

The effect of insulin on excitatory synaptic transmission in the rat dorsomedial hypothalamus

by

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Abstract

Insulin plays a key role in regulating blood glucose levels and it also acts in brain regions involved with appetite regulation. The dorsomedial hypothalamus (DMH) is one such brain region, and although it is a major site of insulin receptors, virtually nothing is known about the effect of insulin on neuronal activity and communication between DMH neurons. Since DMH neurons stimulate appetite and insulin is a satiety hormone, I hypothesized that insulin inhibits both neuronal activity and neuronal communication in the DMH. I used whole-cell patch clamp electrophysiology to record from living DMH neurons taken from young male and female Sprague-Dawley rats. I compared recordings before and after applying 500 nM of insulin to determine the effect of insulin on action potentials (a measure of neuronal excitability) and excitatory synaptic transmission (a measure of neuronal communication). Insulin significantly decreased action potential frequency and excitatory current amplitudes in both sexes, indicating that insulin decreases neuronal excitability and excitatory synaptic transmission in the DMH. Recordings taken with various insulin receptor blockers present suggested that insulin binds to insulin-like growth factor 1 receptors. Insulin signalling in the DMH is also independent of feeding state, suggesting that it is involved with regulating physiological processes beyond appetite regulation. This research contributes to our understanding of how insulin acts in the brain to influence energy metabolism, with direct applications to research on the pathological effects of insulin resistance in obesity and diabetes.

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List of Abbreviations

DMH	Dorsomedial hypothalamus
GABA	Gamma-aminobutyric acid
eEPSC	Evoked excitatory post-synaptic current
PPR	Paired pulse ratio
HNMPA	Hydroxy-2-naphthalenylmethylphosphonic acid
PPP	Picropodophyllotoxin

Introduction

Obesity is a serious health concern in New Brunswick, where the number of adults with obesity is 11% higher than the national average (26.8%) (Statistics Canada, 2019). Obesity is an imbalance in energy intake and energy expenditure that results in excess energy levels in the body (Berthoud & Morrison, 2008). Although most excess energy is stored as fat, long-term periods of excess energy also negatively impact metabolic processes and increase the risk of developing other health conditions (Berthoud & Morrison, 2008), including asthma, type II diabetes, cardiovascular disease, and various cancers (Sarma et al., 2021). Understanding the role of hormones in regulating appetite could lead to the development of new obesity therapies.

Hormones

Hormones are critical for regulating a diverse range of physiological processes such as growth, reproduction, and appetite. When animals are hungry, the stomach releases hormones like ghrelin, which increases the activity of appetite-stimulating neurons to create a desire to find and consume food (Wren et al., 2000). Once an animal has eaten, a new series of hormones enter the brain where they inhibit appetite-stimulating neurons and activate neuronal pathways that induce satiety (Wilding, 2002; Woods et al., 2006). Through their close interactions with digestive system organs and the brain, hormones can respond quickly to short-term energy needs.

Hormones also influence appetite over the long term in response to an animal's energy state. Leptin is one example of a hormone that acts as an indicator of stored energy in the body (Considine et al., 1996). Since adipocytes (fat-storing cells) produce and release leptin, higher bloodstream concentrations of leptin correlate to more body fat, and in turn, more stored energy (Considine et al., 1996). Leptin

stimulates neurons to produce neurotransmitters that eventually decrease appetite (Elias et al., 1999). A second long-term energy indicator is insulin (Wilding, 2002), which is also a key energy regulator.

Insulin

Insulin is a hormone that regulates blood glucose levels, digestive processes, and body weight (De Meyts, 2000). After a meal, the pancreas releases insulin into the bloodstream, where it activates molecular pathways that lower blood glucose levels (De Meyts, 2000). Insulin also interacts with digestive organs to influence metabolic pathways that maximize an animal's available energy resources. Insulin carries out these functions by binding to insulin receptors or insulin-like growth factor-1 receptors (IGF-1Rs) and activating downstream molecular pathways (Figure 1). In addition to acting on body cells, insulin also acts in the brain.

Within the brain, insulin binds to insulin receptors on neurons and alters their activity (Havrankova et al., 1981). Insulin can activate neurons or inhibit them, depending on the signalling pathways that are activated, the type of neuron involved, and/or the location of the neuron. In some neurons, insulin binding activates changes within neurons that make them more easily activated, such as increasing receptors for excitatory neurotransmitters (Liu et al., 1995) or increasing intracellular Ca^{2+} concentrations (Jonas et al., 1997). In other cases, insulin stimulates neurons to express receptors for neurotransmitters like gamma-aminobutyric acid (GABA; Wan et al. (1997)), which makes them more easily inhibited. Through its interactions with insulin receptors, insulin affects individual neurons, which then impacts entire neuronal pathways.

Most brain regions that respond to insulin help animals to find, consume, and enjoy food. The olfactory bulb and the hypothalamus contain the highest numbers of insulin receptors (Corp et al., 1986; Havrankova et al., 1981), making them more sensitive to insulin than other regions. Not only does the olfactory bulb assist with locating and identifying food, but it also influences meal choices, salivation, and appetite (Gaillet-Torrent et al., 2014; Proserpio et al., 2017). Within the hypothalamus, subdivisions called nuclei regulate a diverse range of physiological processes relating to appetite and energy metabolism. Insulin can also amplify leptin signalling in the hypothalamus (Carvalheira et al., 2001), suggesting that

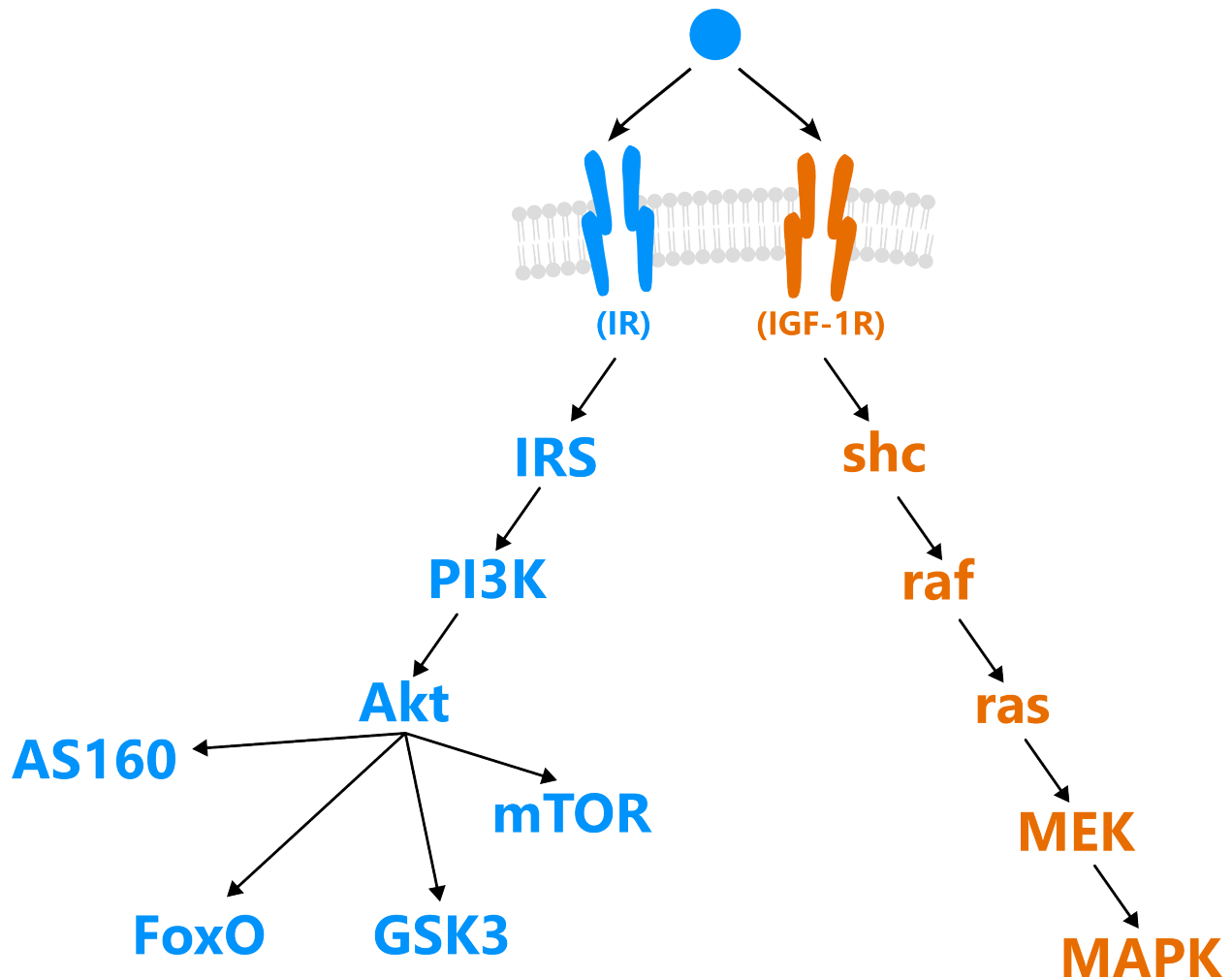


Figure 1: When insulin binds to a cell, it activates a series of molecular pathways involved in energy metabolism and gene expression. Insulin can bind to either an insulin receptor (IR) or an insulin-like growth factor 1 receptor (IGF-1R). The phosphatidylinositol 3-kinase (PI3K) pathway is primarily involved in regulating metabolic processes such as glucose metabolism, lipid metabolism, and gluconeogenesis, while the mitogen activated protein kinase (MAPK) pathway is primarily involved with regulating gene expression and cell division (De Meyts, 2000). Parts of the PI3K pathway can affect synaptic transmission and neuronal activity, with mechanistic target of rapamycin (mTOR) playing a key role in synaptic transmission (McCabe et al., 2020). Although it looks like there are two distinct pathways, there is a lot of overlap between the PI3K and MAPK pathways (De Meyts, 2000).

insulin works with other hormones to inhibit appetite-stimulating hypothalamic pathways. These findings demonstrate that insulin is a satiety hormone that acts on neurons in the hypothalamus to influence a wide variety of processes relating to food intake. Insulin also acts on neurons in regions outside of the hypothalamus. One example is the ventral tegmental area, a brain region involved in reward and pleasure (Labouèbe et al., 2013). All these findings demonstrate that insulin plays an important role in regulating appetite, energy metabolism, and the rewarding feelings associated with eating.

If insulin is so closely linked to food intake, how does it respond to an animal's energy needs? Insulin could act as a marker of feeding state. Since insulin levels in the bloodstream fluctuate with meals and digestion, this could allow for precise changes in neuronal activity in response to an animal's current energy state. Surprisingly, insulin concentrations in the brain are relatively stable compared to the bloodstream (Havrankova et al., 1981). This implies that the brain maintains an independent insulin supply (Gerozissis et al., 1997), either by synthesizing insulin locally or by storing insulin as it enters the brain (Havrankova et al., 1981). The independence of brain insulin levels from the rest of the body suggests that consistent insulin levels are required for neuronal function, not just appetite regulation. Some insulin from the bloodstream does enter the brain, however, and injecting insulin into the bloodstream decreases appetite (Vanderweele, 1994). Insulin likely enters the brain through a structure called the median eminence, where it then has immediate access to the hypothalamus and nearby brain regions (Romanò et al., 2023).

The Dorsomedial Hypothalamus

The dorsomedial hypothalamus (DMH) is a hypothalamic nucleus that regulates appetite (Dalton et al., 1981) and body weight (Bellinger et al., 1979). Neurons in the DMH express receptors for appetite-regulatory hormones including leptin (Zhang et al., 2011) and insulin (Corp et al., 1986). DMH neurons also connect to many brain regions involved with appetite regulation and nutrient sensing (Horst & Luiten, 1986; Thompson & Swanson, 1998). Figure 2 summarizes the major projections to and from the DMH.

Animals require an intact DMH for appetite regulation. When researchers destroyed neurons in the DMH with an electric current, animals experienced decreased food intake and body mass (Bellinger et al.,

1979; Dalton et al., 1981). By sequentially cutting neuronal pathways to and from the DMH, researchers found that DMH neurons require posterior and ventral projections to interact with appetite-regulatory neuronal pathways (Bellinger & Bernardis, 1999). Researchers also learned that DMH neurons are more active after fasting (Groessl et al., 2013), and before and after meals (Angeles-Castellanos et al., 2004). By “turning on” DMH neurons through optogenetic activation, researchers can increase food intake in mice (Jeong et al., 2017; Otgon-Uul et al., 2016). These studies all show that the DMH is vital for appetite regulation.

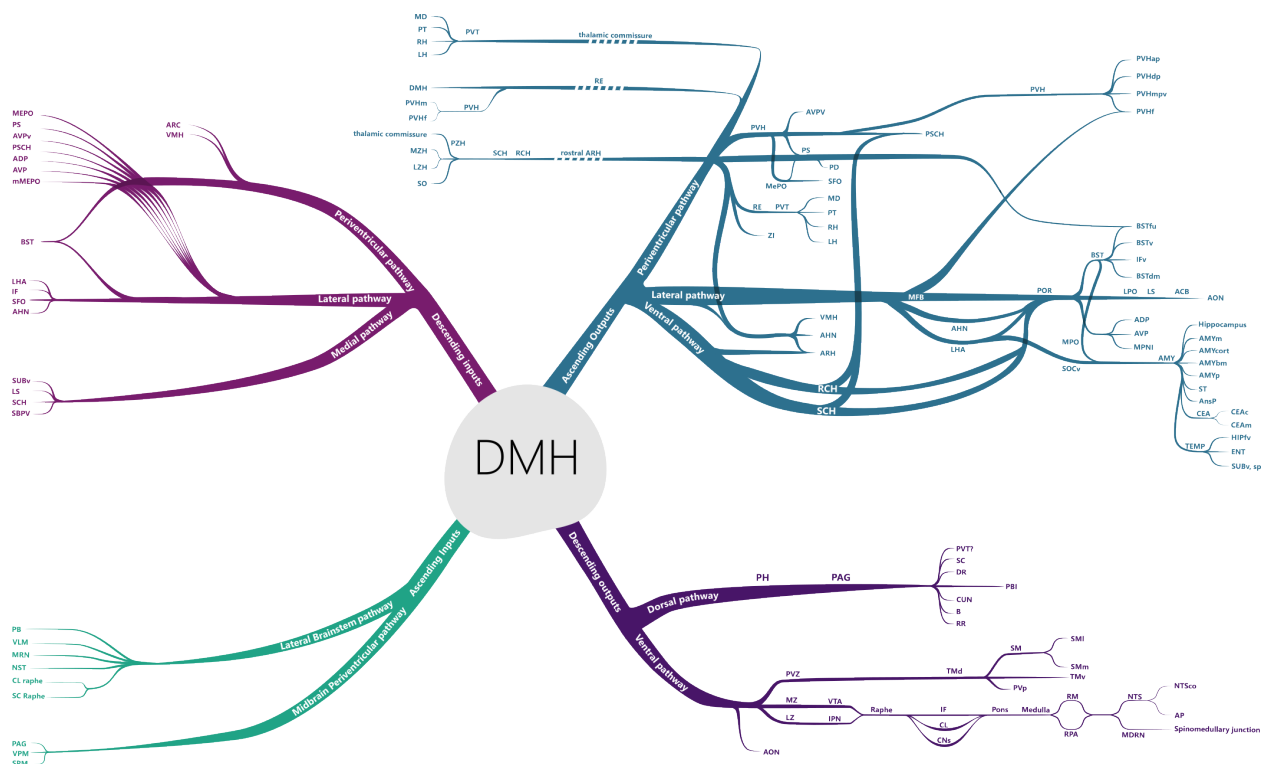


Figure 2: The DMH interacts with many brain regions. Two particularly important regions include the nucleus of the solitary tract (NST) and parabrachial nucleus (PB), which help with nutrient sensing. The DMH has projections leading to and from other brain regions that control appetite, such as the arcuate nucleus (ARC), ventromedial hypothalamus (VMH) and lateral hypothalamus (LHA). Dashed lines indicate locations where neuronal projections cross to the other half of the body. This figure is based on the neuronal tracing studies of Horst & Luiten (1986) and Thompson & Swanson (1998).

Although insulin interacts with neurons in the hypothalamus (Corp et al., 1986) and DMH neurons express insulin receptors (Corp et al., 1986), nothing is known about the effect of insulin on DMH neurons. It is also unclear how insulin binds to DMH neurons, and how it could affect neuronal activity.

The Current Study

The main question of this study is: Does insulin bind to DMH neurons, and if so, how does it affect their activity? Since DMH neurons stimulate appetite, and insulin decreases appetite, I hypothesize that insulin decreases neuronal activity and communication within the DMH. I predict that insulin decreases action potential frequency and current amplitudes associated with excitatory neurotransmitters (a measure of neuronal communication) in DMH neurons relative to control conditions. To see if insulin interacts with DMH neurons, I recorded from living DMH neurons and compared action potential parameters and excitatory current amplitudes before and after adding insulin. I also compared neuronal activity in the DMH neurons of rats who underwent a 24-hour fasting protocol to see if insulin signalling in the DMH varies with feeding state. This research increases our understanding of appetite regulation in the brain, with direct applications to research on the pathophysiological effects of conditions like obesity and diabetes.

Methods

Animals

I used young (28-44 days old) male and female Sprague-Dawley rats from Charles River Laboratories (Québec, Canada). The rats arrived when they were 22 days old, and they had a one-week acclimation period. The rats lived in groups of 4 with free access to standard rat chow and water. All cages had wood shavings for bedding, toys (wooden blocks and a plastic chew toy) for enrichment and Crawl Balls™ (Bio-Serv) for shelter. The animal rooms were maintained at 21°C and 50% ± 10% humidity, with a 12-hour light/dark cycle. The Animal Care Committee at Mount Allison University approved the project (Protocol #103088).

Brain Removal & Slice Preparation

I placed a rat into an anesthetization chamber filled with 5% isoflurane gas in oxygen. After confirming that the rat was unconscious through a toe pinch test, I decapitated it with a guillotine (World Precision Instruments). I transferred the brain to a chilled, freshly oxygenated slicing solution containing (in mM) 87 NaCl, 2.5 KCl, 25 NaHCO₃, 0.5 CaCl₂ · 2H₂O, 7 MgCl₂ · 6 H₂O, 1.25 NaH₂PO₄, 25 glucose and 75 sucrose. I had previously bubbled the solution with carbogen (5% carbon dioxide in oxygen) for 10-15 minutes to ensure full oxygen saturation.

I removed the brain from the solution and cut off the anterior, posterior, and dorsal portions. The brain was glued posterior side down (Krazy Glue) into a vibrating microtome (Leica Biosystems) next to a piece of agar for support. It was then re-immersed in slicing solution with carbogen bubbling. The microtome

created thin slices (250 μm) along the coronal plane, while I identified brain regions with an atlas (Paxinos & Watson, 2009).

On a DMH-containing brain slice, I used a scalpel to remove the lateral edges and then cut the slice along the midline. I transferred the resulting hemisections to a beaker containing artificial cerebrospinal fluid. This fluid contained (mM) 126 NaCl, 2.5 KCl, 26 NaHCO_3 , 2.5 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 15 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.25 NaH_2PO_4 , and 10 glucose. A gas line kept the artificial cerebrospinal fluid bubbled with carbogen, and a water bath maintained the solution at 32°C. I let the slices recover for one hour before recording to ensure maximum cell health.

Recording

I placed a hemisection in a perfusion chamber filled with continuously oxygenated artificial cerebrospinal fluid (32°C; TC-344C Temperature Controller, Warner Instruments) flowing through at 1 mL/minute. The chamber was mounted under an upright microscope (Olympus BX51WI) connected to an Infinity 2 camera and software (Version 6.5.2, Lumenera Corporation), which projected to a computer monitor. I navigated to the DMH, which was located along the border of the third ventricle (Paxinos & Watson, 2009).

I used whole-cell patch clamp electrophysiology to stimulate DMH neurons and record their responses in real-time. The neurons were still alive, so this technique enabled me to observe changes in activity after experimental manipulations. During patch clamping, two pipettes were positioned in the brain slice: a stimulating pipette and a recording pipette (Figure 3). The stimulating pipette used electrical stimuli to stimulate nearby neurons to release neurotransmitters onto the target neuron. The recording pipette recorded the resulting currents from the target neuron.

I used pipettes with resistances ranging from 4.0 to 6.0 M Ω s (Model P-1000 pipette puller, Sutter Instruments). The stimulating pipette contained artificial cerebrospinal fluid, while the recording pipette had internal solution, consisting of (mM) 108 KGluconate, 8 KCl, 8 NaGluconate, 10 HEPES, 2 MgCl_2 , 1

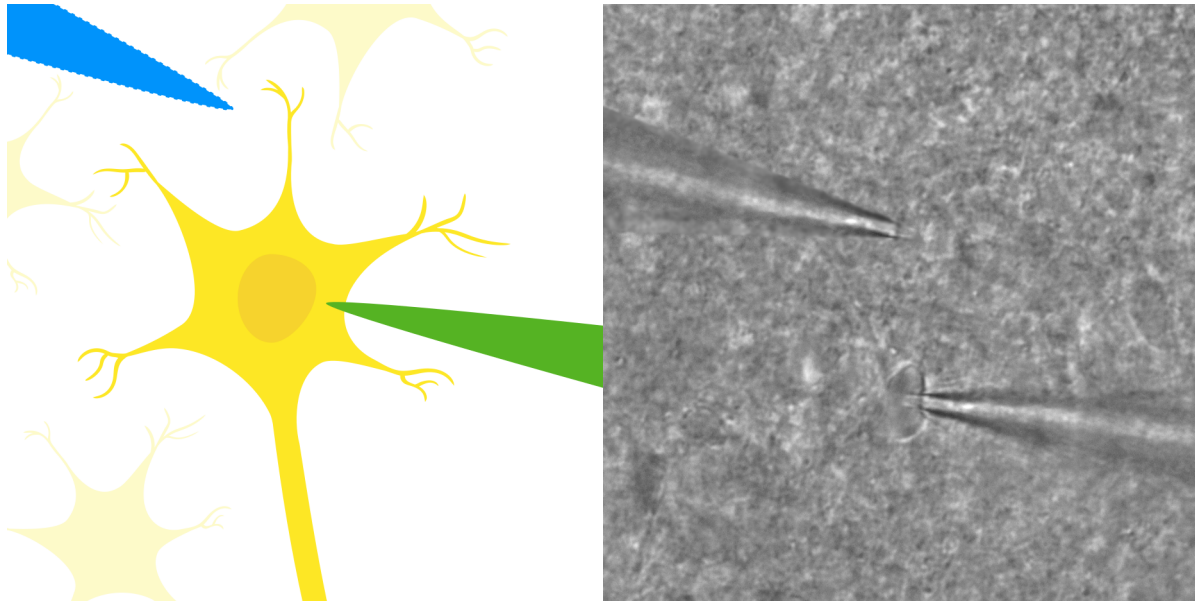


Figure 3: *Left*: In whole-cell patch clamp electrophysiology, the stimulating electrode (dashed blue) stimulates nearby neurons (pale yellow) to release neurotransmitters while the recording electrode (green) records from the target neuron (yellow). *Right*: The recording electrode is inserted directly into a living DMH neuron (40X magnification).

K_2 ETGA, 4 K_2 ATP, 0.3 Na_3 GTP, which simulated the intracellular fluid within neurons. The internal solution was adjusted to pH 7.2 with 1 M KOH and 285-300 mOsm with sterilized water.

This study focused on currents associated with the excitatory neurotransmitter glutamate because DMH neurons receive a large number of excitatory inputs and glutamate-mediated transmission plays a large role in regulating DMH activity (Bailey et al., 2003). Since DMH neurons also receive inhibitory inputs through the inhibitory neurotransmitter GABA (Bailey et al., 2003), I added 50 μ M picrotoxin (Tocris Bioscience) to the perfusion solution. Picrotoxin blocked $GABA_A$ receptors (Newland & Cull-Candy, 1992) and allowed me to isolate and record glutamate currents only.

I lowered both pipettes into the brain slice using micromanipulators (MPC-365 System, Sutter Instruments). I inserted positive pressure into the recording pipette to blow away debris and to keep the tip clean. Once I identified a neuron, I gently pushed the recording pipette against the cell membrane until a dimple formed. Releasing the positive pressure caused the cell to form a seal against the pipette tip. I applied a small pulse of mouth suction to break through the membrane and gain access to the cell.

I used pCLAMP software (Version 10.6.0.13, Molecular Devices) to perform protocols and record the resulting electrophysiological parameters. A MultiClamp700B (Molecular Devices) amplified the neuron's electrical signals and then a Digidata 1550B (Molecular Devices) low-pass filtered and digitized them at 1 kHz and 10 kHz, respectively. I monitored the cell's stability every 5 minutes using access resistance and discarded any recordings with unhealthy or lost cells.

Action Potentials

I used a current clamp steps protocol to observe action potentials and compare their properties. I performed this protocol immediately with each new cell as a baseline, then recorded evoked excitatory currents for 25 minutes after adding insulin (see the *evoked currents* section for a protocol summary). After I finished the evoked currents protocol, I recorded the current clamp steps protocol once again. During the current clamp steps protocol, the software stimulated the cell for 500 ms with electrical current injections of increasing magnitude. The current injections began at -50 pA, and increased by 10 pA with each step until a maximum of 40 pA. The current injections depolarized the neuron to a point where it fired action potentials.

I identified the first current injection that resulted in action potentials and then selected the first action potential for my analysis (Figure 4). I identified the action potential threshold (the depolarization required to induce an action potential; in mV) through the first derivative method (Farries et al., 2010). This method sets the threshold as the membrane potential where the action potential velocity is 10 mV/ms or greater (Farries et al., 2010). The latency to fire was the time (in ms) between the start of the stimulus and the action potential's peak amplitude.

All other action potential properties were relative to the threshold. I measured the action potential's amplitude (mV) and half-width (ms; Figure 4). I also measured the after-hyperpolarization amplitude (mV) and time of the after-hyperpolarization (ms). Lastly, I counted the number of action potentials at each current injection and used these values to obtain the action potential frequency per current injection (Hz). If the recording contained no action potentials, I set the action potential frequency and amplitude to 0, and set all other action potentials to *NA* values. The threshold, amplitude, frequency, and all other

action potential properties conveyed information about the intrinsic excitability of a neuron. For example, the half-width (a measure of the width of the action potential measured at half the amplitude) is an indicator of the repolarization rate (Bean, 2007).

By comparing action potential properties before and after insulin exposure, I was able to explore the effect of insulin on intrinsic neuronal excitability. Since the action potential properties data did not pass the assumptions of normality, even with a log-transformation, I used a paired Wilcoxon signed-rank test to assess differences in action potential parameters across the two states.

Evoked Currents

During the evoked currents protocol, the software maintained the cell at a membrane potential of -70 mV, which is close to a DMH neuron's natural resting membrane potential of -60 mV (Bailey et al., 2003). Through the stimulating pipette, the software delivered two electrical stimuli 50 ms apart, repeated every 5 seconds (Figure 5). The stimuli induced nearby neurons to release glutamate onto the target cell, which then experienced a temporary change in membrane potential. These currents are called evoked excitatory post-synaptic currents (eEPSCs, or "evoked currents") because they are a) artificially induced, b) caused by an excitatory neurotransmitter, and c) present in the cell that receives the neurotransmitters, not the presynaptic cell.

I recorded evoked excitatory currents for 5 minutes under control conditions as a baseline. I then added 500 nM insulin to the perfusion solution and continued recording for 25 minutes. I also applied insulin in the presence of an insulin receptor blocker (Hydroxy-2-naphthalenylmethylphosphonic acid; HNMPA, Abcam Incorporated; 300 μ M) and an insulin-like growth factor 1 receptor blocker (picropodophyllotoxin; PPP, 0.5 μ M; Selleck Chemicals) to explore insulin's binding mechanisms.

To determine if insulin signalling in the DMH varies with feeding state, I placed a rat alone into a cage with no food for 24 hours before brain removal. During this period, the rat had free access to water, toys for environmental enrichment, bedding, and shelter. Removing food creates a hunger state, which would potentially alter insulin signalling at the DMH.

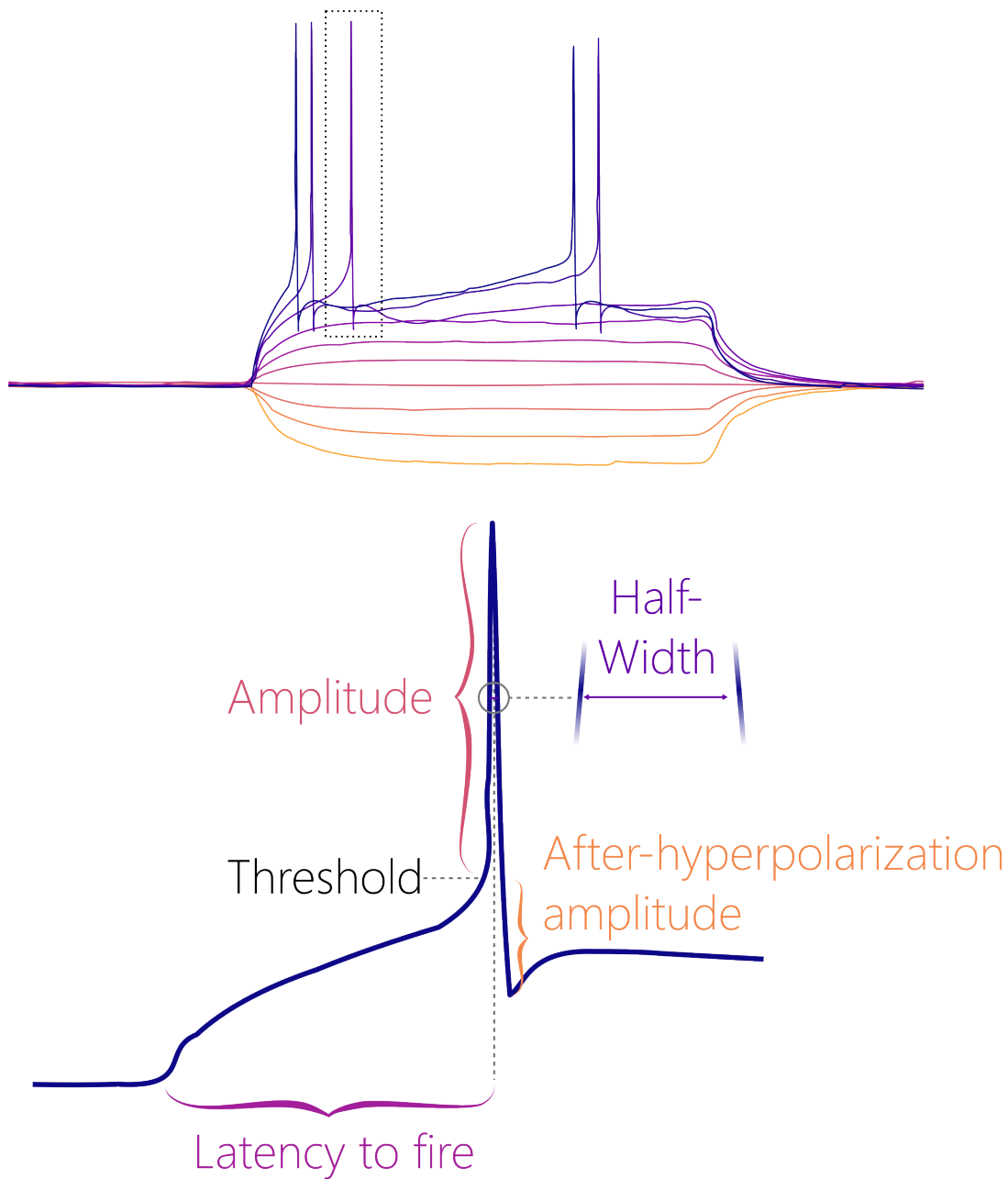


Figure 4: A schematic of the current clamp steps protocol. *Top:* This is a representative recording from a single cell featuring all 10 current clamp steps stacked on top of one other. The current injections ranged from -50 pA (bottom; orange) to 40 pA (top; purple), with a 10 pA increase per step. I used the first action potential at the lowest current injection (boxed outline at a current injection of 20 pA) to analyze action potential properties. All traces have been smoothed through node reduction using the *simplify path* function in Inkscape. *Bottom:* I measured the action potential threshold, amplitude, half-width, and latency to fire. I also measured the after-hyperpolarization amplitude and time of the after-hyperpolarization.

I analyzed the evoked current data for all treatments. I started by normalizing the first evoked current amplitude as a percentage of the cell's mean baseline evoked current amplitudes (100%) for easy comparison across cells with different starting amplitudes. Then, I used a multivariate repeated measures ANOVA to assess changes in current amplitudes over time. The model used a within-subjects factor of time (six 5-minute intervals) and treatment and sex as between-subjects factors. Later, I split the data by treatment and used paired t-tests with Holm's corrections (Holm, 1979) to identify the time when the currents became significantly different than the baseline. I assessed normality using QQ plots and the Shapiro-Wilk normality test or its multivariate equivalent. I used Levene's test or Box's M test (multivariate equivalent) to assess the homogeneity of variances.

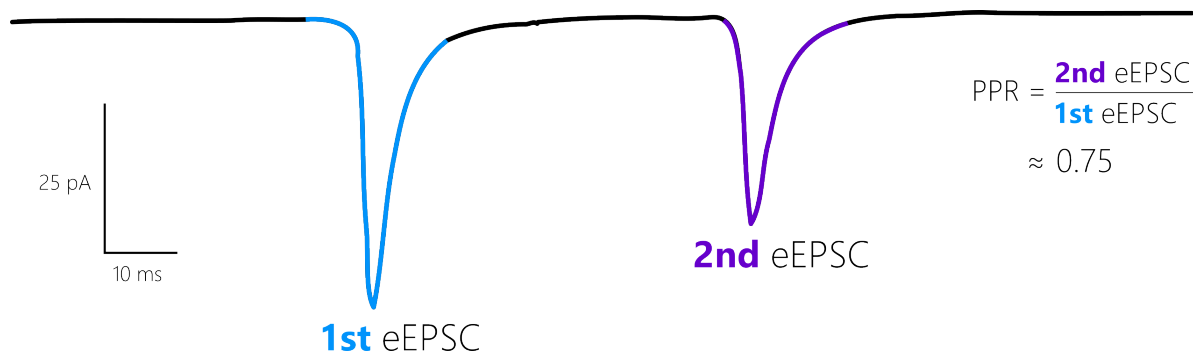


Figure 5: For the evoked currents protocol, the cell experienced two electrical stimuli 50 ms apart, repeated every 5 seconds. This created evoked currents, which I recorded and compared. I measured changes in the amplitude of the first evoked current (blue) over time. I also analyzed changes in the paired pulse ratio (PPR), which is the ratio of the second evoked current to the first evoked current. If the PPR changed throughout the recording, it indicated that the change in evoked current amplitude occurred through a presynaptic mechanism (Manita et al., 2007).

Paired Pulse Ratio

The paired pulse ratio (PPR) is the ratio between the second and first evoked currents (Figure 5). Since the PPR is a measure of the probability of neurotransmitter release (Manita et al., 2007), changes in the PPR over time can provide information about the mechanism of synaptic transmission. If the PPR changes throughout the recording, this indicates that a presynaptic mechanism had changed. If the PPR does not change, this indicates that a change had occurred post-synaptically.

Using the raw data (i.e. the non-normalized data), I divided the amplitude of the second evoked current by the first evoked current to obtain the PPR. I then filtered the PPRs to values within a range of 0 to 5 and averaged the PPR across the baseline period (0 to 5 minutes) and final interval (20 to 25 minutes) within each cell and used a paired T-test to compare the PPR across these two periods.

Software

I measured electrophysiological parameters like evoked current amplitude and action potential properties using *Clampfit* (Version 10.6.0.13, Molecular Devices). I performed all statistical analyses with *R Statistical Software* (Version 4.3.1; R Core Team 2021) in *RStudio* (Build 369; RStudio Team (2020)). I used $\alpha = 0.05$ for all tests.

I used the *abfTools* package (Version 0.9.9; Wu (2019)) to obtain representative traces from raw recording files, and I made all plots with *ggplot2* (Version 3.5.0; Wickham (2016)). I used *Inkscape* (Version 1.3; Inkscape Developers (2023)), an open-source vector editor, to create the explanatory schematics.

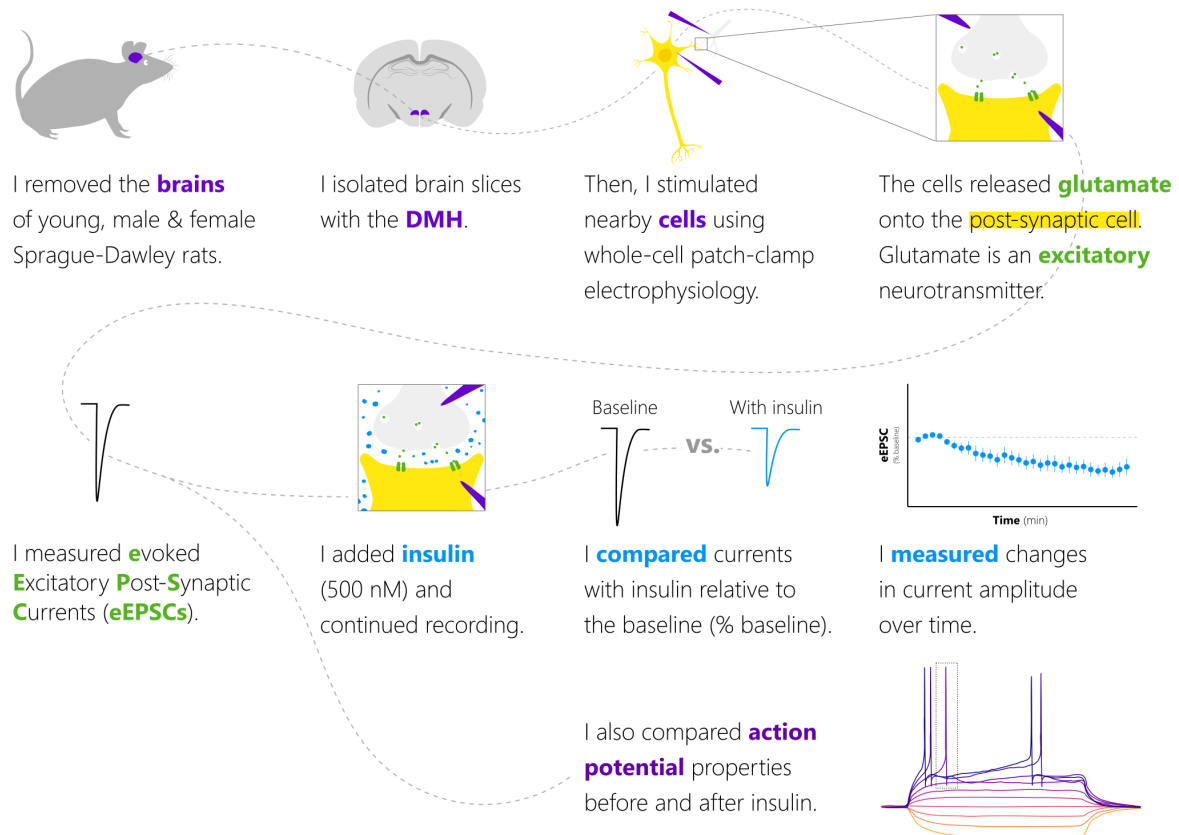


Figure 6: A visual summary of the experiment. I removed rat brains, stimulated DMH neurons, and recorded the resulting currents. This allowed me to compare currents before and after exposing the neurons to insulin.

Results

The primary goal of this study was to examine the effect of insulin on neuronal excitability and communication in DMH neurons. I used whole-cell patch clamp electrophysiology to record action potentials and evoked excitatory currents from 62 unique neurons in the DMH. I analyzed recordings taken before, while and after DMH neurons were exposed to 500 nM insulin. By comparing parameters like action potential frequency and evoked current amplitude across conditions, I was able to draw conclusions about the effect of insulin on intrinsic neuronal excitability, excitatory synaptic transmission, and neurotransmitter release onto DMH neurons.

The second goal of the study was to determine the mechanism of insulin signalling within DMH neurons. I used insulin receptor antagonists like HNMPA and PPP to determine the receptors that insulin binds to, and to explore intracellular signalling pathways that might be activated by insulin binding. I also examined changes in the paired pulse ratio to determine if insulin signalling uses pre- or post-synaptic mechanisms. Lastly, I exposed rats to a 24-hour fasting protocol before tissue extraction to assess if hunger affects insulin signalling within the DMH relative to control experiments from satiated animals.

DMH neurons are less excitable with insulin

A current clamp steps protocol taken before and after insulin exposure showed that insulin decreases action potential frequency (Figure 7A). The difference in action potential frequency was statistically significant from a current injection of -10 pA ($t_{14} = 3.07, p = 0.008$) up to the highest current injection of 40 pA ($t_{14} = 2.75, p = 0.016$).

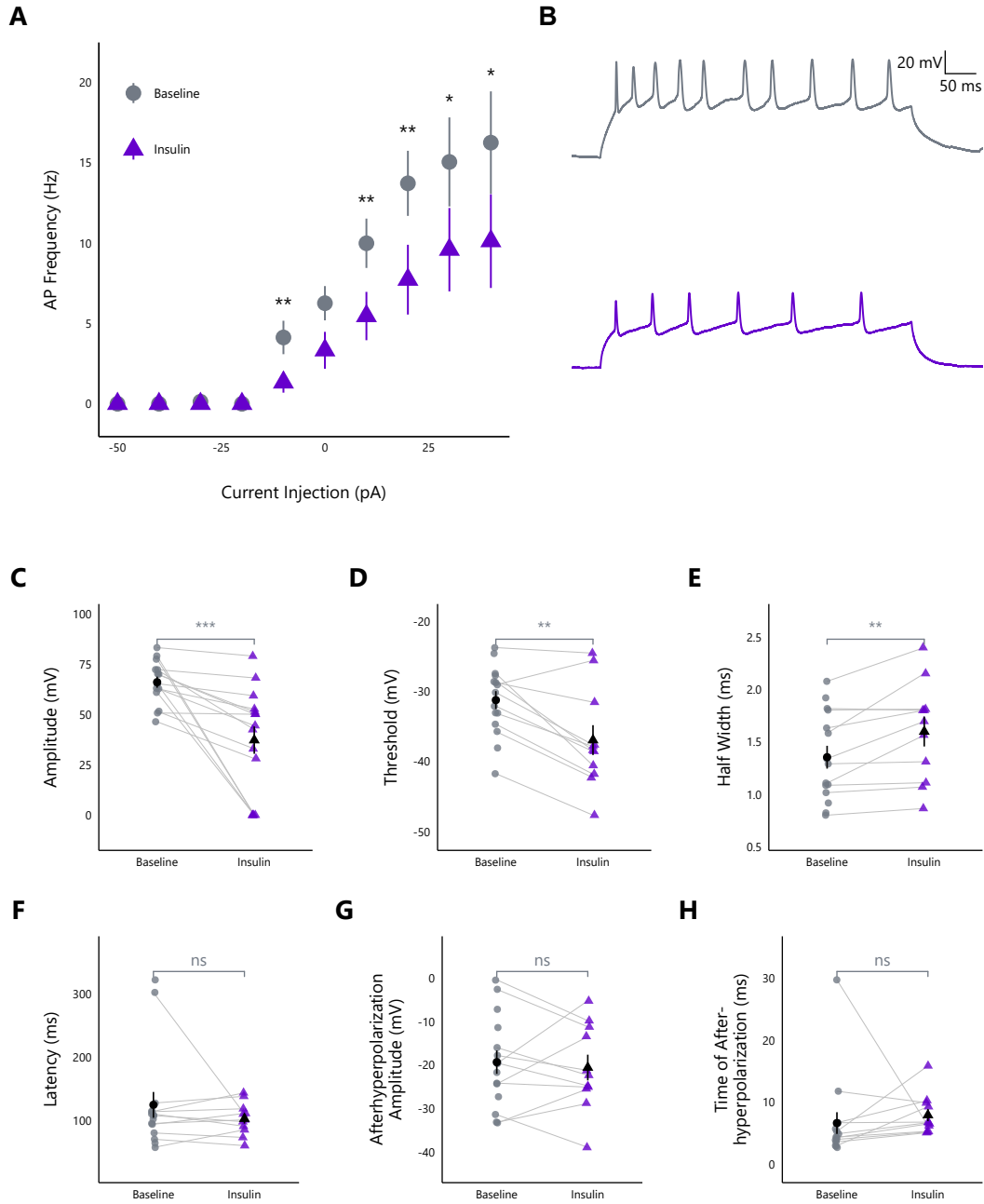


Figure 7: Insulin decreases the excitability of DMH neurons. Action potentials were recorded under baseline conditions (“baseline”) and then 25 minutes after insulin exposure (“Insulin”). *A*) Insulin significantly decreases action potential frequency (mean \pm SE). *B*) Representative action potential traces from a current injection of 40 pA (top: baseline, bottom: after insulin exposure). *C*) Insulin significantly decreases action potential amplitudes *C*) and thresholds *D*), while significantly increasing action potential half-widths *E*). Insulin does not significantly affect action potential latency *F*), after-hyperpolarization amplitude *G*) or after-hyperpolarization time *H*). Overlay on Figures C-H: mean \pm SE. Wilcoxon signed-rank test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 1: A Wilcoxon signed-rank test shows that insulin significantly affects action potential amplitudes, thresholds, and half-widths. Insulin does not significantly affect the latency to fire, after-hyperpolarization amplitude, or the time of afterhyperpolarization.

Parameter	df	Statistic	p -value
Peak amplitude	15, 15	120	< 0.001
Threshold	15, 11	63	0.005
Latency to fire	15, 11	31	0.90
Half-width	15, 11	2	0.003
After-hyperpolarization amplitude	15, 11	35	0.90
After-hyperpolarization time	15, 11	17	0.17

Insulin significantly decreases action potential amplitude relative to baseline amplitudes ($W(15, 15) = 120$, $p = < 0.001$; Figure 7C). Insulin also significantly decreases the threshold required to initiate an action potential ($W(15, 11) = 63$, $p = 0.005$; Figure 7D). Action potential half-widths increased significantly with insulin ($W(15, 11) = 2$, $p = 0.003$; Figure 7E). The latency to fire did not change significantly after insulin exposure ($W(15, 11) = 31$, $p = 0.90$; Figure 7F). The amplitude ($W(15, 11) = 35$, $p = 0.90$; Figure 7G) and time of the after-hyperpolarization ($W(15, 11) = 17$, $p = 0.17$; Figure 7H) also did not significantly change after insulin exposure.

Excitatory synaptic transmission decreases after insulin exposure

To assess the effect of insulin on excitatory synaptic transmission in DMH neurons, I compared eEPSC amplitudes with insulin relative to a baseline period under control conditions. Insulin significantly decreased current amplitude over time in cells with no additional treatments applied ($p < 0.001$; Figure 8). The decrease in evoked current amplitudes was statistically significant within the first five minutes of adding insulin ($t(21) = 2.08$, $p = 0.050$). This indicates that insulin binding quickly activates intracellular processes that decrease excitatory synaptic transmission in the DMH.

The changes in evoked current amplitudes were not significantly different across sexes (Sex*Times, $p = 0.24$), suggesting that insulin acts independently of sex in the DMH. I also found no cross-factor interactions between sex and treatment (Treatment*Sex*Times, $p = 0.41$).

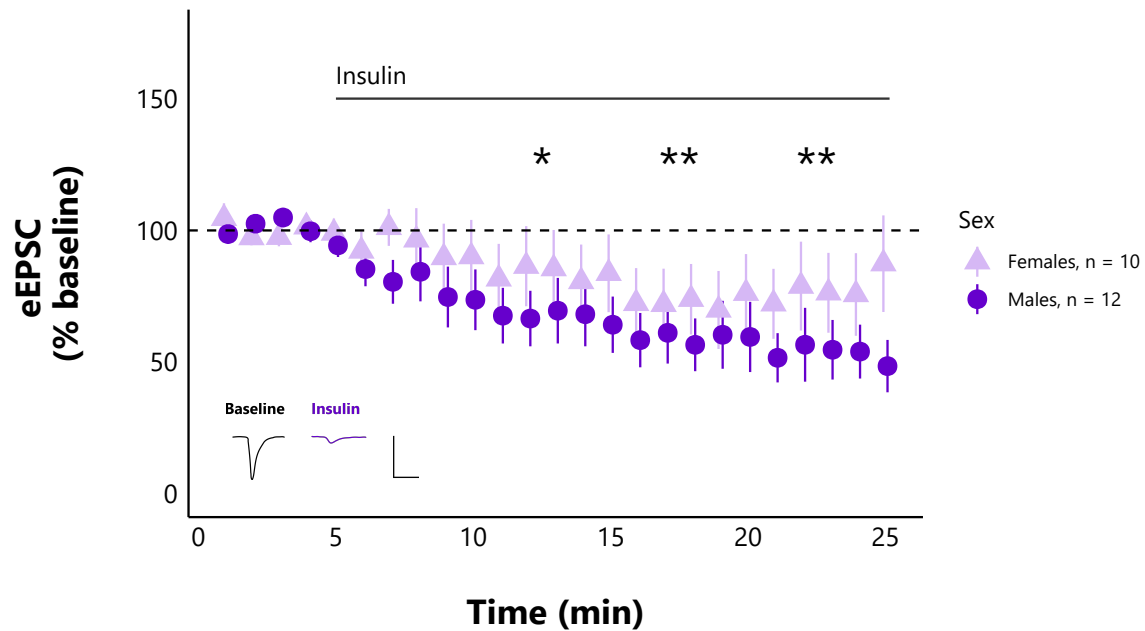


Figure 8: Insulin significantly decreases excitatory evoked post-synaptic current (eEPSC) amplitude over time in both sexes, with no significant differences between the sexes. I exposed DMH neurons to 500 nM of insulin from 5 minutes and onward. Each point represents the mean eEPSC amplitude (\pm the standard error) across all cells and n represents the number of unique cells. The asterisks indicate a statistically significant decrease in current amplitude relative to the baseline (t-test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). The representative traces consist of eEPSCs from one cell averaged over the baseline period (0 to 5 min) and last interval (20 to 25 min) of the recording, and the scale bar represents 50 pA/20 ms.

Insulin may bind to insulin-like growth factor 1 receptors on DMH neurons

After verifying that insulin acts on DMH neurons, the next step was to determine which receptors insulin can bind to. I performed the same evoked currents protocol, but with HNMPA in the intracellular solution. HNMPA is an insulin receptor antagonist that inhibits tyrosine kinase activity (Saperstein et

Table 2: A multivariate repeated measures ANOVA using Pillai's Trace shows that current amplitude varies significantly with time, and there are no significant interactions between factors like sex and treatment.

Parameters	df	Pillai's Trace	F	<i>p</i> -value
(Intercept)	1, 54	0.91	564.93	< 0.001
Treatment	3, 54	0.01	0.27	0.85
Sex	1, 54	0.03	1.44	0.24
Treatment:Sex	3, 54	0.01	0.11	0.95
Times	4, 51	0.46	10.75	< 0.001
Treatment:Times	12, 159	0.20	0.96	0.49
Sex:Times	4, 51	0.10	1.42	0.24
Treatment:Sex:Times	12, 159	0.22	1.05	0.41

al., 1989). Since insulin receptors belong to the tyrosine kinase superfamily (De Meyts, 2000), HNMPA would have blocked insulin from binding to insulin receptors. However, insulin also significantly decreased evoked current amplitudes within the first 5 minutes of exposure ($t_{(11)} = 3.06$, $p = 0.032$; Figure 9) even with HNMPA present. Since DMH neurons show the same response to insulin with HNMPA relative to control conditions, this suggested that insulin acts on DMH neurons through a different receptor.

Insulin-like growth factor receptors have many structural similarities to insulin receptors and they are also responsive to insulin (Nagao et al., 2021). I performed the evoked currents protocol in the presence of an insulin-like growth factor 1 receptor antagonist (PPP). With PPP present, insulin did not significantly decrease evoked current amplitudes over time, even 20 minutes after adding insulin ($t_{(12)} = 1.21$, $p = 0.42$). However, since the changes in evoked current amplitudes over time with PPP was still not significantly different than the other treatments (Treatment*Times, $p = 0.49$; Figure 13), this suggests that insulin is not fully dependent on insulin-like growth factor 1 receptors to bind to DMH neurons.

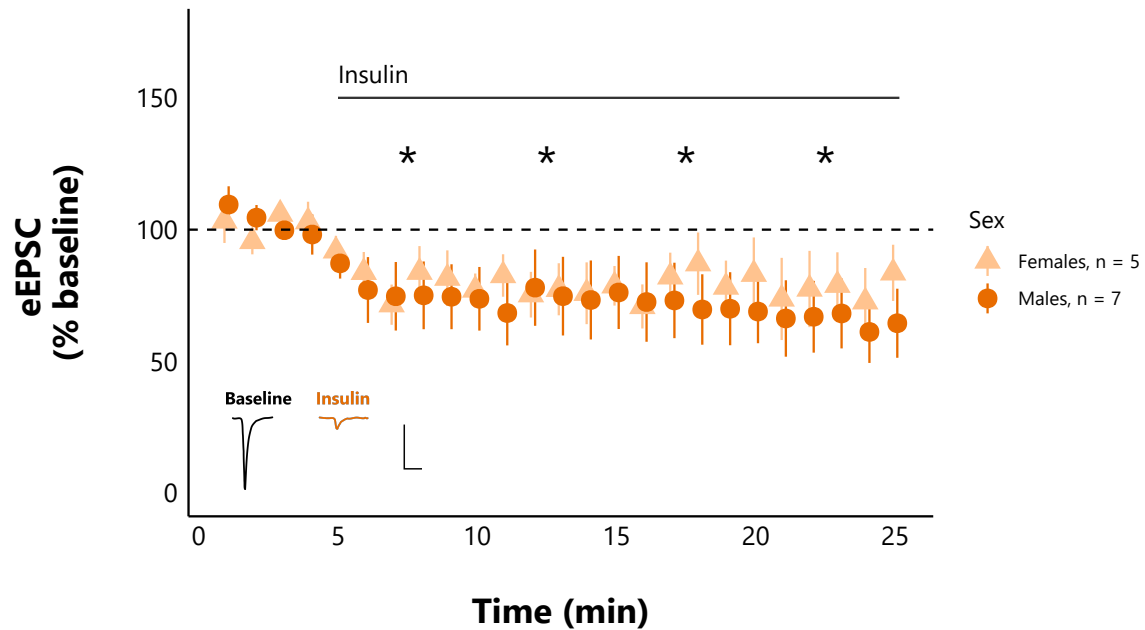


Figure 9: Insulin significantly decreases current amplitude over time in both sexes, even when the insulin receptor blocker HNMPA is applied. This suggests that insulin may not require insulin receptors to bind to DMH neurons and influence excitatory synaptic transmission. Each point represents the mean \pm SE eEPSC amplitude and n represents the number of unique cells. The asterisks indicate a statistically significant decrease in current amplitude relative to the baseline (t-test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). The representative traces consist of eEPSCs from one cell averaged over the baseline period (0 to 5 min) and last interval (20 to 25 min) of the recording, and the scale bar represents 50 pA/20 ms.

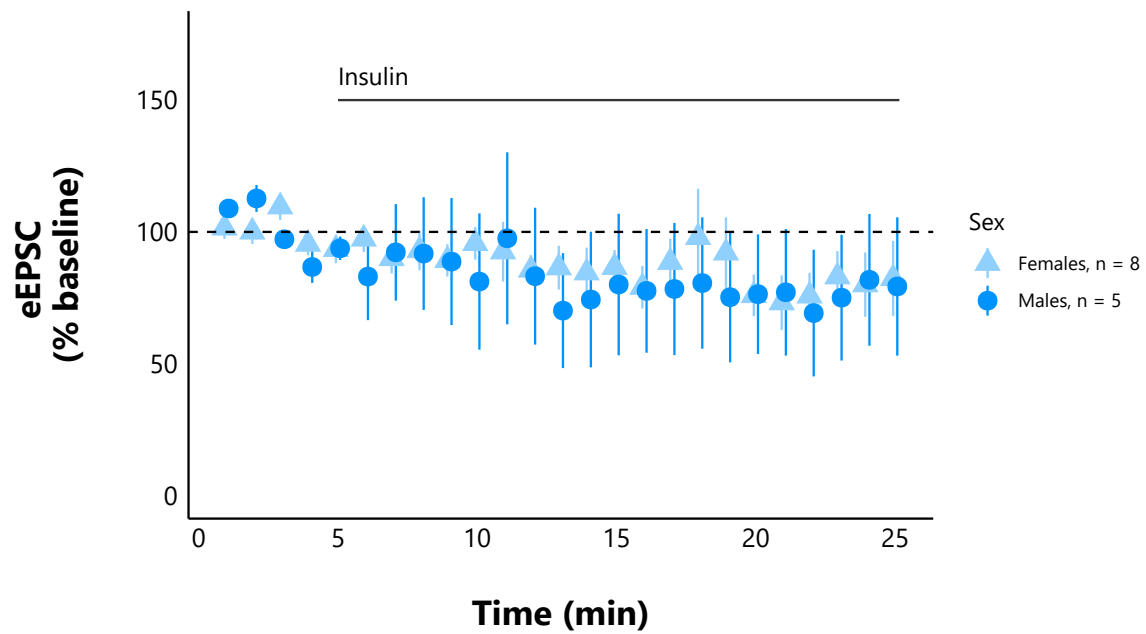


Figure 10: Insulin does not significantly decrease current amplitude over time with PPP present. PPP is an insulin-like growth factor 1 receptor antagonist. Since blocking these receptors reduced the changes in excitatory synaptic transmission seen in control recordings, insulin may require insulin-like growth factor 1 receptors to act on DMH neurons. Each point represents the mean \pm SE eEPSC amplitude and n represents the number of unique cells. The asterisks indicate a statistically significant decrease in current amplitude relative to the baseline (t-test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Feeding state does not affect insulin signalling in DMH neurons

Since insulin and DMH neurons are both involved with appetite and digestion (Dalton et al., 1981; De Meyts, 2000), I measured evoked excitatory currents over time in rats who had fasted 24 hours before brain removal. Hunger increases the activity of DMH neurons (Angeles-Castellanos et al., 2004), and it is also associated with lowered insulin levels in the bloodstream, which may alter insulin signalling in DMH neurons. Within the first 5 minutes of insulin exposure, evoked current amplitudes in the DMH neurons of fasted rats decreased significantly ($t(14) = 2.26$, $p = 0.041$; Figure 11).

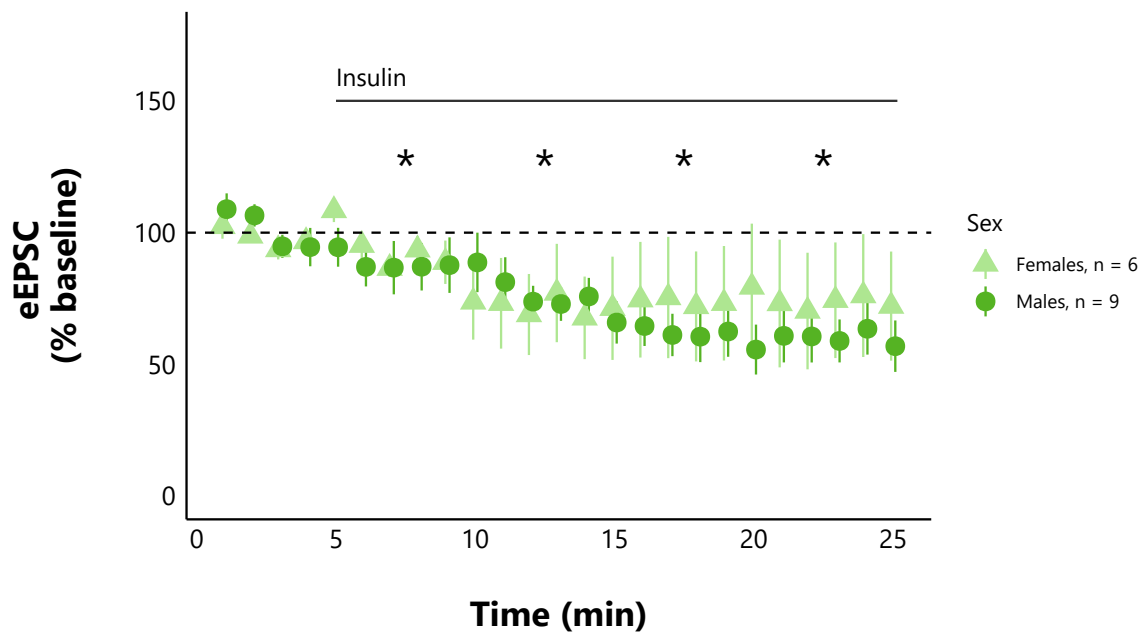


Figure 11: Insulin significantly decreases current amplitude over time in both sexes after a 24 hour fasting protocol. This suggests that insulin signalling in the DMH is associated with processes beyond appetite regulation. Each point represents the mean \pm SE eEPSC amplitude and n represents the number of unique cells. The asterisks indicate a statistically significant decrease in current amplitude relative to the baseline (t-test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Table 3: A paired t-test comparing the mean paired pulse ratio (PPR) per cell during the baseline period (0-5 min) to the mean PPR after insulin exposure (20-25 min) shows that insulin does not significantly affect the PPR during experiments with no additional treatments, HNMPA, or fasting. Insulin significantly increases the PPR when the insulin receptor blocker HNMPA is applied.

Parameter	Statistic	DF	<i>p</i> -value
Control	-1.05	21	0.30
Fasting	0.15	14	0.88
HNMPA	-3.64	11	0.004
PPP	-0.51	12	0.62

Synaptic transmission is likely affected by a postsynaptic mechanism

To further explore the mechanism of insulin signalling at the DMH, I used a paired t-test to quantify changes in the PPR over time (Figure 12). I used PPRs that were averaged per cell over two five-minute intervals: the baseline (0 to 5 minutes) and the last interval of the recording (20 to 25 minutes), which corresponded to 20 minutes of continuous insulin exposure. The PPR did not change significantly relative to baseline values in the control group ($t(21) = -1.05$, $p = 0.30$), fasted group ($t(14) = 0.15$, $p = 0.88$), or PPP-treated cells ($t(12) = -0.51$, $p = 0.62$). Since the PPR indicates the probability of neurotransmitter release from presynaptic cells, and there was no significant change in PPR with insulin, insulin binding to DMH neurons likely activates postsynaptic signalling pathways.

In the HNMPA treatment group, the paired pulse ratio increased significantly relative to the baseline ($t(11) = -3.64$, $p = 0.004$), suggesting that preventing insulin from binding to insulin receptors may activate an alternative presynaptic mechanism that ultimately results in the same decrease in excitatory synaptic transmission.

Neurons with similar insulin sensitivity are not clustered

Neurons within the DMH are not identical. Depending on their location, they can express receptors for different neurotransmitters and receive projections from different brain regions (Bailey et al., 2003; Horst

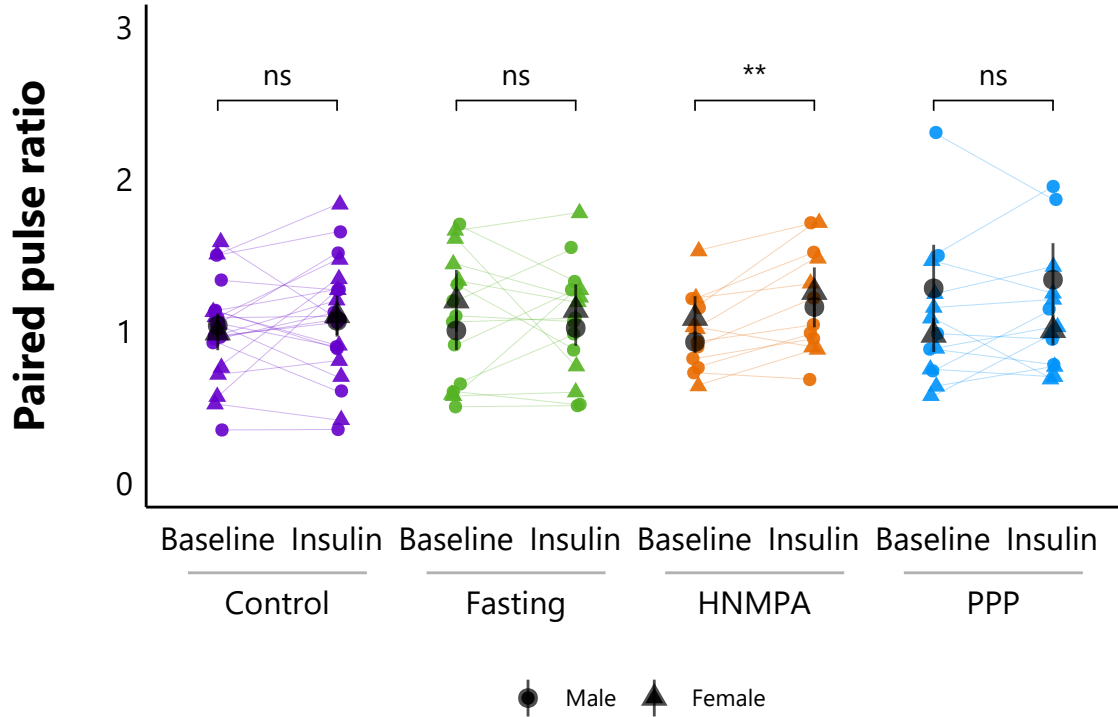


Figure 12: The paired pulse ratio (PPR) did not change significantly after insulin exposure for most of the treatment groups. The change in the PPR relative to the baseline was non-significant for the control, fasting, and PPP treatment groups, but it was statistically significant for the HNMPA-treated cells. Each dot represents the mean PPR of a single cell averaged over the baseline period (0 to 5 minutes) or the last 20 to 25 minutes of the evoked currents protocol. The asterisks indicate a statistically significant difference in the PPR between the two intervals (t-test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

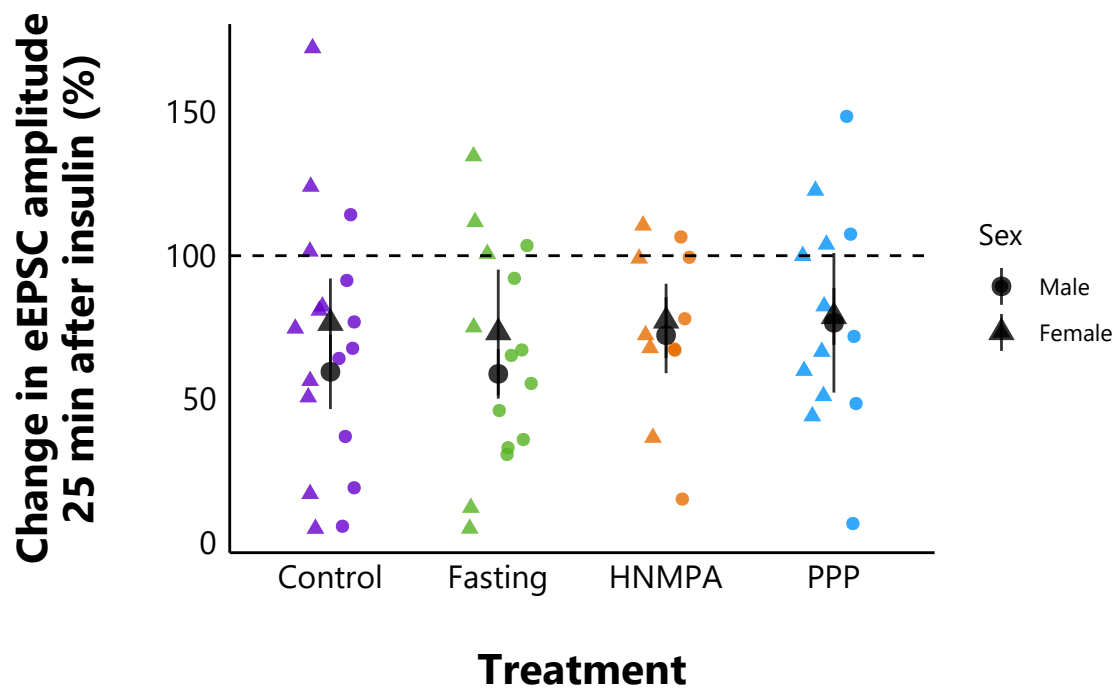


Figure 13: Insulin decreased eEPSC amplitudes in all treatments, with no significant differences between treatments. In this summary figure, each dot represents the mean eEPSC amplitude of a cell relative to the baseline (%) during the last 20-25 minutes of the evoked currents protocol. Black solid shapes indicate the mean \pm SE for each sex.

& Luiten, 1986; Thompson & Swanson, 1998). To see if any sub-populations within the DMH were more sensitive to insulin, I analyzed the spatial distribution of the cells in the study. I plotted the cells on a coordinate system and coloured them according to the percent decrease in evoked current amplitude 20 minutes of insulin exposure (Figure 14). Neurons with similar responses to insulin did not cluster together, indicating that there are no regions within the DMH that have a greater specificity to insulin signalling compared to the rest of the DMH.

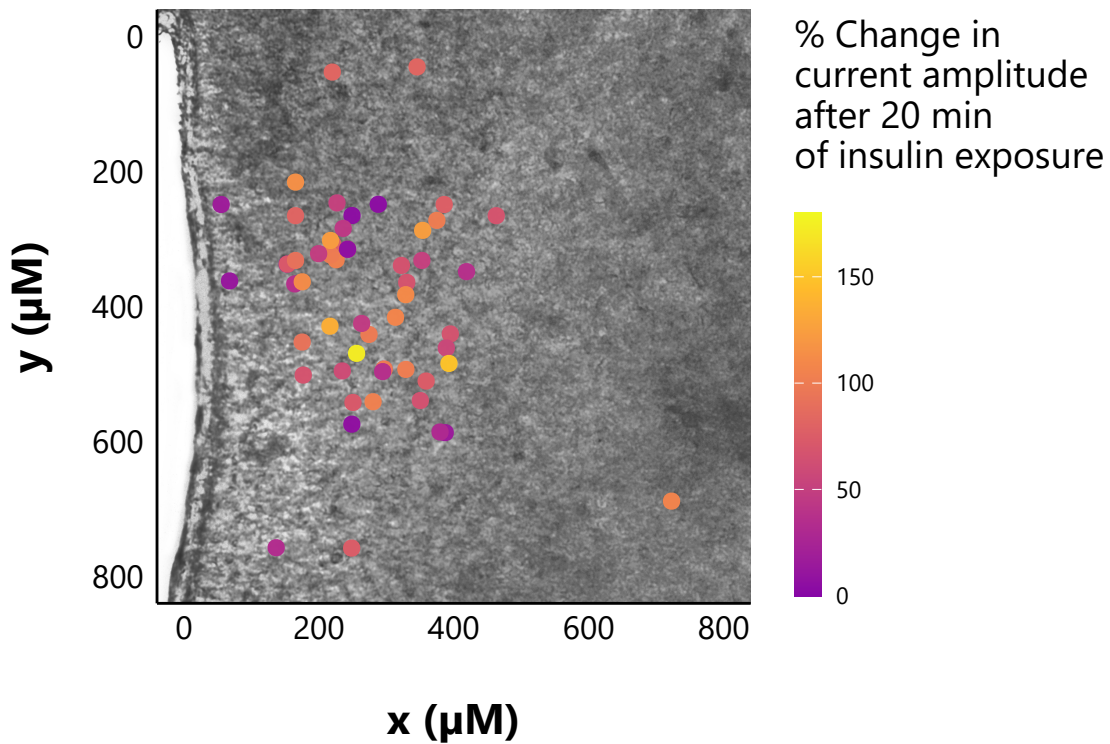


Figure 14: The neurons that I recorded were located in the DMH, and most neurons experienced a decrease in evoked current amplitudes (eEPSC) relative to baseline amplitudes. Each neuron is coloured according to the relative decrease in eEPSC amplitude after 20 minutes of insulin exposure. Neurons with similar responses to insulin did not cluster together, and there were no regions within the DMH that had a greater proportion of highly responsive neurons. In this figure (magnified 5X), the top of the third ventricle is located at (0,0).

Discussion

Insulin is a hormone that is closely associated with food intake, and the DMH is a critical brain region for appetite regulation. Although DMH neurons have receptors for insulin, we know little about the effect of insulin on neuronal activity in the DMH. I compared action potential properties before and after adding insulin to see if insulin affects the intrinsic excitability of DMH neurons. I also recorded current amplitudes in DMH neurons both before and after adding insulin to examine the effect of insulin on excitatory synaptic transmission. Overall, insulin significantly decreased both action potential frequency and evoked current amplitude in DMH neurons. These findings support the hypothesis and predictions of the study, with many implications for future studies on insulin signalling within the DMH.

How does insulin affect neuronal excitability?

After 20 minutes of insulin exposure, DMH neurons fired fewer action potentials, and of the action potentials that did fire, the amplitude was lower. Action potential frequency and amplitude are indicators of intrinsic excitability (Bean, 2007), so these results would suggest that DMH neurons are less excitable with insulin present. However, the action potential threshold decreased after insulin exposure. Since threshold is the depolarization required to induce an action potential, a lower threshold means that a neuron can more easily become depolarized to the threshold required to fire an action potential, suggesting an increase in intrinsic excitability. These findings are unexpected, as they indicate opposing changes in intrinsic excitability after insulin exposure.

One potential explanation for this effect comes from the significant increase in half-width that occurred after insulin exposure. Half-width is a measure of the repolarization rate (Bean, 2007), so a slower repolarization rate could indicate changes in action potential kinetics that decrease the neuron's ability to fire

action potentials. This may include a decrease in the function of A-type potassium ion channels following an action potential (Kim et al., 2005). The K_v4 potassium channel in particular has been shown to play a critical role in regulating action potential repolarization in hippocampal neurons (Kim et al., 2005). However, since the after-polarization amplitude and time of the after-hyperpolarization showed no significant changes, insulin is not likely delaying potassium influx. Further research is required to determine the mechanism leading to decreased AP firing despite a lowered action potential threshold.

How does insulin affect neuronal communication?

After being exposed to insulin, DMH neurons showed a significant decrease in evoked current amplitudes at glutamate synapses. This finding shows that insulin decreased excitatory synaptic transmission between DMH neurons. A decrease in excitatory synaptic transmission between DMH neurons could affect neuronal pathways associated with processes like energy metabolism. Insulin signalling likely does not affect neuronal pathways involved with appetite regulation, since changes in excitatory synaptic transmission were independent of feeding state.

I found that evoked current amplitudes continued to decrease even in the presence of the receptor blocker HNMPA. Since blocking insulin receptors did not change the decrease in evoked current amplitude seen in the control group, insulin likely does not use insulin receptors to bind to DMH neurons. When PPP (an insulin-like growth factor 1 receptor antagonist) was present, the evoked current amplitudes did not decrease significantly after insulin exposure. Since blocking insulin-like growth factor 1 receptors removed the decrease in excitatory synaptic transmission seen in the control group, this suggests that insulin requires insulin-like growth factor 1 receptors to influence synaptic transmission in the DMH.

What is the mechanism of insulin signalling in the DMH?

The paired pulse ratio was unchanged after insulin exposure for the control, fasting and PPP groups, indicating that insulin likely decreases excitatory synaptic transmission through a post-synaptic mechanism. One potential mechanism is the recruitment of mechanistic target of rapamycin (mTOR) through the

phosphatidylinositol 3-kinase (PI₃K) pathway. mTOR is a serine/threonine protein kinase that has been shown to regulate excitatory synaptic transmission (McCabe et al., 2020). mTOR regulates the number of AMPA receptors expressed on the cell surface (Wang et al., 2006), which then affects the ability of a cell to respond to glutamate. If mTOR were to reduce the number of AMPA receptors, this would decrease excitatory synaptic transmission since there would be fewer receptors available for glutamate to bind to.

A second post-synaptic mechanism of decreased excitatory synaptic transmission could be changes in the number of active ion channels on cell surfaces (O'Malley et al., 2003). In the hippocampus, insulin can inhibit neuronal activity through the activation of ATP-sensitive potassium (K_{ATP}) channels (O'Malley et al., 2003), and there is evidence that there may be a body-weight-dependent mechanism of insulin/K_{ATP} signalling in hypothalamic neurons (Spanswick et al., 2000). K_{ATP} channels are activated through the PI₃K pathway, which can be activated through both insulin receptors and insulin-like growth factor 1 receptors (De Meyts, 2000).

When HNMPA was present, the paired pulse ratio increased significantly after insulin exposure. This suggests that blocking insulin receptors causes insulin to use a pre-synaptic mechanism to decrease evoked current amplitudes. This may have been through the activation of the mTORC2 complex, which has been shown to decrease neurotransmitter release through pre-synaptic mechanisms (McCabe et al., 2020).

Does sex matter?

In all treatments, DMH neurons responded similarly to insulin in males and females. When observing changes in evoked current amplitudes, I found no significant differences between the sexes over time. These findings suggest that there are no sex-based differences in the intracellular pathways affected by insulin. However, this does not mean that insulin signalling is completely independent of sex. All rats were younger than 44 days old, which is less than the average onset of puberty (McCutcheon & Marinelli, 2009). At this age, many sex-based differences are not yet present. Experiments with older animals are required before any conclusive sex-based differences can be determined.

What are the next steps?

We now know that insulin decreases excitatory current amplitudes in DMH neurons, which provides opportunities for research on insulin signalling in the DMH. The goal of future studies is to learn which specific processes insulin initiates when binding to DMH neurons, and how this affects neuronal communication, and ultimately physiology. The insulin receptor blocker experiments suggested that insulin-like growth factor 1 receptors are responsible for insulin binding in the DMH. Recordings conducted in the presence of both receptor antagonists could confirm if insulin binds primarily to insulin-like growth factor 1 receptors, insulin receptors, a mixture of both receptors, or a novel receptor not directly associated with insulin.

Since each of these receptors is associated with different pathways within cells (De Meyts, 2000), identifying the binding mechanism of insulin on DMH neurons could provide insight into the specific processes that insulin uses to influence neuronal communication. As mTOR is an important potential source of insulin-signalling in the DMH, studies with antagonists of the $P_{13}K$ pathway, mTOR its downstream effectors could provide information on the specific intracellular processes that influence neuronal activity and synaptic transmission.

There are also opportunities for whole-animal studies, such as injecting insulin into the DMH and monitoring changes in body weight and energy metabolism. This study also raises new questions about the effects of health on insulin signalling in the DMH. The rats used in this study were young and healthy. Could conditions like diabetes, which affects insulin levels in the body, affect DMH neurons? Would DMH neurons respond differently in animals with health conditions like obesity? How would insulin signalling in the DMH change in older animals?

Why does this matter?

This research will contribute to our understanding of the mechanisms of the hormonal control of energy metabolism in mammals, with many potential applications to human health. Exploring insulin signalling within the DMH also contributes to the broader research goal of understanding the actions of insulin in

the brain, and its involvement with energy metabolism. These findings have direct applications to understanding the effects of health conditions like diabetes, where the brain experiences reduced insulin levels and/or impaired insulin signalling (Blázquez et al., 2014). Learning more about the interactions between insulin and DMH neurons could also provide insight into long-term changes in appetite associated with obesity, with the possibility of contributing to the development of new obesity therapies.

Conclusions

Insulin acts in the DMH to decrease the amount of neuronal communication between neurons and alter intrinsic excitability. It achieves this response by binding to a receptor (likely an insulin-like growth factor 1 receptor), to activate an intracellular pathway that decreases excitatory synaptic transmission and action potential frequency. These findings contribute to research on the neurophysiological mechanisms of energy metabolism, with many implications for understanding the pathophysiological effects of diabetes and obesity.

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