2006; Moorman & Aston-Jones, 2009; Shoblock et al., 2011; Smith & Aston-Jones, 2012; Smith, See, & Aston-Jones, 2009). These effects on food and drug intake are mediated through their connections with reward circuitry (Harris, Wimmer, & Aston-Jones, 2005; Valdivia, Patrone, Reynaldo, & Perello, 2014; Zheng, Patterson, & Berthoud, 2007), including the VTA and NAcc (Peyron et al., 1998; Sakurai et al., 2005; Yoshida et al., 2006). Orexin activates VTA dopaminergic neurons (Baimel, Lau, Qiao, & Borgland, 2017; Korotkova, Sergeeva, Eriksson, Haas, & Brown, 2003), which is required for high-fat diet intake (Valdivia et al., 2014). Moreover, the NAcc promotes food intake via orexin signaling (Zheng et al., 2007).

In addition to food intake, orexin neurons also influence energy expenditure through sympathetic outflow and spontaneous physical activity. Orexins increase oxygen consumption, heart rate, respiratory rate, and blood pressure (Shahid, Rahman, & Pilowsky, 2011; J. Wang, Osaka, & Inoue, 2001), while genetic ablation of orexin neurons decreases spontaneous physical activity and induces a late-onset obesity despite consuming less food than wild-type controls (Hara et al., 2001). In agreement with these studies, orexin 2 receptor signaling protects against weight gain through increasing energy expenditure (Funato et al., 2009).

It may seem paradoxical that orexin neurons increase energy intake and energy expenditure concurrently; however, evidence from ablation studies (Hara et al., 2001) suggests that overall their effect on energy expenditure may be more important than energy intake for energy balance.

1.3.1.2 Effects of high-fat diet and obesity on orexin neurons

As expected from orexin neurons' role in food reward processing, they are critical for the consumption of high-fat diet. Orexin A administration selectively increases high-fat diet intake (Clegg, Air, Woods, & Seeley, 2002), while an OX1 receptor antagonist decreases high-fat food consumption (Valdivia et al., 2014; White et al., 2005). Interestingly, high-fat diets can also activate orexin neurons. Specifically, increased expression of cFos in orexin neurons occurs after a 2-hour high-fat diet exposure (Valdivia et al., 2014) and increased orexin mRNA and peptide expression is seen in rats fed high-fat diet for 2-3 weeks (Park et al., 2004; Wortley, Chang, Davydova, & Leibowitz, 2003). This may be due to a direct effect of the diet as intralipid injections also

increase orexin mRNA and protein expression (Chang, Karatayev, Davydova, & Leibowitz, 2004; Wortley et al., 2003). However, this activation does not appear to persist with longer periods of high-fat diet feeding as orexin mRNA expression and peptide levels are decreased in rodents fed a high-fat diet for 1 month compared to chow controls (Novak et al., 2010; Tanno et al., 2013). Similar reduced orexin expression is also seen in genetically obese animals (Cai et al., 2000), suggesting that this may be the result of weight gain or obesity.

1.3.1.3 Other functions of orexin neurons

Orexin neurons are well recognized for their role in the sleep-wake cycle. The most remarkable phenotype of orexin gene deletion is narcolepsy (Chemelli et al., 1999; Hara et al., 2001). Similarly, it has been shown that narcolepsy can result from a loss of orexin neurons in humans (Thannickal et al., 2000) and a mutation in the gene for OX2 receptors in dogs (Lin et al., 1999). Furthermore, orexin neurons promote locomotor and sympathetic activity (Adamantidis, Zhang, Aravanis, Deisseroth, & de Lecea, 2007; Alexandre, Andermann, & Scammell, 2013; Kotz et al., 2006). Therefore, the role of orexin neurons appears to be to coordinate arousal, energy balance, and attention to mediate their various physiological functions (Yamanaka et al., 2003).

1.3.2 MCH neurons

MCH neurons are a distinct cell population that are intermingled with orexin neurons in the lateral hypothalamus and perifornical area but are also found in the zona incerta (Bittencourt et al., 1992). The MCH peptide is a cyclic 19 amino acid

neuropeptide cleaved from its precursor prepro-MCH (Saito et al., 1999). In rodents, there is only one MCH receptor (MCHR1) expressed; however, humans and other species also express a second, MCHR2 (Tan et al., 2002). Therefore, caution must be used when extrapolating data on MCH signaling from rodent models to humans. MCH neurons project to many of the same targets as orexin neurons (Barson, Morganstern, & Leibowitz, 2013); however, their functions are not redundant, rather they are either complementary or antagonistic.

1.3.2.1 Role of MCH neurons in energy homeostasis

MCH neurons promote positive energy balance by increasing food intake and decreasing energy expenditure. This is reflected in an upregulation of MCH mRNA in both fasting and genetically obese mice (Qu et al., 1996). Accordingly, intracerebroventricular administration of the MCH peptide and MCH receptor agonists increase food intake (Della-Zuana et al., 2002; Qu et al., 1996; Shearman et al., 2003), while MCH receptor antagonists reduce food intake (Shearman et al., 2003). Unlike orexin neurons, MCH neurons do not respond to the rewarding value of food but rather its caloric content. This is demonstrated in the lack of effect of MCH to promote intake of non-caloric sweeteners (Karlsson et al., 2012). Consistent with this role in nutrient sensing, MCH neurons are excited by glucose (Kong et al., 2010) and promote the consumption of energy-dense foods (Clegg et al., 2002). Moreover, MCH signaling increases not only the frequency but also the size and duration of meals (Morens, Nørregaard, Receveur, van Dijk, & Scheurink, 2005), while MCHR1 antagonists reduce food intake by decreasing meal size (Kowalski, Farley, Cohen-Williams, Varty, & Spar,

2004). Therefore, in contrast to orexin neurons that are important for the motivation and initiation of eating, MCH neurons support the ongoing consumption of calories suggesting a prominent role in energy homeostasis.

The role of MCH neurons to decrease energy expenditure is also in contrast to the role of orexin neurons. Chronic intracerebroventricular infusion of MCH results in decreased body temperature and oxygen consumption (Glick, Segal-Lieberman, Cohen, & Kronfeld-Schor, 2009). Moreover, acute treatment with an MCH1 receptor antagonist increases energy expenditure, while chronic administration increases physical activity (Zhang et al., 2014).

Collectively, MCH neurons promote weight gain and positive energy balance. This is supported by the genetic ablation of MCH neurons, which results in leanness, reduced food intake, and increased energy expenditure (Alon & Friedman, 2006; Kokkotou et al., 2005).

1.3.2.2 Effects of high-fat diet and obesity on MCH neurons

MCH neurons are known to respond to long exposures to high-fat diet. Animal models of diet-induced obesity have increased levels of MCH mRNA and peptide, as well as an upregulation of MCH1R mRNA expression (Elliott et al., 2004). Similarly, in humans, there is a positive association between MCH serum levels and fat mass (Gavrila et al., 2005). Additionally, mutations in MCH1R have been reported in obese and hyperphagic individuals that are not present in lean controls (Gibson et al., 2004).

These high-fat diet-induced effects on the MCH system is thought to contribute to further high-fat diet intake and its associated weight gain. This is supported by the evidence that intracerebroventricular administration of MCH increases high-fat diet intake over a 2-hour period (Clegg et al., 2002) and overexpression of MCH leads to increased high-fat diet intake and obesity (Ludwig et al., 2001). Conversely, MCH antagonists are effective in reducing weight gain and food intake in animals fed a high-fat diet (Della-Zuana et al., 2012; Kowalski et al., 2006; Zhang et al., 2014), while MCH neuron ablation protects against diet-induced obesity. Moreover, recent evidence suggests that MCH neurons are activated during high-fat diet feeding in an insulin-dependent mechanism that contributes to weight gain by decreasing locomotor activity and insulin sensitivity (Hausen et al., 2016).

In summary, MCH neurons likely contribute to the development of diet-induced obesity during high-fat diet feeding. Due to this compelling evidence, MCH receptor antagonists are currently under development for the treatment of obesity (Moore, Sargent, Guzzo, & Surman, 2014).

1.3.2.3 Other functions of MCH neurons

While MCH neurons are best known for their role in energy balance, they also affect other physiological functions, such as mood and sleep. The effect of MCH neurons on anxiety is not fully understood. MCHR1 antagonists are thought to be anxiolytic and anti-depressive (Borowsky et al., 2002), and in line with this, chronic stress upregulates MCHR1 in the hippocampus (Roy, David, Cueva, & Giorgetti, 2007). However, a separate study suggested that increased MCH mRNA expression and reduced MCHR1

mRNA expression in the hippocampus correlates with depressive behaviours (García-Fuster et al., 2012). Even still, others have found that MCHR1 antagonists do not have any ability to reduce anxiety (Basso et al., 2006). Therefore, while MCH neurons may affect anxiety and depression, more research is required to determine the exact role they play.

While orexin neurons are wake-promoting, MCH neurons instead promote sleep and fire in a reciprocal manner to orexin neurons across the sleep/wake cycle. Accordingly, MCH neurons are sleep active (Hassani, Lee, & Jones, 2009; Verret et al., 2003) and likely contribute to sleep onset as optogenetic activation of MCH neurons initiates sleep (Konadhode et al., 2013), while MCH release is correlated with sleep onset in humans (Blouin et al., 2013).

1.4 Rationale and objectives

Despite the overwhelming evidence connecting orexin and MCH neurons with high-fat diet intake, little is known about how high-fat diets affect the electrophysiological properties of orexin and MCH neurons. Moreover, while it has been suggested that these neurons are promising targets for the treatment of diet-induced obesity, this lack of understanding would limit available treatment options. Therefore, we sought to characterize the effects of high-fat diet on these neurons and investigate their respective underlying mechanisms. Specifically, to better understand the etiology of high-fat diet-induced obesity, we investigated the time course of high-fat diet effects on orexin and MCH neurons by feeding Sprague-Dawley rats for different durations and compared

to those fed a chow control diet. Since this is the first study to assess the time course of high-fat diet effects on these neurons, we used only male rats to lower variability within those groups to have appropriate power. Future studies should address if similar results occur in female rats.

1.5.1 Objective 1 – Characterize electrophysiological properties of orexin and MCH neurons in the post-weaning period

Investigating the effects of different lengths of high-fat diet feeding would inherently lead to differences in the age of rats tested at the various timepoints. This may be particularly problematic since in our study, the feeding period started at 3 weeks of age, which corresponds to the beginning of adolescence and is associated with ongoing development. If orexin and MCH neurons are not yet electrophysiologically mature by 3 weeks of age in rats, this age difference would confound our results. Therefore, our first objective was to determine any age-dependent changes in the electrophysiological characteristics of orexin and MCH neurons from rats fed a control chow (Fig. 1.2A; Chapter 2).

1.5.2 Objective 2 – Determine the time course of high-fat diet effects on orexin neurons

Next, we investigated the effects of high-fat diet on orexin neurons. Since orexin neurons are known to express long-term synaptic plasticity, which can be affected by physiological state (Y. Rao et al., 2007), we tested the effect of high-fat diet on activity-dependent long-term plasticity (Chapter 3) and spontaneous synaptic transmission (Chapter 4) at different lengths of WD feeding (Fig. 1.2B).

1.5.3 Objective 3 – Determine the time course of high-fat diet effects on MCH neurons

Finally, we investigated how high-fat diet affects intrinsic and synaptic properties of MCH neurons at different lengths of feeding (Fig. 1.2B). Specifically, we investigated intrinsic plasticity and its underlying mechanisms (Chapter 5) as well as spontaneous synaptic transmission (Chapter 6).

1.5.4 Objectives summary

Overall, this thesis aims to uncover how the excitability and synaptic plasticity of orexin and MCH neurons are affected by short and long-term exposures to high-fat diet. This is the first comprehensive study to characterize synaptic properties from pre-weaning to adult age and to follow the temporal effects of high-fat diet on these neurons. Therefore, the experiments performed in this thesis will allow a direct assessment of orexin and MCH neuron activity and an investigation of the respective underlying mechanisms with single cell resolution in *in vitro* slice recordings.

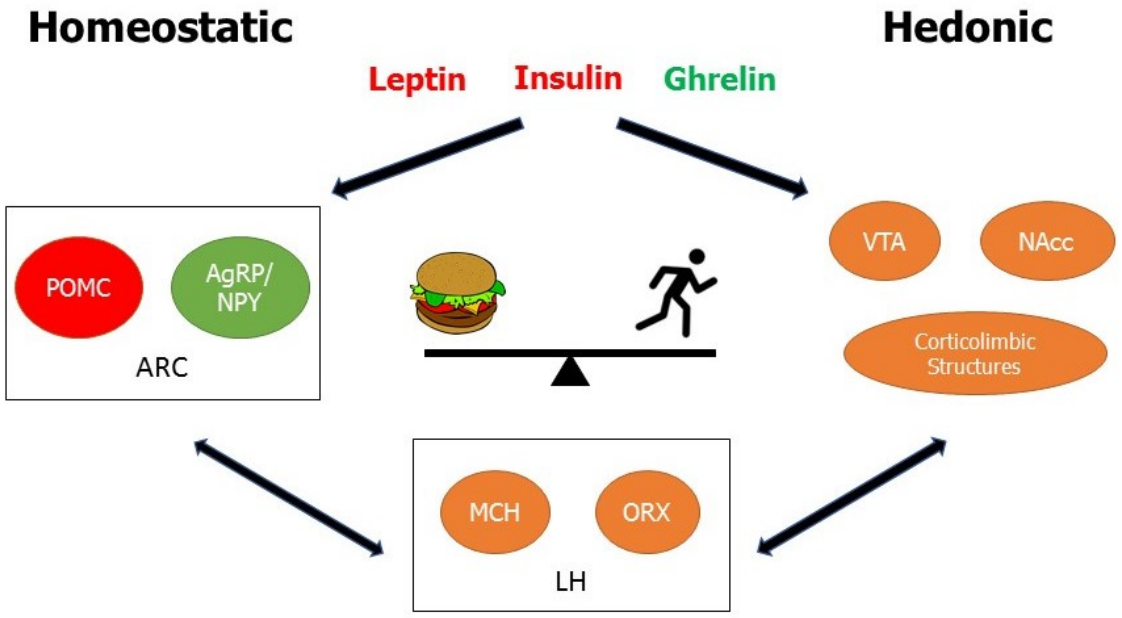

Figure 1.1: Homeostatic and hedonic control of high-fat diet intake.

Within the homeostatic circuitry, the arcuate nucleus (ARC) contains POMC neurons that inhibit food intake and AgRP/NPY neurons that promote food intake. They are modulated by humoural factors such as appetite-suppressing leptin and insulin, and appetite-promoting ghrelin. Within the hedonic and reward circuitry, the VTA and NAcc along with corticolimbic structures, such as the prefrontal cortex and insula, promote reward-based high-fat diet intake. The VTA is also affected by leptin, insulin, and ghrelin, evident of an interaction of homeostatic and reward systems to control food intake and body weight. Finally, both systems have connections with MCH and orexin (ORX) neurons of the lateral hypothalamus (LH), which both preferentially promote high-fat diet intake.
Red: Appetite-suppressing
Green: Appetite-promoting
Orange: Appetite-promoting (specifically high-fat diet)

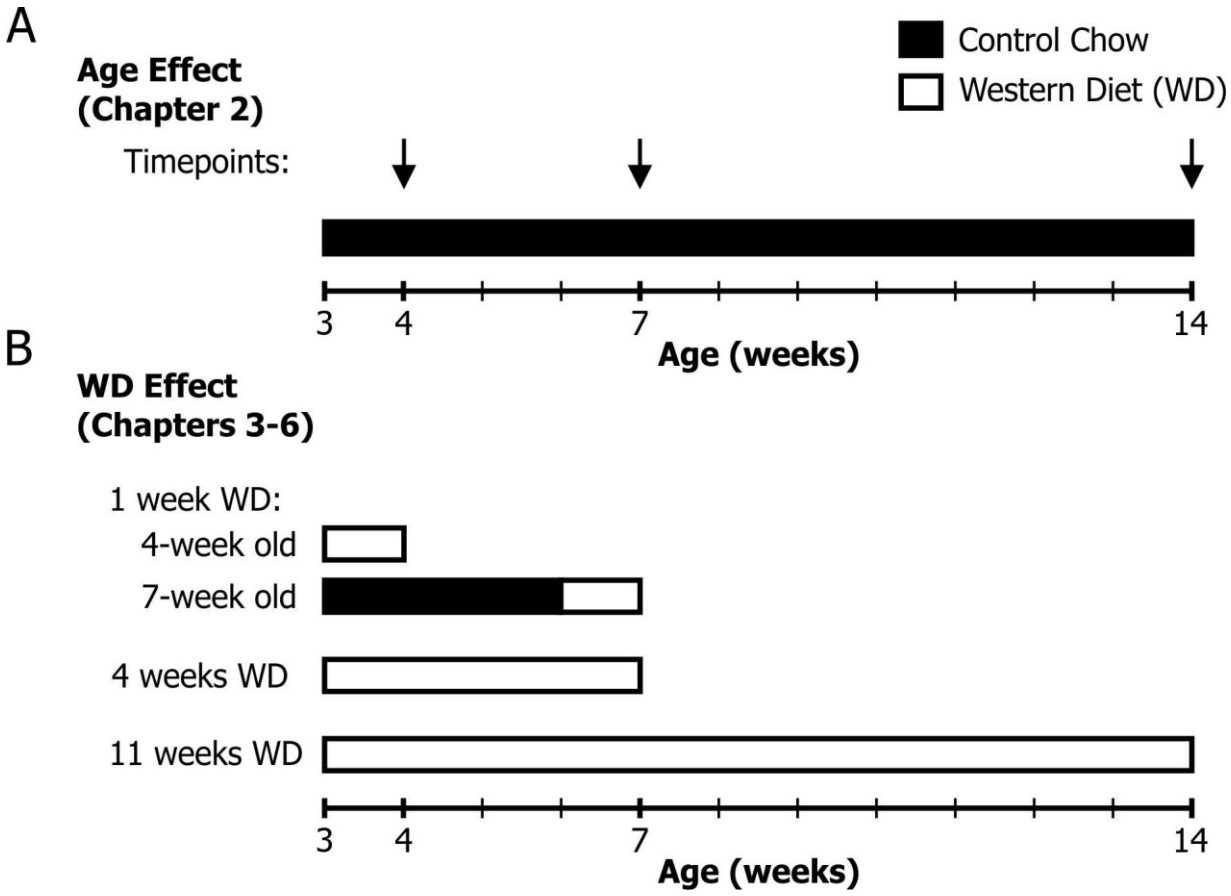

Figure 1.2: Schematic of feeding paradigm.

(A) Three-week old male Sprague-Dawley rats were fed a control chow diet and the electrophysiological characteristics of MCH and orexin neurons are investigated at various ages as indicated by the arrows. (B) The effect of different lengths of Western Diet (WD) feeding were tested on MCH and orexin neurons at the ages characterized in panel A.

CO-AUTHORSHIP STATEMENT

I, Victoria Linehan, designed the experiments in collaboration with Dr. Michiru Hirasawa for all experimental results in this thesis. For Chapters 2-4 and 6, I performed all experimental procedures and data analysis. For Chapter 5, entitled "COX-dependent downregulation of the Na^+/K^+-ATPase underlies high-fat diet-induced activation of Melanin-Concentrating Hormone neurons," I performed most experiments and data analysis, except Dr. Matthew Parsons and Mr. Christian Alberto provided some of the data presented in Fig. 5.2F-J.

CHAPTER 2

CHARACTERIZATION OF AGE-DEPENDENT ELECTROPHYSIOLOGICAL PROPERTIES OF MCH AND OREXIN NEURONS IN POST-WEANING RATS

2.1 Introduction

Orexin and MCH neurons are cell populations co-localized within the lateral hypothalamus that have key roles in several physiological functions including food intake, body weight regulation, motivation, reproductive function, and the sleep/wake cycle (Barson et al., 2013; Hassani et al., 2009; Qu et al., 1996; Sakurai et al., 1998; Skrapits et al., 2015; Wu, Dumalska, Morozova, van den Pol, & Alreja, 2009). To investigate these neurons at the cellular level, *in vitro* electrophysiological studies most commonly use young rodents in the early post-weaning period under the assumption that their nervous system is fully mature. However, the post-weaning period in rodents corresponds to adolescence, a period of ongoing neural development associated with cognitive and behavioural maturation (Paus et al., 2001; Spear, 2000; Steinberg, 2005). In humans, white matter increases continually during development, while gray matter volume reaches a peak in childhood and decreases during adolescence until reaching adult levels, with regional differences in the timing of these changes (Barnea-Goraly et al., 2005; Lebel, Walker, Leemans, Phillips, & Beaulieu, 2008; Pfefferbaum et al., 1994). At the cellular level, these volumetric changes are associated with changes in neuron number, synaptic connections, and dendritic structure in several brain areas including the cortex (Markham, Morris, & Juraska, 2007), hippocampus (He & Crews, 2007; Yildirim et al., 2008), and cerebellum (McKay & Turner, 2005).

As maturation occurs broadly during adolescence in the brain, it is possible that development of the orexin and MCH system also continues in the post-weaning period. If so, this may contribute to development of other physiological systems known to be

controlled by these neurons. For example, both orexin and MCH neurons regulate food intake and energy expenditure (Glick et al., 2009; Qu et al., 1996; Sakurai et al., 1998), thus their functional development during adolescence may influence growth spurts of body weight and height. In support of this idea, MCH deficiency reduces body length and weight gain in adolescent rats (Mul et al., 2010). Orexin and MCH neurons may also have a role in puberty as both are known to regulate gonadotropin-releasing hormone (GnRH) neurons (Gaskins & Moenter, 2012; Skrapits et al., 2015; Wu et al., 2009) and subsequent gonadotropin release (Murray et al., 2000, 2006; Small et al., 2003; Tsukamura et al., 2000).

In this chapter, we characterize the electrophysiological properties of MCH and orexin neurons in rats at several time points post-weaning. Our hypothesis was that both these neurons undergo age-dependent changes over this period. However, our results show that MCH neurons undergo an age-dependent reduction in excitability, while no changes are observed in orexin neurons. These results demonstrate the importance of carefully considering the age of animals used in studies involving MCH neurons and the potential physiological significance of these developmental changes.

2.2 Methods

2.2.1 Animals

All experiments were conducted under the guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Institutional Animal Care Committee. Male Sprague-Dawley rats were weaned at 21 days old (Charles River, Quebec) and fed a standard chow *ad libitum* (Prolab RMH 3000). Food intake and body weight were measured weekly.

2.2.2 Electrophysiological recording

Rats were sacrificed by decapitation under isoflurane anesthesia at 4, 7, and 14 weeks of age (Fig. 1.2A). Coronal slices of the hypothalamus (250µm) were generated using a vibratome (VT-1000, Leica Microsystems) in cold artificial cerebrospinal fluid (ACSF; in mM: 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 18 $NaHCO_3$, 2.5 glucose, and 2 $CaCl_2$) bubbled with 95% O_2/5% CO_2 gas and then incubated in ACSF at 32-34℃ for 30 minutes. Then, hemisected slices were transferred into a recording chamber perfused with ACSF (30-32℃), visualized with infrared-differential interference contrast optics (DM LFSA, Leica Microsystems), and cells in the lateral hypothalamus/perifornical area were selected for study. A glass pipette was filled with an internal solution (in mM: 123 K-gluconate, 2 $MgCl_2$, 1 KCl, 0.2 EGTA, 10 HEPES, 5 Na_2ATP, 0.3 NaGTP, and 2.7 biocytin; tip resistance 3-5MΩ) and whole cell patch clamp recordings were performed using Clampex 9/10 software, and Multiclamp 700B (Molecular Devices).

Once whole cell access was achieved, a series of 600ms hyperpolarizing and depolarizing current injections were applied to cells in 50pA increments. Unique electrophysiological responses to these current injections were used to identify MCH and orexin neurons as described previously (Linehan, Trask, Briggs, Rowe, & Hirasawa, 2015; Parsons & Hirasawa, 2011). Additionally, active membrane properties during this current injection protocol were assessed, including the action potential threshold, frequency, and first spike latency. If a positive current injection did not elicit firing, 600ms was assigned for first spike latency. Voltage measurements were corrected for liquid junction potential (-14.9mV).

The neurochemical phenotype was further confirmed in a subset of recorded neurons filled with biocytin using triple immunohistochemical staining for biocytin, MCH, and orexin A (Fig. 2.1), as previously described (Linehan et al., 2015; Parsons & Hirasawa, 2011). All neurons that were subjected to post hoc immunohistochemistry had matching electrophysiological properties and neurochemical phenotype (91 of 91 MCH neurons, 101 of 101 orexin neurons, total 192 cells confirmed; 100% accuracy).

To study evoked EPSCs, a glass electrode filled with ACSF was used to stimulate afferent fibers. The chloride channel blocker, picrotoxin (50μM), was present for all recordings to isolate glutamatergic excitatory transmission (Li, Gao, Sakurai, & van den Pol, 2002; van den Pol, Acuna-Goycolea, Clark, & Ghosh, 2004) and the voltage-gated Na^+ channel blocker, tetrodotoxin (TTX, 1μM), was used to study miniature EPSCs (mEPSC).

Train stimulation protocols consisted of 50-60 pulses at 10Hz or 50 pulses at 50Hz, every 60 seconds. Paired-pulse ratio (PPR) was calculated as the amplitude of the second EPSC divided by the first EPSC (EPSC2/EPSC1). All EPSCs during train stimulation were normalized to the amplitude of the first EPSC. The plateau of the train was the average amplitude of the last 10 EPSCs normalized to the first EPSC of the train. For all voltage clamp experiments, a 20mV, 50ms square pulse was applied every 60 seconds to monitor access resistance. Signals were filtered at 1kHz and digitized at 5-10kHz.

2.2.3 Statistical analysis

Data are expressed as mean ± SEM. The number of observations is reported in the nested model where N/n represents the number of cells/the number of animals. MiniAnalysis (Synaptosoft) was used to manually analyze mEPSCs by selecting events that had a clear fast rise and exponential decay. For MCH neurons, 283±25 events per cell were analyzed for mEPSC frequency and 134±12 for amplitude. For orexin neurons, 673±93 events per cell were analyzed for the frequency and 456±47 for the amplitude of mEPSCs. Clampfit was used for all other analysis. One- or two-way ANOVA and one- or two-way repeated measures (RM) ANOVA were used with Holm-Sidak post hoc tests as appropriate. $p<0.05$ was considered significant.

2.3 Results

Rats rapidly gained weight and had a steady increase in caloric intake following weaning (21 days old) that tapered over time (Fig. 2.2A,B). Moreover, the rate of weight gain showed an age-dependent decline (n=22 rats; Fig. 2.2C), typical of post-weaning adolescent growth (Spear, 2000). We investigated the excitability of MCH and orexin neurons at three time points: early adolescence (4 weeks old), late adolescence (7 weeks old), and in adulthood (14 weeks old) (Sengupta, 2013).

2.3.1 Intrinsic excitability of MCH neurons during the post-weaning period

MCH neurons underwent an age-dependent decrease in excitability. Compared to those from 4-week old rats, MCH neurons of 7- and 14-week old rats had a hyperpolarized resting membrane potential (RMP; 4wk -77.3±1.6mV, N/n=20/17 vs 7wk -82.4±1.0mV, N/n=31/13, p=0.0090; 4wk vs 14wk -83.8±1.2mV, N/n=25/8, p=0.0020; Fig. 2.3A,B). To investigate action potential properties, positive current injections were applied to elicit firing since MCH neurons are silent at rest. The firing threshold of MCH neurons was lower in 4-week old rats compared to those of older rats (Fig. 2.3C). Additionally, MCH neurons from 4-week old rats showed higher firing frequency (Fig. 2.3A,D) and a shorter latency to first spike (Fig. 2.3A,E). Overall these results suggest that the excitability of MCH neurons decreases with age and reaches the adult level by 7 weeks of age.

2.3.2 Age-dependent changes in excitatory transmission to MCH neurons

Compared to those of 4-week old rats, MCH neurons in 7- and 14-week old rats had lower mEPSC frequency and amplitude (Frequency: 4wk 2.2±0.2Hz, N/n=8/5 vs 7wk 1.2±0.1Hz, N/n=9/8, p=0.0018; 4wk vs 14wk 1.1±0.2Hz, N/n=7/5, p=0.0018; Amplitude: 4wk 13.1±0.8pA vs 7wk 9.1±0.6pA, p=0.0189; 4wk vs 14wk 8.9±1.5pA, p=0.0189; Fig. 2.4A-C). Typically, a decrease in mEPSC frequency represents a presynaptic change, while a decrease in mEPSC amplitude would suggest a postsynaptic change (Han & Stevens, 2009). Therefore, this age-dependent process likely involves pre- and postsynaptic mechanisms. Alternatively, it is possible that the decrease in mEPSC amplitude in 7-week old rats decreased the number of events that were over the threshold of detection resulting in an apparent reduction in frequency.

2.3.3 Characterization of activity-dependent short-term plasticity in MCH neurons

To further investigate their synaptic properties, we applied train stimulation at 10 and 50Hz (N/n=15/8; Fig. 2.5) to excitatory afferents to MCH neurons in 4-week old rats. Paired pulse facilitation, indicative of low release probability synapses, was observed with 10 and 50Hz stimulation but was significantly greater at 50Hz (10Hz 1.44±0.07 vs 50Hz 1.68±0.12, p=0.0160; Fig. 2.5A,B). During a 10Hz train, most MCH neurons tested maintained a synaptic facilitation throughout the train. On the other hand, at 50Hz stimulation, the initial facilitation was brief and followed by synaptic depression (Fig. 2.5C-E), likely due to vesicle depletion. These results suggest that the activity-dependent

short-term plasticity of MCH neurons can be either facilitating or depressing depending on the length and frequency of stimulation.

2.3.4 Age-dependent changes in short-term plasticity in MCH neurons

Interestingly, this activity-dependent plasticity was also affected by age. Namely, unlike the 4-week old group, 10Hz stimulation in the 7-week old group induced a brief facilitation which was then followed by a synaptic depression, resulting in a lower plateau of EPSC amplitude (4wk N/n=15/8, 7wk N/n=17/10, and 14wk N/n=18/10; Fig. 2.6A,B,E). This synaptic depression was no longer present by 14 weeks of age (Fig. 2.6A,B,E). An age-dependent change was also seen with 50Hz stimulation: the initial facilitation was smaller and the overall synaptic depression was more prominent in the 7-week old group compared to 4-week and 14-week old groups (Fig. 2.6A,C-E). This suggests that excitatory synapses may have higher release probability and are more prone to synaptic fatigue in MCH neurons of 7-week old rats relative to 4- and 14-week old rats.

2.3.5 Electrophysiological properties of orexin neurons during the post-weaning period

In orexin neurons, there was no age-dependent change in the RMP, firing threshold, firing frequency, or first spike latency (4wk N/n=58/19, 7wk N/n=40/12, and 14wk N/n=42/14; Fig. 2.7). Likewise, we did not find any differences in the frequency or amplitude of mEPSCs (4wk N/n=10/5, 7wk N/n=8/6, and 14wk N/n=9/6; Fig. 2.8), indicating no change in basal excitatory synaptic transmission.

We also investigated activity-dependent short-term plasticity in orexin neurons. In response to 10 or 50Hz stimulation, orexin neurons in 4-week old rats showed a similar degree of paired pulse depression (N/n=24/12; Fig. 2.9A,B), indicative of high release probability synapses. During train stimulation at 10 and 50Hz, these neurons showed robust short-term synaptic depression. However, the degree of synaptic depression was greater during 50Hz trains than that during 10Hz trains as indicated by significantly lower plateau at the end of the train (Fig. 2.9C-E). Finally, the degree of depression was similar regardless of age (4wk N/n=24/12, 7wk N/n=12/8, and 14wk N/n=14/5; Fig. 2.10). Thus, electrophysiological properties of orexin neurons reach the adult level by 4 weeks of age in rats.

2.4 Discussion

The present study demonstrates that during the post-weaning period in rats, MCH neurons undergo an age-dependent decrease in excitability and reach the adult level by 7 weeks of age. This decrease in excitability is characterized by a hyperpolarization of RMP and a depolarization of the firing threshold, which most likely contributed to the decrease in firing frequency and increased first spike latency. An age-dependent change in the transient outward potassium current is another possible modulator of MCH neuron excitability (Falk et al., 2003), which is a topic of future investigation.

2.4.1 Comparison to previous research

These findings add to a previous study that showed that the excitability of MCH neurons undergo age-dependent changes in mice during pre-weaning development (Li & van den Pol, 2009). In addition to the difference in species used, the results of the present study and theirs differ in two main areas. Firstly, the RMP of MCH neurons in mice plateaus around 4 weeks of age and remains stable into adulthood; contrastingly, in rats, this plateau is reached by 7 weeks old. Secondly, the developmental changes in the excitability of MCH neurons in mice are largely dependent on excitatory GABAergic transmission. This mechanism is unlikely to underlie the excitability changes demonstrated in the present study as they were observed in the presence of the chloride channel blocker picrotoxin and the animals used were older than the age during which GABA is excitatory (Tyzio, Holmes, Ben-Ari, & Khazipov, 2007). Thus, our study shows

that GABA-independent mechanisms are involved in the change in excitability of MCH neurons in post-weaning development.

2.4.2 Synaptic plasticity in MCH neurons during the post-weaning period

Excitatory transmission to MCH neurons also decreased with age. The amplitude of mEPSCs became smaller as animals aged, suggesting a postsynaptic mechanism such as a decrease in the surface expression or conductance of synaptic AMPA receptors (Lüscher et al., 1999; Swanson, Kamboj, & Cull-Candy, 1997). Furthermore, mEPSC frequency also decreased with age, suggesting a presynaptic mechanism such as a decrease in release probability or in the number of active synaptic inputs (Horvath & Gao, 2005; Reim et al., 2001). However, we found that the PPR of MCH neurons decreased between 4 to 7 weeks, suggesting a relative increase in release probability. This discrepancy may be explained by a removal of low release probability synapses through pruning (Hashimoto & Kano, 2003; Huttenlocher & Dabholkar, 1997) or conversion to silent synapses that contain NMDA but not AMPA receptors (Petralia et al., 1999), mainly leaving the higher release probability synapses intact. Alternatively, separate pools of vesicles may exist for evoked and spontaneous EPSCs (Fredj & Burrone, 2009) that can be distinctly affected by intracellular signaling (Katsurabayashi, Kubota, Moorhouse, & Akaike, 2004), calcium concentration (Maeda et al., 2009) or spatial relationship with voltage gated calcium channels (Grauel et al., 2016). Finally, between 7 and 14 weeks of age, mEPSC frequency does not show further change, while the PPR increases. This may indicate a reduction in release probability of existing glutamatergic synapses.

Our characterization of MCH neurons during the post-weaning period adds to the growing body of literature involving maturation and structural plasticity of neural circuitry during adolescence. For instance, over a very similar age range as our study, a subpopulation of neurons within the cingulate cortex show a developmental increase in miniature inhibitory postsynaptic currents (Vandenberg, Piekarski, Caporale, Munoz-Cuevas, & Wilbrecht, 2015). Additionally, synaptic pruning has been reported in the medial prefrontal cortex in early adolescence in rats (Dagher et al., 2001) and in the hippocampus, as dendritic spines decrease during adolescence in female rats (Yildirim et al., 2008).

2.4.3 Activity-dependent short-term plasticity in MCH and orexin neurons

This is the first study to report activity-dependent short-term synaptic plasticity of fast EPSCs in MCH neurons. These neurons show a robust paired pulse facilitation at both 10 and 50Hz, while this is followed by depression of EPSC amplitude with 50Hz stimulation, likely due to synaptic fatigue (Wu & Borst, 1999). In contrast, excitatory synapses to orexin neurons show a strong short-term depression at both 10 and 50Hz, with higher frequency resulting in greater depression as previously reported (Xia et al., 2009). These different forms of synaptic plasticity in two populations of lateral hypothalamic neurons may have distinct functional implications. The synaptic facilitation seen in MCH neurons would act as a high-pass filter, which preferentially allows high frequency signals to trigger postsynaptic firing. On the other hand, short-term depression in orexin neurons would act as a low-pass filter, which preferentially dampens high frequency signals while allowing low frequency signals to trigger postsynaptic firing

(Fortune & Rose, 2001). It is possible that the differences in synaptic responses and age dependence between MCH and orexin neurons may differentially affect network activity within the lateral hypothalamus and consequently, their roles in physiology.

2.4.4 Physiological implications of age-dependent changes in MCH neurons

Altogether, these age-dependent changes likely affect MCH neuron excitability and functional output. Notably, these changes occur between early (4 weeks old) and late adolescence (7 weeks old), where 4-5 weeks of age in rats represents the peak of the growth spurt and normally coincides with the onset of puberty (Sengupta, 2013; Spear, 2000). Given the known role of MCH in energy homeostasis, body growth, and reproductive functions (Barson et al., 2013; Qu et al., 1996; Wu et al., 2009), the electrophysiological maturation of MCH neurons may impact these functions in adolescence. To further support this idea, MCH knockout results in decreased bone mass, body length, and body weight in adolescence and adulthood (Alon & Friedman, 2006; Mul et al., 2010). These findings agree with our results showing higher excitability of MCH neurons during a period of rapid weight gain (4 weeks of age), while older rats show decreases in both MCH neuron excitability and weight gain. Additionally, MCH neurons regulate the release of GnRH and luteinizing hormone (Murray et al., 2006; Segal-Lieberman, Rubinfeld, Glick, Kronfeld-Schor, & Shimon, 2006; Wu et al., 2009), which suggests an involvement of MCH in reproductive function. Finally, adolescents are known to be particularly susceptible to obesity or substance abuse (Chambers, Taylor, & Potenza, 2003; Teegarden, Scott, & Bale, 2009), both of which can be influenced by MCH signaling (Chung et al., 2009; Elliott et al., 2004; Kowalski et al., 2006). Therefore,

the age-dependent change in the excitability of MCH neurons potentially broadly affects their functional output, including energy homeostasis, growth, obesity, addictive behaviours, and hormonal components of adolescent development.

While orexin neurons are also reported to affect energy homeostasis, reward-based behaviour, and the reproductive system (Choi, Davis, Fitzgerald, & Benoit, 2010; Gaskins & Moenter, 2012; González, Jensen, et al., 2016; Skrapits et al., 2015; Small et al., 2003), we found no change in the excitability of orexin neurons during adolescent development. However, our results are consistent with previous studies that reported age-dependent increases in orexin-immunopositive cell number and prepro-orexin mRNA expression before weaning, which reach adult levels between 2 and 3 weeks of age (Iwasa et al., 2015; Sawai, Ueta, Nakazato, & Ozawa, 2010; Yamamoto et al., 2000). Therefore, while orexin neurons may contribute to physiology and physical development during adolescence, this does not involve changes in electrophysiological properties of these neurons.

2.4.5 Conclusions

In summary, during the post-weaning period, MCH but not orexin neurons undergo an age-dependent decrease in excitability. These changes may be important to promote caloric intake and weight gain during growth spurts or to affect hormonal regulation during puberty. Importantly, our findings have implications for designing experiments involving MCH neurons. Namely, studies on the MCH system should account for age-dependent changes in intrinsic excitability and synaptic properties of

MCH neurons, which may indicate that findings in adolescents may not be generalizable to adults. Furthermore, it is possible that these age-dependent changes are species- or strain-specific; therefore, it is important to determine maturational stages of neurons and use age-matched controls within specific animal models.

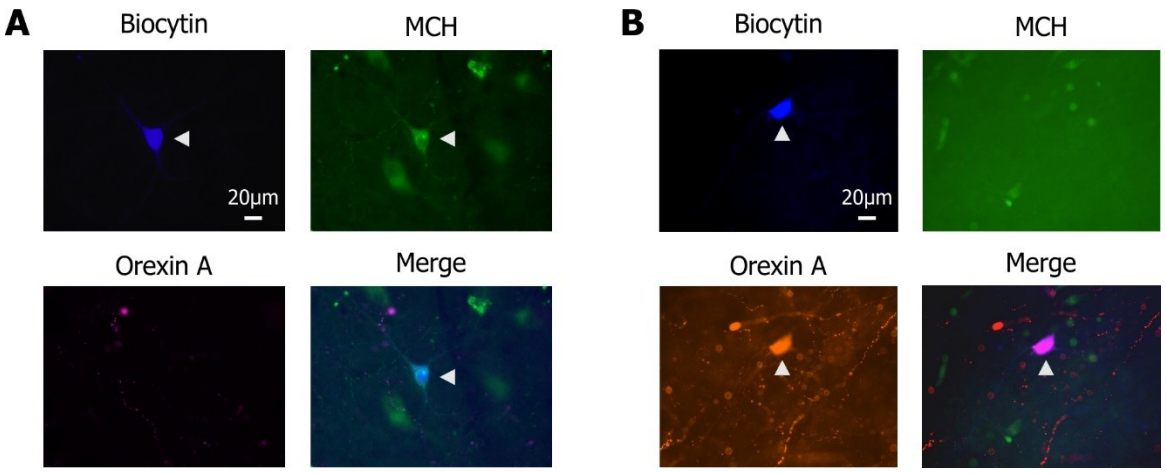

Figure 2.1: Immunohistochemical identification of MCH and orexin neurons.

(A) A sample image of a MCH neuron (adjacent to the white arrow) identified by immuno-histochemistry. (B) A sample image of an orexin neuron (adjacent to the white arrow) identified by immunohistochemistry.

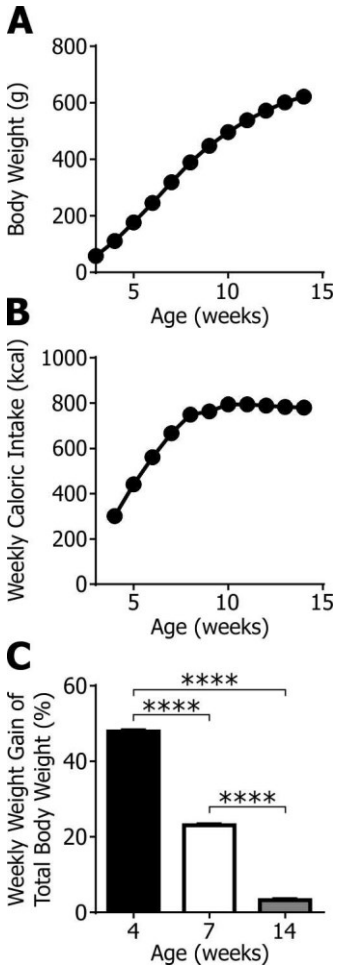

Figure 2.2: Body weight and food intake of post-weaning rats.

(A) Body weight of rats starting at 3 weeks old fed a chow diet *ad libitum*. (B) Weekly caloric intake. (C) Rate of weight gain expressed as a percent increase in body weight compared to total body weight at 4, 7, and 14 weeks old.
*One-way ANOVA with post hoc comparisons: ****p<0.0001*

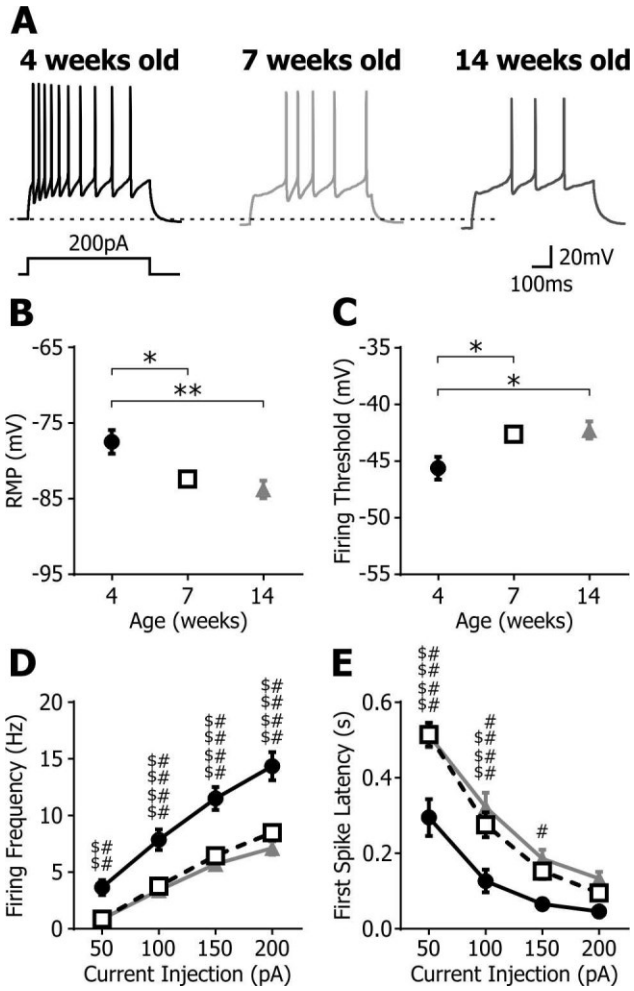

Figure 2.3: MCH neurons undergo an age-dependent decrease in excitability.

(A) Sample traces showing the electrophysiological response of MCH neurons to a 200pA current injection in rats at 4, 7, and 14 weeks old. Dotted line is -80mV. (B) RMP and (C) firing threshold of MCH neurons in rats at 4, 7, and 14 weeks old. (D) Firing frequency and (E) first spike latency with current injections in MCH neurons of rats at 4 (black circles), 7 (open squares), and 14 (grey triangles) weeks old.
*One-way ANOVA with post hoc comparisons: *p<0.05, **p<0.01*
Two-way RM ANOVA with post hoc comparisons: 4wk vs 7wk $$p<0.01, $$$p<0.001, $$$$p<0.0001; 4wk vs 14wk: #p<0.05, ##p<0.01, ####p<0.0001

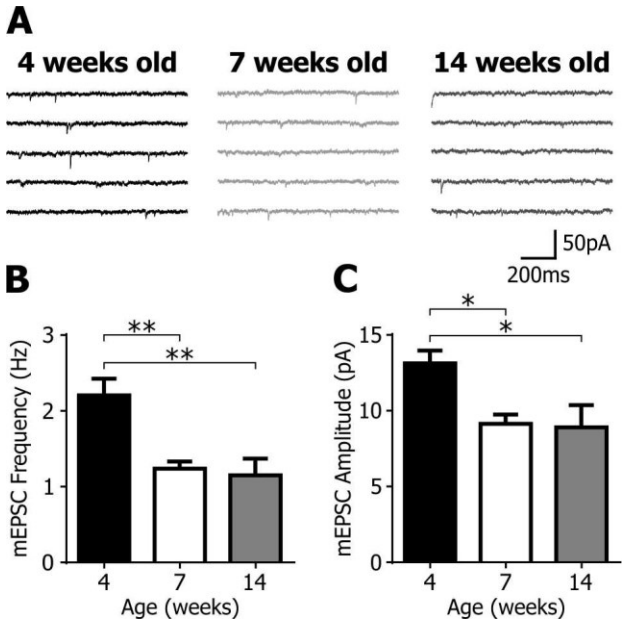

Figure 2.4: Excitatory synaptic transmission to MCH neurons decreases with age.

(A) Sample traces of mEPSCs in MCH neurons of rats at 4, 7, and 14 weeks old. (B) The frequency and (C) amplitude of mEPSCs in MCH neurons.
*One-way ANOVA with post hoc comparisons: *p<0.05, **p<0.01*

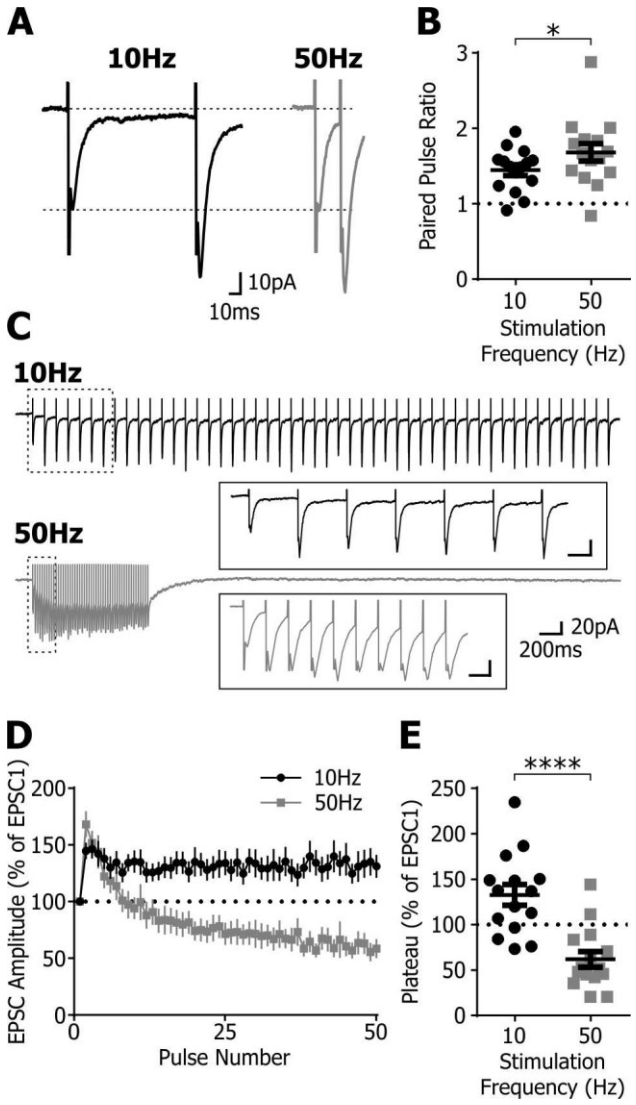

Figure 2.5: Activity-dependent short-term plasticity in MCH neurons of 4-week old rats.

(A) Sample traces of paired EPSCs evoked at 10 and 50Hz. (B) Paired pulse ratio is higher when MCH neurons are stimulated at 50Hz. (C) Sample 10 and 50Hz trains showing 50 evoked EPSCs. Insets represent the initial portion of the train in the dotted box. Scale bars for 10Hz inset is 20pA and 50ms, and 50Hz inset is 20pA and 20ms. (D) Normalized EPSC amplitude of 50 pulses in 10 and 50Hz stimulation trains in MCH neurons. (E) The normalized plateau of 10 and 50Hz stimulation trains.
*Paired t-test: *p<0.05, ****p<0.0001*

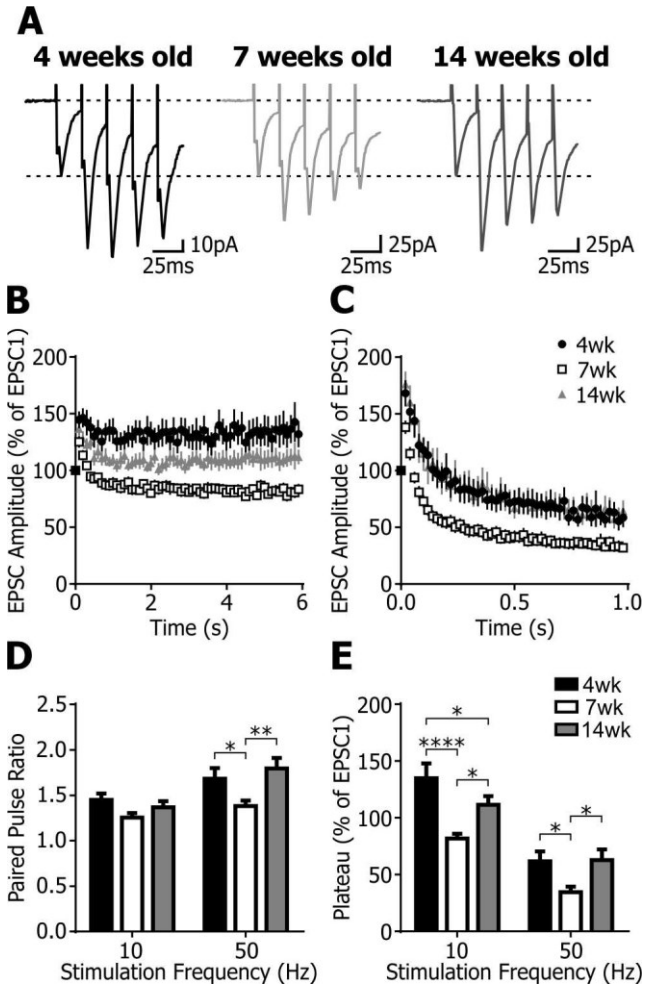

Figure 2.6: Activity-dependent short-term plasticity in MCH neurons is modulated by age.

(A) Sample traces of EPSCs during 50Hz train stimulation, recorded in MCH neurons from rats 4, 7, and 14 weeks old. Normalized EPSC amplitude during (B) 10Hz and (C) 50Hz stimulation train in MCH neurons. (D) Paired pulse ratio at 10 and 50Hz in MCH neurons at various ages. (E) Plateau of 10 and 50Hz stimulation trains in MCH neurons at various ages.

*Two-way RM ANOVA with post hoc comparisons: *p<0.05, **p<0.01, ****p<0.0001*

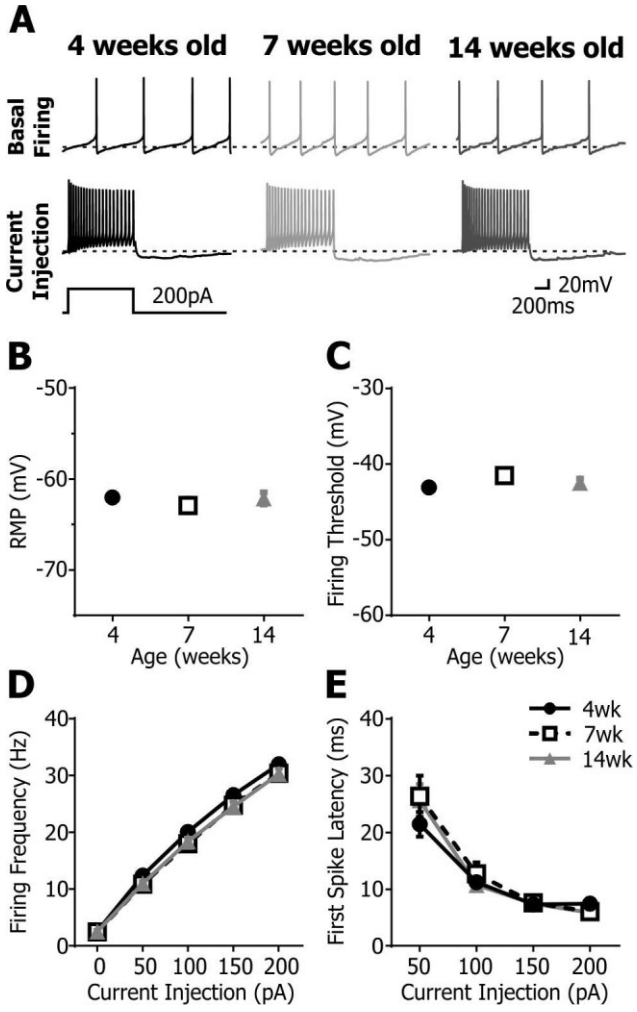

Figure 2.7: Orexin neurons do not undergo a change in excitability over the post-weaning period.

(A) Sample traces of basal firing and firing in response to a 200pA current injection in orexin neurons of rats at 4, 7, and 14 weeks old. Dotted line is -65mV. (B) RMP and (C) firing threshold of orexin neurons. (D) Firing frequency and (E) first spike latency of orexin neurons at baseline and with current injections.

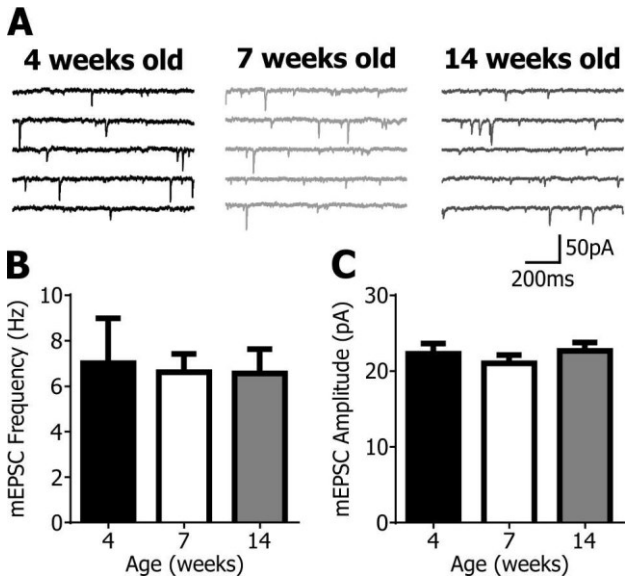

Figure 2.8: Orexin neurons do not undergo changes in spontaneous excitatory transmission after weaning.

(A) Sample traces of mEPSCs in orexin neurons of rats at 4, 7, and 14 weeks old. (B) Frequency and (C) amplitude of mEPSCs in orexin neurons.

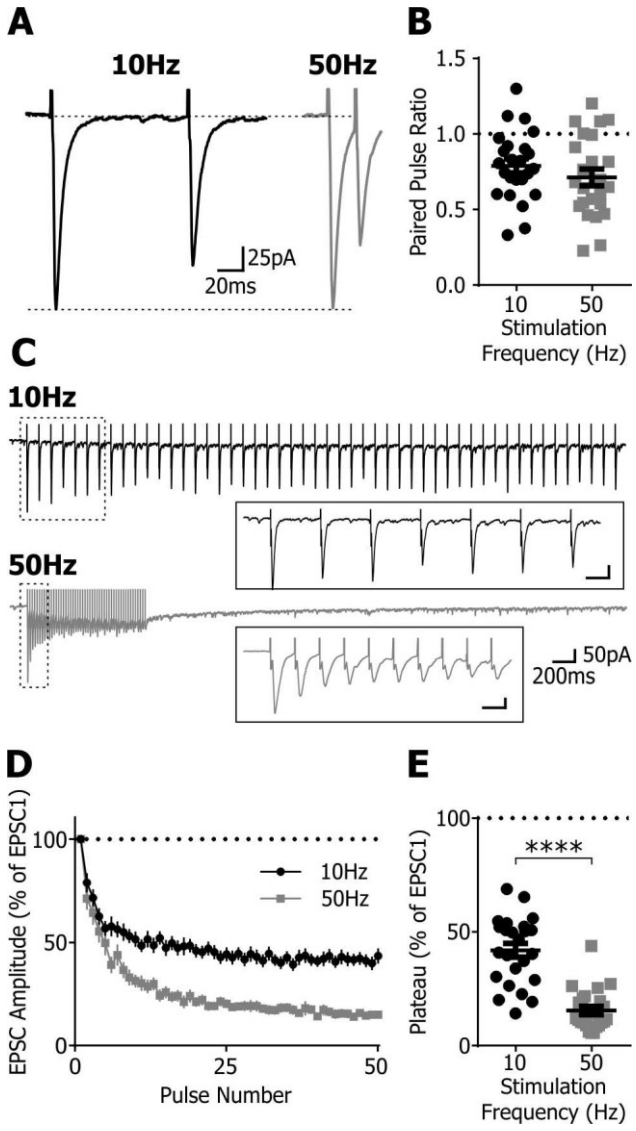

Figure 2.9: Activity-dependent short-term plasticity in orexin neurons of 4-week old rats.

(A) Sample traces of paired EPSCs evoked at 10 and 50Hz. (B) Paired pulse ratio does not differ between 10 and 50Hz stimulation. (C) Sample 10 and 50Hz trains showing 50 evoked EPSCs. Insets represent the initial portion of the train in the dotted box. Scale bars for 10Hz inset is 50pA and 50ms, and 50Hz inset is 50pA and 20ms. (D) Normalized EPSC amplitude of 50 pulses in 10 and 50Hz stimulation trains in MCH neurons. (E) The normalized plateau of 10 and 50Hz stimulation trains.
*Paired t-test: ****p<0.0001*

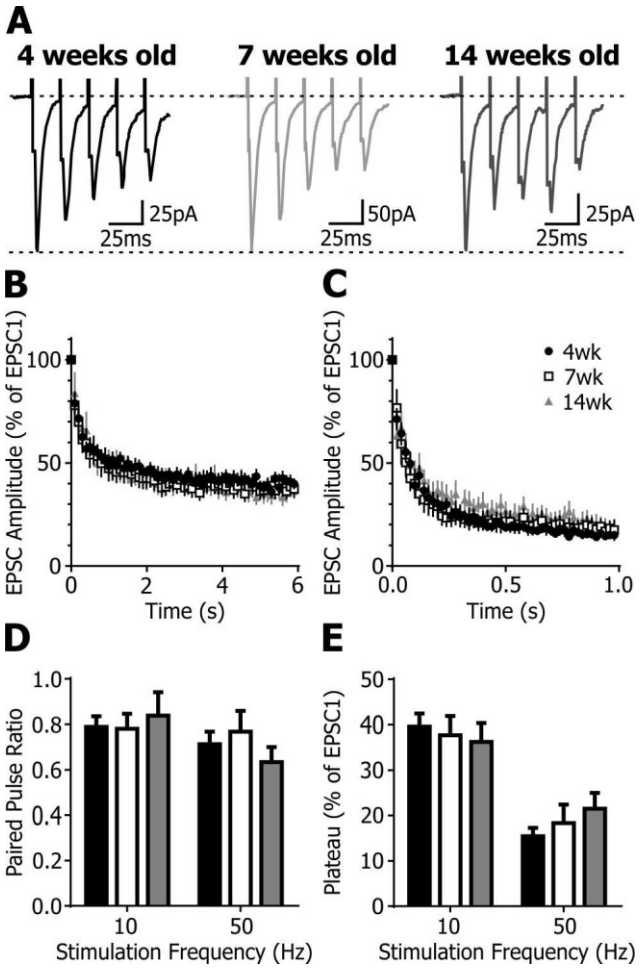

Figure 2.10: Orexin neurons do not undergo changes in activity-dependent short-term plasticity after weaning.

(A) Sample traces of EPSCs evoked at 50Hz in orexin neurons from rats at 4, 7, and 14 weeks old. Normalized EPSC amplitude of a (B) 10Hz and (C) 50Hz stimulation train in orexin neurons. (D) Paired pulse ratio at 10 and 50Hz in orexin neurons at various ages. (E) Plateau of the 10 and 50Hz stimulation trains in orexin neurons at various ages.

CHAPTER 3

HIGH-FAT DIET PRIMES EXCITATORY SYNAPSES TO OREXIN NEURONS FOR LONG-TERM DEPRESSION

3.1 Introduction

Excessive consumption of high-fat diets has become common in many countries around the world, increasing the risks for serious health outcomes such as cardiovascular diseases, metabolic syndrome, and obesity (Cordain et al., 2005). The rewarding value of high-fat food leads to caloric consumption beyond metabolic need and the motivation for this excessive intake is driven by the mesolimbic dopamine pathway (Volkow et al., 2011). Activation of this pathway by high-fat diet involves orexin signaling. Specifically, orexin neurons of the lateral hypothalamus are activated by short-term high-fat diet (Chang et al., 2004; Valdivia et al., 2014) and subsequently stimulate dopamine neurons within the ventral tegmental area (VTA), thereby activating the mesolimbic pathway (Baimel et al., 2017; Valdivia et al., 2014; Zheng et al., 2007). This suggests that overconsumption of high-fat diet is driven by a positive feedback loop between high-fat diet, orexin neurons and VTA dopamine neurons. However, high-fat diet may suppress the orexin system in the long term, as orexin gene and peptide levels are lower in animals after chronic high-fat diet compared to those fed a low-fat diet (Novak et al., 2010; Tanno et al., 2013). Thus, the regulation of the orexin system may change over the course of high-fat diet feeding.

While high-fat diet is known to induce various types of functional and structural plasticity within the reward circuitry (Johnson & Kenny, 2010; Liu et al., 2016; Sharma et al., 2013; Vucetic, Carlin, Totoki, & Reyes, 2012), plastic changes in orexin neurons during high-fat diet feeding are poorly understood. There has been one study that investigated structural synaptic plasticity in orexin neurons after chronic high-fat diet,

which found changes in type 1 cannabinoid receptor (CB1R)-expressing presynaptic terminals apposed to orexin neurons (Cristino et al., 2013). However, to my knowledge there has been no report on synaptic plasticity of orexin neurons during short-term high-fat diet exposure. Such plasticity of excitatory synapses should impact the excitability of orexin neurons, as excitatory synaptic contacts to these neurons significantly outnumber inhibitory ones and influence their basal firing rate (Horvath & Gao, 2005; Li et al., 2002). Moreover, orexin neurons are known to display plasticity of excitatory synapses under various physiological states, such as fasting (Horvath & Gao, 2005) and sleep deprivation (Y. Rao et al., 2007), which has been postulated to affect foraging and arousal, respectively.

In this study, we investigated the effect of short-term exposure to a palatable high-fat Western Diet (WD) on excitatory transmission to orexin neurons. Our hypothesis was that WD induces synaptic plasticity in orexin neurons. We identified a novel form of long-term plasticity of these synapses after one week of WD feeding. This effect was diminished after a prolonged WD exposure, suggesting an adaptation to the diet. These changes may alter orexin network properties and underlie their physiological role in reward-based feeding during short- and long-term high-fat diet.

3.2 Methods

3.2.1 Animals and diets

All animal procedures were conducted as approved by the Memorial University's Institutional Animal Care Committee under the guidelines of the Canadian Council on Animal Care. Male 3-week old Sprague-Dawley rats were obtained either from Charles River (Quebec, Canada) or Memorial University's Breeding Colony. Upon arrival, rats were weighed then singly housed and fed either WD (TestDiet AIN-76A: 4.55kcal/g: 40% fat, 16% protein, and 44% carbohydrates by caloric content) or a standard rodent chow *ad libitum*. Body weight and food intake were measured weekly.

3.2.2 Electrophysiological recording

After 1 or 4 weeks of feeding, rats were sacrificed by decapitation under isoflurane anesthesia and acute 250µm hypothalamic slices were generated on a vibratome (VT-1000, Leica Microsystems) in ACSF composed of 126mM NaCl, 2.5mM KCl, 1.2mM NaH_2PO_4, 1.2mM $MgCl_2$, 18mM $NaHCO_3$, 2.5mM glucose, and 2mM $CaCl_2$. ACSF was chilled on ice for 5-10 minutes before slicing. After dissection, hypothalamic slices were incubated at 32-34℃ for 30-35 minutes in ACSF then left at room temperature until experiments were performed. ACSF was continuously bubbled with 95% O_2/5% CO_2 gas.

Slices were transferred to a recording chamber, perfused with ACSF (1-2mL per minute at 30-32℃) and visualized with infrared-differential interference contrast optics (DM LFSA, Leica Microsystems). Whole cell patch clamp recordings were performed on

neurons of the lateral hypothalamus/perifornical area using Multiclamp 700B and pClamp 9 or 10 software (Molecular Devices). Signals were filtered at 1kHz and digitized at 5-10kHz. An internal solution containing 123mM K gluconate, 2mM $MgCl_2$, 1mM KCl, 0.2mM EGTA, 10mM HEPES, 5mM Na_2ATP, 0.3mM NaGTP, and 2.7mM biocytin was used to fill glass electrodes with a final tip resistance between 3-5MΩ.

A glass pipette stimulation electrode filled with ACSF was placed medial to recorded neurons to stimulate afferent fibers. Evoked EPSCs were pharmacologically isolated using the $GABA_A$ channel blocker picrotoxin (50μM) and recorded every 15 seconds at a holding potential of –70mV. These EPSCs are largely mediated by AMPA receptors but not kainite receptors (Alberto & Hirasawa, 2010). Paired pulses were applied at 10 or 50Hz to assess paired pulse ratio (PPR) defined as the amplitude of EPSC2 divided by that of EPSC1. To induce activity-dependent synaptic plasticity, high frequency stimulation (HFS) was applied to the afferents using the same stimulation electrode while in current clamp mode, consisting of 100 pulses at 100Hz every 5 seconds, 10 times. NMDA receptor mediated EPSCs (NMDAR-EPSC) were obtained as the difference between evoked EPSCs recorded at +50mV in the absence or presence of the NMDA receptor antagonist (DAP5, 50μM). Access resistance was assessed by applying 20mV, 50ms hyperpolarizing pulses every 15 seconds. Cells that showed a change in these parameters by more than 20% were excluded from further analysis.

3.2.3 Identification of orexin neurons

A series of hyperpolarizing and depolarizing currents (600ms steps) were applied in current clamp mode to identify putative orexin neurons. Orexin neurons display a distinct electrophysiological response to these current injections compared to other cell types in the region which allow for high accuracy identification (Alberto, Trask, & Hirasawa, 2011; Linehan et al., 2015). Specifically, orexin neurons display spontaneous firing, uniphasic after-hyperpolarizing potential, H-current, and rebound depolarization following relief from hyperpolarization.

To confirm the neurochemical phenotype, post hoc immunohistochemistry was performed in a subset of cells (114 of 148 cells included in this study). During recording, cells were filled with biocytin via the recording pipette and subsequently brain slices were fixed in 10% formalin for at least 24 hours. Slices were then incubated with a goat anti-orexin A IgG (1:2000; SC8070, Santa Cruz Biotechnology, Santa Cruz, CA) for 3 days, followed by Alexa 594-conjugated donkey anti-goat IgG (1:500) and Alexa 350-conjugated streptavidin (1:500). Colocalization of biocytin with orexin A staining was assessed on an epifluorescence microscope (Fig. 2.1). Using immunohistochemical analysis, the success rate for correct identification of orexin neurons from electrophysiological properties was verified to be over 99% (113 of 114 cells). Therefore, cells that displayed the characteristic electrophysiological fingerprint of orexin neurons were included in the analysis.

3.2.4 Drugs

Drugs were prepared as stock solutions and were diluted with ACSF to the final concentration immediately prior to experiment except for GDPβS, which was added to the internal solution at its final concentration in place of GTP (0.3mM). Picrotoxin, GDPβS, and AM251 were from Sigma Aldrich (Oakville, ON, CA); DAP5 and γDGG were from Abcam (Cambridge, MA, US); and MPEP, DHK, CPPG, and DHPG were from Tocris Bioscience (Minneapolis, MN, US).

3.2.5 Statistical analysis

Data are expressed as the mean ± SEM and $p<0.05$ was considered significant. The number of observations is reported in the nested model where N/n represents the number of cells/the number of animals. Statistical analyses were performed using Prism 6.0 (GraphPad). Unpaired, ratio paired, or paired t-test and one-way or two-way ANOVA with post hoc Holm-Sidak tests were performed on the data as applicable.

3.3 Results

To examine the effects of high-fat diet on orexin neurons, rats were fed with either a WD or a standard chow (Ctrl) for up to 4 weeks. While rats overconsumed calories from WD throughout the entire feeding period compared to chow controls (Ctrl n=14 vs WD n=15; Fig. 3.1A), they only had a significantly higher body weight by the fourth week of feeding (Fig. 3.1B). To study the time-dependent effects of WD on plasticity in orexin neurons, two time points were investigated: 1 week of feeding, when orexin neurons are reported to be activated by high-fat diet (Valdivia et al., 2014; Wortley et al., 2003), and at 4 weeks, when orexin gene expression is first reported to decrease (Novak et al., 2010).

3.3.1 HFS induces a presynaptic LTD of excitatory transmission in the WD condition

After 1 week of feeding, all orexin neurons tested in the WD condition displayed a long-term depression (LTD), resulting in approximately 50% reduction on average in EPSC amplitude (WD: baseline 285.9±60.9pA vs post-HFS 162.3±28.1pA, N/n=6/4, p=0.0115; Fig. 3.2A-C). In contrast, chow control orexin neurons did not display a consistent response. A fraction of chow control cells (3 out of 7 cells) displayed a long-term potentiation (LTP), defined as an increase in EPSC amplitude by more than 20% of baseline values, while others showed no change (Fig. 3.2A-C). Collectively, EPSC amplitude post-HFS was significantly different between chow control and WD (Ctrl 118.7±9.0% vs WD 60.5±7.0%, p=0.0004; Fig. 3.2C). The LTD in the WD group was accompanied by an increase in PPR (WD: baseline 0.93±0.16 vs post-HFS 1.22±0.18,

p=0.0006; Fig. 3.2A,D) but there was no PPR change in chow controls (Ctrl: baseline 0.79±0.08 vs post-HFS 0.78±0.05, p>0.05; Fig. 3.2D). Thus, LTD in the WD condition has a presynaptic locus of expression.

3.3.2 LTD is dependent on metabotropic glutamate receptors

Since a robust LTD occurred only in the WD condition in orexin neurons, we sought to determine the mechanism underlying this plasticity using brain slices from WD-fed rats. NMDA receptors are commonly involved in long-term synaptic plasticity; however, we found that NMDA receptors are not involved in this LTD, as the NMDAR antagonist DAP5 (50μM) failed to significantly influence the HFD-induced changes in EPSC amplitude (HFS N/n=6/4 vs HFS+DAP5 N/n=5/4; Fig. 3.3A,C) or PPR (Fig. 3.3A,D). Another glutamate receptor that could mediate presynaptic LTD is metabotropic glutamate receptor 5 (mGluR5) (Robbe, Kopf, Remaury, Bockaert, & Manzoni, 2002). We found that LTD was attenuated by the mGluR5-specific antagonist, MPEP (20-40μM) (HFS 60.5±7.0%, N/n=6/4 vs HFS+ MPEP 94.6±4.2%, N/n=5/4, p=0.0029; Fig. 3.3B-D). Further supporting mGluR5 involvement, the group 1 mGluR agonist DHPG (50μM) induced LTD on its own (baseline: 301.4±53.5pA vs post-DHPG: 230.1±48.6pA, N/n=13/7, p<0.0001; Fig. 3.3E) and occluded HFS-induced LTD (HFS 60.5±7.0%, N/n=6/4 vs HFS post-DHPG 101.7±4.3%, N/n=6/3, p=0.0005; Fig. 3.3F,G). Taken together, these results suggest that LTD in the WD condition is due to an mGluR5-dependent mechanism.

3.3.3 LTD is dependent on retrograde endocannabinoid signaling

To test whether mGluR5 signaling is pre- or postsynaptic, 2mM GDPβS was added to the pipette solution and allowed to diffuse into the cell for 10 minutes prior to baseline recording. We have shown previously that this concentration of GDPβS and procedure inhibits G-protein signaling within the postsynaptic orexin neuron (Parsons et al., 2012). We found that GDPβS blocked both the reduction in EPSC amplitude (HFS 60.5±7.0%, N/n=6/4 vs HFS+GDPβS 120.9±9.6%, N/n=5/3, $p<0.0001$; Fig. 3.4A,C) and the increase in PPR (Fig. 3.4A,D) in the WD condition, suggesting an involvement of postsynaptic G-protein signaling. This data, along with the result showing that LTD is expressed presynaptically, suggests that a retrograde transmitter such as endocannabinoids must be involved (Robbe et al., 2002). Consistent with this idea, the CB1R antagonist AM251 (5μM) blocked HFS-induced LTD (HFS 60.5±7.0%, N/n=6/4 vs HFS+AM251 98.5±2.8%, N/n=6/4, $p=0.0011$; Fig. 3.4B-D). These results suggest that LTD in orexin neurons is dependent on postsynaptic mGluR5 and retrograde endocannabinoid signaling.

3.3.4 Activation of group 1 mGluRs induces LTD in both Ctrl and WD conditions

It remains unknown why LTD is consistently observed in orexin neurons in the WD condition but not in chow-fed controls. One possibility is that orexin neurons do not express functional mGluR5 receptors in chow conditions. However, pharmacologically activating group 1 mGluRs in chow controls using the group 1-specific mGluR agonist DHPG also led to a presynaptic LTD (Ctrl: baseline 353.9±55.5pA vs post-DHPG

187.3±29.8pA, N/n=7/5, p=0.0008; Fig. 3.5A-C). Thus, the molecular machinery necessary for mGluR5-dependent LTD is intact even in the chow-fed condition.

3.3.5 WD attenuates DHPG-induced LTD

Interestingly, the magnitude of the decrease of EPSC amplitude in DHPG-induced LTD was smaller in WD (Ctrl 53.8±5.7%, N/n=7/5 vs WD 71.6±3.9%, N/n=13/7, p=0.0159; Fig. 3.5A,B). This may be an indication that LTD had been endogenously established in WD rats prior to our recordings and was therefore occluding the effect of DHPG. If so, the baseline release probability would be lower, which should be observed as an increase in PPR. This indeed appeared to be the case since the baseline PPR was higher in the WD group compared to chow controls (Ctrl 0.70±0.05, N/n=20/14 vs WD 0.92±0.07, N/n=17/10, p=0.0131; Fig. 3.6A,B). An alternative explanation for this higher PPR is a tonic activation of presynaptic inhibitory group III mGluRs (Acuna-Goycolea, Li, & van den Pol, 2004); however, the group III mGluR antagonist CPPG (200μM) had no effect on EPSCs in the WD condition (N/n=6/3; Fig. 3.6C). Taken together, it is possible that LTD may have occurred endogenously in orexin neurons in the WD condition and occluded the effects of DHPG.

3.3.6 WD increases synaptic glutamate

Since the signaling components for group 1 mGluR-dependent LTD are present in both chow and WD conditions, unmasking of HFS-induced LTD by WD may involve a process upstream of mGluR5 activation, such as synaptic glutamate clearance. To test any difference in the level of extracellular glutamate during synaptic activity, we used the

compound, γ-D-glutamylglycine (γDGG). It is a low affinity, competitive AMPA receptor antagonist, which competes with endogenous glutamate for AMPA receptors. Therefore, the degree of EPSC inhibition induced by γDGG is negatively correlated to glutamate concentration at the synapse (Budisantoso et al., 2013). We tested different concentrations of γDGG to find a concentration (1mM) that reduced EPSC amplitude by about 50% in chow controls, such that we could equivocally detect either an increase or decrease in the response (Fig. 3.7A,B). In the WD group, the same concentration of γDGG induced significantly less inhibition of EPSCs (Ctrl 45.3±4.4%, N/n=7/3 vs WD 71.7±4.4%, N/n=5/3, p=0.0020; Fig. 3.7A,B), indicating an increase of synaptic glutamate. However, there was no difference in the decay of AMPA or NMDA receptor-mediated EPSCs between WD and chow controls (AMPAR-EPSC decay: Ctrl N/n=13/8 vs WD N/n=10/6; NMDAR-EPSC decay: Ctrl N/n=5/2 vs WD N/n=5/2; Fig. 3.7C-E), suggesting that the time course of the glutamate transient at the synapse is not affected.

3.3.7 Inhibition of glutamate uptake is sufficient to prime synapses for LTD

An increase in synaptic glutamate may lead to more glutamate spillover during HFS to activate perisynaptic mGluR5 receptors and their downstream LTD signaling cascade. To test this idea, we used a low concentration of DHK (10-15μM), an inhibitor of the astrocytic glutamate transporter-1 (GLT-1), to inhibit glutamate uptake and facilitate spillover in chow controls. This concentration of DHK did not affect EPSC amplitude (N/n=8/3, baseline: 302.2±70.1pA, DHK: 276.9±73.3pA, ratio paired t-test, p=0.2466) or decay (Fig. 3.7F); however, in its presence, chow control synapses expressed a presynaptic LTD in response to HFS (HFS 118.7±9.0%, N/n=7/4 vs

HFS+DHK 67.9±7.9%, N/n=6/3, p=0.0016; Fig. 3.7G-I). Thus, increased extracellular glutamate is sufficient for LTD induction.

3.3.8 LTD can no longer be induced by HFS after 4 weeks of WD feeding

Finally, we asked whether these WD-induced synaptic changes are long lasting. After 4 weeks of feeding, we found that there was no longer any difference between WD and age-matched chow control groups in synaptic glutamate as measured by the degree of EPSC inhibition by γDGG (4wCtrl 59.2±5.4%, N/n=5/4 vs 4wWD 48.1±1.3%, N/n=4/3, p>0.05; Fig. 3.8A,B). Accordingly, neither group expressed HFS-induced LTD (4wCtrl N/n=6/2 vs 4wWD N/n=5/2; Fig. 3.8C-E). Therefore, adaptation to WD occurs at excitatory synapses to orexin neurons.

3.4 Discussion

The present study demonstrates that one week of WD feeding primes excitatory synapses to orexin neurons to undergo activity-dependent LTD. WD increases the level of synaptic glutamate, which results in activation of postsynaptic mGluR5 receptors during intense synaptic activity. This leads to endocannabinoid release from orexin neurons, subsequent activation of presynaptic CB1Rs, and the expression of a presynaptic LTD (Fig. 3.9). This priming effect is transient and disappears with longer periods of WD feeding. To our knowledge, this is the first study to describe LTD of excitatory inputs to orexin neurons. Because orexin neurons promote high-fat food intake, it is reasonable to assume that LTD of excitatory synapses to these neurons is a homeostatic response to WD, limiting food intake. On the other hand, a previous study described a postsynaptic LTP at these synapses via a cAMP-dependent mechanism (Y. Rao et al., 2007). In our chow controls, HFS induced LTP without a change in PPR in 43% of cells tested, which may be explained by this mechanism.

3.4.1 LTD is mediated by mGluR5 and endocannabinoid signaling

Orexin neurons have been shown to synthesize and release endocannabinoids in response to membrane depolarization (Cristino et al., 2013), which in turn acutely inhibit excitatory and inhibitory transmission presynaptically (Cristino et al., 2013; Huang et al., 2007). Our results show that endocannabinoids can also be released by mGluR5 activation in orexin neurons and retrogradely induce an LTD of excitatory synapses to these neurons. Glutamate has been reported to induce endocannabinoid synthesis via

other receptors in other brain regions, namely NMDA receptors (Stella & Piomelli, 2001). However, our study indicates that these receptors are unlikely to be involved in LTD induction. Therefore, mGluR5 is the primary glutamate receptor responsible for stimulating endocannabinoid synthesis from orexin neurons during intense activity of excitatory synapses.

3.4.2 DHPG-induced LTD is occluded in the WD condition

Although WD feeding was necessary for HFS to induce LTD, pharmacological activation of group 1 mGluRs could induce LTD in both Ctrl and WD conditions. Therefore, the machinery for LTD, including the mGluR5-CB1R pathway, is present and functional under both conditions. Nevertheless, the magnitude of DHPG-induced LTD was smaller in WD compared to chow controls. It is possible that in WD animals, LTD is induced endogenously and occludes the DHPG-induced LTD. This is supported by our results showing a higher basal PPR in WD cells, which could indicate a decrease in basal release probability. Alternatively, a change in cell surface expression (Knackstedt & Schwendt, 2016) or perisynaptic localization of mGluR5 receptors (Sergé, Fourgeaud, Hémar, & Choquet, 2002) may account for this difference. In addition, chronic high-fat diet has been shown to induce synaptic remodeling in orexin neurons, accompanying a decrease in the number of CB1R-expressing excitatory synapses (Cristino et al., 2013), which could influence this CB1R-dependent LTD. However, whether this type of synaptic remodeling in orexin neurons would occur after 1 week of WD feeding is unknown.

3.4.3 Synaptic glutamate is increased by WD and may underlie unmasking of LTD

Our study suggests that a change in synaptic glutamate levels underlies the WD-induced unmasking of LTD in orexin neurons. Synaptic glutamate concentration is determined by the amount of release, uptake, and diffusion of glutamate. It is unlikely that WD increases glutamate release, since the basal release probability at these excitatory synapses was decreased during WD feeding. Synaptic remodeling is known to occur in orexin neurons due to dietary factors (Cristino et al., 2013; Horvath & Gao, 2005) and this may accompany a change in synaptic morphology, such as the number of synapses, which could result in altered capacity for glutamate diffusion (Piet, Vargová, Syková, Poulain, & Oliet, 2004). Finally, synaptic glutamate could also increase if glutamate uptake through transporter activity is decreased. Indeed, WD may alter glutamate uptake, since hormones influenced by high-fat diet, such as leptin and ghrelin, have previously been described to modulate the expression of astrocytic glutamate transporters GLT-1 and GLAST in the hypothalamus (Fuente-Martín et al., 2012, 2016). Furthermore, we showed that a GLT-1 inhibitor DHK mimics WD and permits HFS-induced LTD in chow control orexin neurons. Thus, a decrease in glutamate transport may be at least partially responsible for increased synaptic glutamate by WD. Nevertheless, we did not detect any effect of WD or DHK on the decay and/or amplitude of EPSCs in orexin neurons. This suggests that the kinetics of fast excitatory transmission at these synapses may not be influenced by glutamate transport, similar to what is seen in the cerebellum and hippocampus (Isaacson & Nicoll, 1993; Marcaggi, Billups, & Attwell, 2003). Limited glutamate uptake may instead have greater influence on glutamate spillover and

subsequent activation of extrasynaptic receptors (Freestone et al., 2014), and facilitate synaptic plasticity that depends on these receptors (Brasnjo & Otis, 2001). Notably, the developing brain is more vulnerable to the effects of high-fat diet (Morin et al., 2017), while the expression of astrocytic glutamate transporters rapidly increases postnatally and continues to increase into adulthood (Furuta, Rothstein, & Martin, 1997). Therefore, it is possible that young animals, such as those used in this study (3 weeks of age at the start of feeding paradigm), are particularly sensitive to modulation of glutamate clearance mechanisms and the induction of LTD by WD.

3.4.4 Physiological implications of LTD in orexin neurons

While the source of excitatory afferents to orexin neurons tested in this study were not defined, these neurons are known to receive diverse inputs from several brain regions involved in energy balance, reward, and arousal. These include the basal forebrain, the limbic system, the brainstem, and the hypothalamus including the arcuate nucleus and the ventromedial, dorsomedial, posterior, and lateral hypothalamus (Acuna-Goycolea et al., 2004; Deurveilher & Semba, 2005; Henny & Jones, 2006; Yoshida et al., 2006). Since LTD was observed in all orexin neurons tested in the WD group, it may not be a mechanism restricted to certain afferents. Thus, LTD at these glutamatergic synapses would be poised to influence the integration of diverse signals received by orexin neurons and hence their functional output relevant for high-fat diet consumption, including arousal, motivation, locomotor activity, and sympathetic outflow (Tsujino & Sakurai, 2013).

The effects of high-fat diet on the orexin system are known to be time-dependent: short exposures to high-fat diet stimulate, while longer exposures suppress the orexin system. For example, after 2 hours of high-fat diet, orexin neurons increase cFos expression (Valdivia et al., 2014), while orexin cell density and mRNA expression increases within 3 weeks of high-fat diet (Wortley et al., 2003; but see Ziotopoulou et al., 2000). The resulting increases in orexin signaling can lead to a positive feedback loop of high-fat diet overconsumption (Valdivia et al., 2014; Zheng et al., 2007). It is possible that the LTD described in the present study represents a homeostatic mechanism to put a brake on this positive feedback loop to prevent overexcitation of orexin neurons. Because orexin neurons are responsible for activating the VTA upon high-fat diet intake (Valdivia et al., 2014; Zheng et al., 2007), plasticity at the level of orexin neurons could have functional consequences within the reward circuitry. Moreover, high-fat diet is also known to induce an endocannabinoid-dependent LTD in the VTA and decreases the salience of high-fat diet (Labouèbe et al., 2013). Therefore, LTD at both sites could act in concert to regulate behavioral responses to high-fat diet.

In contrast to these excitatory effects of short-term high-fat diet, prolonged exposures (1 to 4 months) reduce orexin gene and peptide levels within the hypothalamus (Novak et al., 2010; Tanno et al., 2013). This may be due to obesity per se, as genetically obese animals also show low orexin mRNA expression (Cai et al., 2000). A reduction in orexin expression suggests that the homeostatic mechanism to keep the activity of the orexin system in check may no longer be necessary. In support of this idea, the present study found that LTD was not induced after 4 weeks of WD. While the mechanism

underlying this adaptation to long-term WD remains unknown, it may be secondary to an extended presence of triglycerides or dynamic changes in diet-sensitive hormone levels that have time-dependent effects on brain and behavior (Cansell et al., 2014; Fuente-Martín et al., 2012, 2016).

3.4.5 Conclusions

In summary, the present study demonstrates a novel mechanism by which WD induces metaplasticity in orexin neurons. This plasticity is time-dependent and may underlie changes in the activity of the orexin system over the course of high-fat diet feeding. In a recent paper using a similar WD and animal model as our study, adolescent male Sprague-Dawley rats were shown to display reward hypofunction after 10 days of WD that was normalized by continued WD feeding into adulthood (Rabasa et al., 2016). It is tempting to speculate that priming of orexin neurons to LTD and its reversibility shown in our study contributes to these behavioral responses and adaptation to WD. Furthermore, apart from modulating motivation and intake of high-fat diet, this synaptic plasticity may also underlie other alterations in physiological functions associated with high-fat diet and obesity, such as diminished wakefulness/alertness and energy expenditure that can be explained by inhibition of orexin neurons (Jenkins et al., 2006; Tanno et al., 2013).

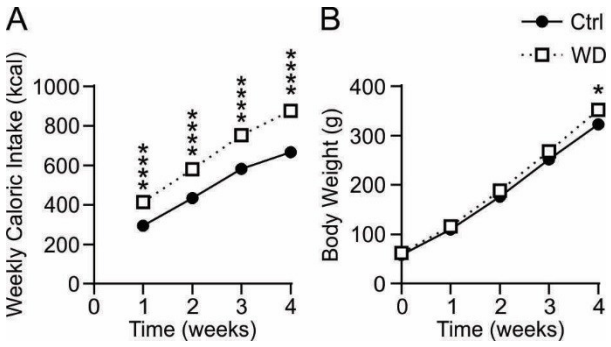

Figure 3.1: Western Diet promotes caloric intake and weight gain.

(A) Caloric intake of rats fed Western Diet (WD, open symbol) or standard chow (Ctrl, filled symbol). (B) Body weight of rats fed WD or chow controls.
*Two-way RM ANOVA with post hoc comparisons: *p<0.05, ****p<0.0001*

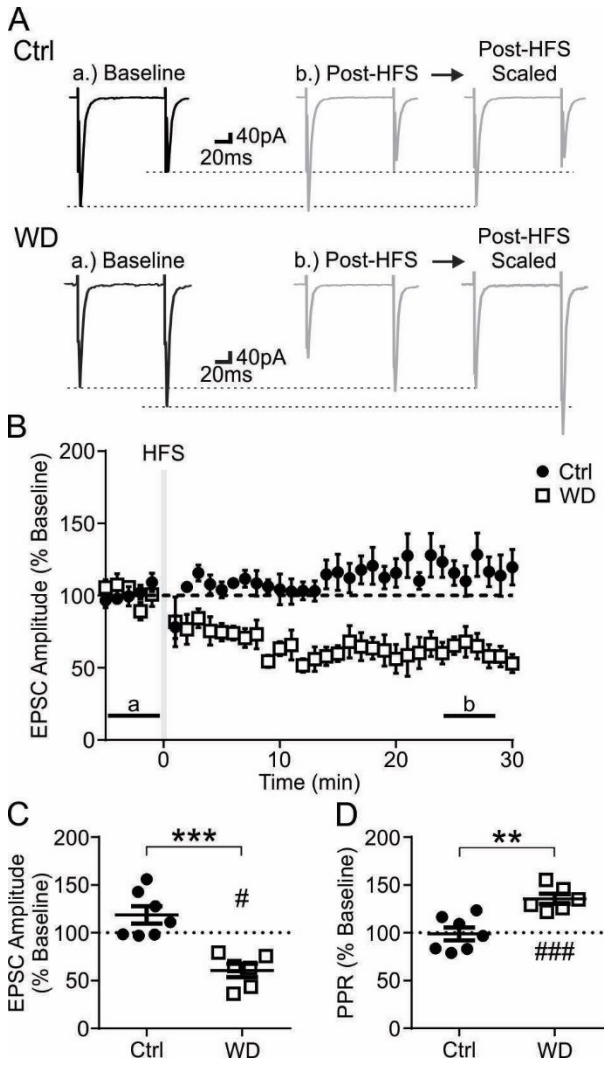

Figure 3.2: One week of WD feeding primes orexin neurons to undergo HFS-induced presynaptic LTD.

(A) Sample traces showing paired EPSCs of orexin neurons in Ctrl and WD groups at baseline (a) and post-HFS (b), recorded at time points indicated in panel B. Post-HFS traces are scaled so that the first EPSC of baseline and post-HFS are comparable (right traces). Dotted reference lines show that the PPR changed in WD but not in Ctrl. (B) EPSC amplitude normalized to respective baseline in WD and Ctrl orexin neurons. HFS was applied at time 0 (grey bar). Normalized (C) EPSC amplitude and (D) PPR 25-30 minutes post-HFS (b in panel B) normalized to a 5-minute baseline (a in panel B) in Ctrl and WD. For C and D, each symbol denotes an individual orexin neuron.

*Unpaired t-test (Ctrl vs WD): **p<0.01, ****p<0.0001*

Paired t-test (baseline vs post-HFS within individual groups): #p<0.05, ###p<0.001

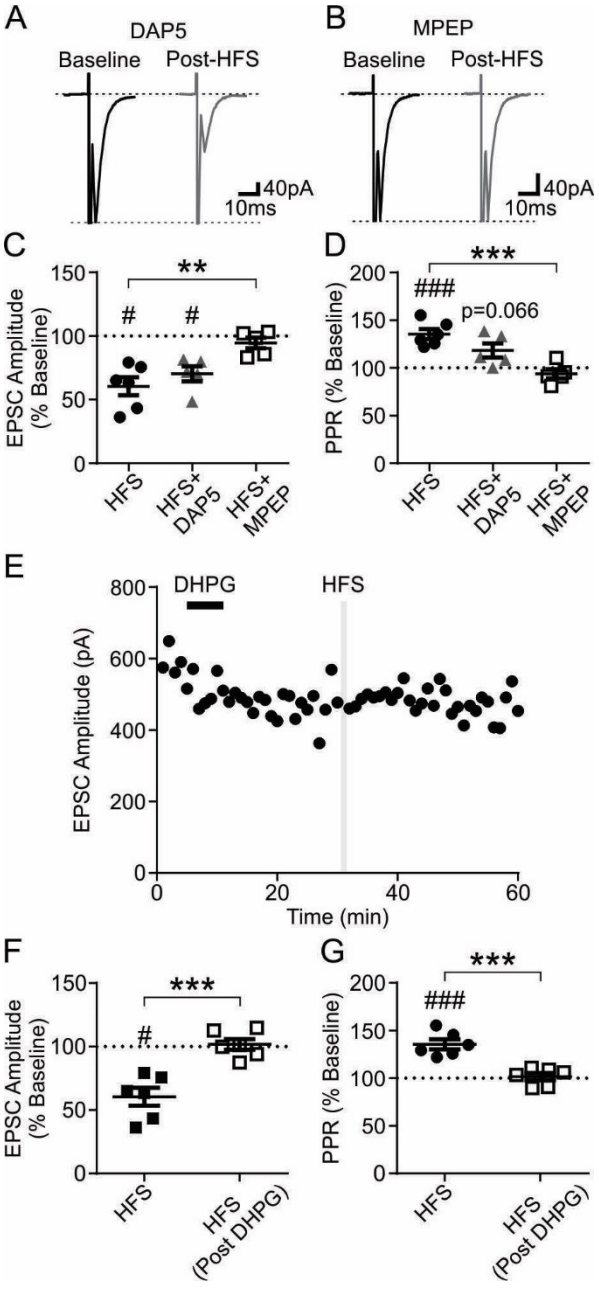
A
DAP5
Baseline
Post-HFS
40pA
10ms
B
MPEP
Baseline
Post-HFS
40pA
10ms
C
EPSC Amplitude (% Baseline)
**
#
#
HFS
HFS+ DAP5
HFS+ MPEP
D
PPR (% Baseline)
***
###
p=0.066
HFS
HFS+ DAP5
HFS+ MPEP
E
DHPG
HFS
EPSC Amplitude (pA)
Time (min)
F
EPSC Amplitude (% Baseline)
***
#
HFS
HFS (Post DHPG)
G
PPR (% Baseline)
***
###
HFS
HFS (Post DHPG)

Figure 3.3: LTD in the WD condition is mGluR5-dependent.

(A) Sample EPSCs showing the effect of HFS on WD cells in the presence of DAP5 (50μM) applied 10 minutes before HFS up until 5 minutes post-HFS. (B) Sample EPSCs pre- and post-HFS in the presence of group 1 mGluR antagonist MPEP (20-40μM) applied 10 minutes before HFS up until 5 minutes post-HFS. Summary of EPSC amplitude (C) and PPR (D) 25 minutes post-HFS normalized to baseline. HFS-induced LTD is not affected by DAP5 but blocked by MPEP. Each symbol denotes an individual orexin neuron. (E) Representative time effect plot of DHPG (50μM) application and subsequent HFS on EPSC amplitude in WD orexin neurons. Summary of EPSC amplitude (F) and PPR (G) post-HFS normalized to respective baseline without or with prior DHPG application. DHPG occludes HFD-induced LTD. Each symbol denotes an individual orexin neuron.
*One-way ANOVA (C,D) and unpaired t-test (F,G): **p<0.01, ***p<0.001*
Paired t-test (baseline vs post-HFS within individual groups): #p<0.05, ###p<0.001

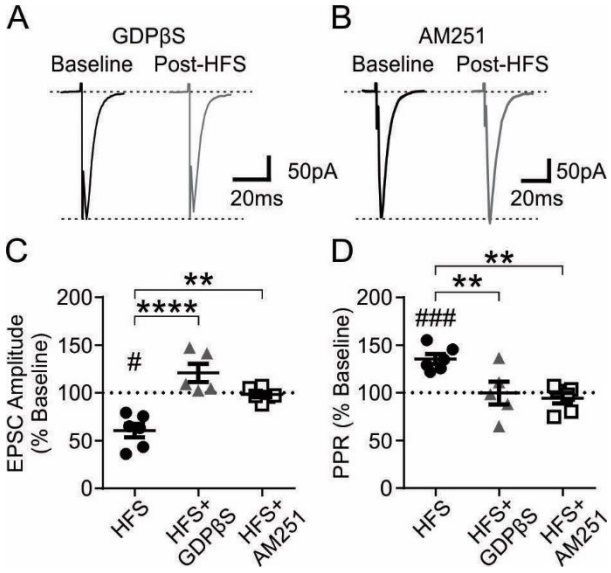

Figure 3.4: LTD is mediated by postsynaptic G protein and retrograde endocannabinoid signaling in orexin neurons.

Sample EPSCs showing the effect of HFS on WD orexin neurons patch clamped with an inhibitor of G-protein signaling GDPβS (2mM) in the recording pipette. (B) Sample EPSCs showing the effect of HFS on WD orexin neurons in the presence of the CB1R antagonist AM251 (5μM). AM251 was applied 10 minutes before HFS up until 5 minutes post-HFS. Normalized EPSC amplitude (C) and PPR (D) 25 minutes post-HFS in orexin neurons treated with GDPβS or AM251. LTD is abolished by both treatments.
*One-way ANOVA with post hoc comparisons: **p<0.01, ****p<0.0001*
Paired t-test (baseline vs post-HFS within individual groups): #p<0.05, ###p<0.001

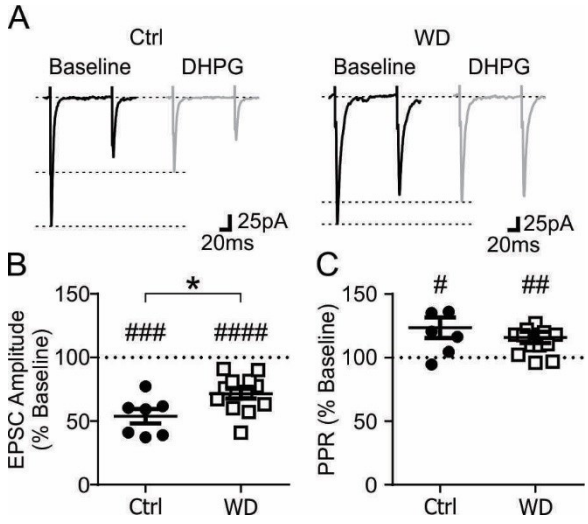

Figure 3.5: Group 1 mGluR activation induces presynaptic LTD regardless of diet.

(A) Sample EPSCs recorded during baseline or 20-30 minutes after DHPG (50μM) application. EPSC amplitude (B) and PPR (C) 20-30 minutes following DHPG application. DHPG induces LTD in both Ctrl and WD conditions, however the magnitude of LTD is greater in Ctrl.

*Unpaired t-test (Ctrl vs WD): *p<0.05*

Paired t-test (baseline vs post-HFS within individual groups): #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001

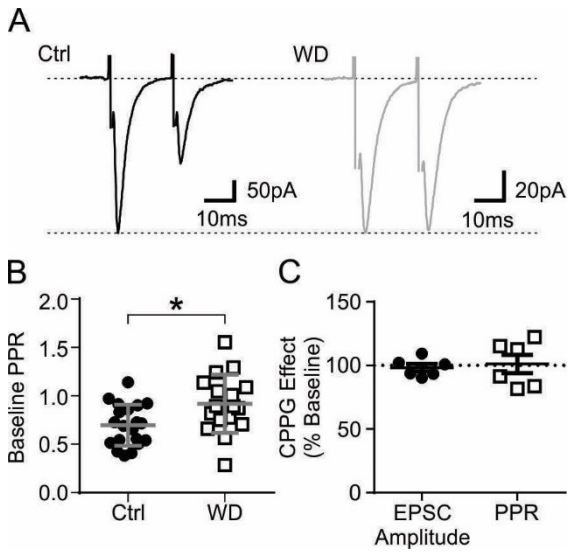

Figure 3.6: Presynaptic release probability is reduced by WD independently of group III mGluR.

(A) Sample traces of paired EPSCs recorded from orexin neurons in Ctrl and WD conditions. (B) WD increases baseline PPR in orexin neurons. (C) Group III mGluR antagonist CPPG (200μM) has no effect on EPSC amplitude or PPR.
*Unpaired t-test: *p<0.05*

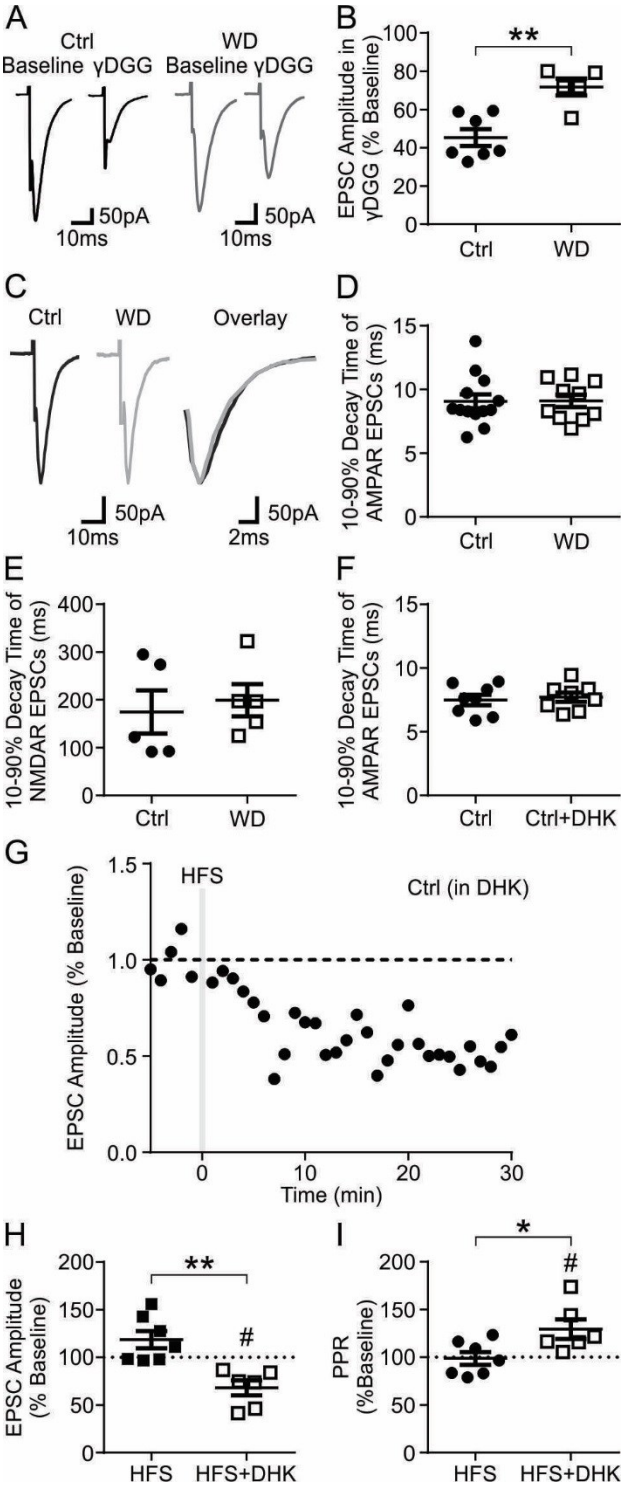
A
Ctrl
Baseline γDGG
WD
Baseline γDGG
50pA
10ms
50pA
10ms
B
EPSC Amplitude in γDGG (% Baseline)
**
Ctrl
WD
C
Ctrl
WD
Overlay
50pA
10ms
50pA
2ms
D
10-90% Decay Time of AMPAR EPSCs (ms)
Ctrl
WD
E
10-90% Decay Time of NMDAR EPSCs (ms)
Ctrl
WD
F
10-90% Decay Time of AMPAR EPSCs (ms)
Ctrl
Ctrl+DHK
G
HFS
Ctrl (in DHK)
EPSC Amplitude (% Baseline)
Time (min)
H
EPSC Amplitude (% Baseline)
**
#
HFS
HFS+DHK
I
PPR (%Baseline)
*
#
HFS
HFS+DHK

Figure 3.7: Reduced synaptic glutamate clearance may underlie the priming of synapses for LTD by WD.

(A) Sample EPSCs showing the effect of the low-affinity, competitive AMPA receptor antagonist γDGG (1mM) on EPSC amplitude in orexin neurons. (B) EPSC amplitude expressed as percent of baseline (before γDGG) and during γDGG treatment. EPSC amplitude is less sensitive to the inhibitory effect of γDGG in WD than in Ctrl. (C) Sample EPSCs (without drug or HFS treatment) and their overlay showing similar decay times in Ctrl and WD conditions. Diet has no effect on the 10-90% decay time of (D) AMPA receptor-dependent EPSCs or (E) NMDA receptor-dependent EPSCs. (F) GLT-1 inhibitor DHK (10-15μM) has no effect on the 10-90% decay time of AMPA receptor-dependent EPSCs. (G) Sample time effect plot showing that HFS induces LTD in the presence of DHK in the chow Ctrl condition. (H) Normalized EPSC amplitude 20-30 minutes post-HFS in Ctrl orexin cells with and without DHK. (I) HFS-induced LTD primed by DHK accompanies an increase in PPR.
*Unpaired t-test: *p<0.05, **p<0.01*
Paired t-test (baseline vs post-HFS within individual groups): #p<0.05

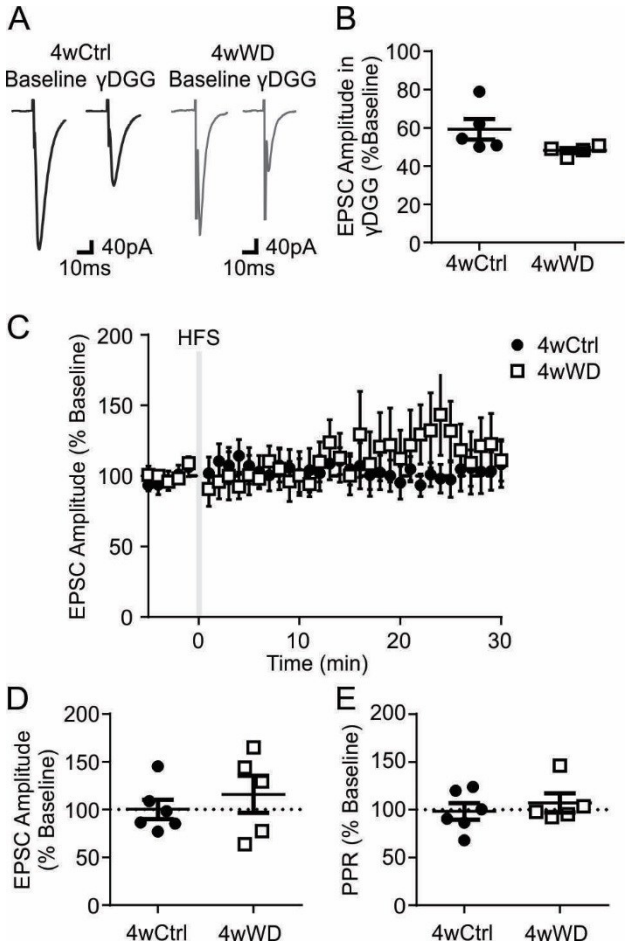

Figure 3.8: Prolonged WD feeding reverses synaptic priming for LTD in orexin neurons.

(A) Sample EPSCs before and during γDGG (1mM) application in orexin neurons from rats fed WD for 4 weeks (4wWD) and age-matched controls (4wCtrl). (B) γDGG effect on EPSC amplitude is not different between 4wWD and 4wCtrl. (C) Time-effect plot of HFS on EPSC amplitude showing a lack of HFS effect in orexin neurons from 4wCtrl or 4wWD groups. EPSC amplitude (D) and PPR (E) are not affected by HFS after 4 weeks of Ctrl or WD.

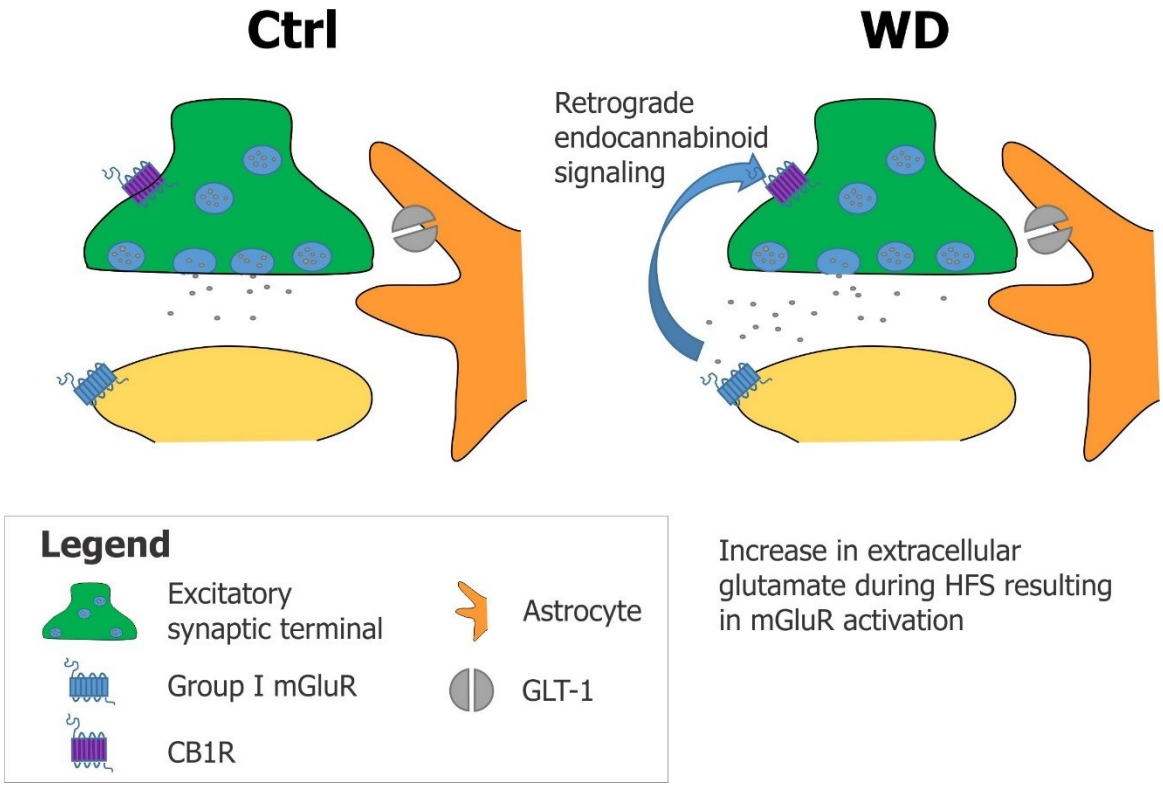

Figure 3.9: Summary of experimental results.

In chow-fed controls (left), synaptically released glutamate is efficiently removed, preventing activation of perisynaptic mGluR5. In WD-fed condition (right), synaptic glutamate is increased, leading to glutamate spillover during HFS, which activates postsynaptic mGluR5 and induces retrograde endocannabinoid signaling to induce a presynaptic LTD.

CHAPTER 4

OREXIN NEURONS UNDERGO TRANSIENT, TIME-DEPENDENT SYNAPTIC PLASTICITY DURING HIGH-FAT DIET FEEDING

4.1 Introduction

Orexin neurons within the lateral hypothalamus are a key component of the circuitry underlying energy homeostasis and reward-based feeding. While orexin neurons promote food intake, particularly so with rewarding, palatable diets (Choi et al., 2010; Perello et al., 2010; Valdivia et al., 2014), they also increase energy expenditure through increased sympathetic activation, locomotion, and spontaneous physical activity (Kotz et al., 2006; Shahid et al., 2011; Wang et al., 2001; Wang, Osaka, & Inoue, 2003). Due to these opposing roles on energy balance and their role in promoting palatable high-fat diet, orexin neurons have a unique role in diet-induced obesity.

Orexin neurons are initially activated by, and then further promote, high-fat diet intake by activating the mesolimbic reward pathway (Valdivia et al., 2014; Wortley et al., 2003). While their role to increase caloric intake would seemingly suggest that orexin neurons are obesogenic, this is not the case – orexin signaling instead protects against weight gain through its actions on energy expenditure (Funato et al., 2009). In fact, a reduction of orexin activity during obesity may contribute to weight gain, as orexin neuron ablation leads to late onset obesity (Hara et al., 2001). Accordingly, in contrast to the activation of orexin neurons seen with short-term high-fat diet exposure, prolonged high-fat diet consumption is associated with decreased orexin mRNA expression and peptide levels (Novak et al., 2010; Tanno et al., 2013), as well as increased inhibitory transmission (Cristino et al., 2013). Together, this evidence suggests that orexin neurons may be regulated in a time-dependent manner during high-fat diet feeding, which may affect energy balance to promote obesity.

However, how high-fat diet affects orexin neurons over the course of feeding is incompletely understood. Cristino and colleagues described synaptic remodeling of orexin neurons in obese mice (Cristino et al., 2013), which they postulated would affect orexin signaling in obesity. In support of this idea, synaptic remodeling has also been reported in other appetite-regulating neurons within the hypothalamus after chronic high-fat diet feeding and is thought to influence body weight regulation (Horvath et al., 2010). Therefore, it is possible that synaptic plasticity also occurs in orexin neurons, and different forms of plasticity may be seen at early and late periods during high-fat diet feeding.

In the present study, synaptic transmission to orexin neurons was characterized over the course of high-fat diet feeding. Our hypothesis was that WD induces synaptic plasticity in orexin neurons. The results show that both excitatory and inhibitory synaptic transmission to orexin neurons are dynamically modulated by high-fat diet and these changes are dependent on the duration of feeding. Given the role of orexin neurons in energy balance, these changes may contribute to the development and maintenance of obesity.

4.2 Methods

4.2.1 Animals and diets

All experiments were conducted following the guidelines of the Canadian Council on Animal Care as approved by Memorial University's Institutional Animal Care Committee. Male 3-week old Sprague-Dawley rats (Charles River, Quebec, Canada and Memorial University Breeding Colony) were fed *ad libitum* for 1, 4, and 11 weeks (Chapter 1, Fig. 1.2B) with a control, low fat diet (LabDiet 5010 – 3.08kcal/g, % by calories: 12.7% fat, 28.7% protein, and 58.5% carbohydrates) or a palatable, high-fat Western Diet (WD; TestDiet AIN-76A– 4.55kcal/g, % by calories: 40% fat, 16% protein, and 44% carbohydrates).

4.2.2 Electrophysiological recording

Rats were sacrificed after feeding by deep isoflurane anesthesia and decapitation. Brains were removed and acute 250μm hypothalamic slices were generated using a vibratome (VT-1000, Leica Microsystems) in chilled ACSF (in mM: 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 18 $NaHCO_3$, 2.5 glucose, and 2 $CaCl_2$) that was bubbled with 95% O_2/5% CO_2. Slices were incubated in ACSF at 32-34℃ for 30 minutes and then left at room temperature until experiments were performed.

For patch clamp recordings, hemisected slices were placed in a temperature-controlled recording chamber (30-32℃) that was perfused with ACSF (1-2mL/min). Differential interference contrast optics (DM LFSA, Leica Microsystems) were used to visualize slices and neurons in the perifornical area and lateral hypothalamus were

targeted for study. Multiclamp 700B and pClamp 9 or 10 software (Molecular Devices, Sunnyvale, CA) were used. Signals were filtered at 1kHz and digitized at 5-10kHz.

Glass pipettes were filled with an internal solution (EPSCs: in mM: 123 K-gluconate, 2 $MgCl_2$, 1 KCl, 0.2 EGTA, 10 HEPES, 5 Na_2ATP, 0.3 NaGTP, and 2.7 biocytin; IPSCs: K-gluconate was replaced with KCl for a total concentration of 131mM), and had a resistance of 3-5MΩ in the bath. Access resistance was 5-20MΩ once whole cell access was obtained. All experiments were conducted in voltage clamp at -70mV and a 20mV, 50ms square hyperpolarizing step was applied every 15-60 seconds to monitor access resistance.

Since blocking glutamatergic and GABAergic channels completely abolishes synaptic currents in orexin neurons (Li et al., 2002), the chloride channel blocker picrotoxin (50μM) could be used to isolate glutamatergic transmission. Moreover, our lab has previously shown that the remaining fast glutamatergic currents are largely mediated by AMPA receptors (Alberto & Hirasawa, 2010). Therefore, to record AMPA receptor-mediated mEPSCs, TTX (1μM) was used to inhibit action potentials. For mIPSCs, in addition to TTX, DAP5 (50μM) an NMDA receptor antagonist, and DNQX (10μM) an AMPA/kainate receptor antagonist was used. EPSCs were also evoked in the presence of picrotoxin by a glass stimulating electrode filled with ACSF and connected to a stimulation box (ISO-Flex, A.M.P.I.).

4.2.3 Identification of neurons

Orexin neurons were identified through a combination of electrophysiological characteristics and immunohistochemical analysis. We have previously established that orexin neurons display a unique combination of responses to positive and negative current injections, which can predict the neurochemical phenotype with a high accuracy rate of identification (>99% in prior studies) (Linehan et al., 2015). Briefly, orexin neurons display an H current and a rebound depolarization following relief from hyperpolarization, which is capped by action potentials in some cells. They also fire spontaneously, and display a uniphasic after-hyperpolarizing potential.

Post hoc immunohistochemistry was also performed in a subset of cells to support initial electrophysiological identification. During recording, cells were labeled with biocytin via the internal solution. Following recording, slices were fixed in 10% formalin for at least 48 hours. Then, slices were washed three times in a PBS solution and then incubated with goat anti-orexin A IgG (1:2000; SC8070, Santa Cruz Biotechnology, Santa Cruz, CA) for 3 days at 4℃. Then slices were washed again and fluorescent conjugated donkey anti-goat (Alexa 488 or 594, 1:500) and streptavidin (1:500) were incubated with slices for 3 hours at room temperature or overnight at 4℃. Slices were then mounted and an epifluorescent microscope was used to check for colocalization of biocytin and orexin A staining (Fig. 2.1).

4.2.4 Data and statistical analysis

MiniAnalysis (Synaptosoft) was used to analyze the frequency and amplitude of miniature postsynaptic currents. Events were manually selected that had a clear fast rise and exponential decay. For mEPSCs, an average of 622±43 (frequency) and 380±27 (amplitude) events were analyzed in each cell. For mIPSCs, an average of 85±7 (frequency) and 90±6 (amplitude) events were analyzed. MiniAnalysis was also used to perform peak-scaled non-stationary noise analysis (NSNA) to estimate the conductance and number of channels that contribute to mEPSCs. Relative frequency distributions of mEPSC amplitude were generated for individual cells and were fitted with a log(Gaussian) non-linear regression to determine the peak of individual distributions in Prism. Distributions were also combined to show an average distribution of mEPSC amplitude. Clampfit (Molecular Devices) was used to analyze evoked EPSC amplitude for paired pulse ratio (PPR) calculations.

Data are expressed as mean ± SEM. The number of observations is reported in the nested model where N/n represents the number of cells/the number of animals. One-way ANOVA with Holm-Sidak multiple comparisons, unpaired t-tests, and descriptive statistics were performed using Prism 6.0 (GraphPad). $p<0.05$ was considered significant. Outliers were identified using the ROUT method in Prism and removed from the data set.

4.3 Results

4.3.1 One week of WD increases the AMPA receptor conductance in orexin neurons

Following a brief WD feeding, the amplitude of mEPSCs was significantly increased. This occurred as early as 1 day of WD feeding (Ctrl 18.9±1.2pA, N/n=8/6 vs 1dWD 26.5±1.2pA, N/n=11/3, p=0.0045; Fig. 4.1B) and at 1 week (Ctrl 18.9±1.2pA, N/n=8/6 vs 1wWD 26.8±1.8pA, N/n=12/6, p=0.0039; Fig. 4.1A,B). In both WD groups, there was a rightward shift in the peak and overall frequency distribution of mEPSC amplitude (Fig. 4.1C,D). Since there was no change in the coefficient of variation (CV) to indicate changes in the skewness of the distribution (Fig. 4.1E), this rightward shift is unlikely to be due to an increase in multivesicular release but rather an increased quantal current. Peak-scaled NSNA indicated that this was due to an increase in the single channel current of AMPA receptors (Ctrl 1.2±0.2pA vs 1wWD 2.1±0.2pA, p=0.0306; Fig. 4.1F,G), rather than a change in receptor number (Fig. 4.1F,H). Together these results suggest that 1 day to 1 week of WD feeding increases AMPA receptor conductance, indicating that there is a postsynaptic potentiation of excitatory input to orexin neurons.

4.3.2 One week of WD differentially affects spontaneous and evoked glutamatergic release

In contrast to the effect on mEPSC amplitude, one week of WD had no effect on mEPSC frequency (Ctrl N/n=8/6 vs 1wWD N/n=12/6; Fig. 4.2A,B), indicating no presynaptic change. On the other hand, we found an increased PPR suggesting a decrease

in release probability (Ctrl 0.67±0.04, N/n=19/15 vs 1wWD 0.87±0.07, N/n=16/10, p=0.0244; Fig. 4.2C,D). This indicates a possible dissociation between presynaptic regulation of evoked and spontaneous excitatory transmission.

4.3.3 Four weeks of WD leads to an adaptation to increased mEPSC amplitude

After 4 weeks of feeding, orexin neurons from WD and chow controls showed no difference in average mEPSC amplitude (Ctrl N/n=8/6 vs 4wWD N/n=9/5; Fig. 4.3A,B); nonetheless, there was still a modest but significant rightward shift in the peak of the distribution of mEPSC amplitude in the 4-week WD group (Fig. 4.3C,D). As there was no change in CV (Fig. 4.3E), the shift is likely a result of a small increase in quantal current, which may be obscured in the averaged amplitude data. Interestingly, peak-scaled NSNA suggested that the single AMPA receptor current remained higher in the WD group than control levels akin to the 1-week WD group (Ctrl 1.4±0.1pA vs 4wWD 1.9±0.2pA, p=0.0338; Fig. 4.3F,G), while AMPA receptor number decreased (Ctrl 15.2±1.0 vs 4wWD 11.7±1.1, p=0.0296; Fig. 4.3F,H). These results suggest that the increase in mEPSC amplitude seen at 1 week is transient and is reversed by 4 weeks of WD; however, this is not through reversal of the increase in AMPA receptor conductance that occurred at 1 week, but rather a decrease in the number of AMPA receptors. This could be the phenomenon of synaptic scaling, a process by which synapses adjust their excitability to maintain their homeostasis (Turrigiano, 2008). Alternatively, the results may be explained by the addition of new excitatory synapses which initially have very few AMPA receptors (Parajuli, Tanaka, & Okabe, 2017) and these receptors could have different kinetic properties.

4.3.4 Four weeks of WD induces remodeling of excitatory synapses

Unlike a lack of change in mEPSC frequency at 1 week, the frequency of mEPSCs was significantly increased after 4 weeks of WD (Ctrl 5.9±0.8Hz, N/n=9/6 vs 4wWD 13.1±2.1Hz, N/n=8/5, p=0.0044; Fig. 4.4A,B). There was no change in PPR (Ctrl N/n=17/11 vs 4wWD N/n=17/9; Fig. 4.4C,D), suggesting no difference in presynaptic release and therefore, there is likely an increase in the number of excitatory inputs to orexin neurons.

4.3.5 Excitatory transmission to orexin neurons is unaffected following 11 weeks of WD

Interestingly, by 11 weeks of WD feeding, all plastic changes of excitatory transmission observed at earlier time points returned to control levels. There was no difference between chow- and WD-fed groups in mEPSC amplitude or frequency (Ctrl N/n=9/6 vs 11wWD N/n=9/7; Fig. 4.5A-G), AMPA receptor conductance or number (Fig. 4.5E,F), or PPR (Ctrl N/n=14/7 vs 11wWD N/n=13/4; Fig. 4.5H,I).

These data suggest that orexin neurons have adapted to the effects of WD on both mEPSCs and PPR with prolonged feeding. Overall, both pre- and postsynaptic mechanisms are in flux early in WD feeding and then settle to baseline levels by 11 weeks of feeding.

4.3.6 Inhibitory transmission is also affected by WD in a time-dependent manner

Next, we investigated inhibitory transmission in orexin neurons and found that it also underwent time-dependent plasticity. While no changes occurred after 1 week of

feeding (Ctrl N/n=7/4 vs 1wWD N/n=8/3; Fig. 4.6A-C), after 4 weeks of feeding, the frequency of mIPSCs was significantly increased (Ctrl 0.60±0.05Hz, N/n=10/3 vs 4wWD 0.88±0.09Hz, N/n=13/3, p=0.0247; Fig. 4.6D,F). This changed to an increase in mIPSC amplitude after 11 weeks of feeding (Ctrl 48.2±4.8pA, N/n=7/5 vs 11wWD 81.1±11.1pA, N/n=8/5, p=0.0228; Fig. 4.6G,H). While these changes were interesting, the low frequency of mIPSCs, particularly in 14-week old rats, made the distribution analysis and NSNA unreliable and therefore these analyses were not conducted.

4.4 Discussion

The present study demonstrated that changes occur in excitatory and inhibitory transmission to orexin neurons through various mechanisms in response to WD, which may interact to determine the output of these neurons. Specifically, orexin neurons first show an increase in mEPSC amplitude at 1 week of feeding, then an increase in mEPSC frequency at 4 weeks, and finally no difference in either at 11 weeks (Fig. 4.7A). At the same time, 4 weeks of WD feeding increased mIPSC frequency, while 11 weeks of WD feeding only increased mIPSC amplitude (Fig. 4.7B). Together, this suggests an increase in excitatory transmission early in feeding (1 week), switching to an increase in inhibitory transmission with longer WD feeding (11 week).

4.4.1 Possible interaction of pre- and postsynaptic changes at 1 week of WD feeding

After 1 week of WD feeding, we found an increase in PPR without a corresponding decrease in mEPSC frequency. This may be explained by a potential bias in the afferents that were stimulated to evoke EPSCs, as the stimulation electrode was always placed medially to the recorded neuron, while mEPSCs could arise from any glutamatergic afferents. Alternatively, there may be a dissociation between evoked and spontaneous glutamate release, including different vesicle pools, calcium dependence, and sensitivity to intracellular signaling pathways (Fredj & Burrone, 2009; Grauel et al., 2016; Katsurabayashi et al., 2004; Maeda et al., 2009).

Changes in the probability of transmitter release usually underlies changes in activity-dependent short-term plasticity, as shown elsewhere (Sippy, Cruz-Martín,

Jeromin, & Schweizer, 2003). Therefore, the combination of increased AMPA receptor conductance and decreased evoked release probability may have interesting repercussions for orexin neuron activity. A reduction in release probability may lessen synaptic fatigue and alleviate short-term synaptic depression during repetitive activity (Xia et al., 2009). While the decreased glutamate release may result in a reduction in EPSC amplitude (but not necessarily, see Chen & Buonomano, 2012), the increased AMPA receptor conductance may compensate for, or even lead to larger evoked EPSC amplitude than chow controls. Overall, this may increase the number of suprathreshold EPSPs during repetitive synaptic activity that would lead to postsynaptic firing. Therefore, together, this may suggest that the changes in excitatory transmission after 1 week of WD could act to change the pattern of presynaptic activity that would activate orexin neurons.

4.4.2 WD affects both excitatory and inhibitory transmission

At 4 weeks of WD feeding, there is a significant increase in both mEPSC and mIPSC frequency. The significance of this finding is unclear but the resultant effect on orexin neuron activity likely depends on input-specific activity, which could change the weight of inhibitory and excitatory synaptic inputs as a function of physiological state. However, since orexin neurons receive about five times more excitatory inputs than inhibitory ones (Horvath & Gao, 2005;), it is possible orexin neurons are more sensitive to the effects seen on excitatory synapses than those on inhibitory transmission.

4.4.3 Physiological implications of these findings

The diet-induced time-dependent electrophysiological changes in orexin neurons described in this thesis correspond well with the biphasic changes in orexin mRNA levels reported in the literature. Specifically, orexin mRNA expression is increased by short-term high-fat diet exposures (<3 weeks) or acute rises in triglycerides (Chang et al., 2004; Valdivia et al., 2014; Wortley et al., 2003) but instead is decreased by longer periods of high-fat diet feeding (>1 month) (Novak et al., 2010; Tanno et al., 2013). Similarly, we show that orexin neuron excitability is increased with short-term high-fat feeding through potentiation of mEPSC amplitude (1 week) and frequency (4 weeks) but is decreased in the long-term by increased mIPSC frequency (4 weeks) then amplitude (11 weeks). Delayed changes in inhibitory transmission suggest that this plasticity is unlikely a direct effect of diet but rather it may be secondary to obesity. Akin to these findings, the reported decreases in orexin mRNA expression and peptide levels after long-term high-fat diet may also be secondary to weight gain as decreased orexin mRNA expression is also seen in genetically obese rats (Cai et al., 2000).

Thus, taken together, the initial activation of these neurons involving increased peptide expression and neuronal excitability may contribute to overfeeding early in high fat feeding, whereas the inhibition with longer high-fat diet feeding may reduce energy expenditure and contribute to diet-induced obesity.

4.4.4 Conclusions

The present study is the first to investigate time-dependent changes in synaptic transmission to orexin neurons over the course of high-fat diet feeding. This study highlights how synaptic plasticity in response to high-fat diet is dynamic and demonstrates how the length of high-fat diet feeding may influence the synaptic plasticity and excitability of feeding-related neurons.

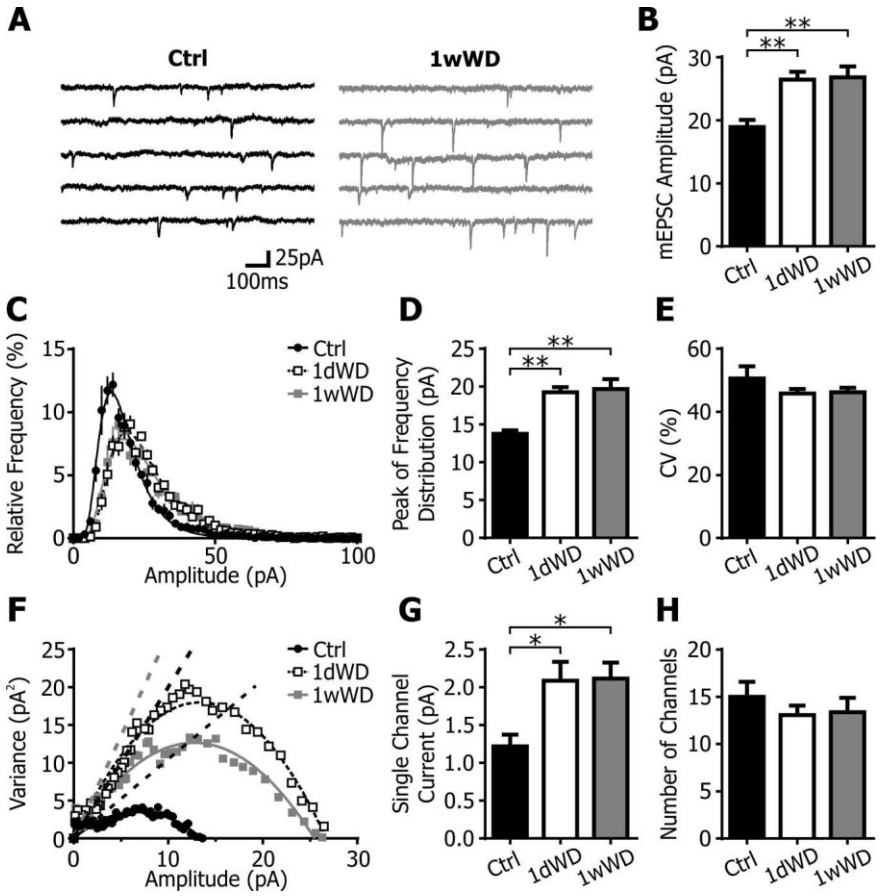

Figure 4.1: The effect of 1 week of Western Diet on mEPSC amplitude in orexin neurons.

(A) Sample traces of mEPSCs in orexin neurons from rats fed a control chow (Ctrl) or WD (1wWD) for 1 week. (B) Average mEPSC amplitude for Ctrl rats, rats fed 1 day of WD (1dWD), and 1wWD rats. (C) Relative distribution of mEPSC amplitudes, averaged by experimental groups. (D) The mEPSC amplitude corresponding to the peak of the distribution histogram for individual cells. (E) The coefficient of variation (CV) of mEPSC amplitude. (F) Sample analysis of peak-scaled NSNA in orexin neurons for Ctrl, 1dWD, and 1wWD. The slope of dotted lines represents the average single AMPA channel current of each cell. Group data for (G) single AMPA channel current and (H) number of channels estimated from NSNA.

*One-way ANOVA with post hoc comparisons: *p<0.05, **p<0.01*

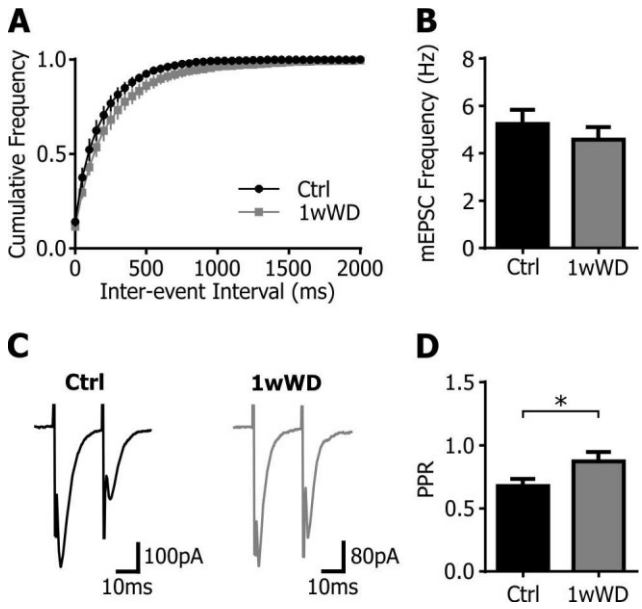

Figure 4.2: The effect of 1 week of WD on mEPSC frequency in orexin neurons.

(A) An average cumulative frequency distribution of inter-event intervals in the Ctrl and 1wWD groups. (B) Average mEPSC frequency for Ctrl and 1wWD. (C) Sample traces of paired pulses evoked at 50Hz in Ctrl and 1wWD. (D) Paired pulse ratio (PPR) of Ctrl and 1wWD.
*Unpaired t-test: *p<0.05*

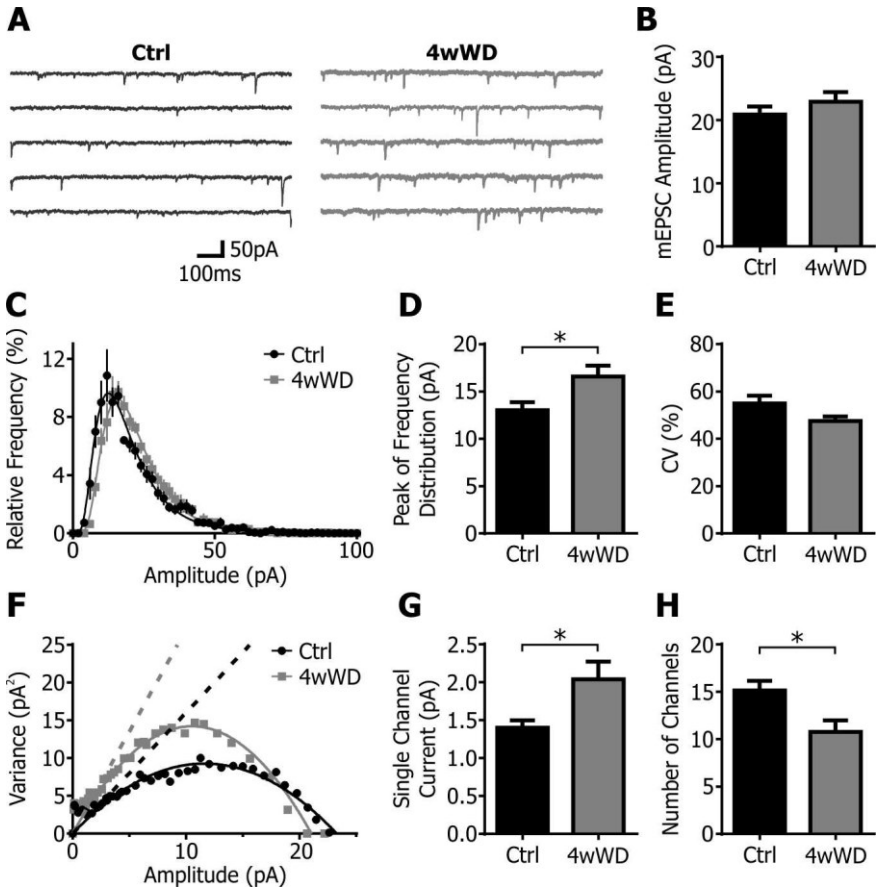

Figure 4.3: The effect of 4 weeks of WD on mEPSC amplitude in orexin neurons.

(A) Sample traces of mEPSCs in orexin neurons from rats fed a control chow (Ctrl) or WD (4wWD) for 4 weeks. (B) Average mEPSC amplitude. (C) Averaged relative distribution of mEPSC amplitudes. (D) The mEPSC amplitude corresponding to the peak of distribution histogram for individual cells. (E) The coefficient of variation (CV) for mEPSC amplitude. (F) Sample analysis of peak-scaled NSNA. The slope of dotted lines represents the average single AMPA channel current of each cell. Group data for (G) single AMPA channel current and (H) number of channels from NSNA.
*Unpaired t-test: *p<0.05*

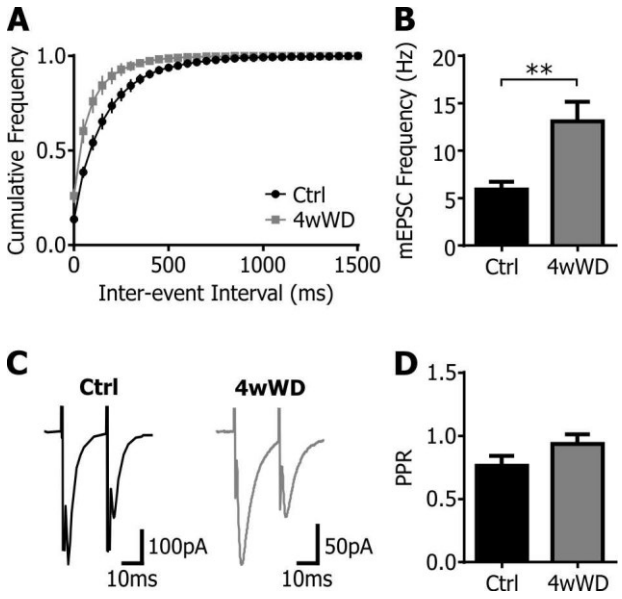

Figure 4.4: The effect of 4 weeks of WD on mEPSC frequency in orexin neurons.

(A) An average cumulative frequency distribution for inter-event intervals in Ctrl and 4wWD. (B) Average mEPSC frequency in orexin neurons of rats in Ctrl and 4wWD groups. (C) Sample traces of paired EPSCs evoked at 50Hz. (D) PPR in Ctrl and 4wWD.
*Unpaired t-test: **p<0.01*

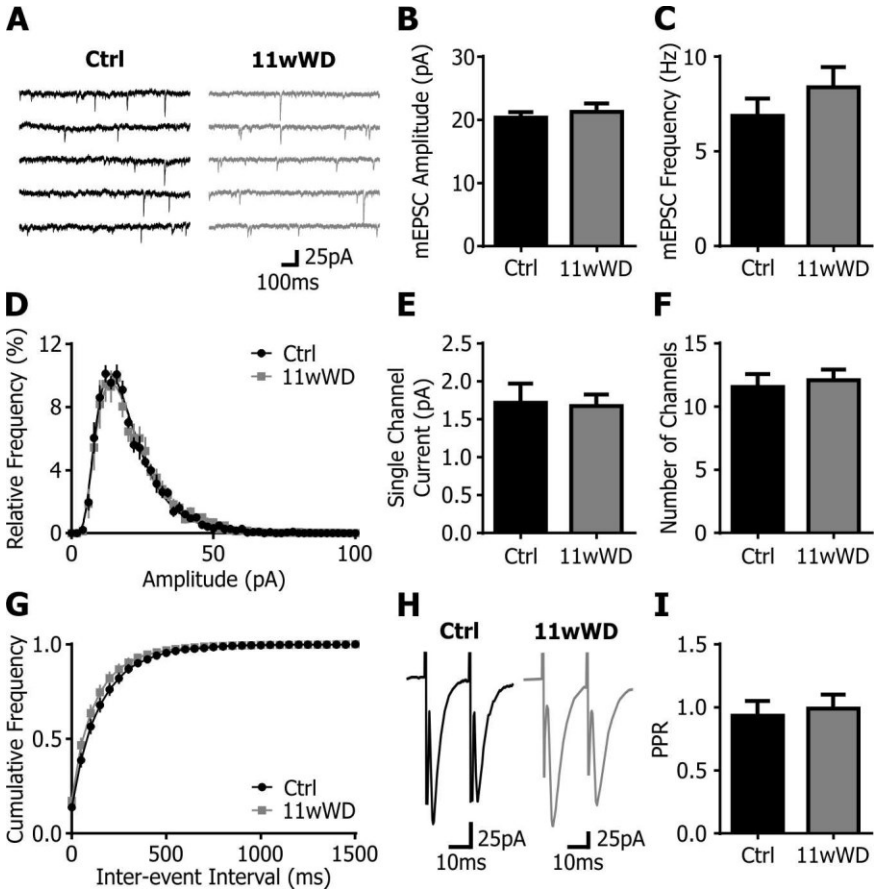

Figure 4.5: The effect of 11 weeks of WD on excitatory transmission to orexin neurons.

(A) Sample traces of mEPSCs in orexin neurons from rats fed a control chow (Ctrl) or WD (11wWD) for 11 weeks. Average mEPSC (B) amplitude and (C) frequency in Ctrl and 11wWD. (D) Averaged relative frequency distribution of mEPSC amplitudes. Group data for the (E) single AMPA channel current and (F) number of channels from NSNA. (G) An average cumulative frequency distribution of inter-event intervals in Ctrl and 11wWD. (H) Sample traces of paired EPSCs evoked at 50Hz. (I) PPR of Ctrl and 11wWD.

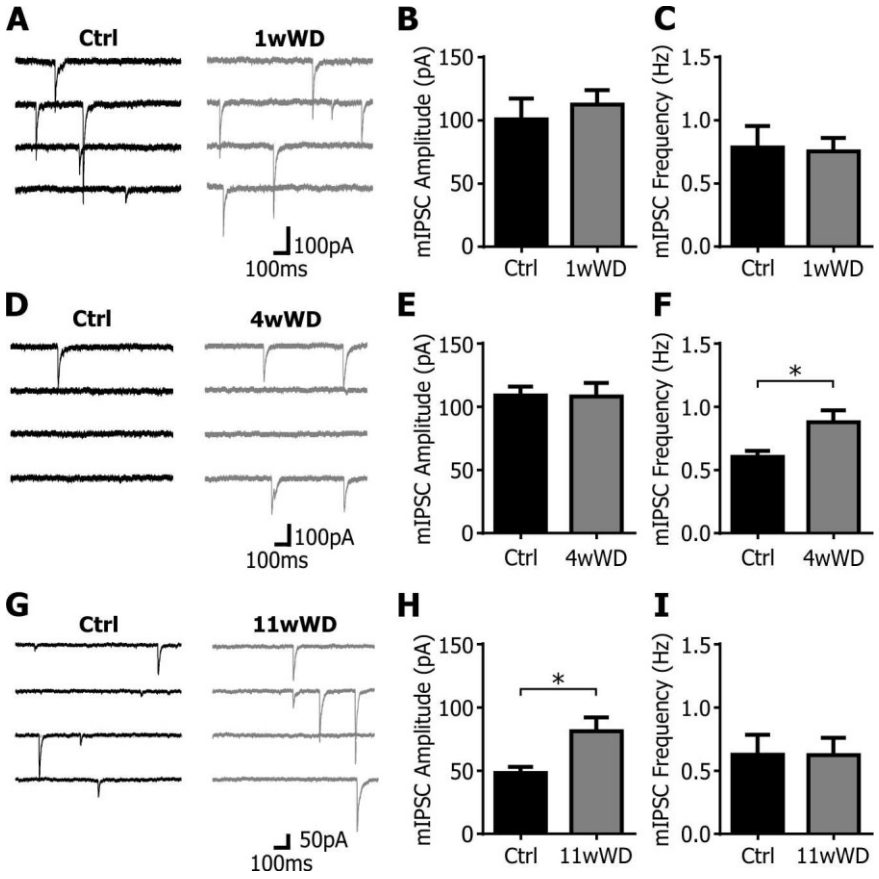

Figure 4.6: The effect of WD on inhibitory transmission to orexin neurons.

(A) Sample traces of mIPSCs in orexin neurons from rats fed a control chow (Ctrl) or WD (1wWD) for 1 week. Average mIPSC (B) amplitude and (C) frequency in Ctrl and 1wWD. (D) Sample traces of mIPSCs in orexin neurons from Ctrl rats and rats fed WD (4wWD) for 4 weeks. Average mIPSC (E) amplitude and (F) frequency in Ctrl and 4wWD. (G) Sample traces of mIPSCs in orexin neurons from Ctrl rats and rats fed WD (11wWD) for 11 weeks. (H) Average mIPSC (H) amplitude and (I) frequency in Ctrl and 11wWD.
*Unpaired t-test: *p<0.05*

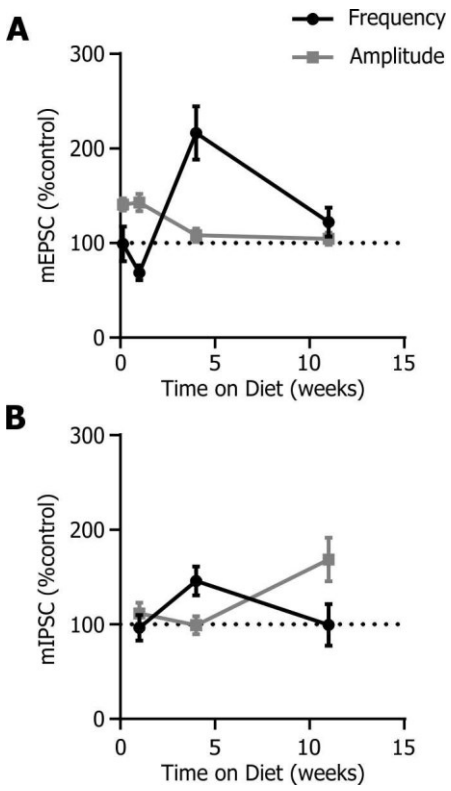

Figure 4.7: Summary of effects of WD on synaptic transmission in orexin neurons.

(A) The frequency (black circles) and amplitude (grey squares) of mEPSCs at 1 day, 1 week, 4 weeks, and 11 weeks of WD expressed as a percent of the chow control average at the corresponding timepoint. (B) The frequency (circle) and amplitude (square) of mIPSCs at 1, 4, and 11 weeks of WD expressed as a percent of the chow control average at the corresponding timepoint.

CHAPTER 5

COX-DEPENDENT DOWNREGULATION OF THE NA^+/K^+-ATPASE UNDERLIES HIGH-FAT DIET-INDUCED ACTIVATION OF MELANIN-CONCENTRATING HORMONE NEURONS

5.1 Introduction

Hypothalamic inflammation is a typical immune response to various illnesses that induce sickness syndrome including fever, anorexia, and weight loss. However, a contrasting role of inflammation has recently emerged in energy balance. Chronic low-grade hypothalamic inflammation has been shown to be one of the hallmarks of diet-induced obesity (De Souza et al., 2005; Thaler et al., 2012). Furthermore, this inflammation has a causal role in caloric overconsumption and weight gain, which is thought to be mainly through leptin and insulin resistance (Araújo et al., 2007; Kleinridders et al., 2009; Klöckener et al., 2011; Posey et al., 2009; Zhang et al., 2008). However, the majority of these previous studies have examined the hypothalamus as a whole or focused only on the arcuate nucleus despite the importance of other hypothalamic nuclei, including the ventromedial, paraventricular, and lateral hypothalamus (Schneeberger, Gomis, & Claret, 2014). Whether inflammation affects these other components of the energy balance circuitry in diet-induced obesity remains obscure.

Within the lateral hypothalamus, MCH neurons are of particular interest in relation to obesity as MCH signaling increases food intake and weight gain (Della-Zuana et al., 2002; Morens et al., 2005). In obese animals, there is an upregulation of MCH mRNA (Qu et al., 1996) and peptide levels (Elliott et al., 2004), while MCH receptor antagonists reduce food intake and body weight (Kowalski et al., 2006; Morens et al., 2005; Zhang et al., 2014). Furthermore, MCH or MCH receptor deficiency protects against weight gain (Chen et al., 2002; Shimada, Tritos, Lowell, Flier, & Maratos-Flier,

1998; Wang, Ziogas, Biddinger, & Kokkotou, 2010). These studies demonstrate a role of MCH in diet-induced obesity. However, two main questions remain: whether the excitability of MCH neurons is indeed increased during high-fat diet feeding and if so, whether that prospective activation is a result of hypothalamic inflammation.

In the present study, we investigated the electrophysiological characteristics of MCH neurons in animals exposed to high-fat diet. Our hypothesis was that MCH neurons are activated by high-fat diet, which could be observed through electrophysiological recording. Our results suggest that high-fat diet depolarizes MCH neurons, which contributes to increased food intake, fat accumulation, and weight gain. This depolarization results from the tonic inhibition of the Na^+/K^+-ATPase (NKA) by prostaglandin E_2 (PGE_2), an inflammatory mediator. Thus, our study provides a novel cellular mechanism by which hypothalamic inflammation can directly activate appetite-promoting neurons to induce weight gain.

5.2 Methods

5.2.1 Animals and diets

All experiments were carried out in accordance with the Canadian Council on Animal Care guidelines and approved by Memorial University's Institutional Animal Care Committee. Three-week old male Sprague Dawley rats were obtained from Memorial University breeding colony or Charles River Laboratories (Quebec, Canada), and 3-week old male C57BL/6NCrl mice were obtained from Charles River Laboratories (Quebec, Canada). Rats were singly housed whereas mice were housed in groups (3-4 mice each) in a temperature and light controlled room.

Animals were fed one of the two high-fat diets: a milk-based high-fat Western Diet (WD) from Research Diets (D12079B: 4.55 kcal/g: 40.0% fat, 15.8% protein, and 44.2% carbohydrates by calories) or a lard-based high-fat diet (HFD) from Research Diets (D12451: 4.73kcal/g: 45% fat, 20% protein, and 35% carbohydrates by calories). The control chow diet was LabDiet autoclavable rodent diet 5010; 3.08 kcal/g; 12.7% fat, 28.7% protein and 58.5% carbohydrates by calories or Prolab diet RMH 3000; 3.16 kcal/g: 14% fat, 26% protein, and 60% carbohydrates. No differences were observed in caloric intake or other parameters between the different chow diets, thus data were combined. Food and water were available *ad libitum*. Body weight and food intake were measured once a week. For the mouse study, weekly food intake was monitored for each cage and divided by the number of mice to obtain an averaged food intake per mouse.

5.2.2 Electrophysiological recording

Following feeding, animals were decapitated under isoflurane anesthesia. The brains were removed and 250µm-thick coronal slices of the hypothalamus were generated in ice-cold ACSF of the following composition (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 18 $NaHCO_3$ and 2.5 glucose. Following dissection, the brain slices were incubated at 33-35°C for 30-35 minutes and then at room temperature until being transferred to the recording chamber. ACSF was continuously bubbled with a mixture of O_2 (95%) and CO_2 (5%) throughout the procedures.

A hemisected slice was transferred into a recording chamber where it was submerged and perfused at 32-34°C with ACSF. Using infrared-differential interference contrast optics, large neurons (10-20µm diameter) in the lateral hypothalamus and perifornical area or zona incerta were selected. Whole cell patch clamp recordings were carried out using a Multiclamp 700B amplifier and pClamp 9 or 10 software (Molecular Devices, Sunnyvale CA). Electrodes had a tip resistance of 3-5MΩ when filled with internal solution and an access resistance of 5-20MΩ when whole cell access was attained. The internal solution contained (in mM): 123 K-gluconate, 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10 HEPES, 4-5 Na_2-ATP, 0.3 Na-GTP, pH 7.3. Biocytin (1-2mg/mL) was also included in the internal solution for post hoc immunohistochemical phenotyping. Some recordings were done in the presence of picrotoxin, a chloride channel blocker. Since it had no effect on the experimental results, the data was combined. For the K^+ free experiment, KCl was replaced by NaCl in the ACSF to correct for osmolarity. To test the role of cyclooxygenase (COX) or PGE_2, hypothalamic slices were incubated in the COX

inhibitor acetaminophen (50μM) for 2 hours or in PGE_2 (0.1nM) for 35-40 minutes then transferred to the recording chamber for recording while being perfused with ACSF only for up to 90 minutes.

For voltage clamp recordings, hyperpolarizing pulses (20mV, 100ms) were applied every 60 seconds to monitor access and input resistance. Signals were filtered at 1kHz, digitized at 5-10kHz and stored for analysis. Electrophysiological parameters were measured using MiniAnalysis 6.0 (Synaptosoft; Decatur, GA) and Clampfit 9 and 10 (Molecular Devices; Sunnyvale, CA). The liquid junction potential was corrected offline (-14.9mV).

5.2.3 Identification of MCH and orexin neurons

To characterize the electrophysiological features of the recorded cells, a series of hyperpolarizing and depolarizing 600ms step pulses were applied in current clamp mode. This protocol has a high success rate of correct identification of orexin and MCH neurons within our laboratory. Typical electrophysiological characteristics of MCH neurons include a lack of spontaneous activity, no H-current, no rebound following hyperpolarizing steps, and spike adaptation during depolarizing steps (Parsons & Hirasawa, 2011). On the other hand, orexin neurons display spontaneous activity, an H-current, rebound following hyperpolarization, and uniphasic afterhyperpolarization potential (Linehan et al., 2015).

To further confirm the phenotype, we used post hoc immunohistochemistry in a subset of cells tested. Immediately after the recording, each slice was fixed in 10%

formalin for at least 48 hours. Slices were then rinsed in PBS and treated with a primary antibody cocktail consisting of goat anti-orexin A (1:2000; SC8070, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-MCH (1:2000; H-070-47, Phoenix Pharmaceuticals, Belmont, CA) for 3 days at 4ºC, followed by appropriate fluorophore-conjugated secondary antibodies (anti-goat and anti-rabbit) and streptavidin (1:500; Jackson ImmunoResearch, West Grove, PA). The sections were examined under a fluorescence microscope for the presence of orexin A or MCH immunoreactivity with biocytin labeling (Fig. 2.1). Only cells that were confirmed by post hoc immunohistochemistry as orexin or MCH-immunopositive and/or had typical electrophysiological characteristics were included in the analysis.

5.2.4 MCH antagonist feeding experiment

For testing the effect of an MCH antagonist *in vivo*, WD was provided via glass feeding tubes attached to a tube holder with an extended spill catcher underneath to catch any crumbs. Rats were given three days to habituate to eating from these tubes.

On the test day, each rat was injected intraperitoneally (I.P.) with the MCH antagonist GW 803430 (30mg/kg) or vehicle (10% Tween 80, 4% HCl and 4% bicarbonate in water; injection volume was 2mL/kg for both solutions) one hour before the dark cycle. Food intake was measured several times a day starting 1 day before until 5 days after the injection, while body weight was measured once a day. At 5 days post-injection, animals were sacrificed and organ weights were measured.

5.2.5 Drugs

Picrotoxin, ouabain, acetaminophen, and GW 803430 were purchased from Sigma Aldrich (Oakville, Canada); PGE_2 was purchased from Tocris Bioscience (Bristol, United Kingdom); and TTX was purchased from Alomone Labs (Jerusalem, Israel). Drugs used for electrophysiology were prepared from 1000x frozen stock aliquots. All stocks solutions were in water except DNQX and PGE_2 (DMSO) and acetaminophen (ethanol); the final concentration of DMSO and ethanol did not exceed 0.1%.

5.2.6 Statistical analysis

Unpaired Student's *t*-test and one- or two-way ANOVA with post hoc Holm-Sidak's tests were used for statistical comparisons as appropriate. $p<0.05$ was considered significant. Data are expressed as mean ± SEM. The number of observations is reported in the nested model where N/n represents the number of cells/the number of animals. Outliers were defined as values exceeding the mean ± 2 SD and excluded from analysis.

5.3 Results

5.3.1 High-fat diets selectively activate MCH neurons

To determine the effect of high-fat diet on the excitability of MCH neurons, patch clamp recordings were performed using brain slices from obese rats fed a high-fat diet (WD) for 11 weeks (Fig. 5.1A). MCH neurons from the WD group had significantly depolarized RMP compared to age-matched controls (Ctrl -82.5±1.0mV, N/n=38/13 vs WD -72.3±1.2mV, N/n=48/16, $p<0.0001$; Fig. 5.2A,B). The majority of these neurons were silent at rest, consistent with previous reports (Hausen et al., 2016); nonetheless, in response to a series of positive current injections, MCH neurons in the WD group fired action potentials at a higher frequency (Fig. 5.2A,C) with a shorter latency to the first spike (Fig. 5.2A,D). However, there was no difference in membrane resistance (Ctrl 423.8±40.7MΩ, N/n=16/5 vs WD 466.8±46.5MΩ, N/n=17/5, p=0.4940) or membrane capacitance (Ctrl 84.1±3.6pF, N/n=16/5 vs WD 79.8 ± 4.4pF, N/n=17/5, p=0.4602) of these neurons. On the other hand, neighbouring orexin neurons in the lateral hypothalamus and magnocellular neurons of the supraoptic nucleus, other feeding-related and non-related cells, respectively, showed no change in RMP (n=28 and n=13, respectively; Fig. 5.2B), firing rate during positive current injection, membrane resistance, or capacitance (data not shown). A similar MCH neuron-specific depolarization was observed in rats fed a lard-based high-fat diet (HFD, Fig. 5.1B) for 8 weeks (Ctrl -79.7±2.3mV, N/n=6/4 vs HFD -71.8±2.2mV, N/n=10/4, p=0.0033; Fig. 5.2E). Moreover, this increase in excitability was also observed in mice fed WD (Fig. 5.1C) for 11 weeks (Ctrl -83.1±1.8mV, N/n=25/10 vs WD -75.2±1.8mV, N/n=27/12,

p=0.0059; Fig. 5.2F-J), which persisted in the presence of the voltage-gated Na^+ channel blocker, TTX, suggesting that it is a direct effect on MCH neurons (Ctrl N/n=4/3 vs WD N/n=9/5; Fig. 5.2H). Thus, high-fat diet induces MCH activation regardless of species or the type of high-fat diet.

5.3.2 Time course of MCH activation during high-fat diet feeding

To determine the time course of MCH neuron activation by high-fat diet feeding, electrophysiological properties of MCH neurons were examined at earlier timepoints (Fig. 5.3A). Rats, starting at 3 weeks of age, consumed more calories from WD throughout a 4-week feeding period compared to chow-fed controls (Ctrl n=18 vs WD n=15; Fig. 5.3B) while their body weight only became significantly different at the fourth week of feeding (Fig. 5.3C). One week of WD feeding (1wWD) did not affect the RMP (1wCtrl -79.1±1.5mV, N/n=18/13 vs 1wWD -79.1±2.1mV, N/n=20/14, p>0.05; Fig. 5.3D) or firing response of MCH neurons to positive current injections compared to age matched chow controls (1wCtrl) (Fig. 5.3E). In contrast, four weeks of WD feeding (4wWD) significantly increased the excitability of MCH neurons (4wCtrl -82.4±0.8mV, N/n=46/14 vs 4wWD -76.6±1.2mV, N/n=40/15, p=0.0002; Fig. 5.3G-J), although the depolarization was not as large as that seen after 11 weeks of WD (Fig. 5.3F). It is possible that this time-dependent progression was due to age, as the RMP of control MCH neurons showed an age-dependent hyperpolarization between 4 and 7 weeks of age (Chapter 2). To determine whether age had any impact on the lack of 1-week WD effect on RMP, an age-matched control was tested, where the 1-week feeding period was staggered to match the age of the 4wWD group at the end of feeding period (age-

matched, AM1wWD). One week of WD was also without significant effect in these age-matched controls (Fig. 5.3H,I), suggesting that one week is not long enough for WD to depolarize MCH neurons. These results indicate that high-fat diet induces a delayed and chronic activation of MCH neurons.

To address whether the WD-induced increase in excitability was solely dependent on RMP depolarization, the membrane response to current injections was examined in both WD and chow control MCH neurons (4 week feeding) while holding the baseline membrane potential at the same level (Fig. 5.4A). We found that compared to controls, WD cells fired at a higher frequency (Fig. 5.4B) and with a shorter first spike latency in response to a depolarizing step pulse (Fig. 5.4C). These results may be explained by the observed negative shift in firing threshold by WD (4wCtrl -40.6±0.8mV vs 4wWD -43.5±0.6mV, p=0.0072; Fig. 5.4D). Unlike the RMP however, the change in the threshold is transient as the difference between the diet groups was no longer present after 11 weeks of feeding (4wWD N/n=20/10 vs 11wWD N/n=48/16; Fig. 5.4E). This shows that MCH neurons are initially activated by WD via multiple mechanisms and the RMP depolarization is the predominant mechanism that persists throughout WD feeding. Thus, we focused on the characteristics and mechanism of RMP depolarization.

5.3.3 High-fat diet-induced depolarization is reversible but can sensitize

To determine whether the depolarizing effect of WD on MCH neurons is reversible, rats were first fed WD for 4 weeks, then switched to standard chow (Diet) (Fig. 5.5A). While these rats initially showed hypophagia following the switch to chow, by the second week, they consumed similar calories to that of the chow-only control rats

(Ctrl n=10 vs Diet n=5; Fig. 5.5B). Similarly, the rate of weight gain, which was increased by WD, was normalized by the switch to chow (Fig. 5.5C). After four weeks of dieting on chow, the RMP of MCH neurons normalized to control levels (Ctrl -83.0±0.7mV, N/n=62/17 vs Diet -84.7±0.9mV, N/n=37/7, $p>0.05$; Fig. 5.5D), indicating that the WD effect is reversible.

To further investigate whether a history of WD feeding has any long-term consequences, we tested the response of MCH neurons to alternating access to WD and chow. Specifically, rats were exposed to WD for 4 weeks, followed by a 4-week dieting period on chow and 1-week re-exposure to WD (Yoyo). During the second WD exposure, rats showed hyperphagia (Ctrl n=10 vs Yoyo n=8; Fig. 5.5B) and gained significantly more weight (Fig. 5.5C) than chow controls. Following this 1-week WD re-exposure, MCH neurons were significantly depolarized (Ctrl -83.0±0.7mV, N/n=62/17 vs Yoyo -77.6±2.5mV, N/n=14/3, $p=0.0391$; Fig. 5.5D), which is unlike the lack of response to a 1-week WD feeding in naïve animals (Fig. 5.3). These results indicate that a previous history of WD exposure may sensitize MCH neurons to the depolarizing effect of WD.

5.3.4 Depolarization of MCH neurons is due to downregulation of the NKA

The depolarization of MCH neurons by WD was not accompanied by a change in membrane resistance in any model tested, including the 4-week feeding group in rats (4wCtrl N/n=30/10 vs 4wWD N/n=19/10; Fig. 5.6A). Therefore, an electrogenic transporter is likely to be involved. The electrogenic activity of the NKA hyperpolarizes the cell membrane; therefore, its downregulation could depolarize MCH neurons. To test if this is the case, we used ouabain, a NKA inhibitor, on MCH neurons after a 4-week

feeding, when a significant difference in RMP was observed. Ouabain (25μM) induced an inward shift in the current-voltage relationship in both diet groups (Ctrl N/n=5/3, WD N/n=8/3; Fig. 5.6B); however, this current was significantly less in the WD condition (Fig. 5.6C). In current clamp, ouabain caused a depolarization within 0.5-2 minutes of application (Fig. 5.6D,E), which reached a similar membrane potential due to a larger change in the control than WD group (Ouabain-induced depolarization: Ctrl 33.1±1.7mV, N/n=6/3 vs WD 18.1±3.5mV, N/n=8/4, p=0.0045; Fig. 5.6E,F). Furthermore, K^+-free ACSF, which non-pharmacologically inhibits the NKA (Senatorov, Mooney, & Hu, 1997), caused a significantly greater depolarization in controls (Ctrl N/n=9/4 vs WD N/n=9/5; Fig. 5.6G-I), consistent with the effect of ouabain. These results suggest that downregulation of NKA activity accounts for the depolarization of MCH neurons by WD.

5.3.5 The NKA is inhibited via a COX- and PGE_2-dependent mechanism

Next, we sought to find the mechanism underlying NKA inhibition in the WD condition. Since COX-2-mediated PGE_2 production is responsible for NKA inhibition in various cell types (Cohen-Luria, Rimon, & Moran, 1993; Kreydiyyeh, Riman, Serhan, & Kassardjian, 2007) and high-fat diet induces chronic low grade inflammation (Thaler, Choi, Schwartz, & Wisse, 2010), we asked whether PGE_2 signaling could mediate the downregulation of NKA in MCH neurons.

We found that incubating brain slices with PGE_2 (0.1nM) significantly depolarized MCH neurons in chow-fed controls (Ctrl: baseline-82.5±1.0mV, N/n=38/13 vs PGE_2 -75.9±1.5mV, N/n=28/9, p=0.0002; Fig. 5.7A). On the other hand, the COX inhibitor acetaminophen (APAP, 50μM) had no effect on Ctrl MCH neurons (baseline vs

APAP N/n=21/9; Fig. 5.7A), while it reversed the WD-induced depolarization to Ctrl baseline levels (WD: baseline -72.4±0.8mV, N/n=48/16 vs APAP -80.5±0.8mV, N/n=25/7, p=0.0001; Ctrl baseline vs WD APAP, p>0.05; Fig. 5.7A,B). Interestingly, incubating brain slices with PGE_2 in the WD condition did not further activate MCH neurons, instead there appeared to be variable responses to the incubation, with an average effect to hyperpolarize RMP relative to the WD baseline (WD: baseline -72.4±0.8mV vs PGE_2 -76.8±2.0mV, N/n=23/6, p=0.0288; Fig. 5.7B). Concentration-dependent bidirectional effects are commonly reported with PGE_2 (Tang, Loutzenhiser, & Loutzenhiser, 2000; Jianying Zhang & Wang, 2014); therefore, we postulated that differences in PGE_2 levels may explain the hyperpolarizing effect of PGE_2 on WD MCH neurons. To test this, brain slices from chow-fed rats were incubated with either 0.1nM or 1nM PGE_2. We found that while 0.1nM PGE_2 depolarized MCH neurons (Ctrl -82.4±0.8mV, N/n=46/14 vs 0.1nM PGE_2 -78.5±1.9mV, N/n=12/3, p=0.0399; Fig. 5.8A), 1nM PGE_2 instead hyperpolarized (Ctrl vs 1nM PGE_2 -86.5±1.4mV, N/n=14/3, p=0.0399; Fig. 5.8A) MCH neurons and decreased action potential firing (Fig. 5.8B), suggesting that PGE_2 can activate or inhibit these neurons depending on its concentration.

Together, these results suggest that in brain slices from WD-fed animals, COX is active and producing PGE_2 that depolarizes MCH neurons. Contrastingly, in control brain slices, the basal level of endogenous PGE_2 appears to be insufficient for activation of these neurons.

To determine whether the observed PGE_2 effect was due to modulation of the NKA, we tested the effect of ouabain on MCH neurons treated with PGE_2 or APAP. In non-treated slices (baseline) from rats fed chow or WD for 11 weeks, ouabain-induced

current was significantly greater in the Ctrl than the WD condition (Ctrl N/n=9/6 vs WD N/n=9/6; Fig.5.9A-C), similar to our results from the 4-week feeding groups (Fig. 5.6). PGE_2 (0.1nM) caused a reduction in the ouabain-induced depolarization of Ctrl MCH neurons (baseline 34.0±0.9mV vs PGE_2 20.8±1.7mV, N/n=9/8, $p<0.0001$; Fig. 5.9A,B), while APAP increased the ouabain-induced current of WD MCH neurons (baseline 20.7±1.8mV vs APAP 29.7±1.3mV, N/n=9/7, p=0.0101; Fig. 5.9A,C). Thus, the effects of these compounds on ouabain currents mirror those on the RMP, suggesting that the PGE_2 effect on RMP is due to modulation of the NKA. Interestingly, PGE_2 incubation in the WD also increased ouabain-current (baseline 20.7±1.8mV vs PGE_2 27.8±3.0mV, N/n=8/6, p=0.0274; Fig. 5.9C), suggesting that PGE_2 bidirectionality regulates the NKA to affect RMP.

5.3.6 Potential physiological significance of MCH neuron activation by high-fat diet

To address the physiological implications of MCH neuron depolarization by WD, the effect of GW 803430, a MCH1R antagonist on food intake and weight was tested *in vivo*. Since a previous study showed that the effect of MCH antagonists on food intake depends on the type of diet (Morens et al., 2005), in this experiment, all the rats were fed with the same diet at the time of experimentation. Specifically, rats were fed with WD for 4 weeks (4wWD) or 1 week (1wWD) before I.P. injection with 30mg/kg GW 803430 or vehicle (Fig. 5.10A). Feeding periods were staggered so that all rats were age-matched (7 weeks old) at the time of injection. These groups were chosen as MCH neurons are depolarized in the 4wWD but not in the 1wWD group (Fig. 5.3).

We found that GW 803430 decreased food intake in both diet groups compared to their respective vehicle controls (4wWD-Veh n=9 vs 4wWD-GW n=8; 1wWD-Veh n=10 vs 1wWD-GW n=7; Fig. 5.10B, Fig. 5.11). However, GW 803430 induced a greater reduction in food intake on Day 2 and 3 post-injection in the 4-week WD group compared to the 1-week group (Day 2: 1wWD-GW 16.94±1.02g vs 4wWD-GW 12.79±1.82g, p=0.0396; Fig. 5.10B). Concurrently, the antagonist significantly suppressed weight gain (Fig. 5.10C) and caloric efficiency (Fig. 5.10D) only in 4-week WD rats on Day 2 post-injection. Moreover, GW 803430 selectively decreased the white fat pad weight (epididymal and retroperitoneal) only in 4-week WD animals (Fig. 5.10E1,E2) without any change in the weight of other organs including the heart, soleus muscle (Fig. 5.10F,G), brain, kidney, testis, liver, and gastrocnemius muscle (data not shown). In contrast, GW 803430 had no effect on any of these measures in the 1-week WD group (Fig. 5.10C-G). Therefore, systemic administration of an MCHR1 antagonist shows that during high-fat diet feeding, MCH signaling significantly contributes to increased food intake and body weight, specifically fat pad weight.

5.4 Discussion

The present study shows a novel mechanism by which high-fat diet induces a significant depolarization of MCH neurons due to a downregulation of the NKA in a COX-dependent manner. This excitatory effect is observed in both mice and rats and by two types of high-fat diet. Moreover, MCH neurons are activated during the onset of excess weight gain and promote food intake and fat accumulation, suggesting that this mechanism has a significant role in diet-induced obesity. Our study is the first to our knowledge to provide a cellular mechanism, independent of leptin and insulin resistance (de Git & Adan, 2015; Kleinridders et al., 2009) that directly links inflammation and excitability of feeding neurons in the hypothalamus.

5.4.1 MCH neurons are depolarized through a COX-dependent inhibition of the NKA

Our study using ouabain and K^+ free ACSF shows that the NKA has a major role in the maintenance of RMP and that its downregulation by high-fat diet is the main underlying mechanism for the depolarization of MCH neurons. NKA downregulation is dependent on continuous presence of COX products such as PGE_2, as high-fat diet-induced depolarization of MCH neurons is reversed with the COX inhibitor acetaminophen in the *in vitro* slice preparation. Furthermore, PGE_2 mimics the inhibitory effect of high-fat diet on NKA in MCH neurons from control animals, consistent with previous studies showing that PGE_2 inhibits NKA in various tissues (Karmazyn, Tuana, & Dhalla, 1981; Kreydiyyeh et al., 2007; Oliveira et al., 2009). This modulation of the

NKA could be through PKC or cAMP signaling, which are known downstream effectors of PGE_2 receptors (EP1-4) (Sugimoto & Narumiya, 2007).

Our proposed mechanism differs from past reports suggesting that leptin or insulin resistance is the mechanism linking hypothalamic inflammation and weight gain since leptin and insulin signaling is unlikely to be involved in the NKA-dependent activation of MCH neurons. First, MCH neurons do not express leptin receptors (Leinninger et al., 2009), whereas the depolarization was a direct effect as it persisted in TTX, and thereby could not be an indirect effect of leptin (Huang, Viale, Picard, Nahon, & Richard, 1999; Sahu, 1998). Secondly, while MCH neurons express insulin receptors (Hausen et al., 2016), insulin is known to activate NKA, not inhibit it (Pirkmajer & Chibalin, 2016). Therefore, the present study provides a novel mechanism by which inflammation directly activates MCH neurons.

While the NKA plays a fundamental role in neuronal function, the depolarizing effect of high-fat diet is specific to certain neurons within the hypothalamus, since neighboring orexin neurons showed no change in RMP. In addition, magnocellular neurons in the supraoptic nucleus, whose regulation of the NKA is known to be sensitive to salt intake (Mata, Hieber, Beaty, Clevenger, & Fink, 1992), was also not affected, further supporting the specificity of the high-fat diet effect on MCH neurons. This may result from a difference in NKA subunit isoforms expressed by different cells. The ouabain concentration (25μM) used in the present study would inhibit all three α subunit isoforms (Mobasheri et al., 2000) expressed in the hypothalamus (Hieber, Siegel, Fink, Beaty, & Mata, 1991). In contrast, lower concentrations of ouabain (0.1-1μM) that would

inhibit the α2 and 3 isoforms, but not the α1 isoform, had no effect on membrane potential (data not shown). Therefore, our data is consistent with the α1 NKA isoform being a target of WD and PGE_2. Another possibility could be the variable expression of PGE_2 receptors (EP1-4) in different neuronal populations (Ek, Arias, Sawchenko, & Ericsson-Dahlstrand, 2000; Zhang & Rivest, 1999), which could also explain the specificity of NKA downregulation in MCH neurons by high-fat diet intake.

5.4.2 Bidirectional effects of inflammatory signaling on MCH neurons

The sensitivity of MCH neurons to PGE_2 suggests an interaction between these feeding neurons and inflammatory processes. Chronic low-grade inflammation is a hallmark of diet-induced obesity and is thought to underlie hyperphagia and weight gain (Kleinridders et al., 2009; Thaler et al., 2012; Zhang et al., 2008). High circulating levels of saturated fatty acids during high-fat diet feeding leads to activation of toll-like receptor 4 and expression of cytokines such as TNFα, IL-1β, and IL-6 (De Souza et al., 2005; Milanski et al., 2009; Thaler et al., 2012). Interestingly, each of these inflammatory mediators are known to induce COX activity (Bouffi, Bony, Courties, Jorgensen, & Noël, 2010; Kim et al., 1998). Thus, COX/PGE_2 may be a common downstream pathway mediating the effect of inflammation on MCH neurons. This is supported by the result that WD-induced depolarization of these neurons was completely abolished by treatment with a COX inhibitor. It is likely that COX-2, rather than the COX-1 isoform is responsible for PGE_2 production during WD feeding as acetaminophen, which blocked the WD effect, has an apparent COX-2 specificity (Graham, Davies, Day, Mohamudally, & Scott, 2013).

Interestingly, COX-2-dependent PGE_2 release is also linked to anorexia during high-grade inflammation associated with fever or sickness. A bidirectional effect of PGE_2 on feeding behaviours may be at least partially explained by its bidirectional effect on MCH neuron activity shown in this study: PGE_2 depolarizes MCH neurons at low concentrations yet hyperpolarizes them at higher concentrations. This data suggests that high levels of PGE_2 may inhibit MCH neurons to mediate fever-induced anorexia. Similarly, MCH neurons have previously been implicated in sickness behaviour through the inflammatory mediator CCL2 (Le Thuc et al., 2016). These results also explain how a relatively low level of PGE_2 that depolarizes MCH neurons in chow fed controls induces an opposite effect on MCH neurons in WD slices. It is possible that endogenous PGE_2 production within WD slices combined with exogenously applied PGE_2 added up to a higher concentration which hyperpolarizes MCH neurons. Correspondingly, PGE_2 also increased the ouabain-sensitive current in the WD condition, indicating that the PGE_2 upregulates NKA at high concentrations. This concentration-dependent effect on the NKA has been observed in cardiac myocytes as low concentrations of PGE_2 inhibit, while higher concentrations activate the NKA (Skayian & Kreydiyyeh, 2006). These contrasting effects may be mediated by different PGE_2 receptors, as all EP receptors have been reported to affect NKA activity but their effects appear to be tissue specific (Oliveira et al., 2009; Skayian & Kreydiyyeh, 2006).

5.4.3 WD affects the firing threshold of MCH neurons

While depolarization of RMP certainly contributes to the increased excitability, this is not the only change induced by WD. Even when MCH neurons are held at a similar

membrane potential, the WD group shows greater action potential frequency and a shorter latency to the first spike during current injections. This RMP-independent increase in excitability could be at least partially explained by the hyperpolarizing shift in firing threshold. This change follows a different time course than that for RMP depolarization since the threshold decrease was only seen at 4 weeks of WD, while the NKA-dependent depolarization progressively increases over time from 4 weeks up to 11 weeks. The lowering of firing threshold may help to facilitate the activity of MCH neurons and hence the rate of weight gain early in the high-fat feeding period.

5.4.4 The depolarization of MCH neurons is amenable to dietary manipulations

Although chronic high-fat diet induces an enduring depolarization of MCH neurons, we found that this effect is reversible if animals are switched to a standard low-fat diet. This reversibility suggests that the excitability of MCH neurons is dynamically regulated and ongoing nutrient-related or inflammatory signaling is required to maintain an activated state. Interestingly, the mechanism for depolarization sensitizes with repeated high-fat diet exposures. Specifically, we show that a 1-week high-fat diet feeding period is sufficient to depolarize MCH neurons only if rats have had a previous exposure to a high-fat diet. This may help explain weight rebound and difficulties in maintaining long-term weight loss following a diet (Mann et al., 2007; Thaiss et al., 2016).

5.4.5 The increased excitability of MCH neurons is likely physiologically significant

Importantly, we show that the increased excitability of MCH neurons may underlie excess food intake and weight gain *in vivo* during high-fat diet feeding. We

found that a systemic injection of the MCH antagonist, GW 803430, induces a significant reduction in food intake, weight gain, and fat accumulation in rats after 4 weeks of WD feeding compared to those fed for only 1 week. The experiment was controlled for age and diet, therefore, the difference in the GW 803430 effect may be related to the MCH neurons depolarization in the 4-week WD condition but not in the control 1-week WD condition. Therefore, these results support that the COX/NKA-dependent activation of MCH neurons contributes to food intake and fat accumulation during high-fat diet feeding, and consequently, the etiology of obesity.

5.4.6 Conclusions

Our study provides a cellular mechanism by which high-fat diet may induce excess food intake and weight gain leading to obesity. Notably, we found that the degree of MCH neuron depolarization increases over time during chronic WD feeding. This may indicate a positive feedback loop, where high-fat diet activates MCH neurons, which in turn induces further caloric consumption. The membrane depolarization involves inhibition of the NKA via the COX-2/PGE_2 pathway, indicating a direct effect of an inflammatory mediator in activating feeding-related neurons. Preventing the high fat diet-induced activation of MCH neurons by targeting the mechanism identified in this study may be an excellent alternative to blocking MCH receptor signaling for treating obesity without interfering with normal functions of the MCH system.

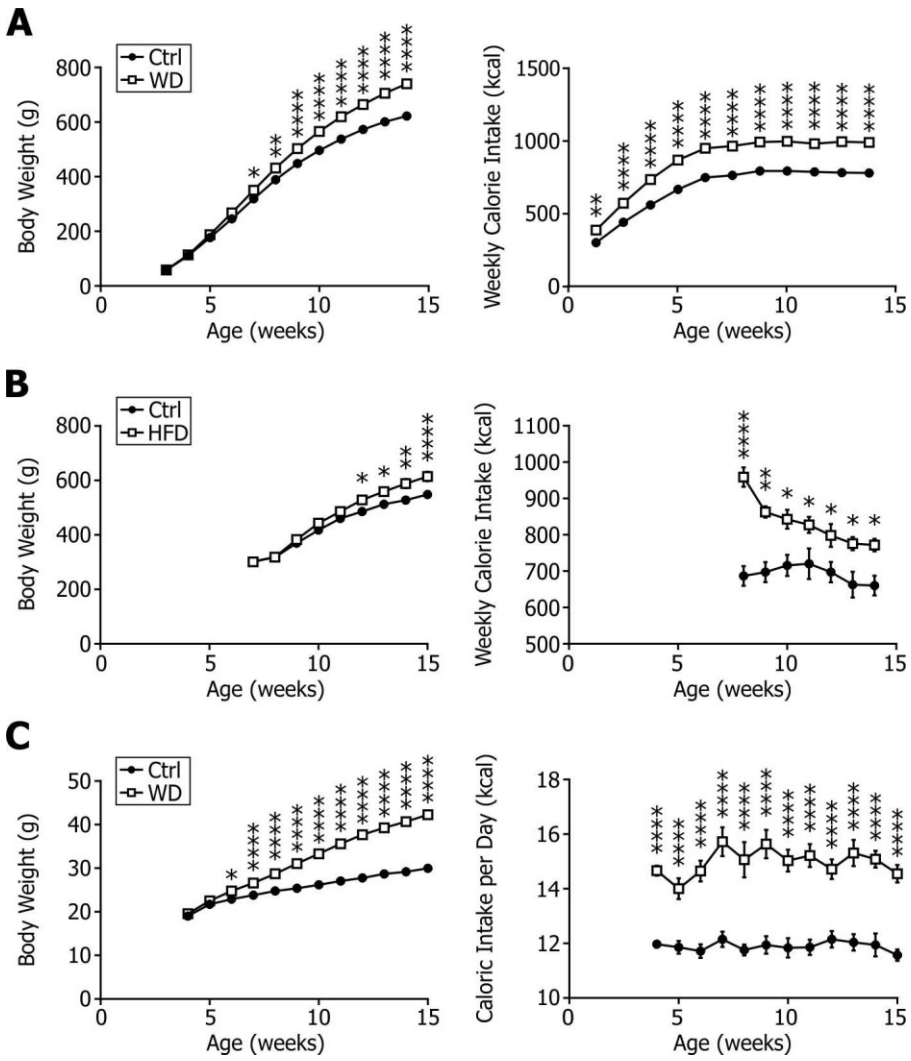

Figure 5.1: Animals models of diet-induced obesity.

(A) Weekly body weight and caloric intake of 3-week old Sprague Dawley rats fed either standard chow (Ctrl) or a milk-based high-fat/high-sugar Western Diet (WD). (B) Weekly body weight and caloric intake for 7-week old Sprague Dawley rats fed either standard chow (Ctrl) or a lard-based high-fat diet (HFD). (C) Weekly body weight and average daily caloric intake for 4-week old C57BL mice fed either standard chow (Ctrl) or WD.
*Two-way RM ANOVA with post hoc comparisons: *p<0.05, **p<0.01, ****p<0.0001*

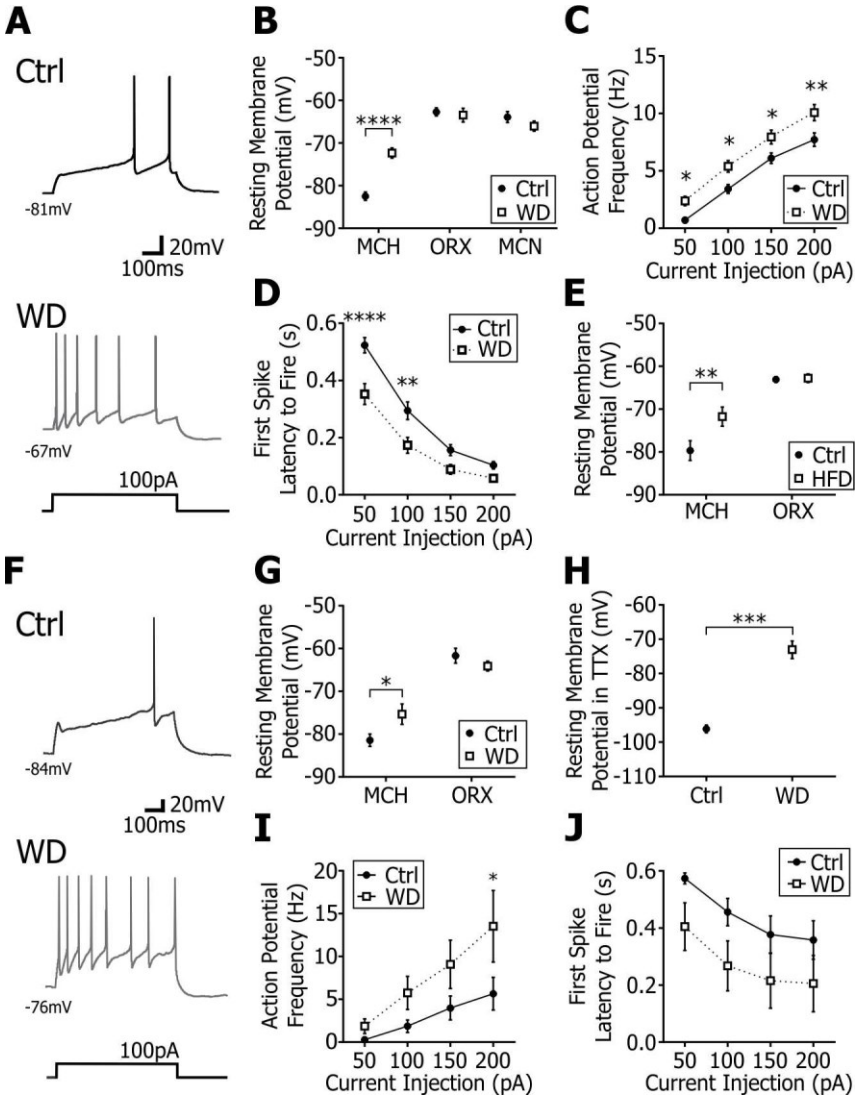
A
Ctrl
-81mV
20mV
100ms
WD
-67mV
100pA
B
Resting Membrane Potential (mV)
-50
-60
-70
-80
-90
****
Ctrl
WD
MCH
ORX
MCN
C
Action Potential Frequency (Hz)
15
10
5
0
*
*
*
**
Ctrl
WD
50 100 150 200
Current Injection (pA)
D
First Spike Latency to Fire (s)
0.6
0.4
0.2
0.0
****
**
Ctrl
WD
50 100 150 200
Current Injection (pA)
E
Resting Membrane Potential (mV)
-50
-60
-70
-80
-90
**
Ctrl
HFD
MCH
ORX
F
Ctrl
-84mV
20mV
100ms
WD
-76mV
100pA
G
Resting Membrane Potential (mV)
-50
-60
-70
-80
-90
*
Ctrl
WD
MCH
ORX
H
Resting Membrane Potential in TTX (mV)
-60
-70
-80
-90
-100
-110
***
Ctrl
WD
I
Action Potential Frequency (Hz)
20
15
10
5
0
*
Ctrl
WD
50 100 150 200
Current Injection (pA)
J
First Spike Latency to Fire (s)
0.6
0.4
0.2
0.0
Ctrl
WD
50 100 150 200
Current Injection (pA)

Figure 5.2: MCH neurons are activated in diet-induced obese rats and mice.

(A) Sample traces recorded during positive current injections in MCH neurons from rats fed WD for 11 weeks (WD) compared to age-matched chow controls (Ctrl). (B) MCH neurons are selectively depolarized by WD, while no change in resting membrane potential is seen in orexin neurons (ORX) of the lateral hypothalamus and magnocellular neurons (MCN) of the supraoptic nucleus. (C) Action potential frequency and (D) first spike latency of MCH neurons during current injections for Ctrl and WD rats (a latency of 600ms indicates that the cell did not fire). (E) MCH neurons are also selectively depolarized in rats fed a lard-based high-fat diet (HFD). (F) Sample traces of MCH neurons from mice fed WD for 11 weeks (WD) and age-matched chow control mice (Ctrl). (G) MCH neurons are selectively depolarized in WD mice compared to Ctrl. (H) The depolarization of MCH neurons in WD mice persists during TTX application. (I) Action potential frequency and (J) first spike latency in MCH neurons during positive current injections in Ctrl and WD mice.
*Two-way ANOVA with post hoc comparisons and unpaired t-test (H): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

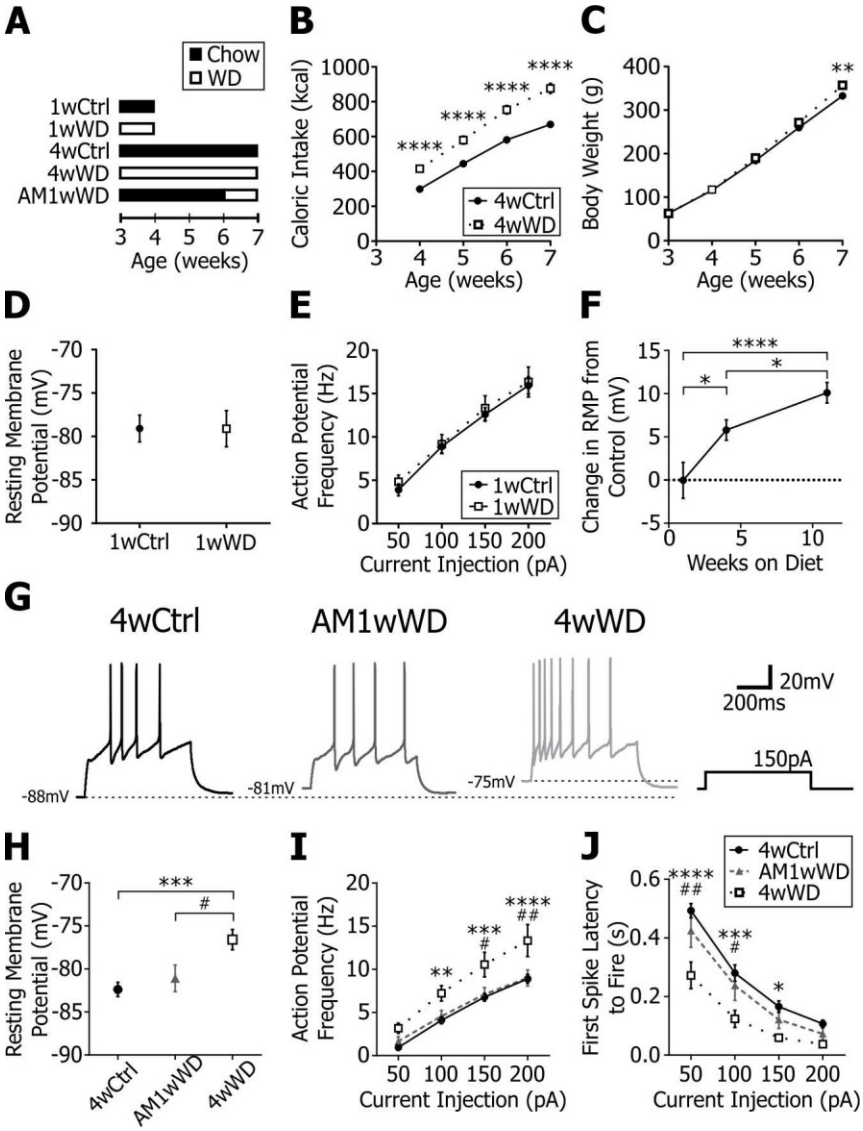
A
Chow
WD
1wCtrl
1wWD
4wCtrl
4wWD
AM1wWD
3 4 5 6 7
Age (weeks)
B
Caloric Intake (kcal)
1000
800
600
400
200
0
****
****
****
4wCtrl
4wWD
3 4 5 6 7
Age (weeks)
C
Body Weight (g)
400
300
200
100
0
**
3 4 5 6 7
Age (weeks)
D
Resting Membrane Potential (mV)
-70
-75
-80
-85
-90
1wCtrl
1wWD
E
Action Potential Frequency (Hz)
20
15
10
5
0
1wCtrl
1wWD
50 100 150 200
Current Injection (pA)
F
Change in RMP from Control (mV)
15
10
5
0
-5
****
*
*
0 5 10
Weeks on Diet
G
4wCtrl
AM1wWD
4wWD
20mV
200ms
150pA
-88mV
-81mV
-75mV
H
Resting Membrane Potential (mV)
-70
-75
-80
-85
-90
***
#
4wCtrl
AM1wWD
4wWD
I
Action Potential Frequency (Hz)
20
15
10
5
0
**
***
#
****
##
50 100 150 200
Current Injection (pA)
J
4wCtrl
AM1wWD
4wWD
First Spike Latency to Fire (s)
0.6
0.4
0.2
0.0
****
##
***
#
*
50 100 150 200
Current Injection (pA)

Figure 5.3: Activation of MCH neurons occurs at the onset of excess weight gain.

(A) Schematic of short-term WD feeding and experimental groups. (B-C) Caloric intake and body weight during the 4-week feeding period. (D) Resting membrane potential for 1wCtrl and 1wWD groups. (E) Action potential frequency during current injections for 1wCtrl and 1wWD groups. (F) Time course of the change in the RMP of MCH neurons during WD feeding. Values represent the difference in the RMP of the WD group relative to the average RMP of age matched controls. (G) Sample traces of MCH neurons responding to current injections from different 4-week feeding groups described in A. (H-J) Measures of excitability for MCH neurons in 4wCtrl, AM1wWD, and 4wWD groups, including RMP (H), and firing frequency (I) and first spike latency (J) during positive current injections.

*One-way (F,H) and two-way ANOVA with post hoc comparisons: * 4wCtrl vs 4wWD: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; # AM1wWD vs 4wWD: #p<0.05, ##p<0.01*

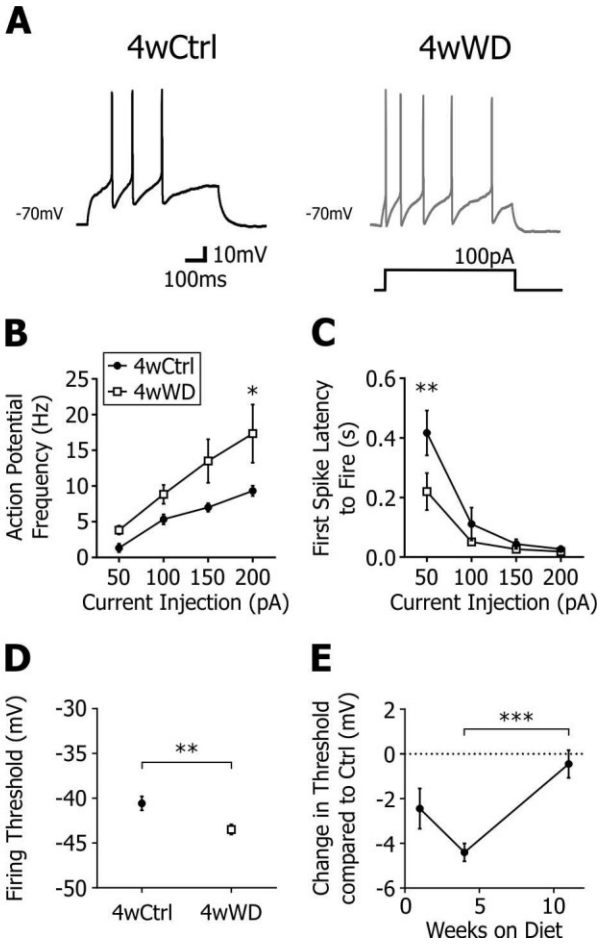

Figure 5.4: WD increases excitability of MCH neurons independent of RMP depolarization.

(A) Sample traces for 4wCtrl and 4wWD MCH neurons held at -70 mV. (B) Action potential frequency and (C) first spike latency during step current injections in MCH neurons maintained at -70mV. (D) Firing threshold for 4wCtrl and 4wWD groups. (E) Time course of the effect of WD feeding on firing threshold in MCH neurons. *Two-way ANOVA (B,C), one-way ANOVA (E), and unpaired t-test (D): *p<0.05, **p<0.01, ***p<0.001*

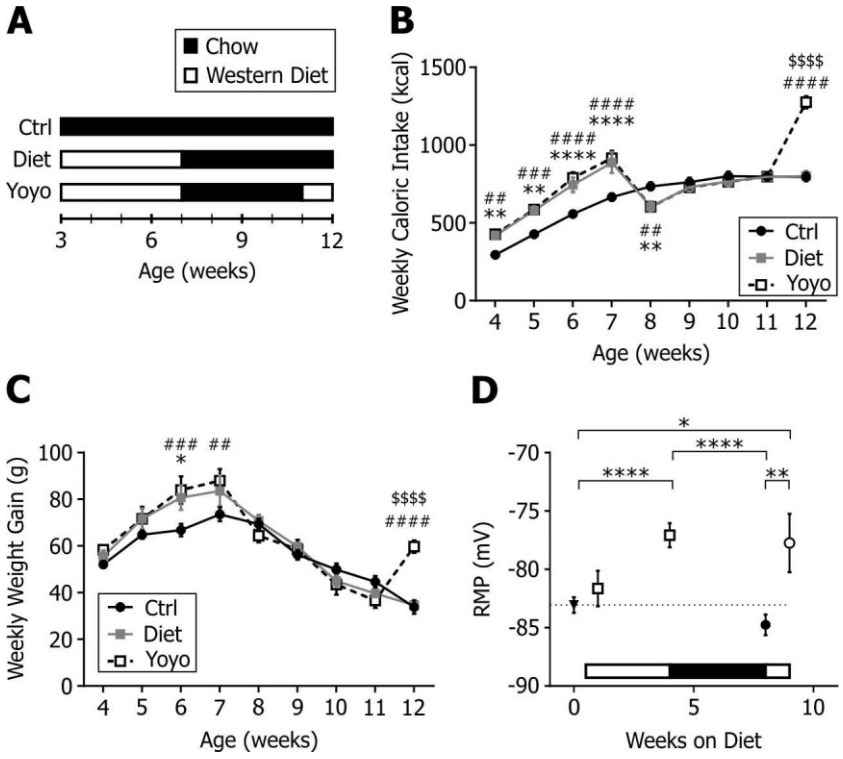

Figure 5.5: Activation of MCH neurons by WD is reversible and sensitizes with further WD exposure.

(A) Schematic of rat groups. Rats were either fed a standard chow (Ctrl), fed WD for 4 weeks and then put on chow (Diet), or cycled between WD and chow (Yoyo). Weekly caloric intake (B) and weight gain (C) for the groups described in panel A.
*Two-way RM ANOVA with post hoc comparisons: * Ctrl vs Diet: *p<0.05, **p<0.01, ****p<0.0001; # Ctrl vs Yoyo: ##p<0.01, ###p<0.001, ####p<0.0001; $ Diet vs Yoyo: $$$$p<0.0001*
(D) RMP in Ctrl rats (Week = 0; dotted line), and rats fed WD for 1 and 4 weeks, rats in the Diet condition, and those in the Yoyo condition.
*One-way ANOVA with post hoc comparisons: *p<0.05, **p<0.01, ****p<0.0001*

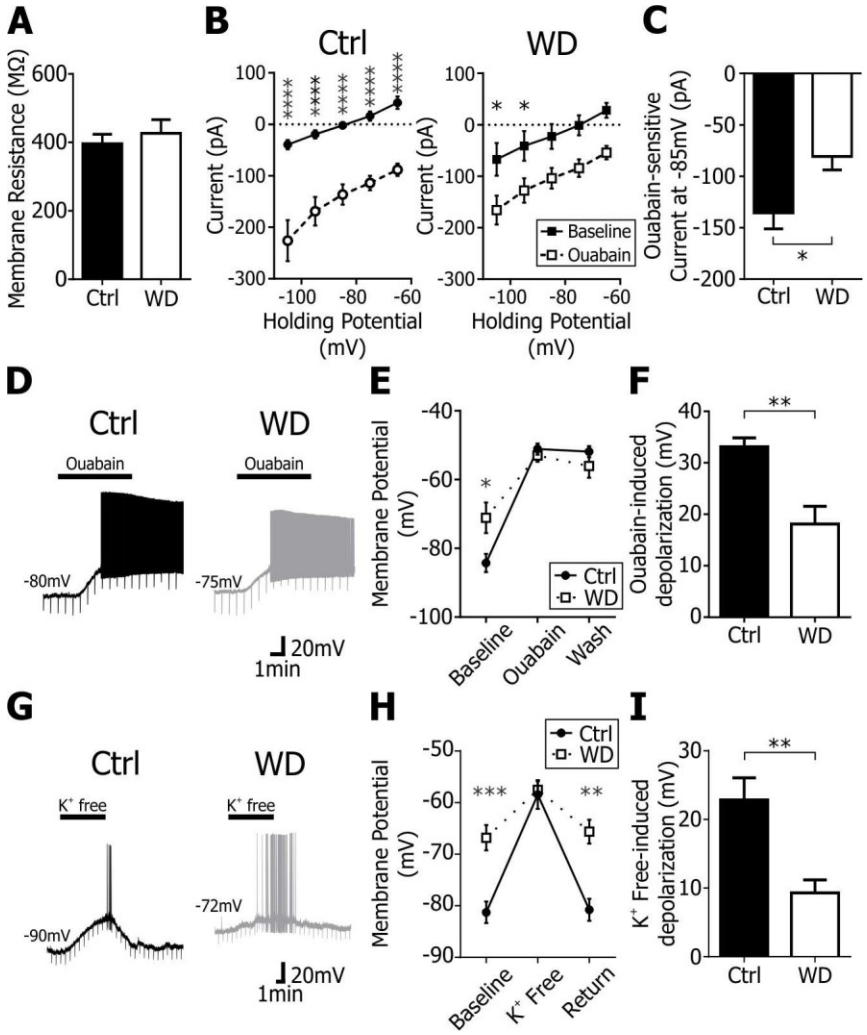
A
Membrane Resistance (MΩ)
600
400
200
0
Ctrl
WD
B
Ctrl
WD
Current (pA)
100
0
-100
-200
-300
****
*
-100
-80
-60
Holding Potential (mV)
Baseline
Ouabain
C
Ouabain-sensitive Current at -85mV (pA)
0
-50
-100
-150
-200
*
D
Ctrl
WD
Ouabain
-80mV
-75mV
20mV
1min
E
Membrane Potential (mV)
-40
-60
-80
-100
Ctrl
WD
Baseline
Ouabain
Wash
F
Ouabain-induced depolarization (mV)
40
30
20
10
0
**
G
Ctrl
WD
K+ free
-90mV
-72mV
20mV
1min
H
Membrane Potential (mV)
-50
-60
-70
-80
-90
***
**
Baseline
K+ Free
Return
I
K+ Free-induced depolarization (mV)
30
20
10
0
**

Figure 5.6: A chronic inhibition of the Na^+/K^+-ATPase underlies depolarization of MCH neurons.

(A) Membrane resistance of MCH neurons from Ctrl and WD groups. (B) Current-voltage relationship of MCH neurons at baseline and with ouabain (25μM) during 5 second voltage ramps. (C) The amplitude of ouabain-sensitive current at -85mV. (D) Sample current clamp traces of ouabain application in MCH neurons of Ctrl and WD rats. (E) Group data showing the effect of ouabain on the membrane potential in MCH neurons. (F) Ouabain causes a greater change in membrane potential in MCH neurons of Ctrl rats. (G) Sample current clamp traces from MCH neurons during K^+ free ACSF application. (H) Membrane potential of MCH neurons before, during, and after K^+ free ACSF. (G) There was a greater change in membrane potential in MCH neurons of Ctrl rats with K^+ free ACSF.
*Two-way RM ANOVA (B,E,H) and unpaired t-test (C,F,I): *p<0.05, **p<0.01, ***p<0.0001, ****p<0.00001*

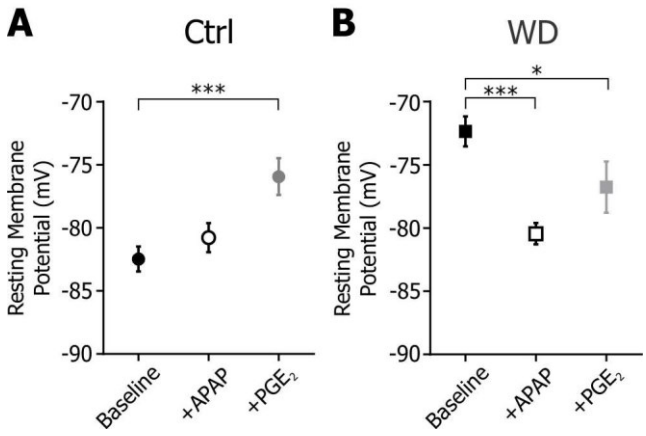

Figure 5.7: WD promotes COX-2 activity to depolarize MCH neurons.

(A) Resting membrane potential of MCH neurons from control rats at baseline or treated with acetaminophen (APAP; 50µM) or PGE_2 (0.1nM). (B) Resting membrane potential of MCH neurons from WD rats at baseline or treated with APAP or PGE_2.
*One-way ANOVA with post hoc comparisons: *p<0.05, ***p<0.001*

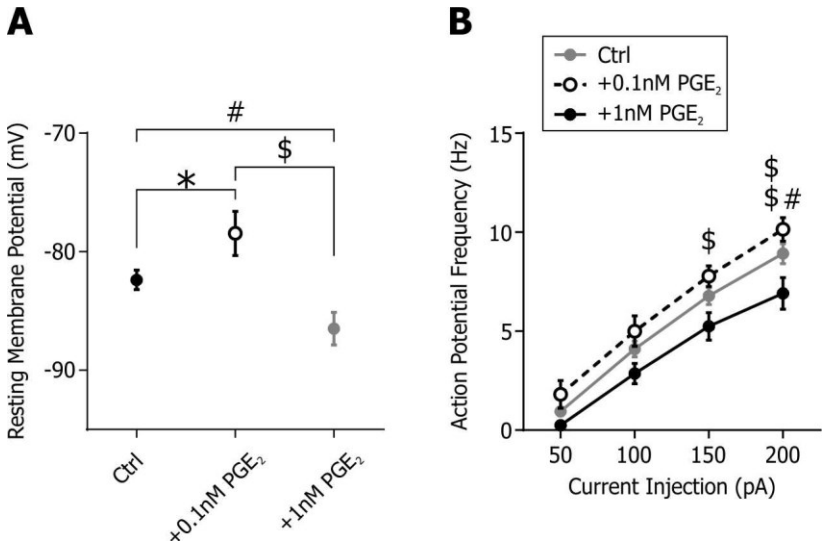

Figure 5.8: Concentration dependence of the PGE_2 effect on MCH neurons.

(A) RMP of 7-week old chow-fed rats (Ctrl) incubated with either 0.1nM or 1nM PGE_2. (B) Action potential frequency of Ctrl rats incubated with either 0.1nM or 1nM PGE_2. *One-way ANOVA (A) and two-way RM ANOVA (B) with post hoc comparisons: *Ctrl vs +0.1nM PGE_2: *p<0.05; #Ctrl vs +1nM PGE_2: #p<0.05; $Ctrl+0.1nM PGE_2 vs Ctrl+1nM PGE_2: $p<0.05, $$p<0.01*

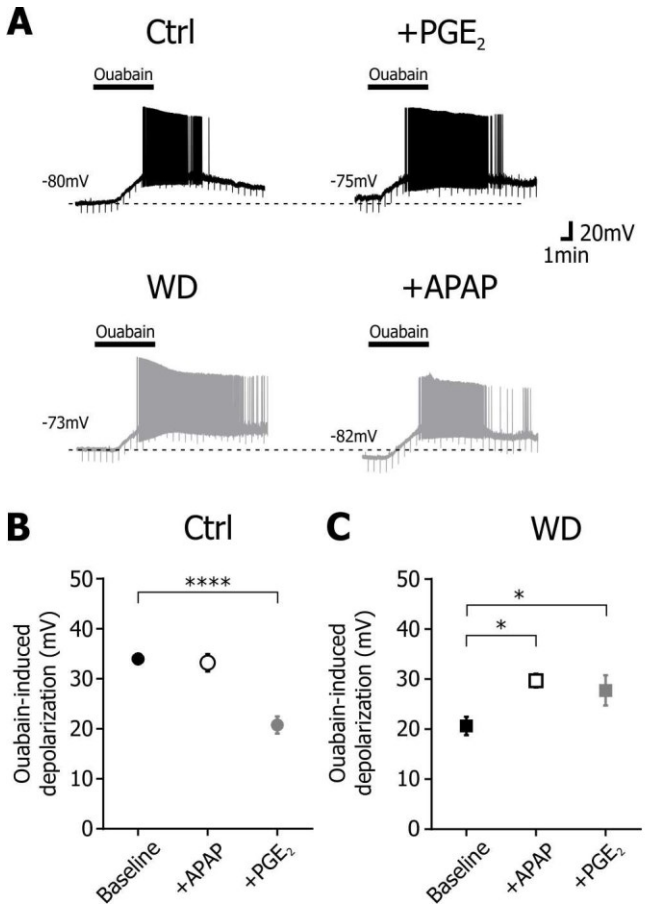

Figure 5.9: WD and PGE_2 modulate NKA function in MCH neurons.

(A) Sample traces showing the effect of ouabain (25μM) on MCH neurons from the Ctrl group at baseline or treated with PGE_2 and from the WD group at baseline or treated with APAP. (B) Effect of APAP or PGE_2 on ouabain-induced depolarization in the Ctrl groups. (C) Effect of APAP or PGE_2 on ouabain-induced depolarization in the WD groups.
*One-way ANOVA with post hoc comparisons: *p<0.05, ****p<0.0001*

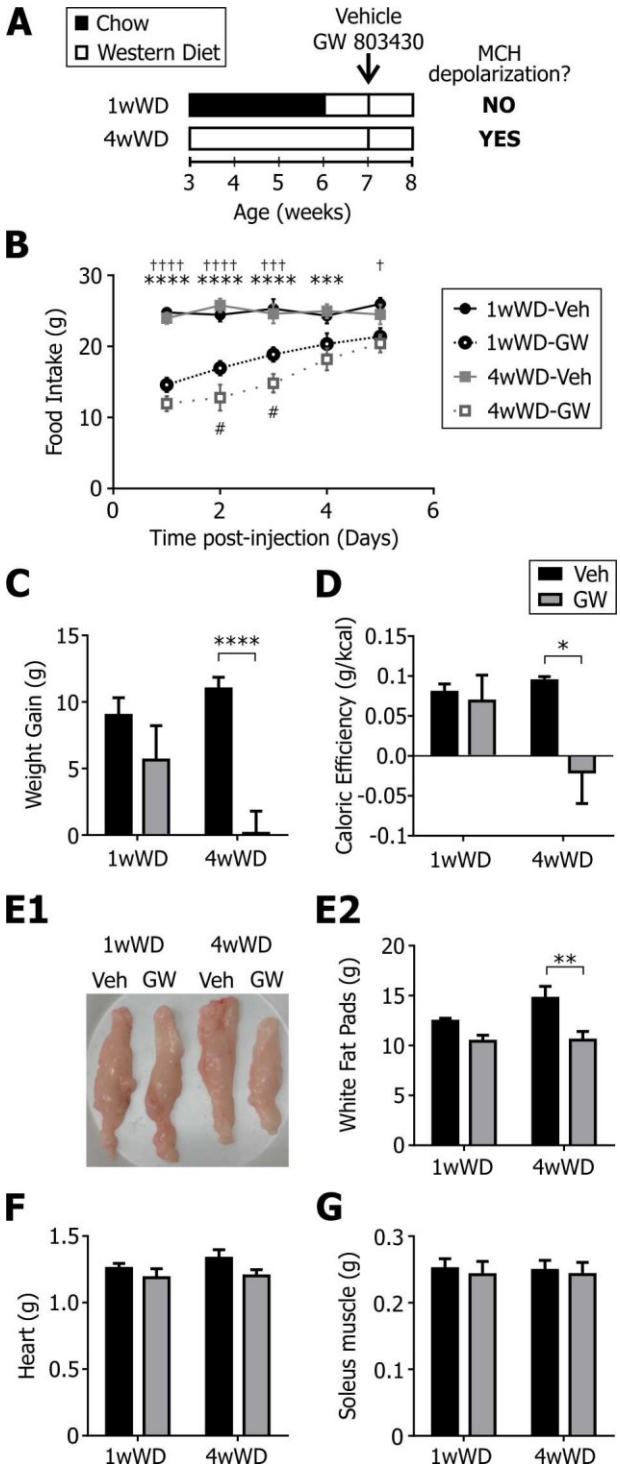
A
Chow
Western Diet
Vehicle
GW 803430
MCH
depolarization?
1wWD
4wWD
NO
YES
3 4 5 6 7 8
Age (weeks)
B
Food Intake (g)
Time post-injection (Days)
1wWD-Veh
1wWD-GW
4wWD-Veh
4wWD-GW
C
Weight Gain (g)
1wWD
4wWD
D
Veh
GW
Caloric Efficiency (g/kcal)
E1
1wWD
4wWD
Veh GW Veh GW
E2
White Fat Pads (g)
F
Heart (g)
G
Soleus muscle (g)

Figure 5.10: WD-induced excitation of MCH neurons is associated with increased MCHR1-mediated food intake, weight gain, and fat accrual.

(A) Schematic of the diet groups and drug or vehicle injections. (B) Daily food intake of rats following the I.P. injection of 30mg/kg GW 803430 (GW) or a vehicle solution (Veh). (C) Weight gain on Day 2 post-injection of the groups described in panel A. (D) Caloric efficiency on Day 2 post-injection. (E1) Photograph of the epididymal fat from rats fed with WD then treated with vehicle or GW. (E2) Weight of fat pads (epididymal and retroperitoneal fat pads) after five days post-injection. (F-G) Weight of the heart and soleus muscle after five days post-injection.
*Two-way ANOVA with post hoc comparisons: † 1wW-Veh vs 1wW-GW: †p<0.05, †††p<0.001, †††† p<0.0001; * 4wW-Veh vs 4wW-GW: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; # 1wW-GW vs 4wW-GW: #p<0.05, ##p<0.01*

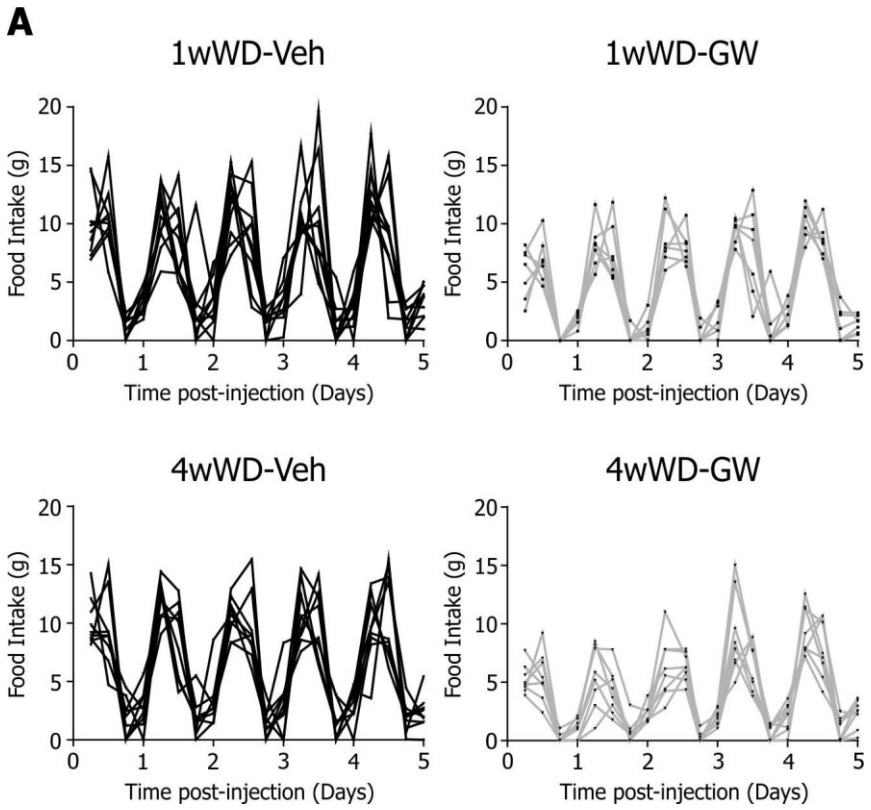

Figure 5.11: Individual food intakes of rats in the GW 803430 experiment.

(A) Individual food intake measurements taken every 6 hours following an I.P. injection of GW 803430 or vehicle in rats either fed for 1 or 4 weeks with WD.

CHAPTER 6

SYNAPTIC PLASTICITY OF MELANIN-CONCENTRATING HORMONE NEURONS DURING HIGH-FAT DIET FEEDING

6.1 Introduction

The rising number of overweight or obese individuals over the past few decades has become an epidemic in developed countries around the world. A major underlying cause of this excessive weight gain is an increased consumption of palatable high-fat and high-sugar food (Cordain et al., 2005). Caloric overconsumption is thought to occur not only because palatable food is energy dense but also because it can disrupt the hypothalamic neural circuitry for energy homeostasis to promote weight gain (El-Haschimi et al., 2000; Woods et al., 2004; Zhang et al., 2008). In particular, MCH neurons of the lateral hypothalamus may be influenced by high-fat diets to promote obesity. In diet-induced obese rats, both MCH mRNA and peptide, as well as MCH receptor mRNA expression are upregulated (Elliott et al., 2004), indicating an overall activation of the MCH system. Since MCH signaling promotes food intake, especially of palatable diets (Morens et al., 2005), and suppresses energy expenditure to overall promote weight gain (Glick et al., 2009; Hausen et al., 2016; Zhang et al., 2014), an increase in MCH signaling may contribute to the development or maintenance of diet-induced obesity.

An intriguing idea in the literature is that palatable diets and obesity trigger plasticity within the hypothalamus and related areas to influence body weight regulation. For instance, synaptic remodeling occurs within two neuronal populations in the arcuate nucleus in obesity that is thought to oppose further food intake and weight gain (Horvath et al., 2010; Pinto et al., 2004). However, despite the evidence implicating MCH neurons in obesity, it is not yet known whether any electrophysiological changes occur in MCH

neurons during high-fat diet feeding. Furthermore, a key question is whether such changes would be a cause or effect of obesity. By investigating time-dependent effects of high-fat diet feeding, one can better understand the etiology of diet-induced obesity and identify which neural changes may be of interest for further investigation.

In the present study, we hypothesized that synaptic plasticity occurs in MCH neurons with WD feeding. Our results show that MCH neurons undergo a time-dependent plasticity which occurs prior to the onset of significant weight gain and persists throughout the remainder of the feeding period. As this change occurs early in high-fat diet feeding, it may contribute to the development of obesity.

6.2 Methods

6.2.1 Animals and diets

All experiments were conducted per the guidelines of Canadian Council of Animal Care under Memorial University's Institutional Animal Care Committee. Male 3-week old Sprague-Dawley rats (Charles River, Quebec or Memorial University breeding colony, Newfoundland) were fed *ad libitum* either a standard chow (LabDiet autoclavable rodent diet 5010; 3.08 kcal/g: (% of total calories) 12.7% fat, 28.7% protein and 58.5% carbohydrates) or a palatable high-fat diet, Western Diet (WD; AIN-76A (TestDiet), 4.55 kcal/g: (% of total calories) 40.0% fat, 15.8% protein, and 44.2% carbohydrates). Body weight and food intake was measured weekly.

6.2.2 Electrophysiology

Rats were deeply anesthetized with isoflurane and the brain was removed. Acute 250μM slices of the hypothalamus were generated in cold ACSF (in mM: 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 18 $NaHCO_3$ and 2.5 glucose) bubbled with 95% O_2/5% CO_2 in a vibratome (VT-1000, Leica Microsystems). Slices were then incubated at 32-34°C for 30 minutes and left at room temperature until experiments were performed. To perform whole cell patch clamp recordings, hemisected slices were placed in a recording chamber perfused at 1-2mL/min with ACSF maintained at 32°C. Glass pipettes electrodes were filled with an internal solution (EPSCs: composition (in mM): 123 K-gluconate, 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10 HEPES, 5 Na_2-ATP, 0.3 Na-GTP, and 2.7 biocytin; IPSCs: K-gluconate was replaced with KCl such that the total concentration of

KCl was 131mM) that had a tip resistance of 3-5 MΩ. After whole cell access was attained, access resistance was 5-20 MΩ and every minute a 20mV, 50ms square pulse was applied to monitor access. Multiclamp 700B, and Clampex 9/10 software (Molecular Devices, Sunnyvale, CA) were used to collect data. Signals were filtered at 1kHz and digitized at 5-10kHz.

All recordings were performed at a holding potential of -70mV. Miniature postsynaptic currents were isolated using TTX (1µM; Alomone Labs, Jerusalem, Israel). Picrotoxin (50µM; Sigma, Oakville, Canada) was used to record EPSCs, while DNQX (10µM; Tocris Bioscience, Minneapolis, MN, US) and DAP5 (50µM; Abcam, Cambridge, MA, US) were used to record IPSCs. As ionotropic glutamate receptor antagonists block EPSCs (Huang & van den Pol, 2007; van den Pol et al., 2004) and $GABA_A$ antagonists block IPSCs (van den Pol et al., 2004), these were referred to as glutamatergic and GABAergic, respectively.

For paired pulse experiments, a pair of EPSCs was elicited at a frequency of 50Hz every 15 seconds using a glass pipette filled with ACSF and placed medially to the recorded cell.

6.2.3 Identification of MCH neurons

MCH neurons have a unique response to positive and negative current injections among other neurons in the lateral hypothalamus. We used a protocol of 600ms 50pA steps from -200pA to +200pA to generate an electrophysiological fingerprint of MCH neurons. Briefly, MCH neurons are not spontaneously active, display spike adaptation,

have uniphasic afterhyperpolarization potential, show no H current, and have no rebound following depolarization. This method of identification has a high accuracy of correct detection in our lab for MCH neurons (Alberto et al., 2011).

Post hoc immunohistochemistry was used in conjunction with electrophysiological identification in a subset of cells. Internal solution is filled with biocytin which diffuses into a cell during recording. Following experiments, slices are fixed in 10% formalin for at least 48 hours. Slices were incubated with a rabbit anti-MCH IgG (1:1000-2000; SC14509, Santa Cruz Biotechnology, Santa Cruz, CA) for 3 days at 4ºC. Then fluorophore-conjugated secondary antibodies to stain MCH (Alexa 488- or 594-conjugated donkey anti-rabbit; 1:500, Invitrogen, Carlsbad, CA) and streptavidin (1:500; Jackson ImmunoResearch, West Grove, PA, USA) to label biocytin-filled cells were applied for 3 hours at room temperature or at 4ºC overnight. Then slices were mounted and imaged on a fluorescent microscope to determine colocalization of biocytin and MCH (Fig. 2.1).

6.2.4 Data and statistical analysis

Data are expressed as mean ± SEM. The number of observations is reported in the nested model where N/n represents the number of cells/the number of animals. Unpaired t-tests for electrophysiological experiments and two-way ANOVA with Holm-Sidak post hoc tests for body weight and food intake were used to test for significance. All statistical analyses were performed with Prism 6.0 (GraphPad). $p<0.05$ was considered significant. Evoked EPSC amplitude was analyzed using Clampfit 10. mPSC frequency and

amplitude were analyzed using MiniAnalysis software (Synaptosoft). Events were manually analyzed and selected visually by those that had a clear fast rise and exponential decay. Peak scaled non-stationary noise analysis (NSNA) was performed using MiniAnalysis to estimate single channel conductance and number underlying mPSCs. NSNA analysis assesses the parabolic relationship between mPSC variance versus amplitude. However, for some cells that had an insufficient number of synaptic events, this relationship could not be properly fit with a parabola and therefore, these cells were excluded from NSNA analysis as indicated in the text. For mEPSCs, an average of 290±24 and 128±8 events per cell were analyzed in each cell for frequency and amplitude, respectively. For mIPSCs, an average of 188±14 and 169±11 events per cell were analyzed for frequency and amplitude. The relative distributions of mEPSC amplitude were fit with a log(Gaussian) nonlinear regression to determine the mEPSC amplitude corresponding to the peak of the distribution.

6.3 Results

Rats fed WD for 11 weeks consumed significantly more calories (Ctrl n=12 vs WD n=12; Fig. 6.1A) and were overweight (Fig. 6.1B) compared to those fed a standard chow (Ctrl). To investigate the time course of synaptic plasticity in MCH neurons, we looked at three key timepoints during WD feeding: 1 week, when rats overeat but do not gain weight; 4 weeks, before the onset of significant weight gain; and 11 weeks, when rats are overweight.

6.3.1 One week of WD feeding decreases excitatory transmission in young rats

After 1 week of WD feeding (4-week old rats), MCH neurons displayed a reduction in excitatory transmission through both pre- and postsynaptic mechanisms. Firstly, there was a decrease in mEPSC amplitude (Ctrl 14.4±1.1pA, N/n=9/7 vs 1wWD 10.4±1.6pA, N/n=7/5, p=0.0493; Fig. 6.2A,B). Accordingly, the average relative distribution of mEPSC amplitude showed a leftward shift (Fig. 6.2C). The peaks of individual distributions also showed a leftward shift to lower amplitudes in the 1wWD condition (Fig. 6.2D). However, this did not accompany a change in the coefficient of variation (CV; Fig. 6.2E), suggesting a lack of change in skewness of the distribution. Thus, the decrease in mEPSC amplitude is unlikely to be due to reduced multiquantal release. To further determine the cause of the reduction in quantal current, we used peak-scaled NSNA and found a decrease in single receptor current (Ctrl 1.41±0.18pA, N/n=9/7 vs 1wWD 0.74±0.17pA, N/n=6/5 (1 cell could not be fit), p=0.0244; Fig. 6.2F,G), with no change in the number of receptors (Ctrl vs 1wWD; Fig. 6.2F,H). These results suggest

that the decrease in mEPSC amplitude was due to a reduction in the conductance of non-NMDA glutamatergic receptors.

We also found a reduction in the frequency of mEPSCs (Ctrl 1.84±0.22Hz, N/n=9/7 vs 1wWD 0.79±0.11Hz, N/n=7/5, p=0.0014; Fig. 6.3A,B). In contrast, there was no change in the PPR, an indirect measure of presynaptic release probability (Ctrl 1.60±0.08, N/n=21/16 vs 1wWD 1.53±0.08, N/n=16/13, p>0.05; Fig. 6.3C,D). These combined results may be due to a reduction in the number of active synapses by synapse removal or silencing. Alternatively, the decrease in mEPSC amplitude may have increased the proportion of mEPSCs that were below the detection threshold, resulting in a lower apparent mEPSC frequency.

6.3.2 Four weeks of WD feeding increases the frequency of excitatory transmission in MCH neurons

After 4 weeks of feeding, WD had no effect on mEPSC amplitude (Ctrl N/n=10/6 vs 4wWD N/n=9/5; Fig. 6.4A,B,D), non-NMDA receptor conductance (Fig. 6.4E) or number (Fig. 6.4F). In contrast, there was a significant increase in the frequency of mEPSCs in the WD group (Ctrl 1.2±0.1Hz vs 4wWD 2.0±0.3Hz, p=0.0087; Fig. 6.4A,C,G), while there was no change in PPR (Ctrl 1.51±0.07, N/n=17/9 vs 4wWD 1.67±0.11, N/n=15/5, p>0.05; Fig. 6.4H,I). Therefore, 4 weeks of WD appears to increase the number of active excitatory synaptic contacts without altering overall release probability.

6.3.3 Age-dependent effects of WD feeding

The opposite effect of WD at 1 week and 4 weeks (a decrease and increase in excitatory transmission, respectively) may be due to the length of WD feeding. However, it is also possible that the age difference had an effect since rats from both groups were 3-week old when feeding began and were therefore tested at different ages. To determine if age had an effect, we staggered 1 week of WD feeding so that the animals were age-matched to the 4-week WD group at the time of testing. In this age-matched group, 1-week WD did not induce any changes (mEPSC amplitude, Ctrl N/n=10/6: 10.55±0.83pA vs 1wWD N/n=6/4: 10.06±0.78pA, $p>0.05$; mEPSC frequency, Ctrl: 1.20±0.10Hz vs 1wWD: 1.00±0.08Hz, $p>0.05$). Therefore, 1 week of WD feeding affects excitatory transmission in 3 - 4-week old but not 6 - 7-week old rats.

6.3.4 Eleven weeks of WD feeding increases excitatory transmission onto MCH neurons

Following 11 weeks of WD feeding, there was no change in mEPSC amplitude (Ctrl n/n=7/4 vs 11wWD N/n=8/4; Fig. 6.5A,B,D), or single non-NMDA receptor current (Ctrl N/n=6/3 (1 cell could not be fit) vs 11wWD N/n=8/4; Fig. 6.5E) and receptor number (Fig. 6.5F). On the other hand, WD induced an increase in mEPSC frequency (Ctrl 1.5±0.3Hz, N/n=7/4 vs 11wWD 3.7±0.4Hz, N/n=8/4, $p=0.0003$; Fig. 6.5A,C,G) with no change in PPR (Ctrl N/n=16/9 vs 11wWD N/n=15/6; Fig. 6.5H,I). This result is similar to what was seen after 4 weeks of feeding; however, the mEPSC frequency at 11 weeks of WD was significantly greater than that after 4 weeks of WD (4wWD N/n=9/5: 2.04±0.84Hz vs 11wWD N/n=8/4: 3.7±1.00Hz, $p=0.0021$), while there was no age-

dependent change in mEPSC frequency in their respective age-matched controls (Ctrl, 7 weeks old N/n=10/6: 1.20±0.30Hz vs Ctrl, 14 weeks old N/n=7/4: 1.49±0.71Hz, $p>0.05$). This suggests that there is a continuous addition of excitatory contacts with longer WD feeding.

6.3.5 WD does not affect inhibitory transmission

In contrast to excitatory transmission, WD induced no change in the amplitude or frequency of mIPSCs at all time points investigated (1 week: Ctrl N/n=7/3 vs 1wWD N/n=8/4, Fig. 6.6A-C; 4 weeks: Ctrl N/n=12/3 vs 4wWD N/n=11/4, Fig. 6.6D-F; 11 weeks: Ctrl N/n=8/4 vs 11wWD N/n=8/4, Fig. 6.6G-I). Therefore, WD-induced synaptic plasticity in MCH neurons appears to be specific to excitatory inputs.

6.4 Discussion

The present study is the first to investigate the synaptic properties of MCH neurons during high-fat diet feeding. Diet-induced plasticity appears to occur specifically on excitatory synapses to MCH neurons. When feeding is commenced from 3 weeks of age (adolescence), WD initially (1 week) induces a transient decrease in excitatory transmission. The effect is reversed if WD feeding continues, as an increase in excitatory transmission occurs around the onset of significant weight gain at 4 weeks of WD feeding and continues to increase in overweight rats by 11 weeks of WD feeding.

6.4.1 WD induced plasticity of excitatory transmission to MCH neurons

While there was a robust increase in excitatory transmission by WD, no changes were observed in inhibitory transmission throughout the WD feeding period. This indicates that MCH neuron excitability may only be gated by modulation of excitatory transmission with high-fat diet. As fast EPSCs in MCH neurons have been shown to be primarily mediated by glutamate receptors (van den Pol et al., 2004), this plasticity involves remodeling of glutamatergic afferents to MCH neurons. The mechanism underlying this synaptic remodeling is unknown; however, it may involve metabolic and hormonal changes known to result from prolonged high-fat diet. In other neuronal populations, high-fat diet associated leptin resistance, insulin signaling, cannabinoid signaling, and reductions in brain-derived neurotrophic factor (BDNF), have all been associated with structural or functional plasticity (Cristino et al., 2013; Labouèbe et al., 2013; Pinto et al., 2004; Stranahan et al., 2008). MCH neurons are responsive to several

of these factors including leptin, cannabinoids, and insulin, which increase MCH neuron excitability or MCH mRNA expression (Hausen et al., 2016; Huang et al., 2007; Huang et al., 1999). However, whether these factors can also affect synaptic plasticity in MCH neurons is unknown. Since there is limited literature available on MCH neurons and their plasticity, further research is required to disseminate the factors underlying this increase in excitatory synaptic contacts.

6.4.2 The WD effect on MCH neurons is age-dependent

Interestingly, there was an age-dependence in the inhibitory effect of 1 week of WD on mEPSCs as the effect was only seen in 4- but not 7-week old rats. Since MCH neurons undergo an age-dependent decrease in excitability between these ages (Chapter 2), it is possible that immature MCH neurons are more amenable to WD-induced changes. Since adolescent brains are still developing, they typically are more susceptible to plasticity, such as expressing greater magnitude of LTP than adults (Swartzwelder, Wilson, & Tayyeb, 1995), or expressing cognitive and structural changes following drug and high-fat diet exposure that are not seen in adults (Labouesse et al., 2016; McDonald et al., 2007; Valladolid-Acebes et al., 2013). Therefore, this interaction of WD- and age-dependent plasticity may have physiological ramifications on MCH functions during early adolescence.

6.4.3 Conclusions

Synaptic remodeling is known to occur within the homeostatic circuitry in response to dietary manipulations. Within the arcuate nucleus, long-term high-fat diet

feeding and obesity reduces excitatory synapses on appetite-promoting NPY/AgRP neurons, while increasing excitatory and decreasing inhibitory synapses on appetite-suppressing POMC neurons. These changes are thought to be homeostatic, suppressing food intake while increasing energy expenditure during times where caloric intake and fat mass is high (Horvath et al., 2010; Pinto et al., 2004). Our findings add appetite-promoting MCH neurons as another hypothalamic target of high-fat diet, where increased excitatory transmission onto MCH neurons would be expected to increase the excitability of these neurons.

Taken together with other studies showing increased MCH mRNA expression and peptide levels during obesity (Elliott et al., 2004), our study suggests that chronic high-fat diet results in activation of the MCH system, unlike the compensatory changes found in the arcuate nucleus. Since an increase in mEPSCs is observed around the onset of weight gain and its degree becomes greater with longer feeding, it may have a role in the development and maintenance of diet-induced obesity.

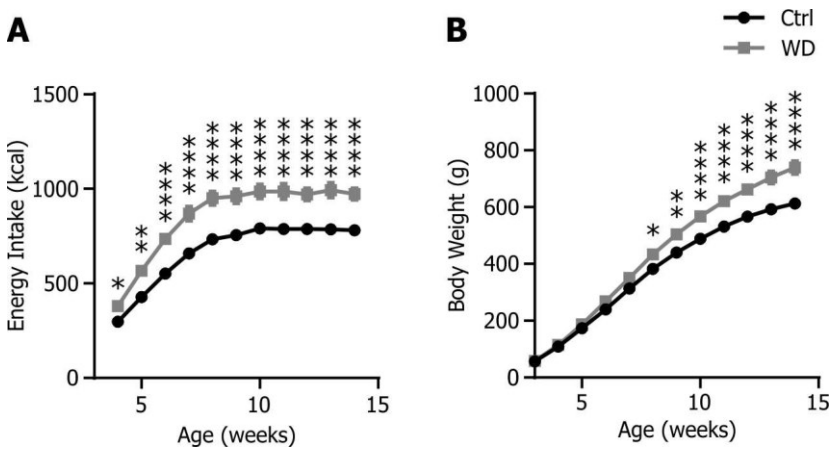

Figure 6.1: Western Diet increases caloric intake and body weight.

(A) Weekly caloric intake of male Sprague-Dawley rats fed a palatable high-fat Western Diet (WD) or a control chow (Ctrl). (B) Weekly body weight of WD and Ctrl rats.
*Two-way RM ANOVA with post hoc comparisons: *p<0.05, **p<0.01, ****p<0.0001*

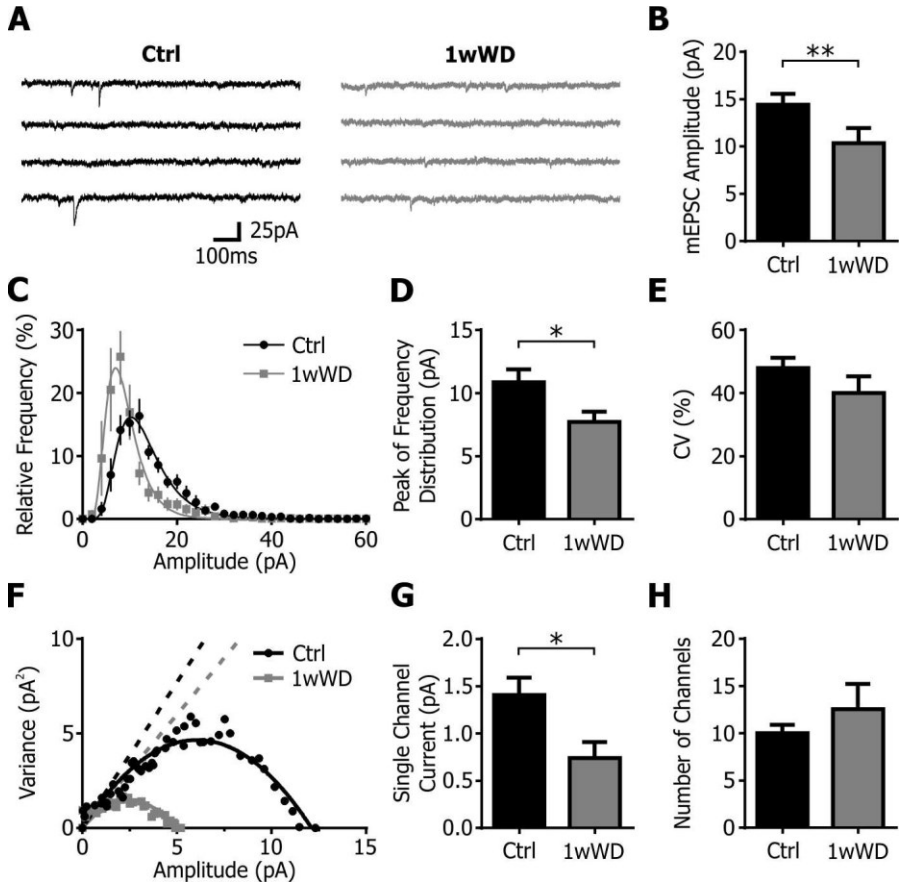

Figure 6.2: One week of WD feeding in early adolescence decreases mEPSC amplitude in MCH neurons.

(A) Sample mEPSC traces from MCH neurons of 4-week old rats fed a control chow (Ctrl) or WD for 1 week (1wWD). (B) Amplitude of mEPSCs in Ctrl and 1wWD. (C) Averaged relative frequency distribution of mEPSC amplitudes. (D) The mEPSC amplitude corresponding to the peak of distribution histograms for individual cells. (E) The coefficient of variation (CV) of mEPSC amplitude. (F) Representative peak scaled NSNA for Ctrl and 1wWD groups. (G) Estimated single channel current and (H) number of channels from NSNA.

*Unpaired t-test: *p<0.05, **p<0.01*

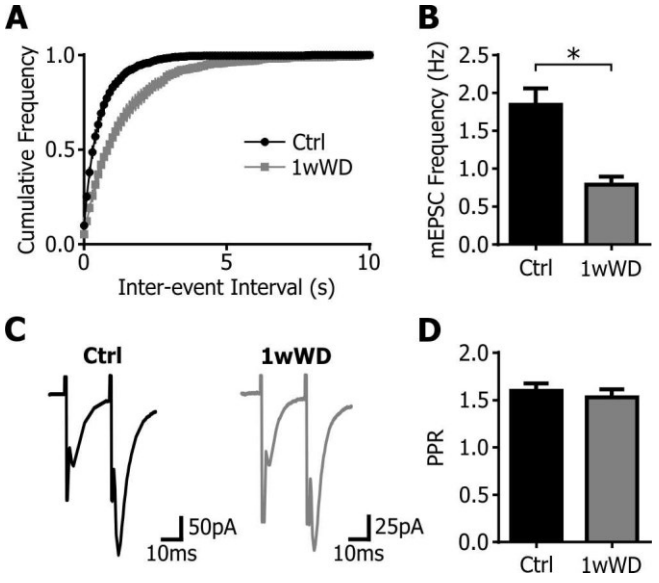

Figure 6.3: One week of WD feeding in early adolescence decreases mEPSC frequency in MCH neurons.

(A) Averaged cumulative relative frequency distribution of inter-event interval. (B) Frequency of mEPSCs in MCH neurons of Ctrl and 1wWD groups. (C) Sample paired EPSCs from Ctrl and 1wWD groups. (D) PPR for Ctrl and 1wWD.
*Unpaired t-test: *p<0.05*

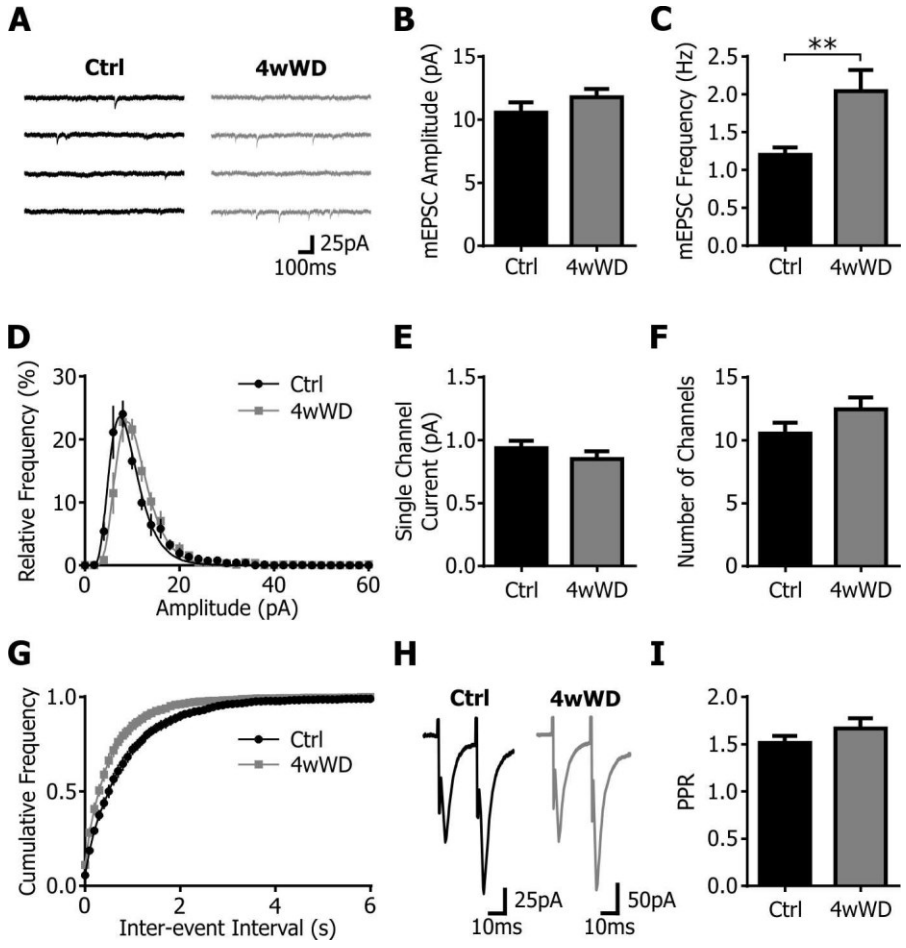

Figure 6.4: Four weeks of WD feeding increases excitatory transmission.

(A) Sample mEPSC traces from MCH neurons in 7-week old rats fed control chow (Ctrl) or WD (4wWD) for 4 weeks. (B) Amplitude and (C) frequency of mEPSCs in MCH neurons of Ctrl and 4wWD. (D) Averaged relative frequency distribution of mEPSC amplitudes. (E) Estimated single channel current and (F) number of channels from NSNA. (G) Averaged cumulative relative frequency distribution of inter-event interval. (H) Sample paired pulse EPSCs from Ctrl and 4wWD groups. (I) PPR for Ctrl and 4wWD.
*Unpaired t-test: **p<0.01*

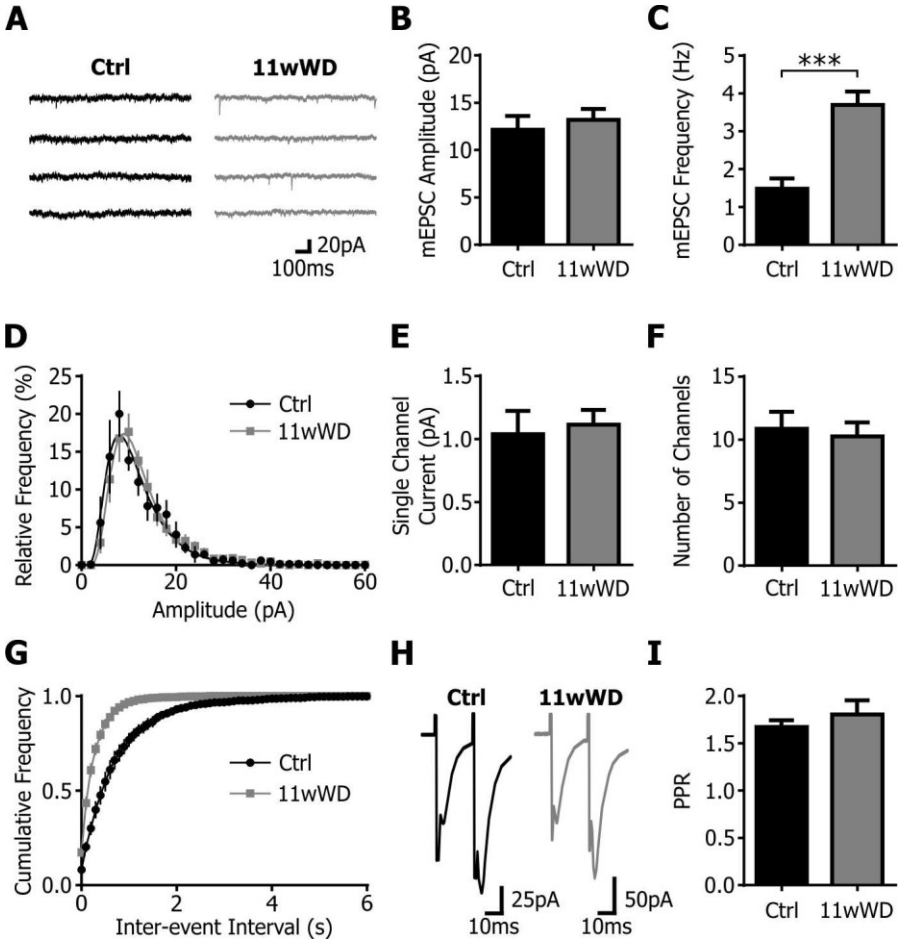

Figure 6.5: Eleven weeks of WD feeding increases excitatory transmission.

(A) Sample mEPSC traces from MCH neurons in 14-week old rats fed a control chow (Ctrl) or WD (11wWD) for 11 weeks. (B) Amplitude and (C) frequency of mEPSCs in Ctrl and 11wWD. (D) Averaged relative frequency distribution of mEPSC amplitudes. (E) Estimated single channel current and (F) number of channels from NSNA. (G) Averaged cumulative relative frequency distribution of inter-event interval. (H) Sample paired pulse EPSCs from Ctrl and 11wWD groups. (I) PPR for Ctrl and 11wWD.
*Unpaired t-test: ***p<0.001*

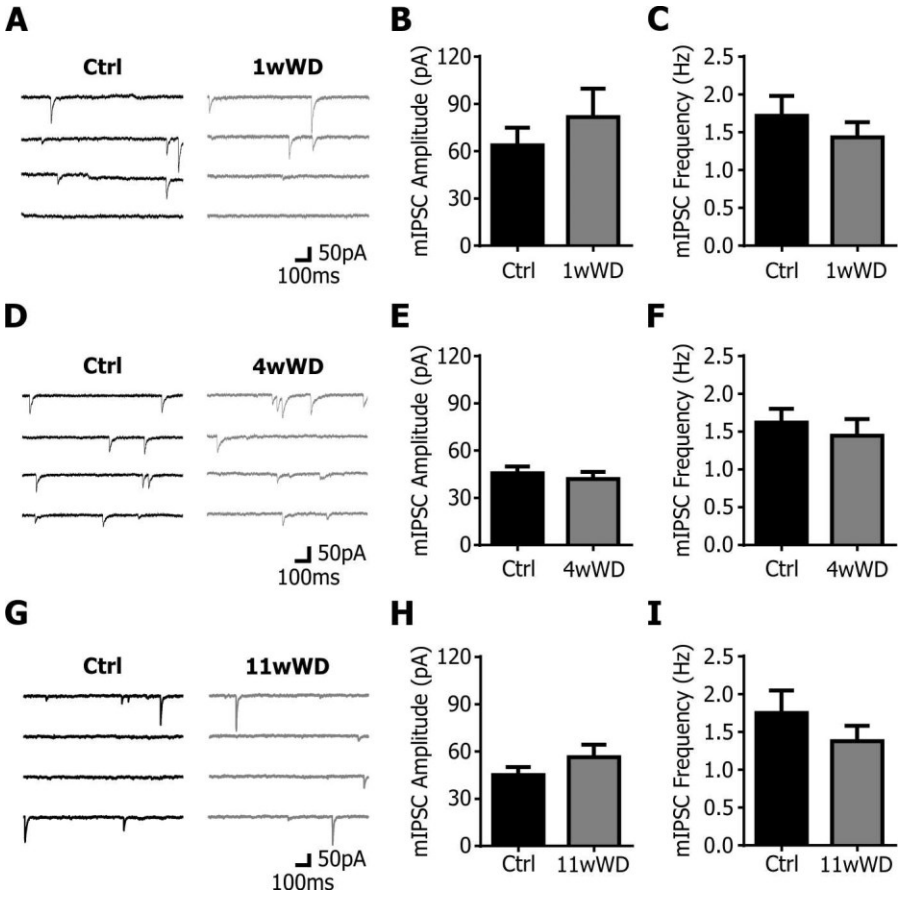

Figure 6.6: WD feeding has no effect on inhibitory transmission to MCH neurons.

(A) Sample mIPSC traces from MCH neurons in 4-week old rats fed a control chow (Ctrl) or WD (1wWD) for 1 week. (B) Amplitude and (C) frequency of mIPSCs in MCH neurons of Ctrl and 1wWD. (D) Sample mIPSC traces from MCH neurons in 7-week old rats fed Ctrl or WD (4wWD) for 4 weeks. (E) Amplitude and (F) frequency of mIPSCs in Ctrl and 4wWD. (G) Sample mIPSC traces from MCH neurons in 14-week old rats fed Ctrl or WD (11wWD) for 11 weeks. (H) Amplitude and (I) frequency of mIPSCs in Ctrl and 11wWD.

CHAPTER 7

SUMMARY

7.1 Summary of main findings

All the main experiments included in this thesis used the same diets, lengths of feeding, age of rats, and rat strain. Therefore, the results from each project are directly comparable with one another. Together, they summate to three key findings. First, orexin neurons undergo various forms of transient plasticity during WD feeding, most notably the unmasking of a presynaptic LTD. Second, MCH neurons show a delayed activation that occurs by the onset of weight gain during WD feeding and involves several mechanisms, including a COX-dependent depolarization. Finally, orexin neurons are electrophysiologically mature by weaning, whereas MCH neurons experience ongoing maturation during the post-weaning period, and perhaps related to this maturation, are modulated by WD in an age-dependent manner.

7.1.1 Effects of WD on orexin neurons

WD dynamically modulated orexin neurons in a time-dependent manner (Table 7.1). Generally, WD appears to activate orexin neurons at early stages of the feeding period, which becomes attenuated by the end of the feeding period. Specifically, WD increased the excitability of orexin neurons after 1 week of feeding as orexin neurons displayed an increase in AMPA receptor conductance (Chapter 4, Fig. 4.1). As an additional observation of increased orexin neuron excitability outside the research chapters included in this thesis, I noted that their RMP was depolarized after 1 week of WD feeding (Fig. 7.1A). However, these excitatory changes may be opposed by a reduction in release probability (Fig. 3.6; Fig. 4.2D) and priming for LTD (Chapter 3) at

excitatory synapses to orexin neurons. This may explain a lack of change in their basal firing frequency despite RMP depolarization (Fig. 7.1B). By 11 weeks of feeding, there was only an increase in the amplitude of inhibitory postsynaptic currents (Fig. 4.7) without any other change (Table 7.1), suggesting a reduction in orexin neuron excitability.

Together, these results suggest that there are multiple mechanisms by which WD affects orexin neuron excitability and that these changes are reversed or compensated for. These time-dependent changes may have interesting repercussions on orexin neuron function during WD feeding. Synaptic plasticity of orexin neurons is known to occur in various physiological states, such as sleep deprivation and fasting (Horvath & Gao, 2005; Y. Rao et al., 2007). Therefore, it is possible that plasticity of orexin neurons is an essential regulator of their activity and downstream physiological functions (Gao & Hermes, 2015). If so, the dynamic synaptic changes in orexin neurons throughout WD feeding may disrupt such routine plasticity and its corresponding regulatory functions in orexin neurons.

7.1.2 Effects of WD on MCH neurons

MCH neurons undergo a delayed but stable set of changes during WD feeding (Table 7.2). The most remarkable finding was a WD-induced depolarization that was present from 4 weeks of WD and became more substantial by 11 weeks of feeding. Interestingly, this time course is paralleled by our finding of increased mEPSC frequency that is also present at 4 weeks and further increases by 11 weeks of feeding. Therefore,

these concurrent intrinsic and synaptic changes may be interconnected or induced by a common mechanism.

The WD-induced depolarization persisted in the presence of TTX (Fig. 5.2H), which suggests an intrinsic change. Nonetheless, since synaptic tone influences RMP in MCH neurons (Huang et al., 2007) it is certainly possible that an increase in excitatory transmission could also contribute to depolarization. Alternatively, depolarized RMP can facilitate synaptic and structural plasticity (Armano, Rossi, Taglietti, & D'Angelo, 2000; Murase, Mosser, & Schuman, 2002), which may underlie the increased mEPSC frequency by WD in MCH neurons.

On the other hand, a potential common mechanism that could explain both synaptic and intrinsic changes is through a COX-dependent pathway. We found that the depolarization of MCH neurons was due to COX activity, which could also act at excitatory synapses onto MCH neurons. COX-2 is expressed at dendritic spines and influences activity-dependent excitatory transmission and plasticity through presynaptic PGE_2 receptors (Chen, Magee, & Bazan, 2002; Kaufmann, Worley, Pegg, Bremer, & Isakson, 1996; Sang, Zhang, Marcheselli, Bazan, & Chen, 2005). While limited data is available on a potential link between COX and structural plasticity, it would be an intriguing future direction for this project.

Regardless of the mechanism, it is likely that RMP depolarization and increased excitatory input would synergistically increase MCH neuron firing. Overall, through both

intrinsic and synaptic mechanisms MCH neurons are activated with long-term WD consumption, which likely promotes weight gain and obesity.

7.1.3 Age-dependent effects of WD on MCH neurons

Chapter 2 demonstrated that MCH neurons continue to mature electrophysiologically during the post-weaning period up to 7 weeks of age through intrinsic and synaptic mechanisms. We postulated that this may affect how MCH neurons respond to various stimuli. Within this thesis, we have provided evidence for this idea as we observed discrepancies between the WD effects on MCH neurons from 4- and 7-week old rats, which may be a result of the ongoing maturation of these neurons.

Specifically, 1 week of WD had differential effects on MCH neurons of 4-week and 7-week old rats. In 4-week old rats, 1 week of WD depolarized the firing threshold, while decreasing firing frequency and excitatory transmission (Fig. 7.2A-C). However, these changes were not observed in MCH neurons of 7-week old rats that were also fed WD for 1 week (Fig. 7.2D-F; and Chapter 6). Interestingly, these WD-induced changes are similar to the age-dependent changes that occur between 4 and 7 weeks of age, i.e. a depolarization of firing threshold, a decrease in firing, and a decrease in excitatory transmission (Fig. 2.3-2.4). The almost complete overlap between the effects of WD and age on MCH neurons suggests that they may share common mechanisms and that WD is accelerating the maturation of MCH neurons. In support of this idea, 4-week old rats fed WD had a similar mEPSC frequency and amplitude as older rats fed a control chow (Chapter 6). This early reduction in MCH neuron excitability may affect many MCH-

dependent functions during the adolescent period including food intake, weight gain, anxiety, reproductive function, and sleep.

It is important to note that the robust depolarization of MCH neurons by 4 weeks of WD is likely not age-dependent, since high-fat diet effectively induced MCH neuron depolarization even when the feeding started at 7 weeks of age (Fig. 5.2E). Therefore, the activation of MCH neurons with long-term WD is not specific to young rats and may be a general mechanism underlying obesity.

7.2 Comparison of the effects of WD on orexin and MCH neurons

Despite the similarities in the anatomical distribution of orexin and MCH neurons, we found that they vary widely in their response to WD feeding. Namely, there were differences in the age-dependence, the timing and nature of intrinsic and synaptic plasticity, and in their adaptation or sensitization to WD. These disparities may indicate that selective WD effects may have a role in the physiological functions of each separate neuronal population.

7.2.1 Age-dependent effects of WD

Unlike the age-dependent effects of WD on MCH neurons discussed in the previous section (7.1.3), orexin neurons did not experience an age-dependent WD effect. 1 week of WD feeding induces the same synaptic changes in orexin neurons from 4- and 7-week old rats (Fig. 7.3). This may be because orexin neurons are electrophysiologically mature by 4 weeks of age (Chapter 2), while MCH neurons are not. Additionally, this

may indicate that orexin neurons may have less contribution to the adolescent-specific vulnerabilities to high-fat diets (Boitard et al., 2012; Rabasa et al., 2016; Teegarden et al., 2009).

7.2.2 Effect of WD on RMP

While both orexin and MCH neurons experienced WD-induced depolarization, this followed distinct time courses. Orexin neurons were depolarized only after 1 week of WD (Fig. 7.1A), while MCH neurons became depolarized by 4 weeks and this further increased by 11 weeks of WD feeding (Fig. 5.3F). While the mechanism underlying the depolarization of orexin neurons was not investigated, it is likely distinct from the mechanism of MCH neuron depolarization since these two cell populations were activated at different time points (Fig.5.2B). Other possible factors that could mediate depolarization of orexin neurons during high-fat diet feeding include: cholecystokinin, glucagon-like peptide 1, leptin, and ghrelin (Acuna-Goycolea & van den Pol, 2004; Sheng, Santiago, Thomas, & Routh, 2014; Tsujino et al., 2005; Yamanaka et al., 2003). Alternatively, the depolarization of orexin neurons at 1 week of WD feeding could be secondary to the observed enhanced glutamatergic transmission, which could significantly contribute to basal firing rate and membrane potential (Li et al., 2002).

7.2.3 Effect WD of synaptic transmission

WD-induced synaptic plasticity of orexin neurons was transient and dynamic throughout the course of feeding. Distinct changes were noted at 1, 4, and 11 weeks of feeding on both excitatory and inhibitory transmission (Table 7.1). However, this was not

the case for MCH neurons. MCH neurons had delayed plasticity of excitatory transmission that was stable over time (Chapter 6). Therefore, the two key differences between these neurons are the transient versus stable nature of plasticity, and the modulation of inhibitory transmission in orexin but not MCH neurons.

High-fat diet feeding results in many metabolic and hormonal changes, such as an increase in the satiety hormone leptin and a decrease in the hunger hormone ghrelin (Handjieva-Darlenska & Boyadjieva, 2009). Interestingly, both leptin and ghrelin have been implicated in synaptic plasticity within the hypothalamus (Cristino et al., 2013; Pinto et al., 2004; Yang, Atasoy, Su, & Sternson, 2011). However, it is unknown whether these hormones are involved in the plasticity described in this thesis or how they would specifically affect orexin or MCH neurons at distinct time periods. Such mechanisms may require both pre- and postsynaptic signaling components to selectively increase relevant input-postsynaptic target connections (Holtmaat & Svoboda, 2009). Therefore, identifying the sources of afferents that underlie WD-induced increased synaptic transmission in both neurons could provide key insights to these underlying mechanisms and their specificity.

7.2.4 Adaptation versus sensitization to WD

Another key difference in the effects of WD in orexin and MCH neurons is that orexin neurons appear to adapt to WD while MCH neurons are progressively activated with longer periods on WD. The transient nature of orexin neuron plasticity indicates the possibility of negative feedback loops that regulate orexin neuron activation. On the other

hand, MCH neurons appear to be a part of a positive feedback loop – activation of MCH neurons promotes further WD intake and weight gain (Fig. 5.10), which leads to further depolarization of MCH neurons. This may be explained by a sensitization in the mechanism underlying the COX-dependent depolarization. In support of this idea, we found that the depolarizing mechanism sensitizes with repeated exposures to WD (Fig. 5.5).

Interestingly, this positive feedback loop or sensitization of the MCH system may occur at several levels. There is also an upregulation of MCH peptide and the MCH receptor mRNA during obesity (Elliott et al., 2004), suggesting that not only are MCH neurons more active (Chapter 5) and releasing more MCH (Elliott et al., 2004), but there is also a sensitization in their efferent targets.

7.2.5 Differences in physiological roles

The key differences discussed throughout this section can be summarized as the following: MCH neurons undergo a positive feedback loop of activation that has a delayed onset but is stable throughout WD feeding, while orexin neurons undergo a variety of transient changes that lean toward increased activation in early WD feeding but instead favour inhibition with prolonged feeding. It is possible that these distinct effects of WD may relate to the differences in the physiological roles of orexin and MCH neurons.

While both neurons promote food intake and specifically that of palatable diets, they do so in distinct ways. Orexin neurons respond not to caloric content but to the

rewarding nature of food (Alcaraz-Iborra et al., 2014). They recruit the mesolimbic reward pathway to mediate hedonic feeding (Valdivia et al., 2014) and as such are particularly involved in behavioral responses to food rewards, such as anticipation and seeking (Akiyama et al., 2004; Mieda et al., 2004). The transient nature of the activation of orexin neurons described in this thesis may reflect heightened reward processing early in WD diet when the stimulus is novel, which decreases with prolonged feeding. Similarly, prolonged activation of the mesolimbic circuitry in obesity leads to reward hypofunction (Johnson & Kenny, 2010).

Contrastingly, MCH neurons respond to the caloric content of food and promote the ongoing consumption of food (Della-Zuana et al., 2012; Morens et al., 2005) while reducing energy expenditure, thus promoting positive energy balance (Alon & Friedman, 2006; Segal-Lieberman et al., 2006). Persistent intrinsic and synaptic plasticity during chronic WD described in this thesis is consistent with their prominent role in long-term body weight regulation.

7.3 Other considerations

7.3.1 Interaction between orexin and MCH neurons

Orexin and MCH neurons have reciprocal connections with one another (Guan et al., 2002). Orexin neurons can have both excitatory and inhibitory effects on MCH neurons through their neuropeptides orexin A and orexin B or dynorphin and nociceptin/orphanin FQ, respectively (Apergis-Schoute et al., 2015; Li & van den Pol, 2006; Parsons & Hirasawa, 2011; van den Pol et al., 2004). On the other hand, the MCH

peptide is reported to inhibit orexin neurons by downregulating AMPA receptors and preventing orexin-induced increases in excitatory transmission in orexin neurons (Y. Rao et al., 2008).

Due to these interactions, the changes in excitability of orexin and MCH neurons during WD feeding may affect how they regulate one another. For instance, it is possible that some changes described in this thesis could occur at synapses connecting orexin and MCH neurons as they both express glutamate and GABA (Apergis-Schoute et al., 2015; Chee, Arrigoni, & Maratos-Flier, 2015; Jego et al., 2013; Schöne, Apergis-Schoute, Sakurai, Adamantidis, & Burdakov, 2014). Additionally, as it has been reported that orexin and MCH neurons fire in a reciprocal manner to one another, it is possible that they may inhibit the other when they are active (Hassani et al., 2009). If true, this may explain the different time courses of activation of orexin and MCH neurons during WD feeding. For example, orexin neurons are activated initially by WD whereas MCH neurons are activated with a later onset. Furthermore, the persistent activation of MCH neurons may underlie the inhibition of orexin neurons during prolonged high-fat feeding and obesity (Cai et al., 2000; Tanno et al., 2013). Therefore, some of the synaptic changes in the lateral hypothalamus could be regulated within local circuitry in a concerted manner.

7.3.2 Other potential functional consequences of plasticity in orexin and MCH neurons

Despite their functional differences, orexin and MCH neurons project to overlapping brain regions (Bittencourt et al., 1992; Peyron et al., 1998). Thus, these two

populations of neurons can independently and often oppositely regulate similar physiological functions. Briefly, orexin neurons are wake-promoting and activate the mesolimbic dopamine pathway (Adamantidis et al., 2007; Valdivia et al., 2014), while MCH neurons are sleep-promoting and may limit dopamine release in the NAcc (Konadhode et al., 2013; Pissios et al., 2008).

Therefore, it is highly likely that the changes induced by WD feeding could also affect these functions. High-fat diet has been linked to increased sleep (Jenkins et al., 2006) and reward hypofunction (Johnson & Kenny, 2010). It is tempting to speculate that the plasticity described in this thesis may have a role in these observations. The increase in inhibitory transmission in orexin neurons after prolonged WD may at least partly underlie the increased sleep and reward hypofunction. Moreover, the activation of MCH neurons with prolonged WD may promote sleep and anhedonia. Therefore, there may be broad functional consequences of the plasticity described in this thesis.

7.4 Future Directions

The work presented in this thesis has provided a strong foundation for future research. While we have electrophysiologically characterized the effect of high-fat diet on orexin and MCH neurons and made several key discoveries, many questions remain unanswered.

Concerning our work on orexin neurons, we have provided a detailed description of how high-fat diet affects their excitability and plasticity; however, how these changes summate to influence orexin neuron activity *in vivo* and its consequent physiological

significance is not fully understood. Future experiments could involve *in vivo* recording or immunohistochemistry for markers of neuronal activation to determine the activity of orexin neurons. Then, modulation of orexin neuron activity through either DREADDs, optogenetics, or siRNA against prepro-orexin mRNA could determine whether the physiological contributions of orexin neurons change over the time course of high-fat diet feeding. Moreover, future experiments should be designed to specifically address the role of LTD in orexin neurons. Intrahypothalamic injections of the antibiotic ceftraxione can increase the expression of GLT-1 and increase glutamate uptake. This treatment is able to modulate the expression of LTD in other brain regions (Omrani et al., 2009) and consummatory behaviours (P. S. S. Rao & Sari, 2014). If this treatment can successfully be applied to orexin neurons, the physiological effects of LTD on food intake and weight gain can be assessed *in vivo*.

For MCH neurons, the next step would be further elucidating the mechanism and ramifications of WD-induced depolarization. First, the receptors that mediate the effect of PGE_2 on the NKA should be identified through application of EP receptor antagonists and agonists. Then the intracellular signaling pathway and its effect on NKA function can be determined through molecular and electrophysiological techniques. These initial findings should provide the foundation to determine the mechanism by which MCH neurons are sensitized with multiple WD exposures. Secondly, the experiments in this thesis should be repeated in older animals and a comparison should be made to determine whether there is a long-term effect attributed to an adolescent vulnerability in the MCH system to the

effects of high-fat diet, since we found a short-term interaction between the effect of WD on 4-week and 7-week old animals on synaptic transmission.

Finally, the work in the thesis suggests that MCH and orexin neurons may be activated at separate times during the course of high-fat diet feeding. This is in line with other work suggesting that these neurons may negatively regulate one another (Hassani et al., 2009), a possibility discussed in greater length in section 7.3.1. Therefore, an important future direction would address how changes in the orexin system influence the MCH system during high-fat diet feeding and vice versa. This could be achieved by modulating the activity of one system at both baseline and during high-fat diet feeding and studying how the other responds.

7.5 Conclusions

WD induces substantial plasticity within orexin and MCH neurons of the lateral hypothalamus. Considering the role of these neurons in high-fat diet intake and energy homeostasis, it is likely that these changes contribute to the overconsumption of high-fat diet and the etiology of obesity. The work presented in this thesis informs on the intrinsic and synaptic response of orexin and MCH neurons, which was limited in the literature. Moreover, this work may provide a new understanding of how the lateral hypothalamus contributes to metabolic and homeostatic dysfunction caused by high-fat diet and provide new therapeutic targets for obesity.

Length of WD Feeding	Age	RMP	EPSC		mEPSC		mIPSC	
			PPR	LTD?	Amp.	Freq.	Amp.	Freq.
1 day	*3-4 weeks*					---		
1 week	*4 weeks*	↑	↑	**YES**	↑	---	---	---
1 week	*7 weeks*		---		↑	---		
4 weeks	*7 weeks*	---	---	**NO**	---	↑	---	↑
11 weeks	*14 weeks*	---	---		---	---	↑	---

Table 7.1: Summary of the effects of WD feeding on orexin neurons.

Green arrows indicate changes that increase excitability of the postsynaptic orexin neuron, while red arrows indicate a decrease in excitability. Dashes signify no change in the parameter and finally, shaded cells indicate no data is available.

Length of WD Feeding	Age	RMP	Threshold	mEPSC		mIPSC	
				Amp.	Freq.	Amp.	Freq.
1 week	*4 weeks*	---	↑	↓	↓	---	---
1 week	*7 weeks*	---	↓	---	---		
4 weeks	*7 weeks*	↑	↓	---	↑	---	---
11 weeks	*14 weeks*	↑	---	---	↑	---	---

Table 7.2: Summary of the effects of WD feeding on MCH neurons.

Green arrows indicate changes that increase excitability of the postsynaptic MCH neuron, while red arrows indicate a decrease in excitability. Dashes signify no change in the parameter and finally, shaded cells indicate no data is available.

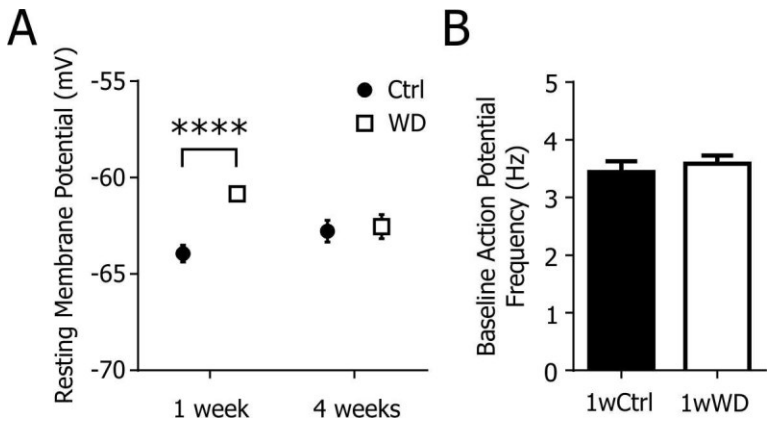

Figure 7.1: Orexin neurons are transiently depolarized by short-term WD feeding.

(A) RMP of orexin neurons from rats fed WD for 1 and 4 weeks and their age-matched chow-fed controls. (B) Baseline action potential frequency of orexin neurons from the 1-week WD condition.
*Two-way ANOVA with post hoc comparisons: ****p<0.0001*

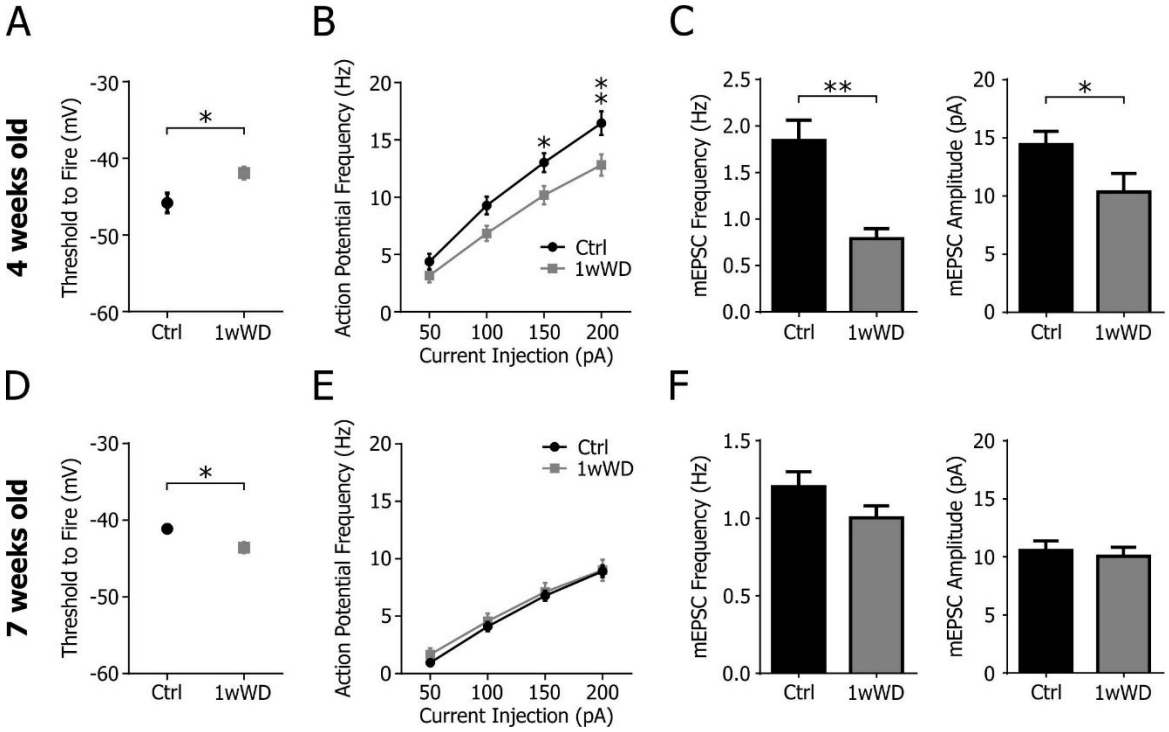

Figure 7.2: Age-dependent effects of 1 week of WD on MCH neurons.

4-week old rats: (A) The threshold to fire is higher in 1wWD and (B) the firing frequency during current injections is lower in the 1wWD condition. (C) WD also reduces excitatory transmission (this data was also reported in the text of Chapter 6). 7-week old rats: (D) Threshold to fire is lower in 1wWD but (E) there is no effect of 1wWD on firing frequency (data is also reported in Chapter 5), or (F) excitatory transmission (this data was also reported in Chapter 6).

*Unpaired t-test and two-way RM ANOVA (B,E): *p<0.05, **p<0.01*

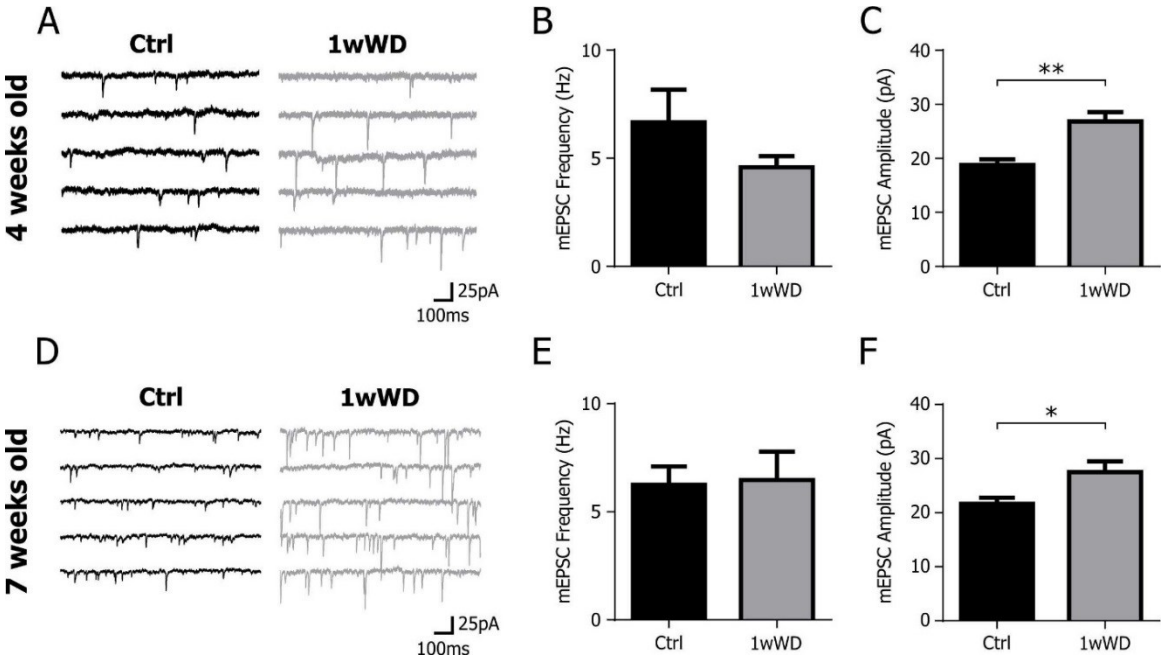

Figure 7.3: 1 week of WD feeding increases mEPSC amplitude in both 4- and 7-week old rats.

4-week old (this data is also reported in Chapter 4): (A) Sample traces of mEPSCs from 1 week of WD (1wWD) or chow controls (Ctrl). (B) mEPSC frequency and (C) amplitude. 7-week old (D) Sample traces of mEPSCs from 1wWD or Ctrl. The frequency (E) and amplitude (F) of mEPSCs.
*Unpaired t-test: *p<0.05, **p<0.01*

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