

THE EFFECT OF FEEDING STATE ON NEURON COMMUNICATION IN THE  
DORSOMEDIAL HYPOTHALAMUS OF FEMALE RATS

BY

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A thesis submitted to the  
Department of Biology  
Mount Allison University  
in partial fulfillment of the requirements for the  
Bachelor of Biology degree with Honours

April 20, 2023

**Abstract**

The dorsomedial hypothalamus (DMH) is a major brain region involved in regulating food intake, body weight, and metabolism. Neurons in the DMH communicate with one another through classical neurotransmitters, primarily glutamate (excitatory) and GABA (inhibitory), as well as non-classical neurotransmitters including endocannabinoids. Endocannabinoids are molecules released from postsynaptic cells that can regulate the release of glutamate and GABA from presynaptic cells by activating type I cannabinoid receptors. Prior research in male rats has found that endocannabinoid signalling at GABA synapses are affected by feeding state. However, little is known about DMH glutamate synapses, and nothing is known in female rats. Therefore, the main objectives of this study are namely: 1) to determine how endocannabinoids affect glutamate signaling in the DMH of naïve female rats, 2) to examine basal synaptic transmission and neuronal excitability in naïve and fasted female rats, and 3) to determine how fasting affects endocannabinoid signaling in the DMH of female rats. To examine the effect of feeding state on DMH neurons, we used young female Sprague-Dawley rats separated into two groups: naïve (no manipulation) and fasted (food deprived for 24-hours). Patch-clamp electrophysiology was used to examine glutamate signaling and neuron excitability in the DMH of naïve and fasted female animals. We observed no difference in basal glutamate transmission in the DMH of naïve and fasted rats. There was also no difference in action potential firing between naïve and fasted female rats. This suggests that basal DMH neuron communication in female rats is not affected by feeding state. We also observed no difference in glutamate release following high frequency stimulation-induced endogenous endocannabinoid release in naïve and fasted animals. A decrease in glutamate release was observed in both naïve and fasted female rats with the application of an exogenous cannabinoid receptor agonist (WIN 55,212-2), suggesting that activation of glutamate transmission of DMH neurons in both feeding states are affected by the application of exogenous endocannabinoids. To the best of our knowledge, this study is the first to focus solely on female rats and the findings indicate that feeding state does not impact DMH glutamate transmission in female animals.

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**List of Abbreviations**Abbreviation:

CCK

DMH

GABA

TCA

VGLUTs

NMDA

AMPA

EPSCs

sEPSCs

eCBs

2-AG

CB

GPCRs

LTP

LTD

HFS

WIN

PPR

Definition:

Cholecystokinin

Dorsomedial hypothalamus

Gamma-aminobutyric acid

Tricarboxylic cycle

Vesicle glutamate transporters

*N*-methyl-D-aspartate $\alpha$ -amino-3-hydroxy-5-methyl-4-  
isoazolepropionic acid

Excitatory postsynaptic currents

Spontaneous excitatory postsynaptic  
currents

Endocannabinoids

2-arachidonoyl glycerol

Cannabinoid receptor

G-protein coupled receptors

Long-term potentiation

Long-term depression

High-frequency stimulation

WIN 55, 212-2

Paired pulse ratio

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## **Chapter 1: Introduction**

### **1.1: Childhood Obesity**

Obesity is a growing concern affecting children, with one in three Canadian children aged six to 17 years identified as overweight or obese (Rao et al., 2016). Childhood obesity is associated with detrimental comorbidities including an increased risk of developing type 2 diabetes mellitus, obstructive sleep apnea, hypertension, and obesity in adulthood (Kumar and Kelly, 2017). Several genetic conditions can contribute to the risk of obesity by often resulting in early-onset obesity (Kumar and Kelly, 2017). Multiple lifestyle factors can also influence the risk for childhood obesity as psychological difficulties, household dietary patterns, and stressful life events can give rise to adverse eating habits (Kumar and Kelly, 2017). Thus, obesity commonly arises from an imbalance between food intake and physical activity, meaning that energy input is greater than energy expenditure (Meister, 2007). In particular, excess consumption of high-caloric food increases the likelihood of developing obesity (Lau et al., 2021). Because hormonal changes associated with obesity can make it very challenging to achieve a normal body weight, therapeutic interventions that target neuronal appetite regulation could help to prevent adverse repercussions of obesity (Fildes et al., 2015; Kühnen et al., 2021).

### **1.2: Neuronal Appetite Regulation**

Food intake is an essential process for survival that is regulated by the integration of hunger and satiety signals in the brain (Delgado, 2013). Energy acquired through food can either be utilized instantly in metabolic processes or accumulated as fat for later metabolic use based on energetic demands (Ahima and Antwi, 2008). Pathways from both the peripheral and central nervous systems interact to control food intake. Signals originating from the gastrointestinal tract, adipose tissue, and pancreas can be relayed to the brain via sensory information, nutrients, or hormones (Stanley et al., 2005). These peripheral signals include insulin, cholecystokinin (CCK), ghrelin, and leptin (Stanley et al., 2005). The concentrations of CCK, ghrelin, and nutrients, along with messages from chemoreceptors and mechanoreceptors in the gut, are some factors involved in the short-term regulation of food intake (Havel, 2001). On the other hand, long-term regulation of food intake is mediated by insulin and leptin that act in the brain to control energy balance and ultimately determine adipose tissue mass and overall body weight (Havel, 2001).

In the central nervous system, the brain plays a critical role in regulating food intake through several circuits that receive peripheral signals (Farr et al., 2016). Multiple factors can affect the communication in the brain to control food intake including reward, sensory information, emotion, attention, cognition, and homeostasis (Farr et al., 2016). In homeostatic appetite regulation, the brain receives peripheral signals from sensory nerves and gut hormones that modulate appetite, such as CCK which invokes satiety (Ahima and Antwi, 2008). The brainstem and hypothalamus are both essential brain regions involved in appetite regulation (Ahima and Antwi, 2008). The hypothalamus is a major site of action for many peripheral signals to regulate energy homeostasis.

### **1.3: The Hypothalamus**

The hypothalamus is a brain structure that plays a major role in maintaining homeostasis within the body (Saper and Lowell, 2014). It is the link between the nervous system and the endocrine system through its connection with the pituitary gland (Hiller-Sturmhöfel and Bartke, 1998). The hypothalamus is involved in regulating energy homeostasis, thermoregulation, circadian rhythms, sleep, stress, and reproduction. Overall, the hypothalamus is vital in integrating information from the body to control functions necessary for survival (Saper and Lowell, 2014). The hypothalamus is a part of the forebrain located below the thalamus in each cerebral hemisphere and is a bilateral brain region separated by the third ventricle (Biran et al., 2015; Saper and Lowell, 2014). Three major regions comprise the hypothalamus, namely the preoptic region, tuberal region, and mammillary complex, which are all further divided into different nuclei (Saper and Lowell, 2014; Meister, 2007).

### **1.4: The Dorsomedial Hypothalamus (DMH)**

The dorsomedial hypothalamus (DMH) is a collection of multipolar neurons in the hypothalamus that is situated along the walls of the third ventricle (Brasil et al., 2020; Ter Horst and Luiten, 1986). It is involved in regulating physiological processes including stress, thermogenesis, circadian rhythms, body weight, and food intake (DiMicco et al., 2002; Zaretskaia et al., 2002; Chou et al., 2003; Bellinger and Bernadis, 2002). The DMH is located within the tuberal region of the hypothalamus and is positioned near the paraventricular, ventromedial, arcuate, and supraoptic nuclei, as well as the lateral hypothalamic area and the fornix (Brasil et al., 2020; Saper and Lowell, 2014).

To conduct its functions, the dorsomedial hypothalamus is connected with other brain regions beyond the hypothalamus including the brainstem and the cerebrum (Thompson et al., 1996). The primary input into the DMH arises from structures within the hypothalamus, particularly the arcuate nucleus and ventromedial nucleus (Ter Horst and Luiten, 1987). The DMH also receives intrahypothalamic projections from the lateral hypothalamic area, paraventricular nucleus, arcuate nucleus, and anterior hypothalamic area (Ter Horst and Luiten, 1987). Besides the hypothalamus, the DMH further receives projections from neurons in the cerebrum and the brainstem (Thompson and Swanson, 1998). The main regions in the hypothalamus that receive extensive projections from the DMH include the lateral hypothalamic area, ventromedial nucleus, and paraventricular nucleus (Thompson et al., 1996; Ter Horst et al., 1984).

### **1.5: The DMH and Appetite Regulation**

The role of the dorsomedial hypothalamus (DMH) in appetite has been investigated using both lesioning and electrical studies (Bellinger and Bernadis, 2002). Early electrolytic lesioning studies in rats revealed that lesions to the DMH resulted in hypophagia (decreased food intake), hypodipsia (lack of thirst), and a reduction in body weight and linear growth compared to control rats (Bernadis et al., 1963). However, Bernardis (1970) found that lesions to the DMH did not cause a change in fat stores as the rats had a normal fat and lean body mass percentage in comparison with control rats. These early findings led to the classification of the DMH as the appetite-regulatory area of the brain (Bernadis, 1970; Jeong et al., 2017).

The DMH is connected to various regions involved in appetite regulation. The arcuate nucleus of the hypothalamus is considered to first sense the peripheral metabolic signals involved in hunger by acting as an integration center between circulating hormones and other brain regions including the DMH (Suyama and Yada, 2018; Roh et al., 2016; Meister, 2007). The arcuate nucleus influences appetite through two main types of neurons namely neuropeptide Y neurons and pro-opiomelanocortin neurons, that stimulate and suppress appetite respectively (Timper and Brüning, 2017). Fasting activates the neuropeptide Y neurons in both hypothalamic nuclei (Bi et al., 2003). The DMH and the lateral hypothalamus are considered areas involved in hunger (Suyama and Yada, 2018). The ventromedial hypothalamus is involved in satiety as lesions to this area result in hyperphagia (Suyama and Yada, 2018; Yadav et al., 2009). The

paraventricular nucleus is involved in controlling food intake through providing autonomic output to the brainstem (Ahima and Antwi, 2008).

Several recent studies have demonstrated the involvement of the DMH in food intake. Jeong et al. (2017) demonstrated that cholinergic neurons in the DMH stimulate food intake. These cholinergic neurons receive both GABAergic and glutamatergic input with overnight fasting decreasing GABAergic synaptic transmission onto cholinergic DMH neurons while increasing the excitability of these neurons (Groessl et al., 2013). Otgon-Uul et al. (2016) also showed that optical stimulation of DMH GABA neurons with leptin receptors increased food intake in rats. Further, Renner et al. (2010) showed through immunohistochemical methods that the DMH neurons are activated in fasted male rats that are refed. Thus, neurons in the DMH are involved in appetite regulation.

### **1.6: Synaptic Transmission**

Neurons in the brain communicate with one another through a combination of electrical and chemical signals. The key chemicals that permit the communication between neurons within the brain are termed neurotransmitters (Pereda, 2014). Neurotransmitters are typically released from a presynaptic axon terminal into the synaptic cleft between neurons and bind to ionotropic and metabotropic receptors on a postsynaptic membrane, usually on the dendrites (Hyman, 2005). Ionotropic receptors are classified as ligand-gated ion channels as they possess a ligand binding domain to which a neurotransmitter can bind leading to the opening of a channel for the passage of ions (Purves et al., 2001). In contrast, metabotropic receptors are classified as G-protein coupled receptors (GPCRs) (Kew and Kemp, 2005). Once a neurotransmitter binds to a receptor, it can typically open an ion channel that allows ions to flow across the membrane. When positively charged ions, such as sodium ( $\text{Na}^+$ ) enters the neuron, it causes an excitatory postsynaptic potential (Hyman, 2005). If the summation of the inputs surpasses the threshold of the neuron, an action potential is initiated and subsequently propagated along the axon of the neuron to the axon terminal (Stuart et al., 1997). This permits the influx of calcium through voltage-gated calcium channels in the axon terminal membrane leading to the release of neurotransmitter vesicles, which allows for further communication with subsequent neurons (Südhof, 2012). In contrast, if the binding of neurotransmitters triggers the influx of negatively charged ions, the neuron hyperpolarizes and action potentials are inhibited (Hyman, 2005).

Glutamate and gamma-aminobutyric acid (GABA) are two major neurotransmitters involved in neuronal communication within the brain (Bak et al., 2006). Following synthesis, glutamate and GABA can both bind to ionotropic or metabotropic receptors to typically excite or inhibit neurons, respectively. Both glutamate and GABA are involved in neuron communication in the DMH and can ultimately affect various functions in the DMH, including food intake (Meister, 2007).

### **1.7: Glutamate**

Glutamate is an amino acid that is considered the main excitatory neurotransmitter in the central nervous system (Zhou and Danbolt, 2014). This neurotransmitter is produced by an intermediate in the tricarboxylic acid (TCA) cycle called  $\alpha$ -ketoglutarate (Bak et al., 2006; Schousboe et al., 2013). Glutamate can additionally be produced through the glutamate-glutamine cycle in which glutamate is transported into astrocytes where it is converted to glutamine by an enzyme called glutamine synthetase (Bak et al., 2006). Glutamine is subsequently moved into neurons where phosphate-activated glutaminase converts the glutamine back to glutamate (Bak et al., 2006; Schousboe et al., 2013). Vesicle glutamate transporters (VGLUTs) located within the membrane of synaptic vesicles result in the movement of glutamate into vesicles for storage (Andersen et al., 2021). The vesicles are found in multiple neuronal structures including dendrites, axon terminals, neuron cell bodies, and glial cells (Danbolt, 2001). Vesicles are stored in an area of the presynaptic neuron called the active zone (Sheng and Hoogenraad, 2007). As a result of exocytosis, glutamate is released from vesicles into the synaptic cleft, resulting in a greater glutamate concentration within the synapse where it can bind to glutamate receptors (Andersen et al., 2021; Danbolt, 2001).

Glutamate can bind to ionotropic and metabotropic receptors that are clustered in an area called the postsynaptic density on postsynaptic neurons (Kew and Kemp, 2005; Sheng and Hoogenraad, 2007). Ionotropic glutamate receptors include NMDA, AMPA, and kainate receptors. The three receptors are termed according to the agonist that activates them namely, *N*-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) (Kew and Kemp, 2005). Glutamate can also bind to one GPCR family that consists of eight receptors divided into three groups based on the similarity of their sequences and their signaling (Kew and Kemp, 2005).

Prolonged activation of NMDA and AMPA receptors can cause long-lasting changes in synaptic strength, in which the synapses either undergo long-term potentiation or depression of neurotransmitter signaling (Sheng and Hoogernraad, 2007). When glutamate binds to receptors on the postsynaptic membrane, it results in excitatory postsynaptic currents (EPSCs) (Song et al., 2015). Excitatory postsynaptic currents (EPSCs) can occur due to an upregulation of postsynaptic glutamate receptors or through greater glutamate release from the presynaptic neuron. The release and subsequent signaling of glutamate can be controlled by another class of neurotransmitters called endogenous cannabinoids (Song et al., 2015).

### **1.8: Endocannabinoids**

Endocannabinoids are endogenous lipid molecules that play a key role in central nervous system communication (Lu and Mackie, 2016). Two major endocannabinoid molecules (eCBs) are anandamide and 2-arachidonoyl glycerol (2-AG). eCBs are found throughout the body, with 2-AG being more abundant in the brain than anandamide (Hillard, 2018; Stella et al., 1997). The eCB molecules are synthesized on demand and unlike neurotransmitters, are not produced prior to release and stored in vesicles (Mackie, 2008). Generally, both eCBs are produced through the cleavage of phospholipid precursors in cell membranes (Freund et al., 2003). 2-AG is synthesized from cells when diacylglycerol lipase converts phospholipase-C to 2-acylglycerols, which includes 2-AG (Hillard, 2018). Anandamide is synthesized from a phospholipid called N-acyl-phosphatidylethanolamine catalyzed by phospholipase-D (Hillard, 2018; Freund et al., 2003).

Endocannabinoids are released from postsynaptic neurons in response to a rise in intracellular calcium and/or activation of Gq/11-linked GPCRs and typically bind to cannabinoid (CB) receptors in the membrane of presynaptic neurons, with both eCBs considered CB receptor agonists (Lu and Mackie, 2016; Hillard, 2018). As eCBs usually act on receptors in presynaptic membranes, they perform their function in a retrograde manner (Song et al., 2015). The cannabinoid receptors are classified as GPCRs and are separated into two main types: CB1 and CB2 (Howlett et al., 2002). CB1 receptors are the primary binding site for eCBs, although they can also bind to CB2 receptors based on their efficacy and location. The receptor location varies, with CB1 receptors located on presynaptic terminals within the brain and peripheral tissue, whereas CB2 receptors are primarily found in the immune tissue (Howlett et al., 2002; Regehr et al., 2009). 2-AG has a high efficacy for cannabinoid receptors, while anandamide is a partial

agonist with low intrinsic efficacy for cannabinoid receptors (Mackie, 2008; Luk et al., 2004). Endocannabinoid release can be inhibited by nitric oxide (Kyriakatos and El Manira, 2007).

CB1 receptors are expressed in several brain regions that regulate food intake, including the brainstem, corticolimbic system, and hypothalamus (Mailleux and Vanderhaeghen, 1992). When eCBs are released from postsynaptic neurons and activate presynaptic CB1 receptors, they reduce the probability of neurotransmitters release resulting in long-term depression (Regehr et al., 2009; Heifets and Castillo, 2009). This long-term depression has been demonstrated through the application of cannabinoid agonists or by delivering high frequency stimulation, which triggers endogenous cannabinoid release (Gerdeman et al., 2002; Stella et al., 1997). Endocannabinoids are further involved in food intake as hypothalamic endocannabinoid levels vary based on feeding state (Kirkham et al., 2002). Thus, synaptic transmission involving endocannabinoids and glutamate can be involved in the neuronal control of food intake.

### **1.9: Current Study**

Previous studies have shown that the DMH is involved in regulating food intake (Jeong et al., 2017; Otgon-Uul et al., 2016; Renner et al., 2010). Endocannabinoids modulate DMH neuron communication and can influence appetite (McGavin et al., 2019; Kirkham et al., 2002). Research in male rats has demonstrated that neuron communication at GABA synapses in the DMH is affected by hunger (induced by food deprivation) (Crosby et al., 2011). Although the previous research has identified the importance of the DMH in regulating food intake, studies have mainly focused on GABA synapses and have used male rats. Therefore, nothing is known about glutamate signalling at DMH neurons in female rats. Thus, the current study aims to examine the neuronal communication and the effect of feeding state on DMH neurons in female rats. This study has three objectives namely: 1) to determine how endocannabinoids affect glutamate signaling in the DMH of naïve female rats, 2) to examine basal synaptic transmission and neuronal excitability in naïve and fasted female rats, and 3) to determine how fasting affects endocannabinoid signaling in the DMH of female rats. The feeding states consisted of naïve (no manipulation) and fasted (restricted from food for 24 hours). Whole-cell patch clamp electrophysiology recordings were conducted to assess glutamatergic synaptic transmission and action potentials. The findings of this study could ultimately contribute to the understanding of neuronal appetite regulation in the DMH and potentially aid in developing efficient interventions for childhood obesity.

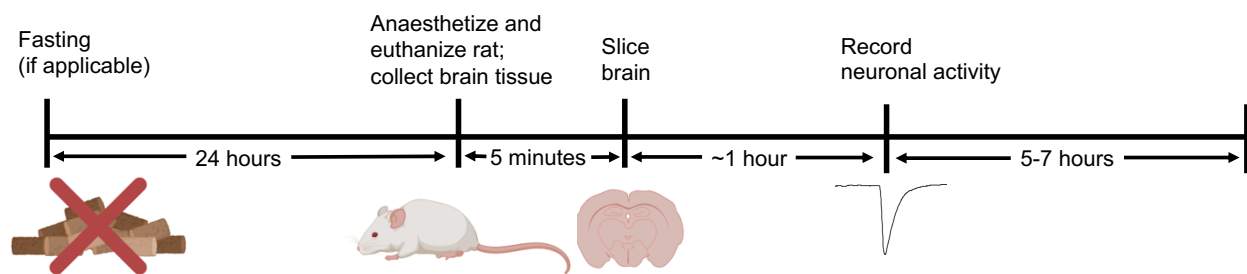
## Chapter 2: Methods

### 2.1: Experimental animals

All experiments were performed according to protocols approved by the Mount Allison University Animal Care Committee (protocol number 103088) in accordance with the Canadian Council on Animal Care Guidelines. Female Sprague-Dawley rats were obtained from Charles River Laboratories (Montreal, QC, Canada). The rats were 22-23 days postnatal upon arrival, were housed in groups of two to four, and were never alone in a cage for more than 24 hours. All rats acclimated to the environment for a minimum of 4 days before any experiments were performed. The rats were housed in clear plastic cages (43cm x 21cm x 20.5cm) in a room with the temperature maintained at 22°C, 50% ± 10% humidity, and a 12-hour light/dark cycle (7:30 am lights on, 7:30 pm lights off). Within the cages, the rats had a wooden block and plastic bone for environmental enrichment, along with shredded paper towel, wood shavings, and two plastic balls for bedding. Rats had *ad-libitum* access to food (regular rat chow) and reverse osmosis water, unless otherwise stated.

### 2.2: Experimental overview

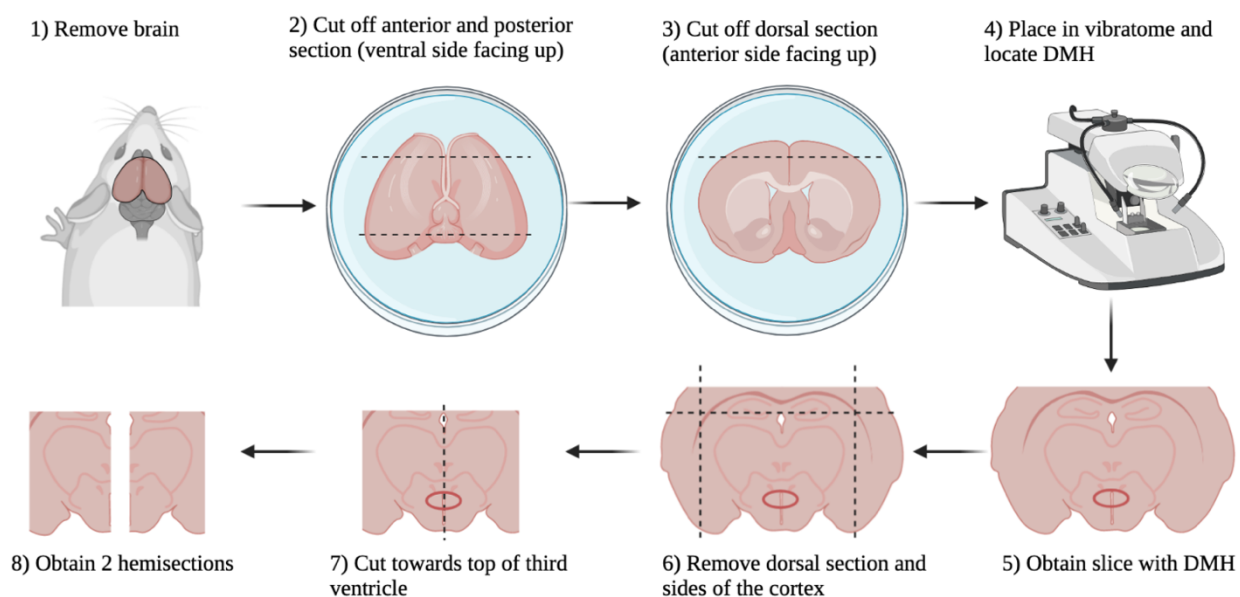
The female rats (~27-40 days postnatal) were separated into two treatments: 1) naïve and 2) fasted. The naïve group received no manipulation and were presumably satiated as they had *ad libitum* access to food, eat during the dark cycle, and tissue collection was conducted in the morning. The rats in the fasted group were deprived from food for 24 hours but still always had access to water. Twenty-four hours of food deprivation has been shown to reduce body weight and induce hunger (Dietze et al., 2016). The fasted rats were weighed prior to and following 24 hours of food deprivation. The rats were subsequently anaesthetized and euthanized to collect brain tissue. The rats were subsequently anaesthetized and euthanized to collect brain tissue to record neuronal activity (Figure 2.1).



**Figure 2.1** Schematic representation of the experimental protocol in chronological order. Images (except the current trace) were obtained from BioRender.

### 2.3: Tissue collection and preparation

Between 8:00am and 10:00am, individual rats were removed from their cage and promptly placed into a plastic induction chamber filled with isoflurane. The plastic chamber was filled with 5% isoflurane in oxygen approximately five minutes prior to placing the animal in the chamber. Rats were exposed to the combination of gaseous and liquid isoflurane until they were deeply anesthetized (confirmed by pinching their paw to look for reflexes). Rats were subsequently euthanized through decapitation with a guillotine. The brain was removed and placed in ice-cold slicing solution containing (in mM): 75 sucrose, 25 glucose, 87 NaCl, 25 NaHCO<sub>3</sub>, 2.5 KCl, 0.5 CaCl<sub>2</sub> \* 2H<sub>2</sub>O, 7 MgCl<sub>2</sub> \* 6H<sub>2</sub>O, and 1.25 NaH<sub>2</sub>PO<sub>4</sub>, oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The brain was submerged in the solution for approximately five minutes. Thereafter, the brain was removed from the solution (ventral side facing up), placed in a petri dish, and portions from the anterior and posterior brain were cut off using a razor blade. The brain was turned with the anterior side facing up, and the dorsal part of the cortex was cut off (Figure 2.2). These cuts are beneficial as it allows for faster location of the hypothalamus while slicing the brain. The brain was then positioned and glued using Krazy Glue with the anterior side facing up onto a small plate, with a small square piece of agar glued beside the ventral side of the brain to prevent the brain from moving during slicing. Using a vibrating blade microtome slicer (VT1000S; Leica Microsystems, Ontario, Canada), the brain was sliced into 250 µm coronal sections while submerged in the ice-cold slicing solution continuously bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. To locate the DMH on the brain slices, the optic chiasm and lateral ventricles were used as landmarks (Paxinos and Watson, 2007). Slices with the DMH were divided into two hemisections by removing a horizontal dorsal section of tissue, the sides of the cortex, and cutting towards the top of the third ventricle (dorsal to ventral) with a scalpel (Figure 2.2).



**Figure 2.2** Schematic of brain slicing to illustrate how the brain and slices containing the DMH are obtained. The dashed lines represent the cuts made with either a razor blade or scalpel and the circle represents the approximate location of the DMH region. Schematic created in BioRender.

These individual slices were subsequently placed on a screen with plastic dividers secured in a 250 ml beaker filled with oxygenated artificial cerebrospinal fluid (aCSF) that consists of (in mM): 10 glucose, 126 NaCl, 26 NaHCO<sub>3</sub>, 2.5 KCl, 2.5 CaCl<sub>2</sub>\*2H<sub>2</sub>O, 1.5 MgCl<sub>2</sub>\*6H<sub>2</sub>O, and 1.25 NaH<sub>2</sub>PO<sub>4</sub>. The slices incubated in the beaker for a minimum of one hour prior to data collection. The beaker was placed in a PolyScience water bath filled with deionized water kept at 32.5°C, and the aCSF in the beaker was continuously bubbled prior to and during experiments with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

#### 2.4: Patch-clamp electrophysiology

After incubating the slices for a minimum of one hour in aCSF, a slice was transferred with a glass pipette into a recording chamber and weighed down with two ~1cm metal weights. The recording chamber that contained the slice was perfused with an aCSF and picrotoxin (GABA<sub>A</sub> receptor antagonist, 50µm) solution at a flow rate of ~1 ml/min and continually oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>). The temperature was kept at 32.5°C through an in-line heater attached to a TC-344C dual temperature controller (Warner Instrument Corporation,

Hamden, CT). An Olympus BX51 upright microscope (Olympus, Canada) equipped with an Infinity 2 camera (Lumenera, Ontario, Canada) allowed for the visualization of DMH neurons.

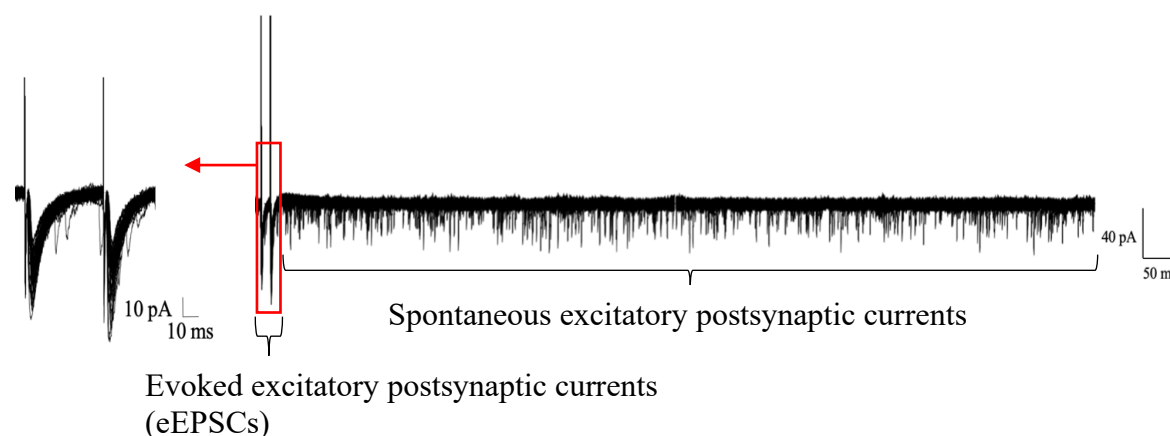
The DMH region was identified under a 40x objective by locating the top of the third ventricle, distinguishable by the appearance of ciliated ependymal cells. Once the DMH was identified, the stimulating and recording electrodes were positioned near the slice. Electrodes were composed of silver wires attached to headstages (CV-7B; Axon Instruments) and their movement was controlled by a multi-manipulator controller (ROE-200, MPC-200; Sutter Instruments, CA, USA). Borosilicate glass micropipettes were pulled with a P-2000 micropipette puller (Sutter Instruments, CA, USA) to surround the silver wire to access cells for recording and stimulation. The silver wire of the stimulating electrode was enclosed by a glass micropipette filled with aCSF, whereas the silver wire of the recording electrode was immersed in an intracellular solution to allow for whole cell electrophysiological recordings. The intracellular solution contained (in mM): 108 potassium gluconate, 8 KCl, 8 sodium gluconate, 1 EGTA, 10 HEPES, 2 MgCl<sub>2</sub>, 4 K<sub>2</sub>ATP, and 0.3 N<sub>3</sub>GTP, which was adjusted to a pH of 7.2 with KOH and an osmolarity of 290 mOsm with deionized water. Household bleach (Clorox) was used to chloride both silver wires to ensure that the electrodes were electrochemically stable and had low impedance (Hunt et al., 2019).

Once the electrodes were positioned near the slice, the tip of the recording electrode was indented on the membrane of the cell of interest. Positive pressure was released to establish a seal between the cell and glass pipette. Suction was subsequently used to break through the cell membrane to conduct whole-cell recordings. The cell was considered for recording once the recording electrode reached a giga-ohm (GΩ) seal on the membrane, and an access resistance (R<sub>a</sub>) between 1.0 and 2.5 in whole-cell configuration. The electrophysiological recordings obtained from the attached cell were amplified by a Multiclamp 700B amplifier (Molecular Devices, CA, USA), filtered at 1kHz, and digitized at 10kHz with a Digidata 1550B digitizer (Molecular Devices, CA, USA). The recorded data was subsequently stored for later analysis.

After a cell was successfully patched, the recording of current clamp steps was conducted while injecting current into the cell to hold it at -70mV. Current clamp mode recorded the action potentials of the neuron by hyperpolarizing and depolarizing the neuron in ten consecutive steps. The protocol in current clamp starts by hyperpolarizing the membrane potential to -100mV by injecting current for 500ms. Nine more 500ms steps are conducted by raising the membrane

potential by -10mV each step, resulting in the last current step recorded at -10mV. Data from the action potential recordings were used to assess the excitability of the DMH neurons.

Subsequently, voltage-clamp mode was used to examine glutamatergic synaptic transmission through spontaneous and evoked excitatory post synaptic currents (sEPSCs and eEPSCs respectively) (Figure 2.3). To produce evoked excitatory currents, the stimulating electrode was positioned in the extracellular space surrounding the target cell. The stimulating electrode stimulates surrounding neurons to release neurotransmitters, which results in currents in the recorded target cell. The stimulating electrode stimulates two postsynaptic currents 50 ms apart every five seconds at 0.2 Hz (Figure 2.3). The amplitude of the resulting postsynaptic currents allows for synaptic transmission to be assessed. The division of the amplitude of the second evoked current by the amplitude of the first evoked current allowed for the calculation of the paired-pulse ratio (PPR), which can indicate the probability of neurotransmitter release and allow for the assessment of synaptic transmission as the PPR is associated with the presynaptic probability of release (Glasgow et al., 2019). The PPR and probability of release is inversely related as a high PPR indicates a lower probability of presynaptic neurotransmitter release, and a low PPR indicates an increased probability of release (Linders et al., 2022). An increase in PPR can indicate weak synapses, whereas a decrease in PPR can suggest synaptic potentiation (Linders et al., 2022). The spontaneous currents (sEPSCs) can provide information on the overall synaptic activity with no stimulation of neurotransmitter release (Glasgow et al., 2019). Changes in the frequency of spontaneous currents can be affected by both presynaptic and postsynaptic mechanisms (Glasgow et al., 2019), whereas changes in the amplitude of spontaneous currents is indicative of postsynaptic changes (Melis et al., 2004).



**Figure 2.3.** Recording showing evoked excitatory postsynaptic currents (eEPSCs) and spontaneous excitatory postsynaptic currents (sEPSCs). The eEPSCs were stimulated twice every five seconds 50 ms apart at 0.2 Hz with a stimulating electrode and recorded from the target cell using a recording electrode. The amplitude of the evoked currents can be used to assess synaptic transmission of neurons. The sEPSCs were currents that occurred with no stimulation.

Evoked excitatory postsynaptic currents (eEPSCs) were recorded for five-minutes to establish a baseline recording. Subsequently, a high-frequency stimulation protocol (HFS; 100 Hz for 4 sec, repeated twice, 20 sec apart) was applied to stimulate surrounding axons and currents were recorded for 25 minutes. HFS has been shown to induce endogenous endocannabinoid release, and this protocol has been used to investigate synaptic transmission at GABA synapses in the DMH (Stella et al., 1997; Crosby et al., 2011). The baseline evoked current amplitude can be compared to the current amplitude after HFS to assess whether feeding states cause changes in synaptic strength through synaptic potentiation or depression at glutamate synapses. Every five minutes, the condition of the cell was examined by filtering at 10Hz and assessing the capacitance ( $C_m$ ), membrane resistance ( $R_m$ ), access resistance ( $R_a$ ), time constant ( $\tau$ ), and the holding current, visible in the membrane test. Capacitance is used to monitor endocytosis and exocytosis, and to normalize whole cell currents (Gillis, 1995). The time constant ( $\tau$ ) indicates the total conductance and is derived from the membrane potential (Berg and Ditlevsen, 2013). Access resistance indicates the quality of access to the cell. Holding current is the amount of current needed to hold the cell at -70 mV.

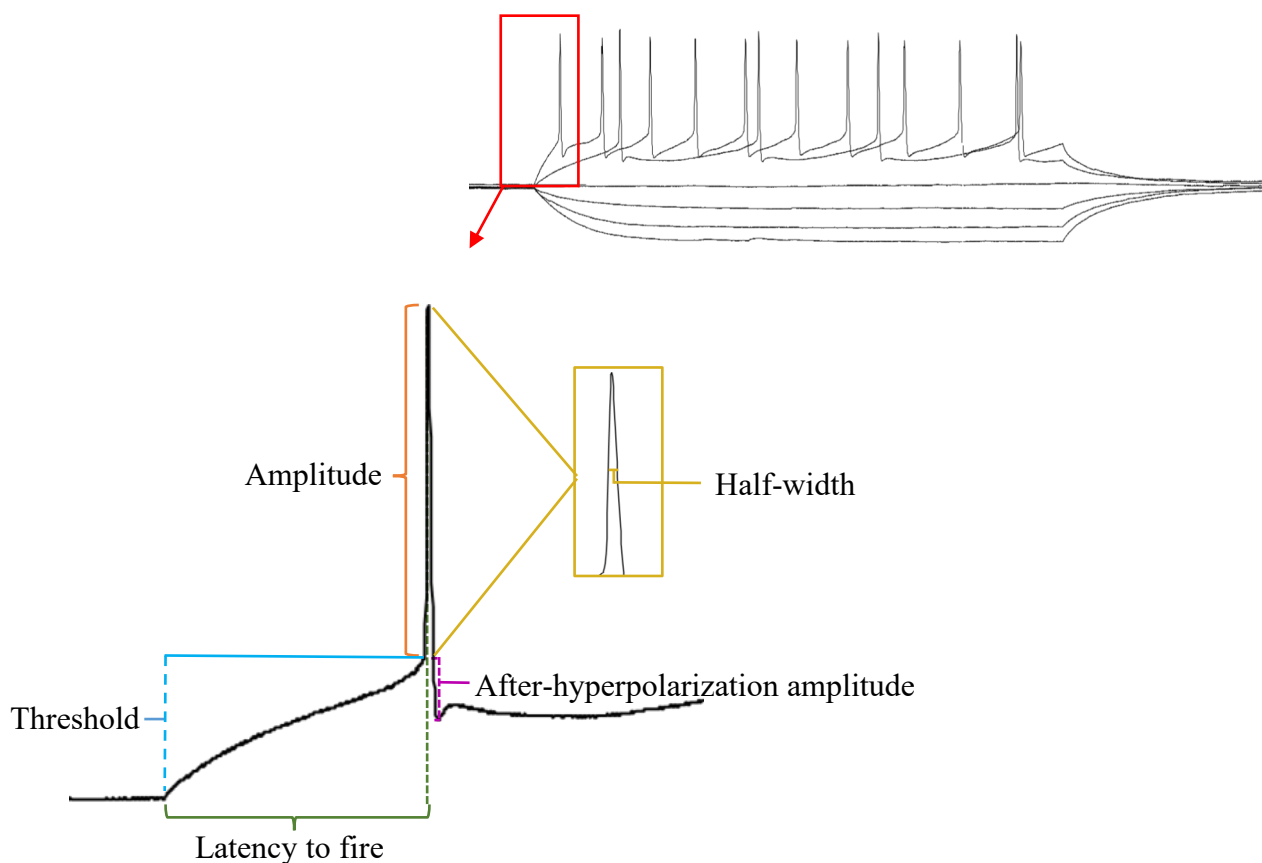
To assess the effect of an exogenous cannabinoid agonist on DMH neurons, a five-minute baseline recording was collected, followed by the application of WIN 55,212-2 (5 $\mu$ m; dissolved in DMSO) added in the aCSF and picrotoxin solution. After the application of WIN 55,212-2, currents were recorded for 25 minutes, and the cell condition was assessed every five minutes in the membrane test. For all experiments, only one cell was recorded per slice and there were generally one to three cells recorded per animal.

## **2.5: Electrophysiological measurements**

Current clamp mode was used to assess action potentials fired by the neurons in the dorsomedial hypothalamus (DMH). First, the number of action potentials fired at each step were counted. To assess the number of action potentials, only action potentials fired at current steps 4, 6, 8, and 10 were examined in the analysis (correlated with depolarization of -50mV, -30mV, and -10mV respectively). Clampfit 10 software (Molecular Devices, CA, USA) was subsequently used to analyze the properties of the first action potential in each recording, which is outlined in Figure 2.4. The action potential threshold was first measured using the first derivative method, where the membrane potential is measured when the derivative is greater than 10mV/ms (Platkiewicz and Brette, 2010). The first derivative of the current trace with the first action potential was created in Clampfit and the threshold was defined as the membrane potential where the derivative was equal to or greater than 10mV/ms. The amplitude of the action potential is the change in membrane potential that occurs during the action potential and is measured from the threshold to the most positive peak (Nagaeva et al., 2020). The after-hyperpolarization amplitude represents the difference between the threshold and the most negative membrane potential reached during the hyperpolarization after the action potential. The half-width is the time between the depolarization and hyperpolarization, assessed at the halfway point of the action potential amplitude (from the threshold to the peak). Lastly, latency to fire represents the time from the start of the current step to the peak of the action potential (Nagaeva et al., 2020).

The glutamatergic neuron communication was examined through the evoked excitatory postsynaptic currents (eEPSCs) in the recorded cells. Clampfit was used to measure the amplitude of the evoked currents by assessing the change in current amplitude from prior to stimulation by the stimulating electrode to the peak of the evoked current after stimulation. The spontaneous currents were analyzed in Mini Analysis software to obtain their frequency and

amplitude. The traces shown with the graphs have the stimulus artifact digitally removed for the five-minute baseline and the 15-to-20-minute period into the recording.



**Figure 2.4** Schematic of action potential properties recorded in Clampfit to examine neuronal excitability. All analysis was conducted on the first action potential in a 10-step recording starting at -100mV to -10mV. The threshold was determined from the first derivative of the action potential. The action potential amplitude, half-width, and amplitude of the after-hyperpolarization was determined using the threshold. The latency to fire was measured from the onset of action potential to its peak.

## 2.5: Statistical analysis

The difference in rat weight prior to and after the 24-hour fasting period was analyzed using a paired t-test. Next, the amplitude of the first evoked excitatory postsynaptic current (eEPSC) was averaged for each individual cell during its 5-minute baseline recording. To investigate the baseline glutamatergic transmission in the DMH neurons before any manipulation, we used a two-sample t-test to compare the average baseline evoked amplitude between naïve and fasted animals. The baseline paired pulse ratio (PPR) was also averaged for the individual cells and examined using a two-sample t-test. Furthermore, the baseline amplitude and frequency of the spontaneous currents (sEPSCs) of naïve and fasted animals were each

analyzed using a two-sample t-test. If the data did not pass the assumptions of normality and homogeneity of variance, it was log transformed prior to analysis.

Endogenous endocannabinoid release was examined using the high-frequency stimulation (HFS) protocol. The average baseline evoked current amplitude was compared with the current amplitude after HFS. A paired t-test was used to compare the average baseline amplitude with the average amplitude post-HFS (15-to-20 minutes into the recording). The 15-to-20-minute period is used as it is the middle of the recording and prevents any impact of a short-term potentiation or a potentiation/depression at the end of the recording. The effect of HFS on PPR was also examined by conducting a paired t-test with the average baseline PPR and the PPR post-HFS (15-to-20 minutes into the recording). The application of an exogenous cannabinoid agonist (WIN 55, 212-2) was examined by conducting a paired t-test comparing the average baseline current amplitude with the average current amplitude following the application of WIN 55,212-2 (15-to-20 minutes into the recording). The effect of WIN 55,212-2 on PPR was also examined by conducting a paired t-test with the average baseline PPR and the PPR post-WIN application (15-to-20 minutes into the recording).

To examine neuron excitability, the number of action potentials at each current step was counted. A linear mixed model was used to compare the number of action potentials fired at approximate depolarizations of -50mV, -30mV, and -10mV (corresponding to current clamp 6, 8, and 10) in the naïve and fasted animals. When conducting the analysis, the cell was the random subject, the feeding state (naïve or fasted) was the between-subjects factor, the current step was the within-subject factor, and the number of action potentials was the dependent variable. The data did not pass the assumption of normality due to autocorrelation, leading to the inclusion of the CorAR1 function in the linear mixed model to fix the autocorrelation in the data. Two-sample t-tests were conducted to compare the number of action potentials between the groups at each step. Two-sample t-tests were conducted to compare the action potential properties of threshold, peak amplitude, after-hyperpolarization amplitude, half-width, and latency to fire between the naïve and fasted animals. If the assumptions of normality and homogeneity of variance were not passed, the data was log transformed. If the log transformation did not work and the data still did not pass the assumptions, a Wilcoxon test (non-parametric) was performed. Data were considered significant if  $p < 0.05$ . All statistical analysis was conducted in R (version 4.2.2) with the R studio interface (version 2022.12.0+353). All graphs were created in Prism.

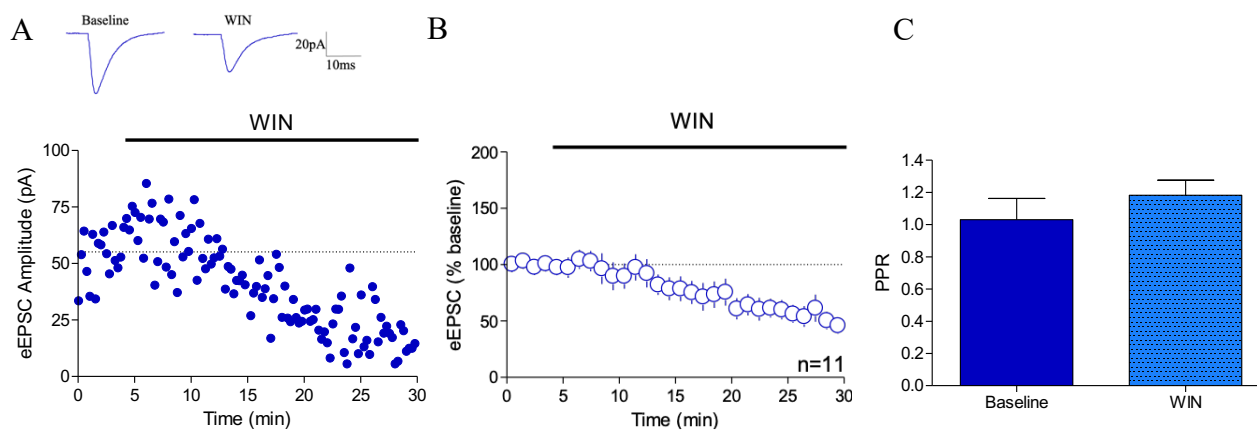
## Chapter 3: Results

The dorsomedial hypothalamus (DMH) is a brain region that plays an essential role in the regulation of food intake (Bernardis et al., 1963), but the underlying mechanisms are not completely understood. Neurons in the DMH receive extensive glutamate and GABA inputs from other hypothalamic and extra-hypothalamic sites, and they in turn project to the paraventricular nucleus and other brain regions to send signals related to hunger/satiety. In the DMH, there is accumulating evidence that endocannabinoids can be released from DMH neurons and subsequently bind to cannabinoid receptors on glutamate and GABA terminals to control their release. Most of this work, however, has been carried out at GABA synapses and in male rats (Crosby et al., 2011). To the best of our knowledge, nothing is known about glutamate synapses in the DMH of female rats.

### **3.1: Activation of cannabinoid receptors with WIN 55,212-2 decreases glutamate release in naïve female rats**

Endocannabinoids control the activity of DMH neurons through regulating neurotransmitter release. Therefore, we asked how the application of a synthetic cannabinoid receptor agonist (WIN 55,212-2) would affect the synaptic transmission of naïve female glutamate synapses in the DMH. Once the DMH was located in the brain slice, a target cell was indented and patched with a recording electrode, and a stimulating electrode was positioned in the nearby surrounding extracellular space to stimulate neurotransmitter release. When evoked currents from the target cell were located, a five-minute baseline recording was recorded. After recording a baseline of evoked excitatory postsynaptic currents (eEPSCs) for five minutes, a solution of WIN 55,212-2 (5  $\mu$ m), aCSF, and picrotoxin was perfused through the recording chamber containing the brain slice. WIN 55,212-2 caused a significant decrease in glutamate release in the naïve female rats ( $t = 3.1265$ ,  $p = 0.01075$ ,  $df = 10$ ) (Figure 3.1B). The average baseline amplitude in the naïve group was  $57.16 \pm 35.85$  pA and after 15-to-20-minutes with the application of WIN, the average amplitude was  $38.45 \pm 30.85$  pA, resulting in a difference variable (baseline – WIN) of  $18.72 \pm 19.86$  pA. We also examined the paired-pulse ratio (PPR) to see whether the probability of neurotransmitter release is affected by the application of WIN (Glasgow et al., 2019). PPR is obtained by calculating the ratio of the amplitude of the second evoked current to that of the first evoked current. The application of WIN did not alter the paired-pulse ratio (PPR) of the naïve rats ( $-0.17 \pm 0.32$ ,  $t = -1.3827$ ,  $p = 0.1969$ ) (Figure 3.1C).

Overall, these results indicate that the activation of cannabinoid receptors with WIN 55,212-2 decreases glutamate release in naïve female rats.

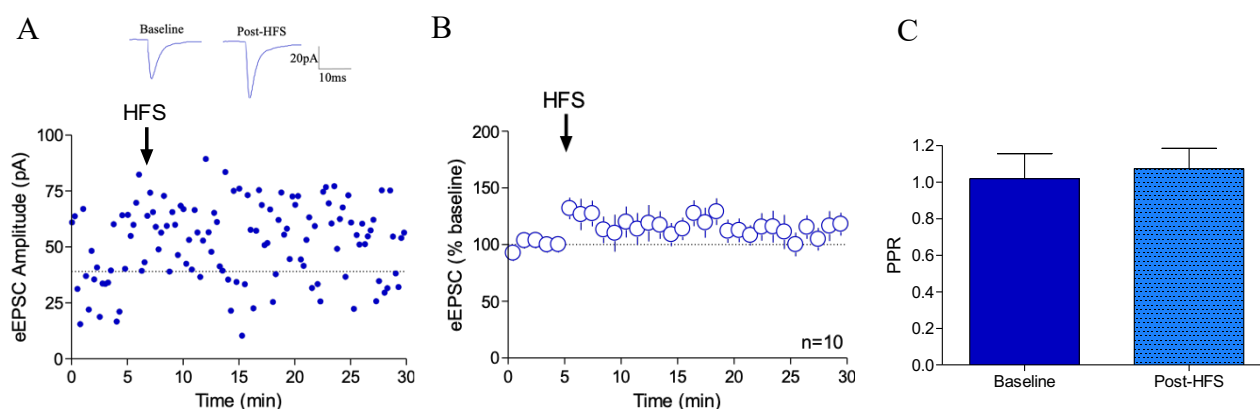


**Figure 3.1 WIN 55,212-2 decreases glutamate release onto DMH neurons in naïve female rats.** Evoked excitatory postsynaptic currents (eEPSCs) in the DMH of naïve female rats following the application of WIN 55,212-2 after a five-minute baseline recording. Application of WIN 55,212-2 is indicated by the line. (A) individual representative cell. (B) summary of all cells. (C) paired pulse ratio (PPR) for baseline and after the application of WIN 55,212-2. Traces represent the average current amplitude for the five-minute baseline recording, and 15-to-20 minutes into the recording of the individual cell.

### 3.2: Endogenous cannabinoid release through high frequency stimulation does not trigger a long-lasting decrease in glutamate release in naïve female rats

Since the cannabinoid agonist (WIN 55,212-2) caused a decrease in glutamate release, we were interested in how endogenous endocannabinoid release would influence glutamate synapses in the DMH in naïve female rats. A high frequency stimulation (HFS) protocol has previously been shown to release endogenous endocannabinoids from neurons in the brain, particularly within the DMH (Stella et al., 1997; Crosby et al. 2011). After recording a baseline of evoked excitatory postsynaptic currents (eEPSCs) for five minutes, a four second HFS protocol was applied twice at 100 Hz, produced 20 seconds apart. The effect of HFS on synaptic transmission was examined by comparing the amplitude of eEPSCs from the five-minute baseline to the amplitude of eEPSCs recorded during the 15-to-20-minute period (10 minutes after the application of HFS). The 15-to-20-minute period is used as it is the middle of the recording and prevents any impact of a short-term potentiation or a potentiation/depression at the end of the recording. High frequency stimulation did not cause a significant change in the amplitude of

eEPSCs in DMH neurons in naive female rats ( $t = 1.6247$ ,  $p = 0.1387$ ,  $df = 9$ ) (Figure 3.2B). The average baseline evoked current amplitude was  $41.15 \pm 5.82$  pA, and the average amplitude was  $47.21 \pm 5.83$  pA after the application of HFS (15-to-20 minutes into the recording). The difference variable (baseline – post-HFS) was  $6.05 \pm 3.73$  pA. HFS further did not affect the paired pulse ratio (PPR) of the neurons in naïve animals ( $-0.04 \pm 0.43$ ,  $t = -0.297$ ,  $p = 0.7732$ ) (Figure 3.2C). Four cells were removed from the analysis due to loss of access to the cell. Overall, endogenous cannabinoid release through high frequency stimulation does not trigger a long-lasting decrease in glutamate release in naïve female rats.

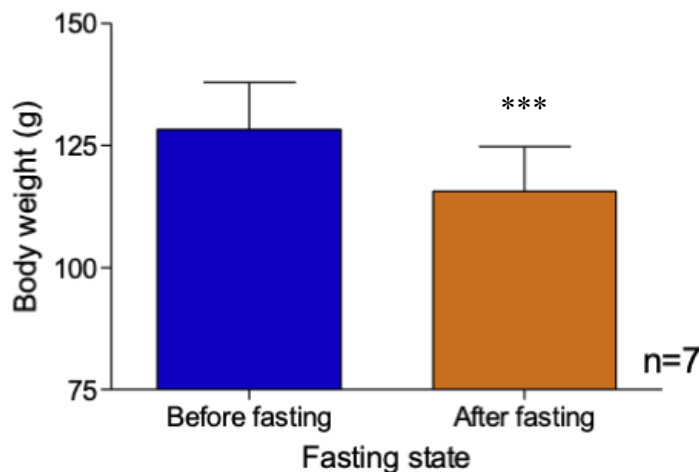


**Figure 3.2 High frequency stimulation (HFS) does not trigger changes at glutamate synapses in naive female rats.** Evoked excitatory postsynaptic currents (eEPSCs) in naive female rats following HFS (100Hz for 4 sec, repeated twice 20 sec apart). Application of HFS is indicated by the arrow. (A) individual representative cell. (B) summary of all cells. (C) paired pulse ratio (PPR) for baseline and post-HFS. Traces represent the average evoked current amplitude for the five-minute baseline recording, and 15-to-20 minutes into the recording of the individual cell.

### 3.3: A 24-hour fasting period decreases female rat body weight

As the dorsomedial hypothalamus (DMH) is involved in regulating food intake, we were interested in how the feeding state of female rats affects glutamate synaptic transmission in DMH neurons. Therefore, we conducted experiments on fasted rats, who were food deprived for 24 hours. A 24-hour fasting period is sufficient to induce body weight loss and hunger in rats (Dietze et al., 2016). Before and after 24 hours of fasting, the rats were weighed. Once the rats were weighed, they were promptly anaesthetized and euthanized for brain removal. The weight of the rats decreased significantly with a mean weight of  $128.26 \pm 9.63$  g prior to fasting and

115.63 ± 9.16 g after fasting ( $n = 7$ ,  $p = 0.000034034$ , Figure 3.3). The difference variable for the weight (before fasting - after fasting) was 12.63 ± 1.13g.

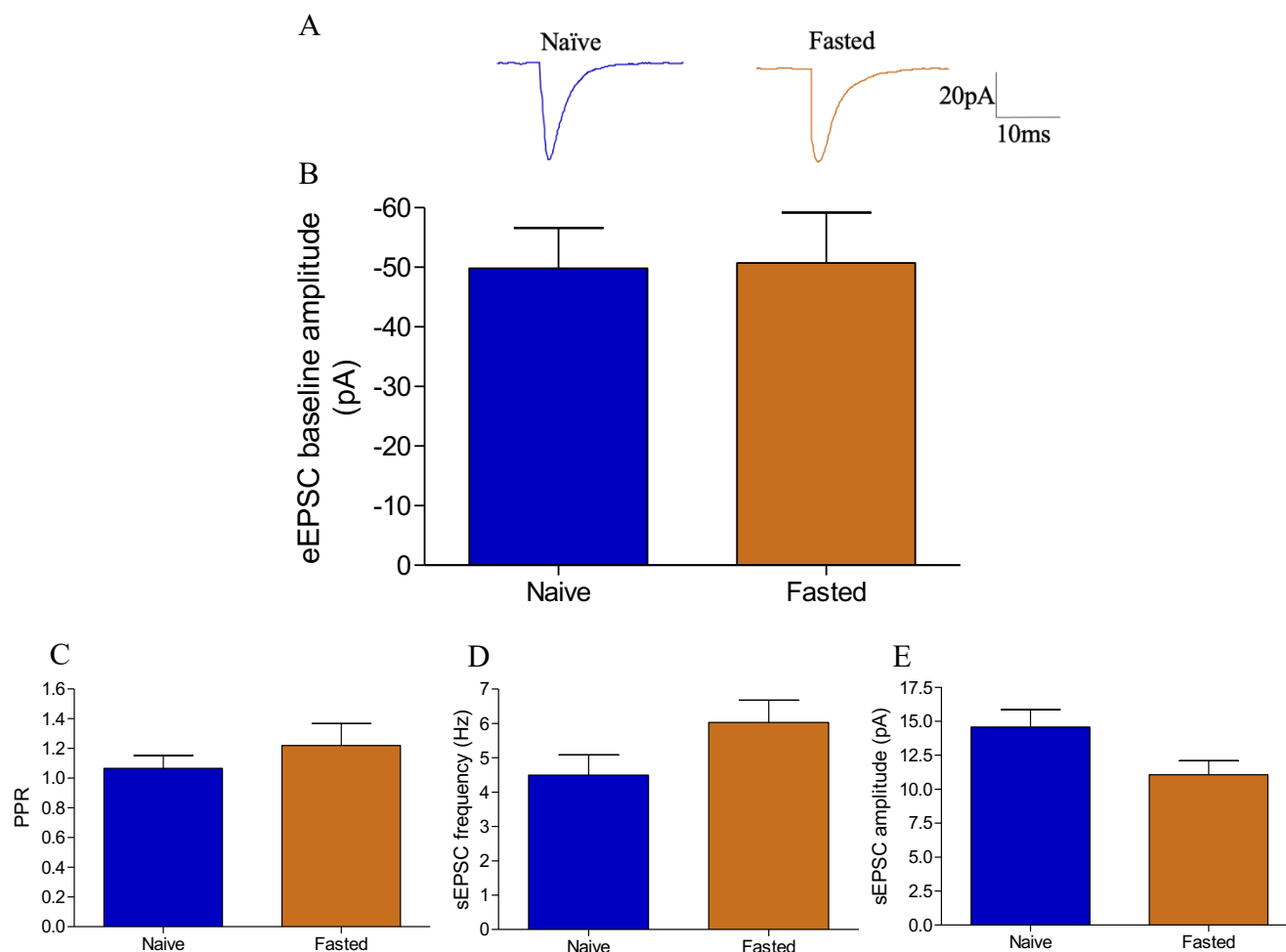


**Figure 3.3 A 24-hour fasting period significantly decreases female rat body weight.** The mean rat weight before and after fasting is represented by bars with the error bars representing the standard error of the mean. \*\*\*  $p < 0.001$ .

#### 3.4: Feeding state does not influence basal glutamate transmission in female rats

Next, we examined the basal synaptic transmission in the DMH of naïve and fasted female rats. When evoked currents from a target DMH cell were located in the brain slice, a five-minute baseline recording was obtained. This baseline recording can provide information on the basal synaptic transmission in DMH neurons. There was no significant difference in baseline evoked current amplitude between control and fasted rats ( $t = 0.1926$ ,  $p = 0.8486$ ,  $df = 29$ ). The average evoked current amplitude was  $49.78 \pm 30.11$  pA for the naïve group, and  $50.65 \pm 28.12$  for the fasted group (Figure 3.4B). We also examined the paired-pulse ratio (PPR) to see whether the probability of neurotransmitter release is affected by feeding state (Glasgow et al., 2019). There was no difference in baseline PPR in the DMH of naïve and fasted female rats (naïve:  $1.07 \pm 0.39$ ,  $n = 20$ ; fasted:  $1.22 \pm 0.49$ ,  $n = 11$ ;  $t = 9628$ ,  $p = 0.3436$ ) (Figure 3.4C). We further examined the basal spontaneous current activity of the DMH neurons to see whether the spontaneous activity (with no stimulation) differs. A difference in the frequency of spontaneous currents could indicate a change in the inputs to, or amount of neurotransmitter released on, DMH neurons. There was no significant difference in baseline spontaneous current frequency between the naïve and fasted female rats, with an average frequency of  $4.49 \pm 2.59$  Hz for the

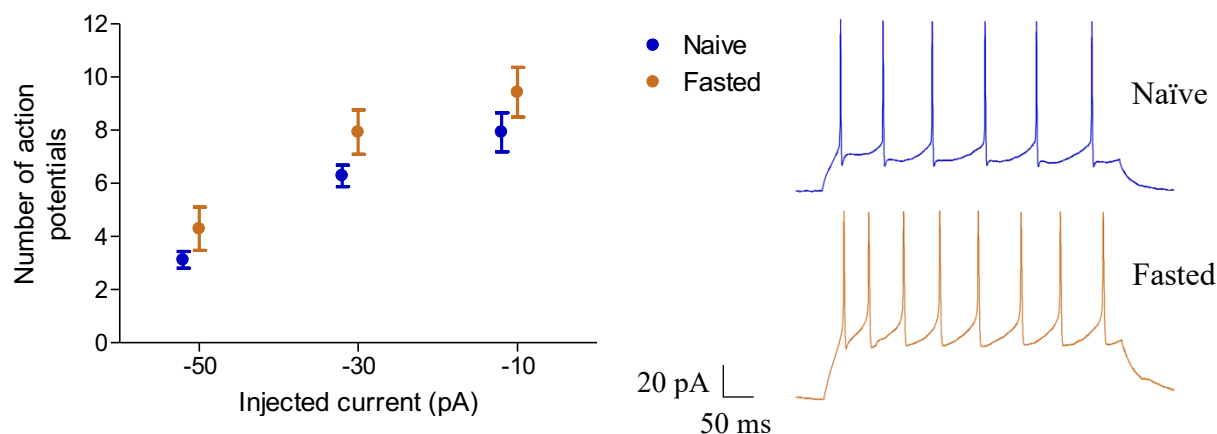
naïve group and  $6.03 \pm 2.15$  Hz for the fasted group ( $t = 1.662$ ,  $p = 0.1077$ ,  $df = 28$ ) (Figure 3.4D). There was also no significant difference in baseline spontaneous current amplitude between naïve and fasted female rats, with an average amplitude of  $14.57 \pm 5.58$  pA for the naïve group and  $11.06 \pm 3.43$  pA for the fasted group ( $t = -1.8831$ ,  $p = 0.07011$ ,  $df = 28$ ) (Figure 3.4E). One cell from the naïve group was excluded from the spontaneous current analysis due to noise in the recording as it resulted in difficulty distinguishing spontaneous currents from the noise.



**Figure 3.4 Basal glutamate transmission does not differ between naïve and fasted female rats.** Baseline data for naïve and fasted female rats. (A) sample traces representative of the average baseline eEPSC amplitude of an individual representative cell. (B) baseline eEPSC amplitude for naïve and fasted rats. (C) baseline paired-pulse ratio (PPR) for naïve and fasted rats. (D) baseline spontaneous current frequency. (E) baseline spontaneous current amplitude.

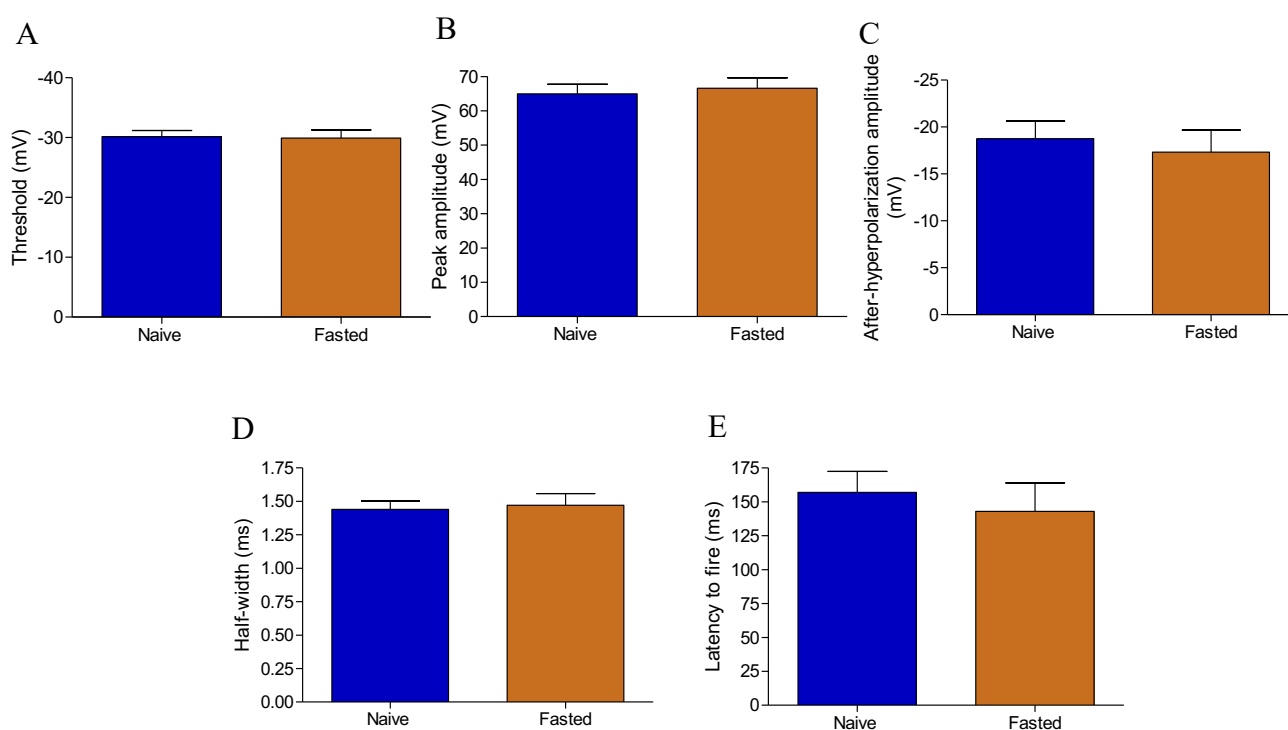
### 3.5: Feeding state does not alter neuronal excitability of DMH neurons in female rats

Action potentials can indicate the excitability of neurons within the brain. Activity of membrane voltage-gated sodium channels can influence the firing of action potentials, which influence neurotransmitter release for neuron communication (Hyman, 2005; Südhof, 2012). Action potentials are measured in current clamp mode and the number of action potentials can indicate the excitability of the DMH neurons based on their feeding state. Action potentials from neurons were measured by injecting current into the neuron to hold it at -70 mV while recording in Clampex. The neuron was then depolarized and hyperpolarized through injecting current in a series of 10 steps, each 500 ms, starting at approximately -100 mV (step 1) and ending at approximately -10 mV (step 10). We examined the number of action potentials in DMH neurons of naïve and fasted animals at depolarizations of -50 mV, -30 mV, and -10 mV (corresponding to current clamp steps 6, 8, and 10). A linear mixed model indicated that there is no significant difference in the number of action potentials across the feeding states ( $F_{1,37} = 3.746$ ,  $p = 0.0606$ ) and no significant interaction between the current step and the feeding state ( $F_{2,74} = 0.122$ ,  $p = 0.886$ ) (Figure 3.5). However, the number of action potentials across feeding state was approaching significance. There was additionally no significant difference in the number of action potentials between the feeding states at each step (step 6: naïve:  $3.12 \pm 1.56$ , fasted:  $4.29 \pm 3.05$ ,  $W = 209.5$ ,  $p = 0.3094$ ; step 8: naïve:  $6.28 \pm 2.03$ , fasted:  $7.92 \pm 3.12$ ,  $t = 1.9984$ ,  $p = 0.0531$ ; step 10: naïve:  $7.92 \pm 3.67$ , fasted:  $9.43 \pm 3.50$ ,  $t = 1.2505$ ,  $p = 0.219$ ). Although not of direct interest to this study, there was a significant difference in the number of action potentials fired between the three current steps (action potentials fired across the two conditions at step 6:  $3.54 \pm 2.25$ , step 8:  $6.87 \pm 2.57$ , and step 10:  $8.46 \pm 3.64$ ,  $F_{2,74} = 54.137$ ,  $p = 3.27 \times 10^{-15}$ ). We further examined intrinsic action potential properties that can indicate changes in neuron excitability based on feeding state. There was no significant difference found in threshold (naïve:  $-30.10 \pm 5.21$ ,  $n = 25$ ; fasted:  $-29.88 \pm 5.14$ ,  $n = 14$ ;  $t = 0.1304$ ,  $p = 0.897$ ), peak amplitude (naïve:  $64.94 \pm 14.09$ ,  $n = 25$ ; fasted:  $66.6 \pm 11.21$ ,  $n = 14$ ;  $W = 174$ ,  $p = 0.9885$ ), after-hyperpolarization amplitude (naïve:  $-18.73 \pm 9.43$ ,  $n = 25$ ; fasted:  $-17.30 \pm 8.77$ ,  $n = 14$ ;  $t = 0.46588$ ,  $p = 0.644$ ), half-width (naïve:  $1.44 \pm 0.32$ ,  $n = 25$ ; fasted:  $1.47 \pm 0.11$ ,  $n = 14$ ;  $t = 0.29697$ ,  $p = 0.7681$ ), and latency to fire (naïve:  $157.05 \pm 76.9$ ,  $n = 25$ ; fasted:  $143.03 \pm 78.14$ ,  $n = 14$ ;  $t = -0.714$ ,  $p = 0.4797$ ) in DMH neurons from naïve and fasted rats (Figure 3.6). Overall, these results indicate that feeding state (naïve or fasted) does not alter neuronal excitability.



**Figure 3.5 Feeding state does not impact DMH neuronal excitability in female rats.**

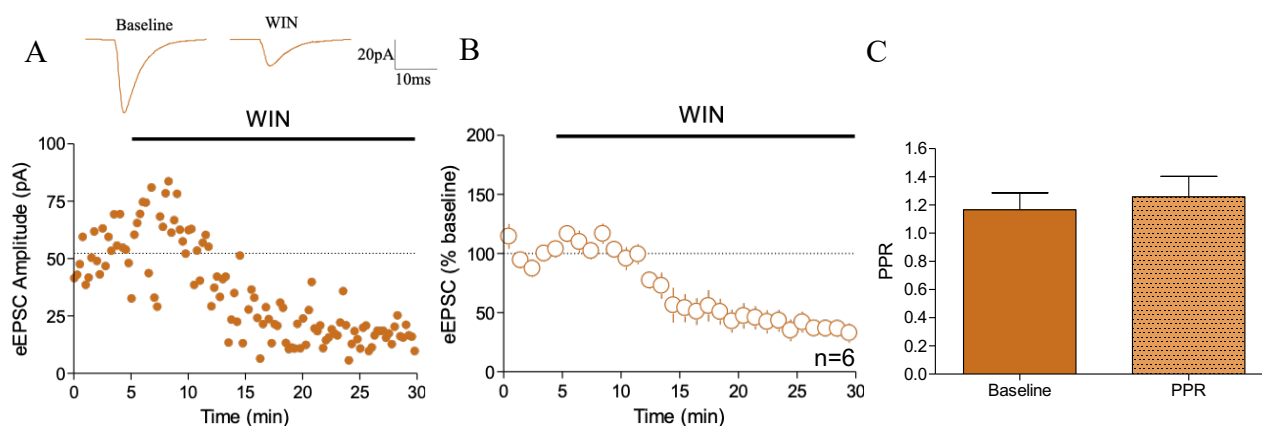
Number of action potentials in naïve and fasted female rats. Action potentials were recorded in current clamp mode while injecting the cell with current to hold it at  $-70\text{mV}$ . The action potentials of neurons were recorded by hyperpolarizing and depolarizing the neuron in ten consecutive steps from  $-100\text{mV}$  to  $-10\text{mV}$  (step 1 to step 10). The  $-50\text{mV}$ ,  $-30\text{mV}$ , and  $-10\text{mV}$  injected current is representative of step 6, 8, and 10 respectively. The representative action potential traces show step 8 ( $-30\text{mV}$ ) in the recording.



**Figure 3.6 Feeding state does not alter the properties of action potentials.** Properties of action potentials used to assess DMH neuronal excitability in naïve and fasted female rats. The results are representative of the first action potential fired in current clamp mode. There was no difference in threshold, peak amplitude, after-hyperpolarization amplitude, half-width, or latency to fire in the naïve and fasted rats.

### 3.6 Activation of DMH cannabinoid receptors through WIN 55,212-2 decreases glutamate release in fasted female animals

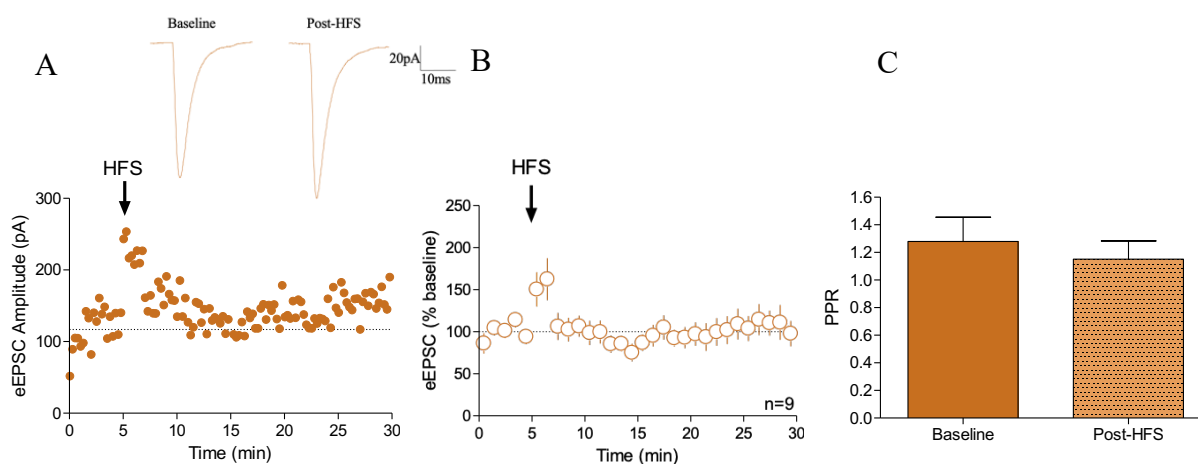
As the DMH is involved in regulating food intake and endocannabinoids are involved in appetite, we examined the effect of the cannabinoid agonist (WIN 55,212-2) in fasted female rats. After recording a baseline of evoked excitatory postsynaptic currents (eEPSCs) for five minutes, a solution of WIN 55,212-2 (5  $\mu$ m), aCSF, and picrotoxin was perfused through the recording chamber containing the brain slice. There was a significant reduction in glutamate release in fasted rats ( $t = -4.7055$ ,  $p = 0.00531$ ,  $df = 5$ ) (Figure 3.7B). The average baseline amplitude in the fasted group was  $74.75 \pm 29.96$  pA and after 15-to-20-minutes with the application of WIN, the average amplitude was  $48.64 \pm 25.28$  pA, resulting in a difference variable (baseline – WIN) of  $26.11 \pm 5.55$  pA. The application of WIN further did not alter the paired-pulse ratio (PPR) of fasted rats ( $-0.11 \pm 0.20$ ,  $t = -1.7738$ ,  $p = 0.1265$ ) (Figure 3.7C). One cell was removed from the analysis due to variation in access to the cell. Overall, these results indicate that the activation of cannabinoid receptors with WIN 55,212-2 decreases glutamate release in fasted female rats.



**Figure 3.7 WIN 55,212-2 decreases glutamate release onto DMH neurons in fasted female rats.** Evoked excitatory postsynaptic currents (eEPSCs) in the DMH of fasted female rats following the application of WIN 55,212-2 after a five-minute baseline recording. Application of WIN 55,212-2 is indicated by the line. (A) individual representative cell. (B) summary of all cells. (C) paired pulse ratio (PPR) for baseline and after the application of WIN. Traces represent the average current amplitude for the five-minute baseline recording, and 15-to-20 minutes into the recording of the individual cell.

### 3.7: Endogenous cannabinoid release through high frequency stimulation (HFS) does not trigger a long-lasting decrease in glutamate release in fasted female rats

The cannabinoid agonist, WIN 55,212-2, decreased glutamate release in the fasted female rats, therefore we were interested in how their DMH neurons would respond to endogenous endocannabinoids which can be released by HFS (Stella et al., 1997; Crosby et al, 2011). After recording a baseline of evoked excitatory postsynaptic currents (eEPSCs) for five minutes, a four second HFS protocol was applied twice at 100 Hz, produced 20 seconds apart. The effect of HFS was examined by comparing the amplitude of eEPSCs from the five-minute baseline to the amplitude of eEPSCs recorded during the 15-to-20-minute period of the recording. High frequency stimulation did not cause a significant change in the amplitude of eEPSCs in DMH neurons in fasted female rats ( $t = 0.4713$ ,  $p = 0.65$ ,  $df = 8$ ) (Figure 3.8B). The average baseline eEPSCs amplitude in the fasted rats was  $53.30 \pm 10.02$  pA, and the average eEPSCs amplitude following HFS (after 15 to 20 minutes) was  $50.54 \pm 11.40$  pA, with a difference variable (baseline – post-HFS) of  $2.76 \pm 5.86$  pA. HFS further did not affect the paired pulse ratio (PPR) of the neurons in fasted animals ( $0.051 \pm 0.38$ ,  $t = 0.4949$ ,  $p = 0.634$ ) (Figure 3.8C). One cell was removed from analysis due to a loss in access. Overall, endogenous cannabinoid release through HFS does not trigger a long-lasting decrease in glutamate release in fasted female rats.



**Figure 3.8 High frequency stimulation (HFS) does not trigger changes at glutamate synapses in fasted female rats.** Evoked excitatory postsynaptic currents (eEPSCs) in fasted female rats following HFS (100Hz for 4 sec, repeated twice 20 sec apart). Application of HFS is indicated by the arrow. (A) individual representative cell. (B) summary of all cells (C) paired pulse ratio (PPR) for baseline and post-HFS. Traces represent the average current amplitude for the five-minute baseline recording, and 15-to-20 minutes into the recording of the individual cell.

## Chapter 4: Discussion

The dorsomedial hypothalamus (DMH) is a brain area that is essential in regulating food intake and body weight. The synapses in the DMH, particularly GABA synapses, have been studied in male rats (Crosby et al., 2011), however, to our knowledge nothing is known in female rats. Therefore, the main objective of this study was to examine the effect of feeding state on DMH glutamate transmission and endocannabinoids in female rats. We used patch-clamp electrophysiology to examine glutamate transmission in the DMH of naïve (no manipulation) and fasted female rats. The female rats were fasted for a 24-hour period, which has previously been shown to trigger changes in synaptic transmission in male rats (Crosby et al., 2011). No difference was observed in the baseline glutamate transmission between the naïve and fasted rats, assessed through evoked currents, spontaneous currents, and paired-pulse ratio. Additionally, no difference in neuronal excitability was observed in the DMH neurons of naïve and fasted animals. The application of an exogenous cannabinoid receptor agonist (WIN 55,212-2) caused a significant depression in both naïve and fasted rats. Furthermore, endogenously released endocannabinoids through high frequency stimulation (HFS) did not cause any long-lasting changes in glutamate release in naïve or fasted female rats.

As the DMH is involved in regulating food intake and endocannabinoids are involved in appetite (Bernardis et al., 1963; Kirkham et al., 2002), we examined the application of a synthetic cannabinoid agonist (WIN 55,212-2) on DMH glutamate synapses of female rats. The CB1 receptor agonist caused a depression at DMH glutamate synapses in both naïve and fasted female rats. This result is consistent with glutamate synapses in naïve male rats as there is a significant depression observed after the application of WIN 55,212-2 (Sukkar, 2021), which show that the activation of CB1 receptors decreases glutamate release from presynaptic DMH neurons onto postsynaptic DMH neurons in female rats. Endocannabinoids have further been shown to influence glutamate release in other hypothalamic nuclei including the supraoptic nucleus and paraventricular nucleus (Di et al., 2005), as well as other regions in the brain such as the ventral tegmental area (Ostlund et al., 2023). At dopamine neurons in the ventral tegmental area, WIN 55,212-2 causes a depression of eEPSCs in both naïve and fasted female mice indicating that glutamate release is decreased (Godfrey and Borgland, 2020). At the dopamine neurons, a similar depression was observed in both feeding states following the application of WIN 55,212-2 indicating that feeding state did not influence the response to the cannabinoid

agonist (Godfrey and Borgland, 2020). Likewise, the results from the current study are also consistent with inhibitory transmission in the DMH as the application of WIN 55,212-2 caused a depression in evoked inhibitory postsynaptic currents at GABA synapses in naïve male rats (Crosby et al., 2011). However, in fasted male rats, WIN 55,212-2 does not affect the amplitude of evoked inhibitory currents or the paired pulse ratio of DMH GABA neurons (Crosby et al., 2011). Crosby et al. (2011) proposed that WIN 55,212-2 did not influence GABA synapses in fasted male rats as CB1 receptors were downregulated due to stress. We did not observe a similar finding in this study; in fasted female rats, WIN 55,212-2 caused a decrease in glutamate release, suggesting that there is no downregulation of CB1 receptors. WIN 55,212-2 further did not affect the paired pulse ratio of DMH neurons in naïve or fasted rats, suggesting the probability of neurotransmitter release is not altered by feeding state in the presence of endocannabinoids. This is contrary to what was expected with the observed decrease in glutamate release, as an increased PPR is associated with a decrease in neurotransmitter release probability. However, this lack in PPR change has been observed with WIN 55,212-2 in the DMH of male rats (Crosby et al., 2011).

As the exogenous cannabinoid agonist (WIN 55,212-2) decreased glutamate release in the DMH of naïve and fasted female rats, we were interested in the effect of endogenous endocannabinoids on DMH neurons in female rats. High-frequency stimulation (HFS) is a protocol that endogenously releases cannabinoids from the membranes of presynaptic neurons and has previously been shown to release endogenous endocannabinoids in the DMH (Stella et al., 1997; Crosby et al., 2011). When HFS was applied to DMH neurons in both naïve and fasted female rats, no significant change in current amplitude from baseline was observed in either group. The results collected from female rats partially agree with what has been demonstrated in male rats. The DMH neurons in naïve male rats have shown no change from baseline with the application of a HFS protocol (data collected by Tenea Welsh and included in Sukkar, 2021). However there appeared to be a slight potentiation following HFS in the naïve female rats in the current study. Even though there was no significant change, the slight potentiation above baseline could be due to a few cells that potentiated due to greater glutamate release. Sex differences in glutamate concentrations have been found within the rat brain, particularly within the hypothalamus where naïve females were found to have higher concentrations of glutamate in the medial preoptic area (Frankfurt et al., 1984). Outside of the hypothalamus, naïve females

have greater glutamatergic transmission within the medial prefrontal cortex evident through greater spontaneous excitatory current amplitude and frequency (Knouse et al., 2022). However, DMH neurons from fasted male rats exhibit a long-term potentiation (LTP) from baseline after the HFS protocol (unpublished data collected by Tenea Welsh). This is a possible sex difference as the fasted female rats show no change following HFS. This LTP in the fasted male rats could be due to nitric oxide inhibiting endocannabinoid release from presynaptic neurons which would allow for greater glutamate release onto the DMH neurons (Kyriakatos and El Manira et al., 2007). Furthermore, the paired-pulse ratio was not different from baseline following the HFS. As the evoked current amplitude after HFS did not differ from baseline, it is likely that the probability of glutamate release remained similar during baseline and after HFS (Glasgow et al., 2019).

Since the DMH is crucial in regulating food intake and body weight, we were interested in examining the change in body weight in female rats before and after fasting for 24-hours. This fasting protocol has previously been used in studies examining the effect of feeding state on DMH neuron communication in male rats (Crosby et al., 2011). In male rats, a 24-hour fasting period induced changes in synaptic transmission at GABA synapses in the DMH (Crosby et al., 2011). Food-deprivation for 24 hours or greater is considered to be chronic caloric restriction in rats (Nowland et al., 2011). In this study, there was a significant decrease in body weight when the female rats were fasted for a 24-hour period. This suggests that a 24-hour fasting period causes a decrease in body mass and is presumably sufficient to induce hunger in female rats. This coincides with Dietze et al. (2016) as they suggested that 24 hours of food-deprivation induces body weight loss and hunger in rats.

As nothing is known about basal conditions of DMH neurons in female rats, we wanted to establish whether there was a difference in baseline glutamate transmission in the DMH of naïve and fasted female rats. We assessed the basal activity by examining the evoked (stimulated) currents and spontaneous currents (occur without stimulation) in each group. There was no difference observed in the baseline glutamate synaptic transmission between the naïve and fasted female rats, as evident through their evoked and spontaneous excitatory synaptic activity as well as their paired-pulse ratio (PPR). As no significant change was observed in DMH spontaneous currents between rats in the naïve and fasted groups, we can conclude that the neurons do not vary in their overall basal synaptic activity (no stimulation of neurotransmitter

release). Overall, these findings suggest that fasting does not influence the basal evoked or spontaneous glutamate activity at DMH synapses in female rats. Similar results have been found at dopamine neurons in the ventral tegmental area with no difference in spontaneous current amplitude and frequency as well as PPR of eEPSCs between naïve and fasted female mice (Godfrey and Borgland, 2020).

The excitability of neurons can influence neurotransmitter release. Recordings of neuronal excitability further did not reveal any significant differences between naïve and fasted female rats, which suggests that feeding state does not alter neuronal excitability of DMH neurons. Glutamate input influences action potential firing; therefore, the similar action potential firing observed in naïve and fasted rats indicates that they are receiving similar glutamate input. Past studies examining the effect of feeding state on neuronal excitability have found that fasting mice for 24 hours causes an increase in the excitability of neuropeptide Y/Agouti-related protein neurons in the arcuate nucleus, which is another key nucleus in the hypothalamus involved in food intake (Takahashi and Cone, 2005).

A limitation to this study is that food-deprivation is considered a stressor. Nowland et al. (2011) found that a 24-hour food deprivation period significantly increased corticosterone levels in fasted male rats compared to naïve rats. As the DMH is involved in regulating stress, the recorded activity could be mediated by both stress and fasting (DiMicco et al., 2002). In addition, the study was conducted on young female rats, therefore the results might not be generalizable to female rats who have reached puberty, which is generally at 50 days postnatal (Sengupta, 2013). Females who have started their estrous cycle might have variations in their neuronal activity. Frankfurt et al. (1984) found that the glutamate and GABA concentrations differed in multiple hypothalamic nuclei based on the stage of the estrous cycle (proestrus, estrus, and diestrus). GABA levels in the DMH differed between the stages whereas glutamate levels were different in the lateral septum, diagonal band of Broca, and the anterior hypothalamic area (Frankfurt et al., 1984). Endocannabinoid levels in the hypothalamus can further differ based on the estrous cycle with 2-AG levels higher during diestrus than proestrus and metestrus (Bradshaw et al., 2006). Therefore, the results of the current study might not be applicable to rats who have reached their estrous cycle (Bradshaw et al., 2006).

Along with a 24-hour fasting period, other common fasting periods include 16 hours of fasting (short-term food-deprivation), and long-term fasting such as a 48-hour fasting period

(Nowland et al., 2011; Dietze et al., 2016). Acute fasting for 16-hours has been shown to influence the amplitude and frequency of spontaneous currents of dopamine neurons in the ventral tegmental area in male mice (Godfrey and Borgland, 2020). A future direction for this study could be to examine DMH neuron communication during different fasting periods as only 24-hours was utilized in this study. Although no changes were observed in DMH synapses between the naïve and fasted group, longer periods of fasting might influence DMH glutamate transmission, potentially through greater glutamate release. Fasting has further been shown to influence GABA synapses in the DMH in male rats (Crosby et al., 2011). Therefore, future studies could look at the effect of feeding state at GABA synapses in the DMH of female rats. Furthermore, normal chow was used in the current study, however obesity commonly results from high-caloric food intake. Therefore, future studies could examine the impact of a high fat diet on DMH neurons in female rats. Differences in neuronal activity based on age has further been observed as Dhanrajan et al. (2004) found that older male rats had a long-term potentiation following HFS at neurons in the hippocampus. Thus, future studies could consider examining neuronal activity in older female rats.

Overall, we found that fasting does not influence basal glutamate synaptic transmission in female rats. We also demonstrated that endogenous endocannabinoid release does not alter DMH glutamate synaptic strength in either naïve or fasted female rats. However, the activation of CB1 receptors through an exogenous cannabinoid agonist does decrease the amplitude of excitatory currents of DMH neurons in both naïve and fasted female rats. Feeding state further does not influence DMH neuronal excitability in female rats. To the best of our knowledge, this is the only study to specifically examine DMH glutamate synapses in female rats. Understanding how the DMH synapses of female rats are affected by feeding states can contribute to the growing data collected on male rats. This information can further help with understanding neuronal appetite regulation as a thorough understanding of how appetite is regulated by the brain is important to help optimize interventions to prevent adverse repercussions of obesity.

**Acknowledgments**

First and foremost, I want to thank my supervisor, Dr. Karen Crosby, for her continuous support, encouragement, and guidance throughout the past year. I would also like to thank Dr. Andrea Morash for being my committee member. Further, I would like to thank Jackie Jacob-Vogels for helping us take care of the animals and creating a positive environment in the animal wing. I am further grateful to my lab partners, Diwan Minocha and Logan Grossman, for all the help and support they have provided in the past year. I am especially thankful for my parents, sister, and friends who have provided unwavering support and encouragement over the past four years. Finally, I would like to express my appreciation to the New Brunswick Health Research Foundation for the funding for my research.

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