

THE EFFECT OF NITRIC OXIDE ON ENDOCANNABINOID SIGNALING AT  
GLUTAMATE SYNAPSES IN THE RAT DORSOMEDIAL HYPOTHALAMUS

BY

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## Abstract

Endocannabinoids (eCBs) and nitric oxide (NO) are both retrograde neurotransmitters that are made in the postsynaptic cell in response to increases in intracellular calcium. eCBs target type I cannabinoid receptors (CB1Rs), located on the presynaptic membrane, while NO binds soluble guanylate cyclase (sGC) inside the presynaptic cell. Both neurotransmitters can act presynaptically to regulate GABA and glutamate transmission. Previous studies have shown an interaction between NO and eCB signaling. NO prevented eCB-mediated decrease in glutamate transmission in the dorsomedial nucleus of the hypothalamus (DMH). Furthermore, another study within the DMH found that NO was required for eCB-mediated decrease in GABA transmission. There is little research, however, on the mechanism behind the interactions between eCBs and NO. The DMH has been a major focus for research due to its importance in appetite regulation. We aimed to determine how NO affects eCB signaling at glutamatergic synapses in the rat DMH. We hypothesized that NO affects eCB-mediated decrease in glutamate transmission through an NMDA, and cGMP, -dependent pathway. To test our hypothesis, male Sprague Dawley rats were used as subjects, and whole-cell electrophysiological recordings were taken at glutamate synapses in live DMH neurons. Our results show that NO disrupts eCB-mediated depression through an NMDA receptor-dependent pathway. Additionally, we further targeted the NO pathway and concluded that NO is likely affecting eCB signaling from the postsynaptic cell. We also performed an experiment targeting CB1R activation by an agonist. Previous data showed that activation of CB1Rs by an agonist significantly decreased glutamate release. We repeated this experiment in the presence of L-arginine, a NO precursor, and observed that the agonist was still able to decrease glutamate signaling. Finally, we wanted to determine if NO is affecting short-term eCB signaling. Previous research has suggested that NO does not affect short-term synaptic plasticity at glutamate synapses in the hippocampus. Our data was consistent with this as we did not see a change in short-term glutamate transmission. Overall, figuring out how these two retrograde neurotransmitters interact to affect synaptic transmission can extend well beyond appetite regulation as these neurotransmitters are ubiquitously produced at synapses in various parts of the brain.

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## List of Important Abbreviations

Abbreviation	Definition
2-AG	2-Arachidonoylglycerol
aCSF	Artificial cerebrospinal fluid
AEA	Anandamide
AM251	CB1R antagonist
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	2-amino-5-phosphonopentanoic acid
Carboxy-PTIO	carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CB1R	Cannabinoid type I receptor
DSE/DSI	Depolarization-induced suppression of excitation (DSE) or inhibition (DSI)
eCB	Endocannabinoid
eEPSC	Evoked excitatory postsynaptic current
GABA	$\gamma$ -aminobutyric acid
HFS	High-frequency stimulation
L-NAME	N- $\omega$ -nitro-l-arginine methyl ester
LTD	Long-term depression
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PPR	Paired pulse ratio
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
sEPSC	Spontaneous excitatory postsynaptic current
TRPV1	Transient receptor potential vanilloid type I

## **1. Introduction**

### **1.1 The Brain**

The brain is arguably the most important and most studied organ in the human body. Its complexity is what makes it an intriguing organ for researchers. Our understanding of the function of different brain regions largely stems from early lesioning studies (Müller and Knight, 2006). More recently, however, the discovery of new techniques, such as optogenetics and neuroimaging, has allowed us to further our understanding of the brain (Batista-García-Ramó and Fernández-Verdecia, 2018; Guru et al., 2015). The brain is structured bilaterally and consists of the cerebellum, medulla oblongata, pons, midbrain, diencephalon, and cerebral hemispheres (Kandel et al., 2000). The diencephalon, however, is of particular interest for this study, as it contains the hypothalamus, which is important in regulating autonomic, endocrine, and visceral systems (Kandel et al., 2000).

### **1.2 The Hypothalamus**

The hypothalamus is located below the thalamus and is comprised of several nuclei (Gao and Sun, 2016). The hypothalamus regulates a myriad of vital functions such as food and water intake, energy expenditure, osmoregulation, thermoregulation, arousal, stress responses, and endocrine responses (Saper and Lowell, 2014). It can regulate these functions by integrating sensory information and inducing physiological changes to maintain homeostasis. For example, it can do this to change body temperature, blood salt and glucose levels, and hormonal imbalances (Saper and Lowell, 2014). There are also studies that highlight the importance of the hypothalamus in regulating satiety signaling (Larsson, 1954; Bernardis et al., 1970). Within the hypothalamus, there are many hypothalamic nuclei that regulate food intake, such as the lateral, ventromedial, dorsomedial, paraventricular, and arcuate nuclei (Roger et al., 2022). We targeted a cluster of these neurons, the dorsomedial nuclei of the hypothalamus.

### **1.3 The DMH and Satiety Signaling**

The dorsomedial nucleus of the hypothalamus (DMH) is known to regulate appetite in vertebrates. The DMH is located adjacent to the walls of the third ventricle, posterior to the paraventricular nucleus (PVN), dorsal to the ventromedial nucleus (VMN), and ventral to the zona incerta (Thompson et al., 1996). The projections in the DMH have been widely studied, with most of these projections being intrahypothalamic (Thompson et al., 1996). The DMH has

been documented to be involved in food intake. Larsson (1954) sought to determine the role of the DMH in the feeding behaviour of sheep and found that electrical stimulation of the DMH increased their feeding activity (Draper et al., 2010). Furthermore, Bernardis et al. (1970) strengthened the findings of Larsson (1954) by demonstrating in their study that DMH-lesioned rats had growth regression, ate less, and drank less than their non-lesioned counterparts (Draper et al., 2010). Moreover, an optogenetic study performed by Jeong et al. (2017) found that cholinergic neurons in the rat DMH increased feeding intake. Altogether, these findings propose that the DMH regulates appetite control. This appetite regulation is presumably mediated by the release of neurotransmitters, which regulate the way that neurons in the DMH communicate with each other (Bernardis, 1970; Draper et al., 2010; Jeong et al., 2017; Larsson, 1954; Thompson et al., 1996).

#### **1.4 Classical Neurotransmitters**

Neurons in the DMH communicate with each other through the release of neurotransmitters (Luján et al., 2005). Neurotransmitters are released from the presynaptic terminal and typically act on postsynaptic membrane receptors. Prominent examples of neurotransmitters include  $\gamma$ -aminobutyric acid (GABA) and glutamate. The former is the brain's primary inhibitory neurotransmitter, while the latter is the primary excitatory neurotransmitter. These neurotransmitters act on fast-acting ion channels, as well as G-coupled protein receptors, to regulate synaptic transmission. Synaptic transmission is different from other forms of cellular communication (such as electrical conduction in myocardial cells) as it provides more variance in its ability to mediate communication. Synaptic communication can be mediated by changes in neurotransmitter composition, breakdown, intensity of effect, and speed of release (Fon and Edwards, 2001; Hyman, 2005).

Classical neurotransmitters, such as GABA and glutamate, are packaged into synaptic vesicles in the axon terminal. The contents within the vesicles are released into the synaptic cleft in response to an increase in intracellular  $\text{Ca}^{2+}$  through an exocytotic process (Fon and Edwards, 2001). Once released into the synaptic cleft, these neurotransmitters typically target postsynaptic membrane-bound receptors. Both of these neurotransmitters can bind more than one receptor: GABA acts on:  $\text{GABA}_A$ , and  $\text{GABA}_B$ , while glutamate can bind: *N*-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), metabotropic, and kainate receptors (Chebib and Johnston, 1999; Greengard, 2001). Although most

neurotransmitters target the postsynaptic membrane, there exist a special class of neurotransmitters that are released postsynaptically, and subsequently act on presynaptic receptors. These special molecules are classified as retrograde neurotransmitters due to their directionality of effect.

### **1.5 Retrograde neurotransmitters**

Retrograde neurotransmitters are a special class of neurotransmitters that are synthesized in response to increases in postsynaptic  $\text{Ca}^{2+}$  and travel backwards across the synapse to exert their effects. Endogenous cannabinoids (eCBs) fall within this class of neurotransmitters. With the widespread use of cannabis, eCBs have become a major focus for research. The molecule responsible for the physiological changes occurring due to cannabis consumption is  $\Delta^9$ -tetrahydrocannabinol (THC). THC is an exogenous cannabinoid that binds to cannabinoid receptors (Mechoulam et al., 1998). There are two receptors that are expressed in vertebrates, type I and type II cannabinoid receptors (CB1Rs and CB2Rs, respectively) (Dietrich and McDaniel, 2004). The former are mostly expressed in the central nervous system (CNS), while the latter are usually restricted to the periphery (Dietrich and McDaniel 2004; Biringer, 2021). Within the CNS, CB1Rs are shown to be expressed in the cortex, hippocampus, basal ganglia, amygdala, hypothalamus, and the cerebellum (Dietrich and McDaniel, 2004). Since receptors are made to bind endogenous compounds, studies began to search for endogenous molecules specific for these receptors (Pacher et al., 2020; Zou and Kumar, 2018). This led to the discovery of endogenous cannabinoids; of these, anandamide (AEA), and 2-arachidonylglycerol (2-AG) are the most well-studied (Dietrich and McDaniel, 2004; Pacher et al., 2020; Zou and Kumar, 2018). Endocannabinoids are lipophilic (or hydrophobic) molecules that act on receptors located on the presynaptic membrane (Zou and Kumar, 2018). Although 2-AG and AEA are both present in the CNS, concentrations for 2-AG exceed that of AEA by 200-fold (Dietrich and McDaniel, 2004). Thus, 2-AG may be largely responsible for the eCB-mediated effects in the CNS. eCBs are made postsynaptically in response to increases in intracellular calcium by  $\text{Ca}^{2+}$ -dependent enzymes including diacylglycerol lipase  $\alpha$  (DGL $\alpha$ ) and N-arachidonoyl phosphatidyl ethanol phospholipase D (NAPE-PLD), for 2-AG and anandamide synthesis, respectively (Gambino et al., 2020; Lu and Mackie, 2016). Upon release from the postsynaptic cell, these neurotransmitters can target CB1Rs expressed at GABAergic and glutamatergic synapses in the brain (Pacher et al., 2006).

Once eCBs bind CB1Rs, subsequent changes are modulated through an eCB-mediated signaling cascade that inhibits adenylyl cyclase and subsequent production of cyclic AMP (cAMP) (Gambino et al., 2020). More specifically,  $\beta\gamma$  subunits of G-proteins coupled to CB1Rs, decrease intracellular  $\text{Ca}^{2+}$  in the presynaptic cell through inhibition of voltage-gated  $\text{Ca}^{2+}$  channels or activation of  $\text{K}^+$  channels; both of which make the cell less likely to release neurotransmitter (Dietrich and McDaniel, 2004). As a result, eCBs decrease neurotransmitter release from the presynaptic axon terminal, this is referred to as eCB-mediated depression (Chevalleyre et al., 2007). This process can be triggered by depolarizations or high-frequency stimulation (HFS). The former triggers minor increases in intracellular postsynaptic  $\text{Ca}^{2+}$  and can trigger depolarization-induced suppression of inhibition or excitation (DSI and DSE; respectively), while the latter triggers a more pronounced increase in calcium (Chevalleyre et al., 2007). Unlike, DSI and DSE, long-term changes to synaptic plasticity require long induction protocols such as HFS (Diana and Marty, 2004). HFS can lead to long-lasting changes in synaptic strength that are mediated by eCBs. Specifically, eCBs can trigger long-term depression (LTD), that is mediated by CB1R activation and a long-lasting decrease in neurotransmitter release. This has been documented at glutamate synapses in many brain regions including the striatum, nucleus accumbens, and in the cortex (Diana and Marty, 2004; Gerdeman et al., 2002; Robbe et al., 2002; Sjöström et al., 2003). Finally, AEA and 2-AG are broken down by their respective enzymes, fatty acid amide hydrolase (FAAH), and monoacylglycerol lipase (MAGL) (Abán et al., 2018).

eCBs are not the only retrograde neurotransmitters that can affect synaptic transmission in the DMH. Nitric oxide (NO) is a retrograde signaler that also controls the release of other classical neurotransmitters, such as glutamate and GABA (Lipina and Hundal, 2017). NO is a signaling molecule produced in many organisms, including mammals (Stefano et al., 2003). NO is well known for its role as a vasodilator (Levine et al., 2012); however, it also acts as a neurotransmitter in the CNS to affect a wide range of physiological functions.

NO is produced from the precursor L-arginine by the enzyme nitric oxide synthase (NOS) (Stefano et al., 2003). NOS is found in different cells and exists as different isoforms: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) (Stefano et al., 2003). eNOS and nNOS are  $\text{Ca}^{2+}$ -dependent isozymes while iNOS is induced by cytokines through a  $\text{Ca}^{2+}$ -independent mechanism (Prast and Philippu, 2001).

In the brain, NO is synthesized in response to nNOS function. nNOS function is stimulated by increases in intracellular  $\text{Ca}^{2+}$  which are induced by activation of NMDA receptors (Prast and Philippu, 2001). Subsequently, NO targets soluble guanylyl cyclase (sGC), the enzyme responsible for producing cyclic guanosine monophosphate (cGMP) (Abán et al., 2018; Prast and Philippu, 2001) which is located inside the cell. Subsequent changes in synaptic transmission can occur due to NO-induced activation of cGMP-dependent protein kinases (Prast and Philippu, 2001). Previous research suggests that NO also modulates the release of specific neurotransmitters (Kuriyama and Ohkuma, 1995). NMDA receptor activation, which subsequently triggers NO synthesis, led to increases in GABA and acetylcholine (ACh) release at striatal neurons near the basal ganglia (Kuriyama and Ohkuma, 1995).

### **1.6 Nitric Oxide and Endocannabinoid Interactions**

eCBs and NO are both retrograde neurotransmitters synthesized in response to increases in intracellular calcium and accumulating evidence suggests that they can interact with one another to influence neuronal function. Crosby et al. (2011) demonstrated that NO increases GABA release while eCBs decrease GABA release at synapses in the DMH with NO being required for the effects of eCBs (Crosby et al., 2011). NO has been shown to enhance glutamate release onto DMH neurons (Poole et al., 2020), but whether eCBs and NO interact to modulate glutamate release remains unknown.

The role of NO in the eCB pathway remains to be discovered. Previous research from the Crosby laboratory suggests that NO prevents eCB-mediated depression at glutamate synapses in the DMH; however, the mechanism behind this has not yet been investigated (Sukkar, 2021).

### **1.7 Current Study**

In this study, we investigated the relationship between NO and eCB at glutamate synapses in the DMH. Previous research has suggested an intricate link between NO and eCBs at GABA synapses (Crosby et al., 2011). NO has also been shown to affect glutamate signaling onto DMH neurons and preliminary data suggests that NO prevents eCB-mediated depression at these same synapses (Sukkar, 2021). Therefore, we used these preliminary findings as a gateway to determine how NO is involved in eCB signaling at glutamate synapses in the DMH of male Sprague-Dawley rats.

We hypothesized that NO affects eCB signaling through an NMDA, and cGMP, - dependent pathway at glutamate synapses in the DMH. We conducted patch clamp

electrophysiology studies and examined the effect of eCB signaling in the presence of various inhibitors of the NO pathway. As such, eCBs were stimulated, due to an influx of  $\text{Ca}^{2+}$ , caused by high-frequency stimulation (HFS) or depolarizations, under different conditions of drug treatment to understand the pathway through which NO modulates eCB signaling. These findings will better our understanding of the mechanism behind the effect of NO on eCB signaling in the rat DMH.

## **2. Methods**

### **2.1 Test Subjects**

The research for this study was conducted on male Sprague-Dawley rats obtained from Charles River Laboratories in Montréal, Québec, Canada. The rats were received from Charles River Laboratories at 23 days postnatal (p23) and were housed in enclosed polycarbonate cages in groups of three to four to promote socialization. The relative humidity was held constant varying between  $50\pm 10\%$ , and light exposure was also held constant through daily 12-hour light/dark cycles beginning at 7:30 am. Rats were provided with food and water daily and cages were cleaned before and after housing animals. Furthermore, bedding and leisure were provided using wooden shavings, and items such as wooden blocks and nylabones, respectively. Rats were monitored daily for any signs or symptoms of discomfort or stress.

This research design followed the code of conduct outlined by the Canadian Council for Animal Care and this protocol was approved by the University's Animal Care Committee (#108033).

### **2.2 Slice Preparation**

Beginning between the hours of 7:00am-10:00am in the morning, a rat, aged between 25-39 days postnatal, was removed from its cage and placed in a chamber containing 5% isoflurane in oxygen; a piece of paper towel wetted with 3-4 mL of isoflurane was also placed within the chamber. Upon anesthetization, which was determined to be the point at which no signs of breathing were noticeable, the rat was placed in a guillotine (Kent Scientific) and decapitated. Using scissors and forceps, the brain was quickly removed and placed in a slicing solution. The contents of the slicing solution were as follows (in mM): 87 NaCl, 2.5 KCl, 25  $\text{NaHCO}_3$ , 0.5  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ , 7  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ , 1.25  $\text{NaH}_2\text{PO}_4$ , 25 glucose, and 75 sucrose. This solution was prepared in the morning prior to brain removal and bubbled for 20 minutes with 95%/5%  $\text{O}_2/\text{CO}_2$  gas.

The brain was removed from the slicing solution and placed ventral side down on a petri dish. The brain was cut to isolate the DMH using a razor blade. Swift cuts were performed, removing coronal sections of the brain, as well as a horizontal cut along the dorsal side of the brain. The brain was then placed on a stage to proceed with slicing. To stabilize the brain, crazy glue was applied to glue the brain posterior side down. A piece of agar was also glued to the ventral side of the brain to further prevent sliding of the brain on the stage. The stage was secured into a microtome (Leica, Nussloch, Germany), filled with slicing solution while being bubbled by the 95%/5% O<sub>2</sub>/CO<sub>2</sub> gas, and the brain was sliced into 250 μM coronal slices. Slices containing the DMH were identified using The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 2009) and were cut down the midline and along the third ventricle to produce two hemi-sections. The slices produced from the microtome were stored in a beaker containing a plastic mesh rack. This beaker was filled with artificial cerebrospinal fluid (aCSF), which was constantly oxygenated with 95%/5% O<sub>2</sub>/CO<sub>2</sub> gas to ensure healthy brain cells. The contents of the aCSF were as follows (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub>•2H<sub>2</sub>O, 1.5 MgCl<sub>2</sub>•6H<sub>2</sub>O, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, and 10 glucose (Sigma Aldrich, Ontario, Canada). aCSF was kept refrigerated and was used no more than two days in a row. The beaker, containing the aCSF and slices, was held at 32°C in a water bath.

### **2.3 Patch Clamp Electrophysiology**

After a one-hour recovery period, an individual slice containing the DMH was removed with a pipette and weighed down in a bath. The bath was perfused with a steady flow of aCSF at a rate ranging between 0.5 – 2 mL/min. The DMH and third ventricle were positioned along the centre of the bath for better visualization. Visualization was obtained using a water immersion microscope (Olympus, Center Valley, PA) and Infinity 2 camera (Lumenera, Ottawa, ON) which was connected to a computer monitor. Micropipettes were generated with a P-2000 micropipette puller (Sutter Instruments, Novato, CA). This device heated borosilicate glass to produce two micropipettes of equal length with a tip resistance of approximately 5.0-8.0 MΩ.

Using a 1 mL syringe, a recording micropipette was filled with an internal solution which contained (in mM): 108 potassium gluconate, 8 KCl, 8 sodium gluconate, 1 potassium EGTA, 10 HEPES, 2 MgCl<sub>2</sub>, 4 potassium ATP, 0.3 sodium GTP, and sterilized water (Sigma Aldrich, Ontario, Canada). To simulate *in vivo* conditions, this solution was brought up to pH 7.2 with KOH and was also corrected to be within the osmolality range of 285-300 mOsm. Upon addition

of the internal solution, the recording microelectrode was placed in the bath and the tip resistance (in  $M\Omega$ ) was recorded. If the micropipette's access did not meet the suggested parameters, it was discarded. Picrotoxin (100  $\mu$ M; Tocris, Ellisville, MO) was administered along with the aCSF to block GABA<sub>A</sub> receptors, which allowed us to focus on glutamatergic terminals in the DMH. Using 40x magnification, cells in the DMH were located adjacent to the third ventricle. Once a cell was targeted, a stimulating electrode filled with aCSF was applied to stimulate activity at glutamatergic terminals in the DMH.

Excitatory post synaptic currents (EPSCs) were triggered by the stimulating electrode at a rate of 0.2 Hz. Furthermore, to evaluate the paired-pulse ratio (PPR) (the ratio between the first and second current), pairs of evoked currents were applied 50 ms apart. In some of the experiments, a high-frequency stimulation (HFS) protocol was used to stimulate nearby axons for 4s at 100 Hz, and again, 20s later. In a different subset of experiments, DMH neurons were depolarized to +20 mV for 5s, and again, 5 minutes later. We then repeated the latter experiment, except the depolarization period was extended to 10s. Finally, in a different experiment, we activated CB1Rs by administering WIN 55,212-2 (CB1R agonist) onto the brain slice 5 minutes after baseline recording.

Once the recording electrode achieved access to the cell, 1 kHz-filtered current traces were picked up on the Multiclamp700B amplifier (Molecular Devices, Union City, CA) and displayed on a computer monitor. These traces were digitized at 10 kHz using the Digidata 1322 device (Molecular Devices). To determine the cell's resting membrane potential and action potential firing, current-clamp mode was applied. This allowed us to determine if a neuron was healthy enough before continuing with current amplitude measurements. Current-clamp mode controls the amount of current within the cell to hold the cell at a desired voltage. Upon reaching a membrane potential of  $-70$  mV, the *in vivo* resting membrane potential, the cell was hyperpolarized and subsequently depolarized over 10 fast steps ranging from  $-100$  mV to  $-10$  mV. Voltage-clamp was then used to record EPSCs while holding each cell at  $-70$  mV. At least five minutes of baseline recording was taken before stimulating protocols (HFS or depolarizations) or WIN 55,212-2 (CB1R agonist) were administered, and then 25 minutes (35 minutes for the WIN 55,212-2 group) of recording was taken place thereafter. Furthermore, at intervals of 5 minutes, access to the cell was determined by the access resistance and if access resistance increased by 20% or more throughout the recording, that cell was not included in

analysis. Trace recordings were stored in Clampfit 10 software (Molecular Devices) for further analysis. Peak amplitudes of currents (pA) taken after stimulation were then compared to those as part of the baseline recording period.

## **2.4 Investigating the Mechanism between Nitric Oxide and Endocannabinoid Signaling**

Several drugs were used as part of this experiment to investigate the mechanism between NO and eCBs. To target the NO and eCB interaction, the following drugs were administered: N- $\omega$ -nitro-L-arginine methyl ester (L-NAME; 200  $\mu$ M; a nitric oxide synthase inhibitor), WIN 55,212-2 (5  $\mu$ M; CB1R agonist), L-arginine (10  $\mu$ M; nitric oxide precursor), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10  $\mu$ M; soluble guanylate cyclase inhibitor), 2-amino-5-phosphonopentanoic acid (APV; 5  $\mu$ M; NMDA receptor antagonist), carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy-PTIO; 30  $\mu$ M; nitric oxide scavenger), and AM251 (5  $\mu$ M; a CB1R antagonist).

## **2.5 Statistical analysis**

Statistical analysis was conducted using RStudio. Baseline measurements were taken for 5 minutes before HFS or WIN 55,212-2 administrations. Post-baseline measurements were taken 20-25 minutes into the recording of HFS cells, and 35-40 minutes into the recording of WIN 55,212-2 cells. To analyze short-term changes in synaptic plasticity, depolarization groups had statistical analysis conducted on 1 minute of baseline before depolarization and 2 minutes after depolarization. This was averaged and baseline corrected over both the first, and second, depolarization. All our results are expressed as means  $\pm$  S.E.M. The Shapiro-Wilk normality test was used to test assumptions of the data. The L-NAME 5-second depolarization group did not pass normality, therefore, it had to be log-transformed to meet assumptions of normality. To test mean differences in current amplitude between baseline and post-baseline measurements we used a paired t-test. Values were considered statistically significant if  $p < 0.05$ .

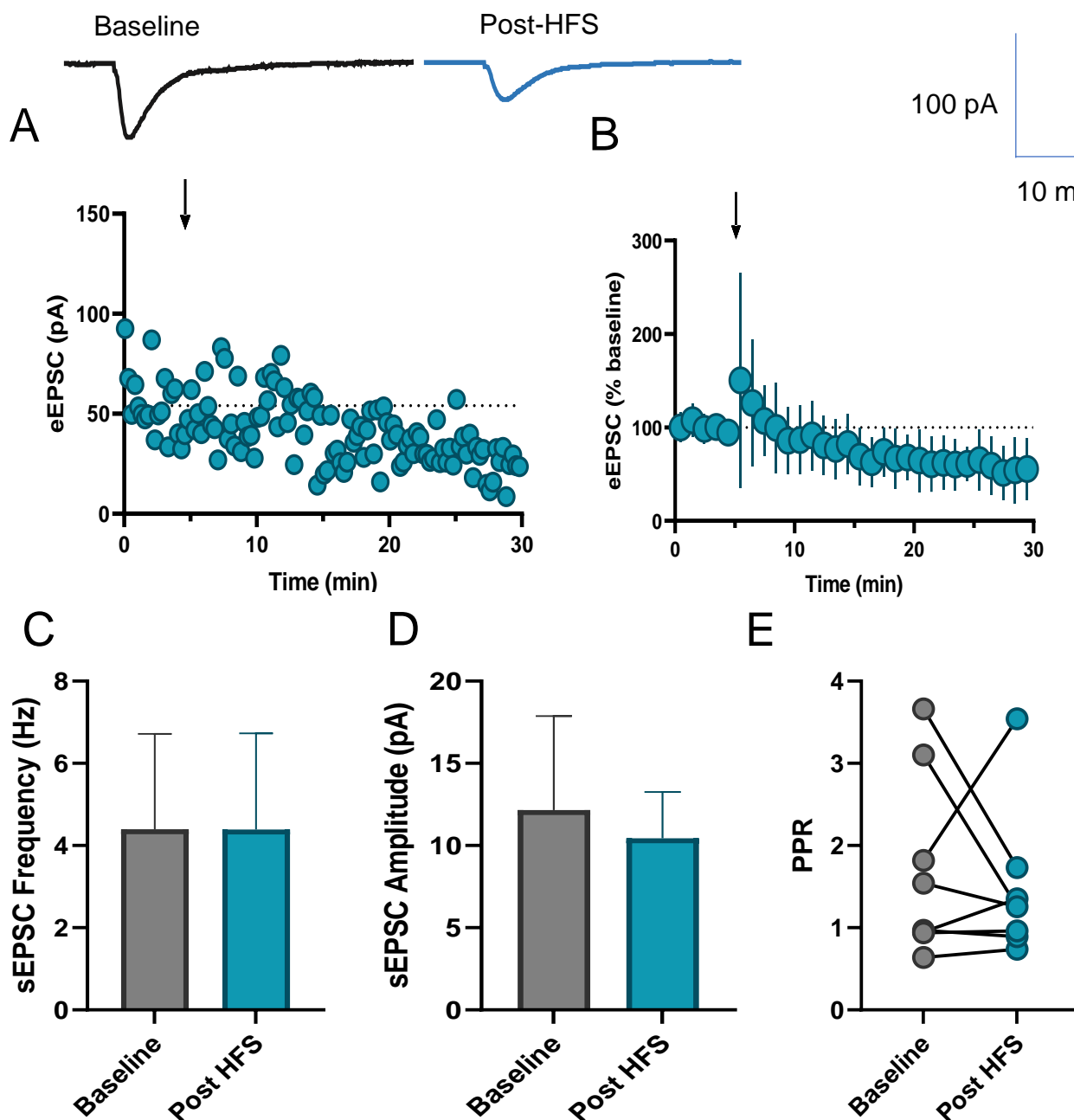
## **3. Results**

eCBs and NO have been shown to interact at GABA synapses in the brain. Although NO is needed for eCB-mediated depression at GABA synapses, less is known about its effects at glutamate synapses (Crosby et al., 2011). This research aimed to investigate the effects of NO on eCB signaling at glutamate synapses in the DMH. Understanding how these ubiquitous signals interact in the DMH could help us understand how DMH neurons become excited or inhibited, and how that potentially regulates food intake.

### 3.1 Blocking NMDA Receptors With APV Leads to LTD at Glutamate Synapses

There is compelling evidence that suggests that NO affects eCB signaling at synapses in the DMH (Crosby et al., 2011; McGavin et al., 2019; Poole et al., 2020). Previous research in the Crosby Laboratory shows that when NO production is inhibited with L-NAME (a NOS inhibitor), eCBs decrease glutamate signaling at synapses in the DMH. However, this effect does not occur when NO synthesis isn't blocked (Sukkar, 2021). Therefore, we sought to determine how NO affects eCB signaling in the DMH. NO production is suggested to be mediated by an NMDA-dependent pathway. Thus, we blocked NMDA receptors with the antagonist DL-2-Amino-5-phosphonopentanoic acid (APV; 5  $\mu$ M) to investigate the effects of eCBs on glutamate signaling. Brain slices containing the DMH were incubated with APV for 5 minutes of baseline recording, followed by delivery of two 100 Hz, 4-second stimulations, 20-seconds apart (HFS). Administration of HFS, in the presence of APV, significantly decreased the amplitude of currents ( $60.58\% \pm 9.64\%$  of baseline,  $n = 8$ ,  $p = 0.0067$ ; Figures 1A and 1B). Thus, blocking NMDA receptors with an antagonist significantly decreased glutamate transmission, suggesting that NO is working through an NMDA-dependent pathway. Spontaneous excitatory postsynaptic currents (sEPSCs) provide insight into synaptic activity without extracellular stimulation; this information can help determine whether long-lasting changes in synaptic strength are due to presynaptic or postsynaptic effects (Glasgow et al., 2019). To determine if the effect was occurring pre- or postsynaptically, sEPSCs were examined during baseline recording and 20-25 minutes into the recording, after HFS. To determine the locus of the effect, we analyzed the frequency and amplitude of sEPSCs. We saw no change in the frequency (baseline: 4.40 Hz  $\pm$  0.88 Hz, post-HFS: 4.39 Hz  $\pm$  0.88 Hz,  $p = 0.9921$ ; Figure 1C) or amplitude (baseline: 12.15 pA  $\pm$  2.16 pA, post-HFS: 10.44 pA  $\pm$  1.06 pA,  $p = 0.3018$ ; Figure 1D) of sEPSCs. We expected that the administration of APV, and subsequent HFS, would decrease glutamate release, consistent with a presynaptic effect (Glasgow et al., 2019; Melom et al., 2013). Therefore, to further investigate the locus of effect, we analyzed the paired-pulse ratio (PPR). PPR represents the ratio of the second current amplitude over the first. The PPR is directly related to the probability of release from the presynaptic terminal. If PPR exceeds 1, it is assumed that the probability of neurotransmitter release has increased and that changes in PPR are due to a presynaptic effect of neurotransmitter release from the axon terminal (Glasgow et al., 2019; Kim and Alger, 2001; Letellier et al., 2019). There was no significant difference in the paired-pulse ratio at the 20-25-

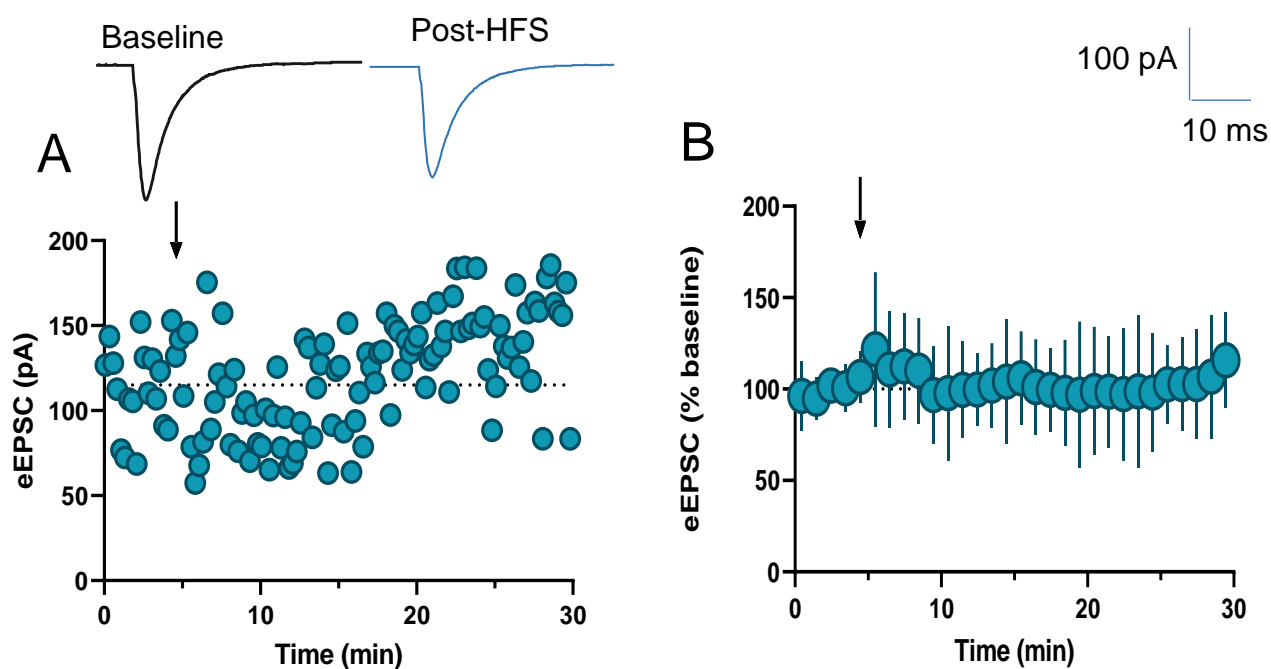
minute interval of the recording when compared to baseline recording (baseline:  $1.70 \pm 0.39$ , post-HFS:  $1.47 \pm 0.31$ ,  $p = 0.5911$ ; Figure 1E).



**Figure 1. Blocking NMDA receptors with APV significantly decreases glutamate transmission onto DMH neurons.** (A) Representative cell comprised of evoked excitatory postsynaptic currents (eEPSCs) before and after administration of high-frequency stimulation (HFS) (post-HFS recording taken during the 20-25-minute interval). Averaged traces of the representative cell are also represented above the graph. (B) Summarized cell graph demonstrating percentage change of eEPSCs compared to baseline recording. Changes in the frequency (C) and amplitude (D) of spontaneous excitatory postsynaptic currents (sEPSCs) comparing baseline to post-HFS. (E) Changes in the paired-pulse ratio (PPR) between baseline and post-HFS. Arrows (on A and B) indicate HFS. The values are expressed as mean  $\pm$  SEM; \* $p < 0.05$ .

### 3.2 Blocking CB1Rs with AM251 Prevents NMDA-Mediated Interference with Glutamate Signaling

Administration of an NMDA receptor antagonist onto DMH slices led to a significant decrease in glutamate signaling, after HFS. To determine if this effect was eCB-mediated, we repeated the same experiment, except we also added a CB1R antagonist. In the presence of APV and AM251 (CB1R antagonist; 5  $\mu$ M), we found that, after HFS, the LTD was prevented ( $98.33\% \pm 14.02\%$  of baseline,  $n = 6$ ,  $p = 0.7628$ ; Figures 2A and 2B). Together, these results show that LTD, when NMDA receptors are blocked by APV, is mediated by eCBs. Therefore, NO is preventing eCB-mediated LTD at glutamate synapses through an NMDA-dependent pathway.

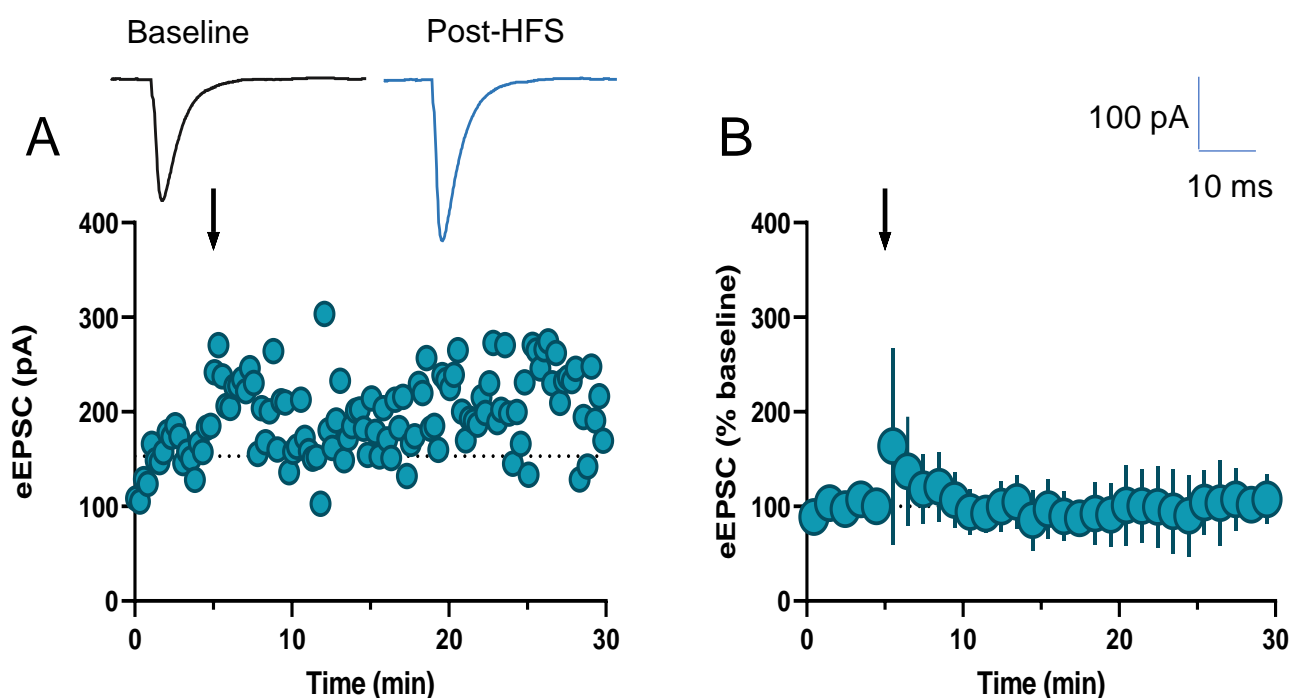


**Figure 2. Adding a CB1R antagonist blocks LTD at glutamate synapses.** (A) Representative cell comprised of evoked excitatory postsynaptic currents (eEPSCs) before and after administration of high-frequency stimulation (HFS) (post-HFS recording taken during the 20-25-minute interval). Averaged traces of the representative cell are also represented above the graph. (B) Summarized cell graph demonstrating percentage change of eEPSCs compared to baseline recording. Arrows (on A and B) indicate HFS. The values are expressed as mean  $\pm$  SEM; \* $p < 0.05$ .

### 3.3 Blocking Soluble Guanylate Cyclase with ODQ Prevents LTD at Glutamate Synapses

NO is also known to activate soluble guanylate cyclase (sGC) as part of its retrograde pathway in the presynaptic cell. NO binds sGC as part of a mechanism to increase intracellular cyclic guanosine

monophosphate (cGMP) (Schmidt et al., 1992). Therefore, we aimed to determine if the activation of this enzyme is required for the NO-mediated disruption in eCB signaling. Blocking sGC with an inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10  $\mu$ M) did not significantly reduce glutamate signaling onto DMH neurons after delivery of HFS ( $97.24\% \pm 18.74\%$  of baseline,  $n = 5$ ,  $p = 0.7853$ ; Figures 3A and 3B). This suggests that NO is affecting eCB signaling independent of its sGC receptor. Therefore, it is possible that NO is working postsynaptically to affect eCB signaling and/or through a pathway that is independent of cGMP. To explore this idea, we incubated slices with an NO scavenger to determine if NO needs to cross the synapse to exert its effects.



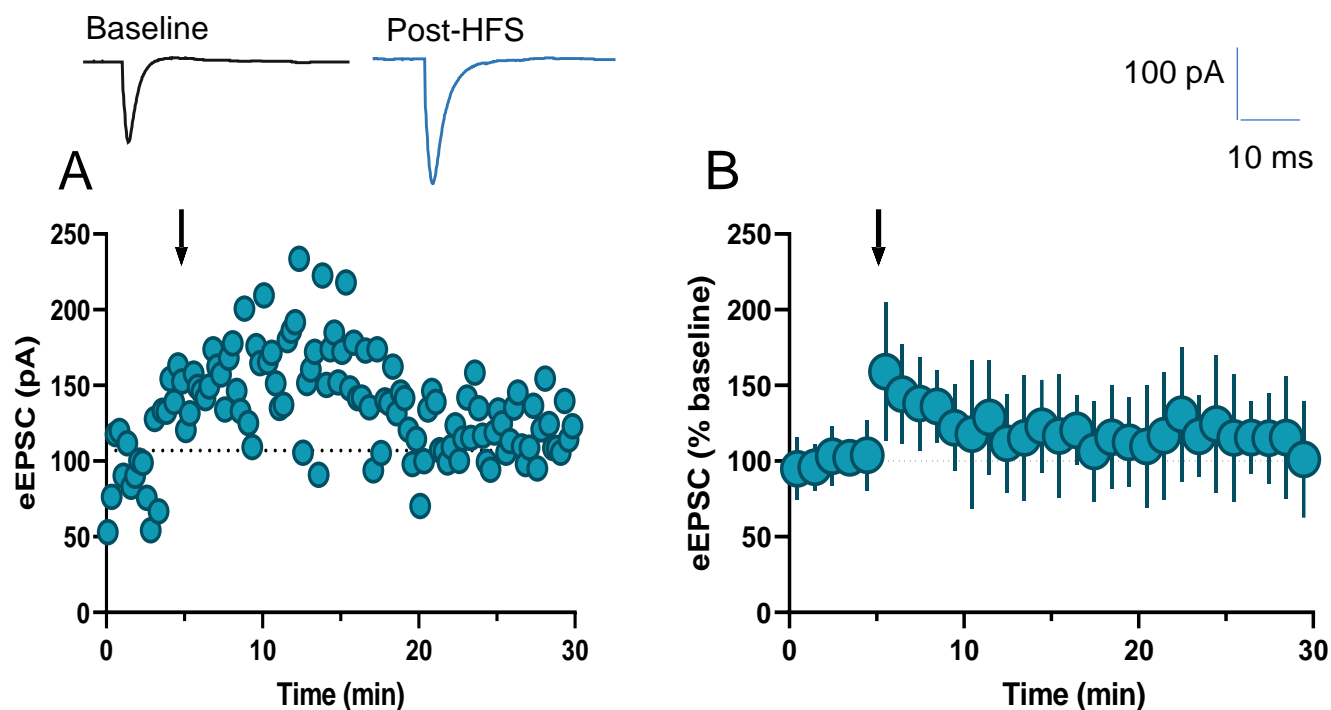
**Figure 3. Inhibiting soluble guanylate cyclase with ODQ does not unmask eCB-mediated LTD.**

(A) Representative cell comprised of evoked excitatory postsynaptic currents (eEPSCs) before and after administration of high-frequency stimulation (HFS) (post-HFS recording taken during the 20-25-minute interval). Averaged traces of the representative cell are also represented above the graph. (B) Summarized cell graph demonstrating percentage change of eEPSCs compared to baseline recording. Arrows (on A and B) indicate HFS. The values are expressed as mean  $\pm$  SEM; \* $p < 0.05$ .

### 3.4 Scavenging NO in the Synaptic Cleft Does Not Unmask LTD

NO, a retrograde neurotransmitter, is produced postsynaptically and typically acts on sGC in the presynaptic terminal (Stefano et al., 2003). As such, based on data hinting that NO may be exerting postsynaptic effects on eCB signaling, we interrupted the retrograde pathway across the

synapse by scavenging NO in the synaptic cleft with 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, monopotassium salt (carboxy-PTIO; 30  $\mu$ M). After 5 minutes of baseline recording during carboxy-PTIO incubation, HFS was applied and current measurements were taken throughout the whole-cell recording. Scavenging NO in the synaptic cleft did not unmask eCB-mediated LTD at glutamate synapses ( $119.62\% \pm 14.62\%$  of baseline,  $n = 6$ ,  $p = 0.4058$ ; Figures 4A and 4B). This suggests that NO is preventing eCBs from decreasing glutamate transmission via actions in the postsynaptic (DMH) cell. Collectively, these findings highlight the importance of NO in regulating eCB signaling. We unraveled this mechanism and found that this effect is NMDA-mediated since eCB-mediated LTD was unmasked once NMDA receptors were blocked with APV. Furthermore, we found that this mechanism might be occurring in the postsynaptic cell. When we blocked sGC with ODQ, or when we scavenged NO in the synaptic cleft, we did not see eCB-mediated depression at glutamate synapses. Altogether, our results demonstrate that NO is interrupting eCB signaling from the postsynaptic cell. These experiments focused on endocannabinoid signaling in response to HFS, however, endocannabinoid signaling can also be studied by activating its receptor with an agonist.

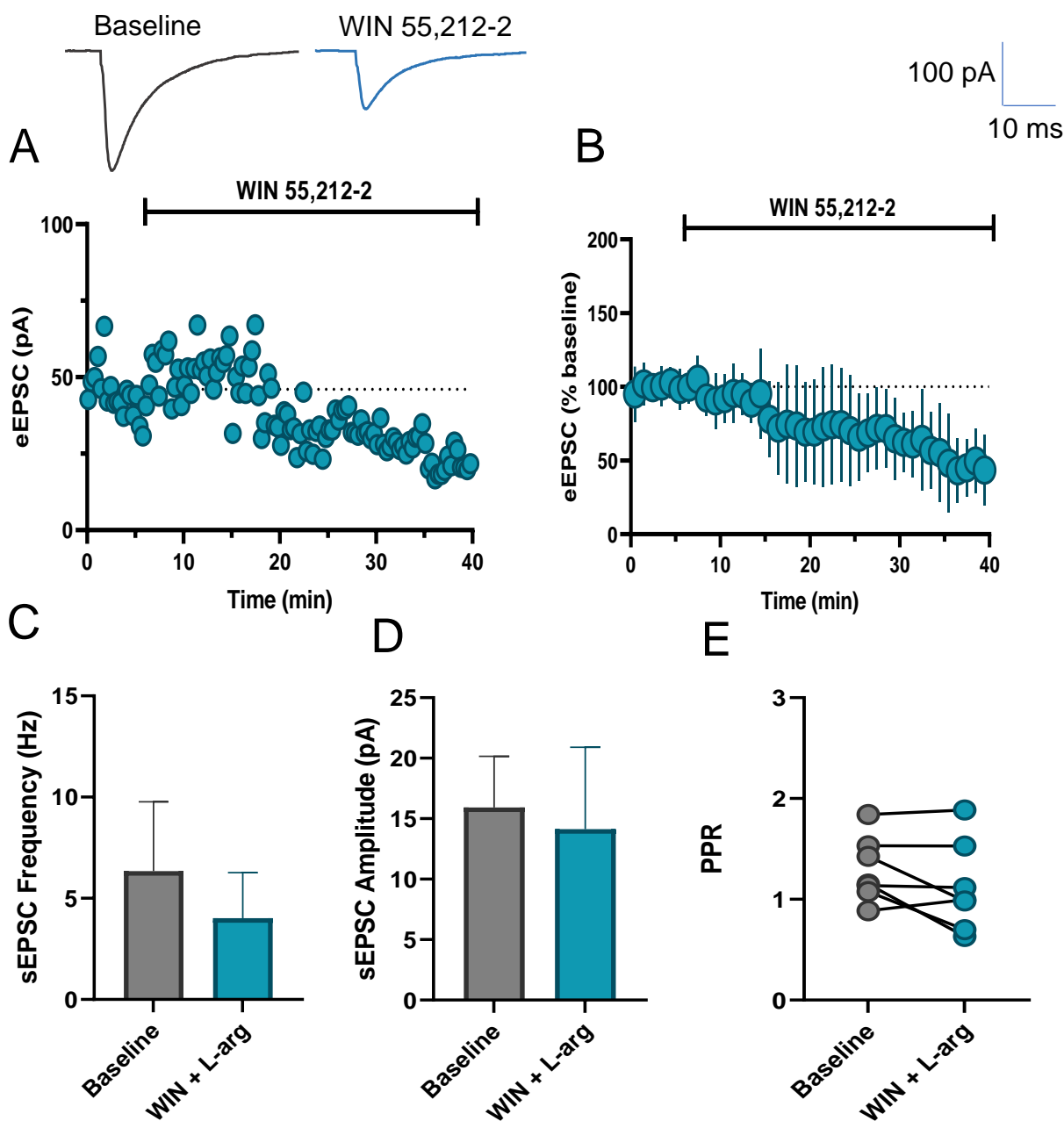


**Figure 4. Scavenging NO in the synaptic cleft by Carboxy-PTIO does not unmask eCB-mediated LTD.** (A) Representative cell comprised of evoked excitatory postsynaptic currents (eEPSCs) before and after administration of high-frequency stimulation (HFS) (post-HFS recording taken during the 20-25-minute interval). Averaged traces of the representative cell are also represented above the graph. (B) Summarized cell graph demonstrating percentage change of eEPSCs compared to baseline recording. Arrows (on A and B) indicate HFS. The values are expressed as mean  $\pm$  SEM; \* $p < 0.05$ .

### 3.5 Pairing an NO Precursor with CB1R Agonist Results in LTD

According to earlier research, activation of CB1Rs by an agonist decreases glutamate release onto DMH neurons (Sukkar, 2021). To determine if NO may be affecting CB1R activation, brain slices containing the DMH from male Sprague-Dawley rats were incubated with L-arginine (10  $\mu$ M; NO precursor) and WIN 55,212-2 (5  $\mu$ M; CB1R agonist). L-arginine was applied for 5-minutes of baseline recording and WIN 55,212-2 was added in the presence of L-arginine for the duration of the whole-cell recording. The results show that the CB1R agonist decreases evoked EPSCs even in the presence of an NO precursor, L-arginine. The administration of WIN 55,212-2 onto the brain slices significantly decreased current amplitude ( $45.86\% \pm 8.95\%$  of baseline,  $n = 7$ ,  $p = 0.0096$  Figures 5A and 5B). To aim to discover if the effect was occurring pre- or postsynaptically, sEPSCs were examined during baseline recording

and 35-40 minutes during the administration of WIN 55,212-2. Analysis of sEPSCs revealed that neither amplitude (baseline:  $15.93 \text{ pA} \pm 1.60 \text{ pA}$ , WIN 55,212-2:  $14.13 \text{ pA} \pm 2.56 \text{ pA}$ ,  $p = 0.3969$ ; Figure 5D) nor frequency (baseline:  $6.33 \text{ Hz} \pm 1.30 \text{ Hz}$ , WIN 55,212-2:  $4.024 \text{ Hz} \pm 0.85 \text{ Hz}$ ,  $p = 0.1226$ ; Figure 5C) of spontaneous currents taken during the 35-40-minute interval significantly differed from baseline recordings. However, even though a significant difference was not present, there appears to be a trend towards a decrease in spontaneous current frequency after the brain slice was incubated with the CB1R agonist (Figure 5C). We expected WIN 55,212-2 to decrease glutamate release, consistent with a presynaptic effect (Glasgow et al., 2019; Melom et al., 2013). Therefore, to further investigate the locus of effect, we analyzed the PPR. This study found no significant difference in PPR between baseline and during the addition of WIN 55,212-2 (baseline:  $1.29 \pm 0.12$ , WIN 55,212-2:  $1.12 \pm 0.17$ ,  $p = 0.1328$ ; Figure 5E). The results from this experiment demonstrate that WIN 55,212-2 still decreases glutamate signaling onto DMH neurons even in the presence of L-arginine. However, no change in the PPR or amplitude and frequency of eEPSCs, makes it harder to determine if this effect is occurring pre- or postsynaptically. Furthermore, this effect may be taking longer than what preliminary data suggests but more research is needed to conclude that suggestion. Therefore, in the initial stages, L-arginine may be mitigating the effects of the agonist. eCBs have also been shown to affect short-term changes in synaptic plasticity (Chevalleyre et al. 2007). This experiment focused on long-term responses to eCB signaling, however, we wanted to investigate if eCB signaling can be affected in the short-term.



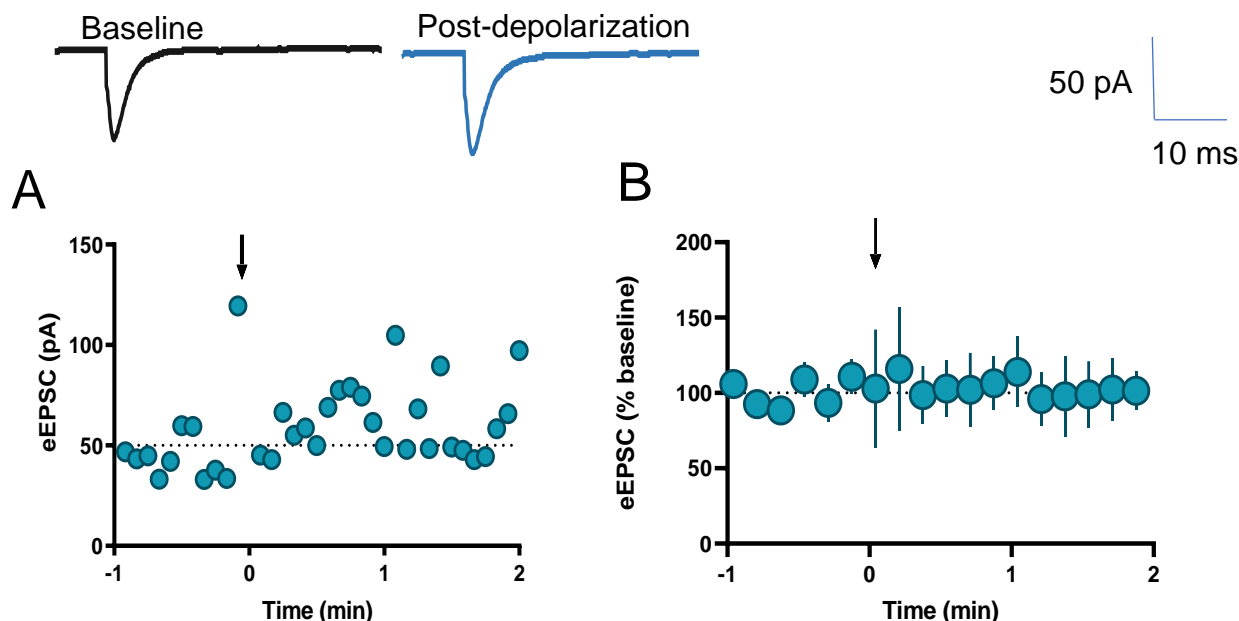
**Figure 5. eCBs decrease neurotransmitter release at glutamate synapses in the DMH even in the presence of L-arginine, an NO precursor.** (A) Representative cell comprised of evoked excitatory postsynaptic currents (eEPSCs) before (5 minutes of baseline recording with L-arginine alone) and during the administration of WIN 55,212-2, a CB1R agonist (post-WIN 55,212-2 recording taken during the 35–40-minute interval). Averaged traces of representative cell also represented above graph. (B) Summarized cell graph demonstrating percentage change of eEPSCs compared to baseline recording. Changes in the frequency (C) and amplitude (D) of spontaneous excitatory postsynaptic currents (sEPSCs) comparing baseline (L-arginine) to 35–40-minutes during the addition of WIN 55,212-2. (E) Changes in the paired-pulse ratio (PPR) between baseline recordings (L-arginine) and 35–40-minutes during the addition of WIN 55,212-2. All of the values are expressed as mean  $\pm$  SEM; \* $p < 0.05$ .

### 3.6 Administration of L-NAME Doesn't Unmask Depolarization-Induced Suppression of Excitation in the DMH

eCBs can also trigger changes to short-term synaptic plasticity. These short-term changes in plasticity occur within seconds to minutes after CB1R activation and are due to the inhibition of voltage-gated  $\text{Ca}^{2+}$  channels (Chevaleyre et al., 2007). Depolarizations induce small increases in intracellular calcium; this can trigger depolarization-induced suppression of inhibition (DSI) or excitation (DSE), at GABAergic and glutamatergic terminals, respectively. DSI has been detected at various locations of the brain such as the cerebellum and hippocampus (Llano et al., 1991; Makara et al., 2007; Pitler and Alger, 1992). Glutamatergic terminals in hippocampal neurons experienced DSE. Moreover, DSE induced depression of EPSCs to nearly the same extent as the CB1R agonist, WIN 55,212-2 (Straiker and Mackie, 2005). This suggests that glutamatergic synapses can experience eCB-mediated short-term depression; however, less is known about the effects of NO on eCB-mediated short-term depression in the DMH. We focused on changes in short-term synaptic plasticity at glutamatergic terminals in the DMH.

Preliminary data suggests that glutamatergic synapses in the DMH do not undergo short-term changes in synaptic plasticity when DMH neurons are depolarized in the presence of NO (Sukkar, 2021). However, it is unknown if NO is preventing short-term eCB signaling in the DMH in a similar manner to its effects on long-term synaptic changes. Therefore, we aimed to determine if NO affects short-term eCB signaling at glutamate synapses in the DMH. Brain slices were incubated with L-NAME (200  $\mu\text{M}$ ) for 5 minutes of baseline recording before being exposed to depolarizations. Subsequently, the cell of interest was depolarized to +20 mV for 5 seconds, and again 5 minutes later. Whole-cell recordings were taken for the duration of the time. Both post-depolarization recording intervals were averaged for statistical analysis and compared to baseline recording.

The results indicated that there was no significant decrease in current amplitude immediately after depolarizing the cell to +20 mv ( $103.27\% \pm 4.53\%$  of baseline,  $n = 8$ ,  $p = 0.7278$ ; Figures 6A and 6B). This finding suggests that in the presence of the NOS inhibitor, L-NAME, eCBs do not trigger short-term depression at glutamatergic terminals after depolarization.

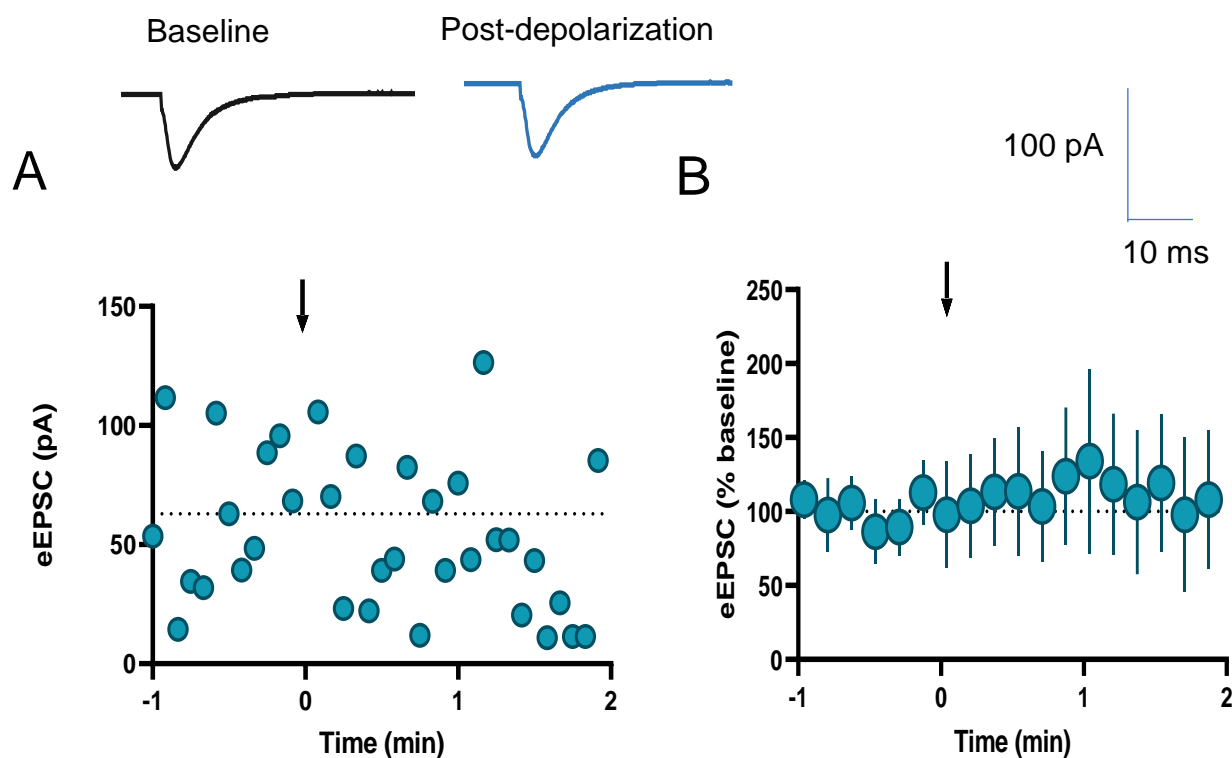


**Figure 6. L-NAME does not unmask eCB-mediated depolarization-induced suppression of excitation.** (A) Representative cell comprised of evoked excitatory postsynaptic currents (eEPSCs) before and after depolarizing the cell to +20 mV for 5 seconds. Averaged traces of representative cell also represented above graph. (B) Summarized cell graph demonstrating percentage change of eEPSCs compared to baseline recording; cells were averaged over both depolarizations and baseline corrected. Arrows (on A and B) indicate depolarization. The values are expressed as mean  $\pm$  SEM; \* $p < 0.05$ .

### 3.7 Delivering a Longer Depolarization Does Not Unmask Depolarization-Induced Suppression of Excitation in the DMH

Neurons in the brain are known to experience short-term changes in synaptic plasticity (Llano et al., 1991; Makara et al., 2007; Pitler and Alger, 1992; Straiker and Mackie, 2005; Sukkar, 2021). We did not observe a significant difference in glutamate release after a 5-second depolarization (+20 mV) in the presence of L-NAME at glutamatergic synapses in the DMH. We therefore tried a longer depolarization (10 seconds) to determine if that would invoke short-term changes in synaptic plasticity at glutamate synapses in the DMH. We incubated brain slices containing the DMH, in artificial cerebrospinal fluid (aCSF), to use as our control group. Baseline recording was taken for 5 minutes. Subsequently, a 10-second depolarization of +20 mV was delivered onto the brain slice, and again 5 minutes later. Currents during the post-depolarization recording were baseline corrected and averaged. The results show that there is no significant decrease in glutamate release after the 10-second depolarization ( $112.29\% \pm 17.03\%$

of baseline,  $n = 6$ ,  $p = 0.4717$ ; Figures 7A and 7B). Therefore, delivering a longer depolarization does not invoke short-term changes to synaptic plasticity at glutamate terminals in the DMH.

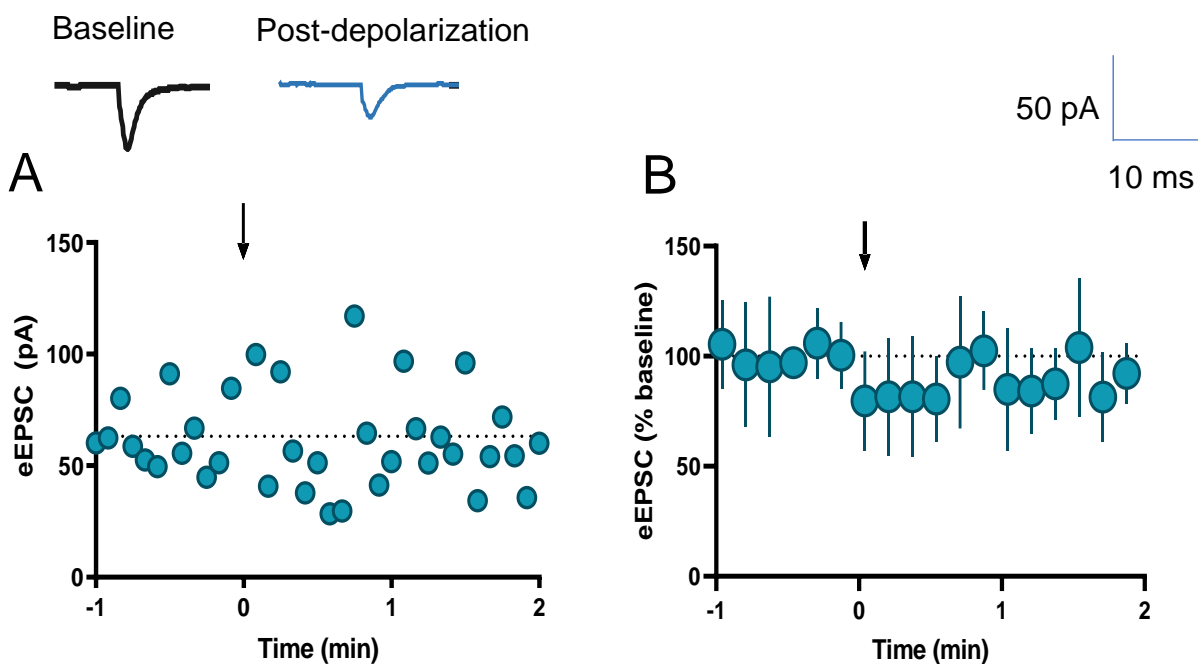


**Figure 7. Increasing the length of the depolarization does not result in changes to short-term synaptic plasticity.** (A) Representative cell comprised of evoked excitatory postsynaptic currents (eEPSCs) before and after depolarizing the cell to +20 mV for 10 seconds. Averaged traces of representative cell also represented above graph. (B) Summarized cell graph demonstrating percentage change of eEPSCs compared to baseline recording; cells were averaged over both depolarizations and baseline corrected. Arrows (on A and B) indicate depolarization. The values are expressed as mean  $\pm$  SEM; \* $p < 0.05$ .

### 3.8 Increasing the Length of Depolarization Does Not Unmask eCB-Mediated Short-Term Depression Even in the Absence of NO

Finally, the 10-second depolarization experiment was completed again, however, in the absence of NO. Brain slices were incubated with L-NAME (200  $\mu$ M) for 5 minutes of baseline recording before 10-second depolarizations (+20 mV) were delivered 5 minutes apart. The post-depolarization recordings were baseline corrected and then averaged. The results indicated that there was no significant difference in current amplitude after depolarization of the postsynaptic cell ( $89.22\% \pm 6.98\%$  of baseline,  $n = 5$ ,  $p = 0.1636$ ; Figures 8A and 8B). Thus, short-term

plasticity changes are not occurring at glutamate synapses after both 5-second and 10-second depolarizations of +20 mV



**Figure 8. Increasing the length of the depolarization does not result in changes to short-term synaptic plasticity even when NO synthesis is blocked. (A)** Representative cell comprised of evoked excitatory postsynaptic currents (eEPSCs) before and after depolarizing the cell to +20 mV for 10 seconds. Averaged traces of representative cell also represented above graph. **(B)** Summarized cell graph demonstrating percentage change of eEPSCs compared to baseline recording; cells were averaged over both depolarizations and baseline corrected. Arrows (on A and B) indicate depolarization. The values are expressed as mean ± SEM; \*p<0.05.

#### 4. Discussion

This study aimed to discover the effects of nitric oxide on endocannabinoid signaling at glutamate synapses in the rat DMH. Previous data shows that endocannabinoids (eCBs) decrease glutamate release onto DMH neurons when nitric oxide (NO) synthesis is inhibited with L-NAME (Sukkar, 2021). Therefore, we targeted the NO pathway to determine where and how it is affecting eCB signaling. After administration of HFS, eCBs produced in response to HFS trigger long-term depression (LTD), meaning that synaptic strength is weakening over time. However, this only occurs when NO synthesis is inhibited with L-NAME (Sukkar, 2021). We showed that blockage of NMDA receptors resulted in eCB-mediated LTD. This was evident since glutamate signaling decreased after administration of HFS onto DMH neurons incubated with APV (an

NMDA receptor antagonist). We confirmed that this was eCB-mediated LTD by repeating the same experiment, except we added AM251 (CB1R antagonist), along with APV, to the brain slice. The results showed that when we blocked CB1Rs with AM251 we no longer observed LTD. We also found that NO is interrupting eCB signaling from the postsynaptic cell. We concluded this because when we blocked its receptor on the presynaptic cell (sGC), we did not see LTD after delivering HFS to the slice. Furthermore, our data obtained from applying carboxy-PTIO also supported this conclusion: scavenging NO in the synaptic cleft with carboxy-PTIO did not result in LTD after administering HFS. Furthermore, we also looked at how NO may be affecting CB1R activation by WIN 55,212-2, an agonist for the receptor. We showed that even in the presence of L-arginine, an NO precursor, glutamate transmission significantly decreased onto DMH neurons. We then evaluated if short-term changes to synaptic plasticity occur at glutamate synapses in the DMH after depolarizations. Depolarization induced suppression of excitation (DSE) has been detected in the in areas of the brain such as the hippocampus (Straiker and Mackie, 2005), but to the best of our knowledge, it had yet to be detected in the DMH. Previous work in the Crosby Laboratory did not detect DSE at glutamatergic synapses in the DMH (Sukkar, 2021). Research suggests that NO may be affecting short-term synaptic plasticity. For example, NO has been shown to affect DSI at GABA synapses in the hippocampus (Makara et al., 2007). To investigate NO's effect on short-term synaptic plasticity, we delivered 5-second +20 mV depolarizations onto DMH neurons in the presence of L-NAME and found that glutamate release did not alter from baseline. Subsequently, we increased the length of the depolarization to 10 seconds and found that once again, glutamate release did not change in the short-term for our control data, or in the presence of L-NAME. Overall, our research suggests that NO is affecting long-term eCB signaling at glutamate synapses in the DMH and that it is exerting its effects from the postsynaptic cell. Our data collected from depolarization groups proposes that NO is not affecting short-term changes to synaptic plasticity. Understanding how NO affects eCB signaling after being endogenously produced (due to HFS) will assist global efforts to target orexigenic DMH neurons.

We showed that blocking NMDA receptors with APV decreased glutamate release onto DMH neurons. This can be attributed to NMDA receptors triggering an increase in intracellular calcium resulting in increased nitric oxide synthase (NOS) activity (Garthwaite et al., 1989; López-Colomé and López, 2003; Skeberdis et al., 2006). Thus, blocking NMDA receptors with

APV can prevent NO formation and ultimately its interference with eCB signaling. We, however, did not see any difference in the frequency nor amplitude of sEPSCs, which makes it harder to determine the locus of effect. Moreover, paired-pulse ratio (PPR) did not significantly differ from baseline after delivering HFS to the slice (Glasgow et al., 2019; Kim and Alger, 2001; Letellier et al., 2019). Even though we did not see a decrease in the PPR, frequency nor amplitude of sEPSCs, there are cases where eCBs can activate membrane-bound receptors on the postsynaptic cell. This is referred to as non-retrograde endocannabinoid signaling and happens when eCBs can activate postsynaptic CB1Rs. eCBs can also activate transient receptor potential vanilloid-type 1 (TRPV1) channels on the postsynaptic cell (van der Stelt et al., 2005). TRPV1 channel activation has been shown to exhibit similar effects to CB1R activation. For example, research has shown that anandamide (AEA) can trigger LTD via postsynaptic TRPV1 channels in the nucleus accumbens, dentate granule cells, and in the bed nucleus of stria terminalis (Chávez et al., 2010; Grueter et al., 2010; Puente et al., 2011; Castillo et al., 2012). Altogether, this illustrates how a postsynaptic effect is possible without changes in PPR or sEPSC frequency and amplitude. We were able to confirm that this effect was eCB-mediated since the effect no longer persisted when slices were incubated with both AM251 (CB1R antagonist) and APV. Thus, eCBs are likely responsible for decreasing glutamate release in DMH neurons, which they accomplish by blocking voltage-gated  $\text{Ca}^{2+}$  channels (Jones et al., 2008). However, since we did not see a change in the frequency or amplitude of sEPSCs, or in the PPR, more research may be needed.

Knowing that NO affects eCB signaling through an NMDA-dependent pathway, we then wanted to determine NO's locus of effect. We started by first investigating if blocking soluble guanylate cyclase (sGC; the NO receptor), can influence the effect of NO on eCB signaling. We incubated brain slices with ODC (an sGC inhibitor) before and after applying HFS, while recordings were taken throughout the process. NO is known to be released in response to activation by NMDA receptors, and target sGC inside the postsynaptic cell (Bellamy et al., 2002; Jones et al., 2008; Krumenacker et al., 2004). We did not observe a significant decrease in glutamate signaling after HFS was delivered. These data are not consistent with research performed at GABAergic terminals in the DMH. NO was needed to bind to sGC to induce  $\text{LTP}_{\text{GABA}}$  (Crosby et al., 2011; Nugent et al., 2007). This is the first study to conclude that the effects of NO on eCB signaling at glutamate synapses do not occur through an sGC-dependent

pathway. However, two isoforms of NO-sensitive sGCs exist, NO-GC 1, and NO-GC2 (Neitz et al., 2011). Previous research found that glutamate release decreased in mice that had the knockout gene for NO-GC1, but not for NO-GC2 (Neitz et al. 2011). Therefore, sGC activation may only increase glutamate release for one of its isoforms in the DMH, which may explain why depression was not observed when ODQ targeted sGC in our study. Thus, subsequent research should focus on the expression of these isoforms in the DMH, and if NO exhibits different effects on eCB signaling when binding to these two isoforms. This data suggests that NO is affecting eCB signaling independent of its receptor on the presynaptic cell.

To further rule out a presynaptic effect, we completed a similar study but incubated the DMH slice with carboxy-PTIO, a NO scavenger. Scavenging NO from the synaptic cleft did not unmask eCB-mediated depression at glutamatergic synapses in the DMH. Current amplitude did not decrease after administration of HFS, therefore NO appears to be affecting eCB signaling from the postsynaptic cell. This is consistent with our results obtained from incubating DMH slices with ODQ (sGC inhibitor). A common concern when using scavengers is that low concentrations of scavengers may not reliably remove their targets from the synaptic cleft. However, we are confident in the concentration of scavenger used since previous research found that the concentration of carboxy-PTIO reliably affected signaling in hippocampal slices when the concentration was 30  $\mu$ M (Ko and Kelly, 1999). Overall, our results show that NO is affecting eCB signaling through a postsynaptic effect.

We demonstrated that administering WIN 55,212-2, a CB1R agonist, significantly decreased glutamate signaling, even in the presence of L-arginine. L-arginine was incubated for 5 minutes of baseline recording before WIN 55,212-2 was added, and recordings were taken for the remainder of the experiment. We found that current amplitude significantly decreased within the 35–40-minute interval when compared to baseline. However, this was different than preliminary research that showed that WIN 55,212-2 expedites agonist-induced depression. According to previous data in the Crosby Laboratory, WIN 55,212-2 significantly decreased glutamate release (Sukkar, 2021). Contrary to these findings, we observed that L-arginine, an NO precursor, may be delaying the effects of the agonist and subsequent decrease in glutamate release, but further analysis needs to be performed. This isn't the first research to propose that NO may have a role in mitigating the effects of the CB1R agonist. A study conducted by Spina et al. (1998) found that tolerance to WIN 55,212-2-induced hypothermic and cataleptic effects

was obtained by control mice, but not mice who had repeatedly been administered L-NAME. This proposes the idea that NO may be affecting the downstream signaling of CB1Rs, which seems consistent with our results and the variability seen in our data (Spina et al., 1998). To determine if the effect was pre- or postsynaptic, we analyzed sEPSCs and the PPR of our results. There was no significant decrease in amplitude nor frequency of sEPSCs. Furthermore, analysis of the PPR suggested that there was no significant difference in PPR after administration of WIN 55,212-2. Despite all of this, the effect is likely presynaptic since previous research on CB1R activation in the DMH suggested a presynaptic effect even without changes in the frequency or amplitude of sEPSCs, or PPR (Crosby et al., 2011). This study should be repeated with L-NAME (NOS inhibitor) in the presence of L-arginine, and WIN 55,212-2. L-arginine is converted to NO through nitric oxide synthase (NOS), while producing L-Citrulline and nitrite in the process (Neilly et al., 1994). Thus, to ensure that the by-products of L-arginine conversion are not responsible for this effect, a future experiment should focus on the by-products of this conversion and see if they alter eCB signaling.

We finally investigated if NO affects short-term synaptic plasticity in the DMH. To determine this, we depolarized neurons to observe short-term changes in synaptic plasticity at glutamatergic terminals in the DMH. We found that depolarizing DMH neurons for 5 seconds at +20 mV did not result in DSE in the presence of L-NAME. This is consistent with preliminary data in the Crosby Laboratory that did not detect DSE when DMH neurons were depolarized for 5 seconds at +20mV with NO present (Sukkar, 2021). Furthermore, even when we increased the length of depolarization to 10 seconds at +20 mV, we did not observe DSE with, and without, L-NAME. These results were not like those obtained from Straiker and Mackie (2005), who found that depolarizing neurons in the hippocampus for 1-10s resulted in DSE. On the other hand, they also showed that administration of L-NAME had no effect on DSE when compared to the control group. This suggests that NO may not be interfering with DSE in the brain. Our results were also not consistent with results obtained from GABA synapses in the DMH. The Crosby Laboratory found that NO prevented DSI at GABA synapses in the DMH, and that DSI was noticeable when L-NAME was administered onto the slice (Sukkar, 2021). Altogether, our data further hints that NO may not be interfering with short-term plasticity in the DMH, but upcoming research should aim to conduct depolarizations with higher voltage to trigger greater  $\text{Ca}^{2+}$  influx.

Overall, we show that NO affects long-term eCB signaling at glutamatergic synapses in DMH neurons. Previous research showed that NO prevents eCBs from decreasing glutamate release after HFS. We explored this and found that after administering HFS to brain slices, eCBs only decreased neurotransmitter release when NMDA receptors were blocked. We were able to conclude that this effect was eCB-mediated by repeating the same experiment with a CB1R antagonist. Furthermore, according to sEPSC analysis, PPR analysis, and results from our studies using a NO scavenger, and sGC inhibitor, we conclude that NO is affecting eCB signaling from the postsynaptic cell. We also found that NO, in the form of its precursor (L-arginine), does not prevent LTD. Our data suggests that L-arginine may be mitigating the effects of the agonist and delaying eCB-mediated depression due to activation of CB1Rs, however, further analysis should be performed to confirm this. Finally, we concluded that there was no significant change in synaptic plasticity after depolarizing DMH neurons for 5 and 10 seconds. This work furthers our understanding of the interactions between endocannabinoids and nitric oxide and can lead to future discoveries which may assist in finding out how DMH neurons are regulating appetite. This research can also be monumental in discovering how these ubiquitous signals interact at other synapses in the brain.

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