

THE IMPACT OF *L*-ARGININE ON CANNABINOID 1 RECEPTOR EXPRESSION,
NEURONAL EXCITABILITY, AND SYNAPTIC TRANSMISSION IN THE
HYPOTHALAMUS OF YOUNG RATS
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Abstract

Interactions between nitric oxide (NO) and endogenous cannabinoids (eCBs) have been reported in various brain regions including dorsomedial hypothalamus (DMH). Such interactions include that eCBs diminish NO signaling and NO is required for eCB signaling. I aimed to determine if the requirement of NO for eCB signaling is mediated by CB1R expression. To determine the effects of NO on CB1R expression, young male Sprague Dawley rats were divided into two groups: (1) acute robust exposure to *L*-arginine (100mg/kg) by intraperitoneal injection or vehicle (saline) injection and (2) prolonged modest exposure to *L*-arginine by supplemented water (2.25%) and control distilled water for control. It was determined by RT-PCR that the hypothalamus of animals receiving an acute exposure to *L*-arginine showed a significant increase in CB1R mRNA, but not the cortex or hippocampus. Prolonged treatment with *L*-arginine water did not cause a significant change in CB1R expression. Results from RT-PCR experiments lead to obtaining electrophysiological recordings from the DMH, a region known to exhibit interactions between NO and eCBs. Electrophysiological recordings were used to determine the effects of *L*-arginine on neuronal excitability and synaptic transmission at glutamate synapses. To do this, animals received the same acute and robust exposure to *L*-arginine that was found to increase CB1R mRNA in the hypothalamus. Whole cell recordings from DMH neurons were performed to determine the effect of *L*-arginine on neuronal excitability and the ability of glutamate synapses to undergo long-term changes in synaptic strength in response to high frequency stimulation (HFS). It was found that glutamate synapses from animals receiving *L*-arginine injections were less excitable than those from vehicle-treated animals. In animals receiving a vehicle treatment, there was a short-term potentiation of glutamate signaling observed in the first five-minutes after HFS. This short-term potentiation was abolished in animals which received *L*-arginine. No long-term changes in synaptic strength were observed with either treatment. These findings suggest that *L*-arginine may increase CB1R expression in the DMH, causing a decrease in neuronal excitability as well as masking short-term potentiation post-HFS at glutamate synapses. Future research is required to confirm if the *L*-arginine effects on neuronal excitability and synaptic transmission are CB1R mediated.

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List of Abbreviations

Abbreviation	Definition
DMH	Dorsomedial nucleus of the hypothalamus
CB1R	Cannabinoid 1 Receptor
CNR1	Cannabinoid Receptor 1 Gene
NO	Nitric oxide
eCBs	Endogenous cannabinoids
THC	Delta-9-Tetrahydrocannabinol
HFS	High frequency stimulation
GABA	Gamma-aminobutyric acid
CNS	Central nervous system
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
cGMP	Cyclic guanosine monophosphate
MAP	Mitogen-activated protein
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
RT-PCR	Real time polymerase chain reaction
IP	Intraperitoneal
MAPK	Mitogen activated protein kinase

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Chapter 1: Introduction

1.1 Obesity

In recent years, the prevalence of obesity worldwide has increased. The rise in obesity is cause for concern as excess weight gain leads to an increased risk of several diseases including cardiovascular disease, diabetes, and cancer (Engin, 2017). Obesity is characterized by an excess of adipose tissue resulting from increased food intake and decreased energy expenditure (Al-Goblan *et al.*, 2014). Recent studies have reported that 64% of Canadian adults and 30% of children are overweight or obese (Public Health Agency of Canada, 2017). Research into pathways involved in food intake may lead to the understanding of complex interactions implicated in the obesity epidemic. Food intake is primarily controlled by the brain and relies on a complex integration of nutrient and hormonal signals to maintain homeostasis (Schwartz *et al.*, 2000). One main brain region involved in the regulation of food intake is the hypothalamus.

1.2 The Hypothalamus

A paper by Stellar in the 1950s examined the role of the hypothalamus in food intake and body weight. These studies defined the ventromedial hypothalamic nucleus as the satiety centre and the lateral hypothalamic nucleus as the hunger centre. Lesioning studies of the hypothalamus have led to knowledge of many nuclei that regulate food intake and neuronal pathways that may integrate multiple inputs in relation to metabolic fuel (Schwartz *et al.*, 2000). These studies led to the discovery of the role of the dorsomedial hypothalamus (DMH) as an area implicated in food intake.

1.3 Dorsomedial Hypothalamus

Earlier lesioning studies have implicated the DMH in the regulation of food intake and body weight (Bellinger & Bernardis, 2002). Stimulation of the DMH is found to induce eating behaviour while lesioning decreased feeding behaviour, when food is provided to rats *ad libitum*. In food-restricted animals with a lesion in the DMH, an increase in food intake was reported (Bellinger & Bernardis, 2002). This data suggests that lesioning of the DMH induces regulation of appetite. Subsequent protein studies found animals fed high fat diets and subjected to diet-induced obesity showed increased c-fos staining, a protein product related to neuronal activity, in

the DMH. When animals were returned to a standard diet their c-fos levels decreased in the DMH (Xin *et al.*, 2000). More recent optogenetic studies have shown that the activation of GABAergic neurons in the DMH increases food intake. These neurons are controlled by the metabolic signals of leptin and glucose. Once activated, the inhibitory synaptic transmission of DMH GABAergic neurons onto the periventricular nucleus promotes food intake (Otgong-Ul *et al.*, 2016).

1.4 Neuronal Communication

In the DMH and other brain regions, neurons communicate through the release of neurotransmitters, resulting in either excitation or inhibition of downstream neurons. Two main neurotransmitters are involved in this communication: glutamate is the main excitatory neurotransmitter and gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter (Myers *et al.*, 2014). Interestingly, the release of glutamate and GABA from axon terminals is mediated by signaling molecules released from postsynaptic cells in a process called retrograde signalling (Alger, 2002). Retrograde signalling is a precise, fast, and flexible form of neuronal signalling. The most widely studied form of retrograde signalling in the brain is by endogenous cannabinoids (endocannabinoids; eCBs) (Alger, 2002). Nitric oxide (NO) is also a gaseous retrograde signaling neurotransmitter (Palmer *et al.*, 1988). Retrograde signalling by eCBs and NO is found ubiquitously throughout the brain and may underlie appetite regulation in the DMH. The interaction of eCBs and NO has previously been reported in the DMH; however, the mechanism of this interaction is still unknown (Crosby *et al.*, 2011).

1.5 Endocannabinoids

The discovery of eCBs came in the early 1990s, long after the discovery of neurotransmitters, on the tail of research on plant-based cannabinoids from the plant *Cannabis sativa*. Δ^9 -Tetrahydrocannabinol (THC), the main psychoactive ingredient in cannabis, was the first chemical found to stimulate cannabinoid 1 (CB1R) and 2 receptors. CB1Rs are found in high density in brain tissue and their activation exhibits the typical cannabis effects, while cannabinoid 2 receptors are found throughout the body and involved in immune function (Martin *et al.*, 1999).

It was not until the turn of the century that the two main endogenous cannabinoids were discovered and found to stimulate the same receptors as THC (Devane *et al.*, 1992; Mechoulam *et al.*, 1995). N-arachidonylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) are readily synthesized by the human body through remodelling of membrane phospholipids (DiMarzo, 2006). These endogenous cannabinoids are able to bind to CB1R and cannabinoid 2 receptors, however this thesis will focus on CB1Rs.

Endogenous cannabinoids have a wide range of implications in pain, mood and anxiety disorders, hypertension, cancer, and obesity (Pacher *et al.*, 2006). Pre-clinical trials have found that excess endocannabinoid signalling can result in an increase in intake of high-calorie foods and increased risk of obesity (Hillard, 2018).

1.6 Cannabinoid 1 Receptors

The human gene encoding the CB1R protein, cannabinoid receptor 1 (CNR1) gene, is located on chromosome 6 in the 6q14-q15 location (Zhang *et al.*, 2004). These receptors are primarily found on presynaptic neurons but have also been observed on astrocytes, as well as on mitochondria within postsynaptic cells (Meng *et al.*, 2014; Bénard *et al.*, 2012). CB1R are G-protein type Gi/o-coupled receptors which contain a 7-transmembrane domain. On neurons, eCBs bind to these receptors by retrograde signalling from the postsynaptic cell to the presynaptic cell. The activation of CB1R lowers cyclic AMP (cAMP) production. This is accomplished by coupling of CB1R to the G protein on the presynaptic neuron stimulation of mitogen-activated protein kinase (MAPK) and the inhibition of adenylyl cyclase (Svíženská *et al.*, 2008). A decrease in cAMP then reduces the influx of calcium ions into the presynaptic cell and increases the efflux of potassium ions from the cell due to ion channel coupling. The decreased production of cAMP caused by CB1R activation, results in decreased activity of protein kinase A (PKA). In the presence of cannabinoids, PKA, which is responsible for phosphorylating potassium efflux channels, is inhibited (Svíženská *et al.*, 2008). This has a net result of decreasing the excitation of the presynaptic neuron and decreasing overall neurotransmitter release (Hillard, 2018). Cannabinoids have been shown to inhibit glutamate, acetylcholine, and noradrenaline release. CB1Rs are expressed in the central nervous system (CNS) in regions influencing mood, motor coordination, autonomic function, memory, food intake, sensation and cognition (Alger, 2002; Schurman *et al.*, 2020).

The expression of CB1R can be up- and down-regulated by a variety of environmental factors including; stress, feeding state, and the presence of drugs like THC (Hillard, 2014; Hirvonen *et al.*, 2012; Crosby *et al.*, 2011). Tolerance to cannabinoids can be caused by downregulation of receptors, conformational changes to receptors, or internalization of receptors. The CNR1 mRNA density and CB1R binding is reduced in the hippocampus of human cannabis users. After approximately 4 weeks of abstinence from cannabis, chronic users' cortical density of CB1R returns to normal levels (Hirvonen *et al.*, 2012). This data suggests that the expression of CB1R may fluctuate based on environmental factors. However, the mechanisms of downregulation of CB1R are not yet well understood.

1.7 Nitric Oxide

Nitric oxide (NO) is a gaseous retrograde neurotransmitter. First recognized in the late 1980s for its role in vasodilation, its synthetic pathway was soon after identified (Palmer *et al.*, 1988). NO is produced by the nitric oxide synthase enzyme (NOS), as a by-product of the transformation of *L*-arginine to *L*-citrulline (Alger, 2005). NOS enzymes have a highly conserved structure and are present in most organisms from bacteria to mammals. Throughout the human body, there are three known forms of NOS enzymes including neuronal, inducible, and endothelial. Neuronal nitric oxide synthase (nNOS) is the enzyme primarily responsible for NO production in the CNS (Lipina & Hundal, 2017).

NO is a molecule well suited for signaling; due to its gaseous nature, it is able to dissolve in both lipid and aqueous environments. NO increases calcium influx in presynaptic neurons, increasing neurotransmitter release (Alger, 2005). In the CNS, NO increases the concentration of cyclic GMP (cGMP) by activating soluble guanylyl cyclase (Fiel & Kleppisch, 2008). An increase in cGMP causes activation of the multitude of cGMP receptors, including c-GMP dependent protein kinases and cyclic nucleotide gated cation channels. Activation of cyclic nucleotide gated cation channels result in an influx of calcium ions in the presynaptic cell, leading to increased excitation and increased neurotransmitter release from the presynaptic terminal (Fiel & Kleppisch, 2008). NO has been found, through cGMP-dependent mechanisms, to regulate the release of glutamate, which in turn regulates the release of other transmitters in brain areas like the hippocampus and hypothalamus. The effects of NO depend on the

concentration of NO released and the brain region in which it is released. These factors determine whether a neurotransmitter release is inhibited or enhanced (Fiel & Kleppisch, 2008).

1.8 Interactions Between Nitric Oxide and Endocannabinoids

Although NO and eCBs have conflicting effects at synapses, there is evidence to suggest an interaction between the two signalling pathways. Previous studies have shown that NO is required for eCB signalling in rats in both *in vitro* brain slices and *in vivo* behaviour (Crosby et al. 2011; McGavin et al., 2019; Makara et al., 2007). A study conducted by Crosby *et al.* (2011) found through electrophysiological experiments that eCBs failed to alter GABA signaling if NO synthesis was blocked. This demonstrated that nitric oxide is required for endocannabinoid signalling in the DMH. The mechanisms by which NO is required for eCB signaling, however, still remain poorly understood.

Although the mechanism of interaction between NO and CB1R signalling in the DMH is currently unknown, several mechanisms of interaction have been proposed in other brain areas. NO may act downstream of CB1R in the cerebellum and striatum. In contrast, in the hippocampus NO is required upstream of the CB1R to exhibit eCB-mediated plasticity of synapses (Safo and Regehr, 2005; Makara et al., 2007). There is also evidence that NO may act directly on the CB1R to increase eCB signalling, by preventing receptor desensitization and internalization (Kokkola et al., 2005; Whalen et al., 2007). Preventing desensitization and internalization will lead to more CB1R present in the membrane with a greater capacity for signalling. This study, however, will focus on the possibility that NO has a role in increasing CB1R expression.

1.9 Current Study

The overall goal of the current study was to begin to determine the mechanisms by which NO is required for eCB signaling. My objective was to determine if NO may be interacting with eCB signaling in the DMH by increasing the expression of CB1R. I hypothesized that (1) treatment with *L*-arginine, a nitric oxide precursor, would alter the expression of CB1R in the hypothalamus. RT-PCR was used to assess changes in CB1R expression mediated by either a robust and acute, or modest and prolonged, exposure to the NO precursor *L*-arginine. As NO and CB1Rs are known to modulate appetite, food intake was also recorded in each treatment group. I hypothesized that (2) treatment with *L*-arginine would alter food intake. Results from RT-PCR

experiments lead to a third hypothesis: (3) *L*-arginine would alter neuronal excitability of glutamate synapses in the DMH, a region known to exhibit interactions between NO and eCB signaling. Uncovering the mechanism of interaction between NO and eCBs may lead to the development of novel drugs to combat diseases affected by the eCB system, such as anxiety and obesity.

Chapter 2: Materials and Methods

2.1 Subjects

Male Sprague Dawley rats obtained from Charles River Laboratories (Quebec, Canada) arrived between postnatal day 21-23. Rats were housed in groups of 2-4 animals and allowed to habituate to the environment for at least 5 days prior to experimentation. Animals were housed in enriched environments in polycarbonate cages at an ambient temperature of $21\pm 2^{\circ}\text{C}$. Animals were kept on a 12hr light-dark schedule (lights on from 0730h to 1930h). Food and water were available *ad libitum*. All animals were postnatal day 26-40 at time of experimentation. All protocols were approved by the Mount Allison Animal Care Committee in accordance with the Canadian Animal Care Guidelines.

2.2 Acute *L*-Arginine Exposure

Animals were given a one-time intraperitoneal (IP) injection of *L*-arginine to mimic a brief and robust influx of nitric oxide. Animals were randomly assigned to either vehicle (0.9% saline, n=6) or *L*-arginine (Sigma Aldrich, Ontario, Canada, 100mg/kg, n=6) injection groups. *L*-arginine was dissolved in sterile saline (100 mg/ml in 0.9% NaCl). Animals in the vehicle were injected with sterile saline (0.1mL, 0.9% NaCl). Body weight of the injected animals was measured prior to injection and post one-hour incubation. Food intake of injected animals was measured one-hour post injection, prior to sacrifice.

2.3 Prolonged *L*-Arginine Exposure

L-arginine was dissolved in the animals' drinking water to allow for a prolonged, but moderate, *L*-arginine exposure. Previous studies have shown a moderate concentration of 2.25% *L*-arginine to have effects on protein expression (Moretto *et al.*, 2017). Animals were randomly assigned to either control or *L*-arginine (Sigma Aldrich, 2.25%) groups. Control groups were given their usual distilled water, with no *L*-Arginine. Food intake and body weight of animals given control water or *L*-arginine water (2.25%) was measured over 24hrs at 0, 1, 2, 4, 6, 12, and 24hrs. After 24hrs animals given control or *L*-arginine water were sacrificed. All animals were given *ad libitum* access to food and water. Food intake was measured by weighing food pre and

post incubation period for both acute and prolonged treatment, as well as checking the cage for any food residue.

2.4 Tissue Sample Collection

Animals were deeply anaesthetized using sodium pentobarbital injections (130mg/kg) until no reflex reaction was observed following a toe pinch. The animal was then decapitated using a guillotine. Brains were then removed and placed on filter paper dampened with PBS, ventral side up. The hypothalamus was removed by scooping the tissue away from the underlying structures. The brain was then flipped dorsal side up and the cerebellum was removed by slicing across the inferior posterior colliculus. A slice was made along the longitudinal fissure of the brain and the hippocampus was removed by scooping away the thalamus and midbrain from the corpus callosum to expose the hippocampus and scooping out the hippocampus. A section of cortex was taken from the opposite side of the brain from the section with the hippocampus removed, by removing the mid brain and hippocampus and slicing a piece of the cortex. All brain sections were flash frozen immediately in liquid nitrogen and then stored at -80°C.

2.5 Real Time Reverse Transcription PCR

RNA was isolated from the hippocampus, the hypothalamus, and the cortex samples using TRIzol reagent (Fischer Scientific, Cat: A33250, Massachusetts, USA). All samples weighed between 20-50mg. Tissue was sonicated in TRIzol reagent and RNA was extracted according to manufacturer's protocol for RNA extraction from tissue samples. RNA resuspended in nuclease free water was then quantified using a Nanodrop (ND-1000). The cDNA was then synthesized using the ThermoFisher High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Cat: 4374966), according to manufacturer's protocol with 2ug of total RNA. Reverse transcription was performed in a thermocycler and after synthesis the cDNA samples were stored at -20°C.

Quantification of CNR1 mRNA was carried out by real-time PCR (TaqMan). Real-time PCR was performed on all cDNA samples using the TaqMan Fast Advanced Master Mix (Cat: 4444556) in accordance to the manufacturer's protocol. TaqMan β -actin primers (VIC, Rn00667869_m1) were used as an endogenous control and TaqMan CNR1 primers (FAM, Rn00562880_m1) were used to measure levels of CB1R mRNA. PCR reactions were performed

in the QuantStudio7 Flex. The quantity of cDNA in the sample was controlled for by measuring CNR1 and β -actin genes simultaneously to adjust for differences between samples.

2.6 Brain Slice Preparation

Animals underwent a brief exposure to the NO precursor *L*-arginine. Animals received an IP injection of either *L*-arginine (Sigma Aldrich, Ontario, Canada, 100mg/kg, n=5) or vehicle treatments (0.9% saline, n=6). Animals were allowed to incubate for a period of one hour. All treatments occurred before noon. One-hour post-injection the animals were promptly placed into a chamber filled with 5% isoflurane gas in oxygen, with some liquid isoflurane in the chamber. The flow of isoflurane was sustained until the animal was anesthetized, at which point the animal was rapidly decapitated using a guillotine. The brain was then quickly removed and placed into a semi-frozen slicing solution (87mM NaCl, 2.5mM KCl, 0.5mM CaCl₂•2H₂O, 7 MgCL₂•6H₂O, 1.25mM NaH₂PO₄•H₂O, 25mM NaHCO₃, 10mM glucose, and 75mM sucrose) saturated with 95% O₂ and 5% CO₂. The brain remained in the solution for 3-5 minutes before slicing. The brain was removed from the slicing solution and the section containing the hypothalamus was then isolated by removing the rostral, caudal, and dorsal portions of the brain. The brain was then glued to a stage rostral side up, in front of a piece of agar to prevent movement of the brain during slicing. The stage was then placed into a vibrating microtome (Leica, Nussloch, Germany) and then submerged in ice-cold slicing solution saturated with oxygen (95%/5%, O₂/CO₂). The brain was sliced into 250 μ m coronal sections. Brain slices containing DMH were cut into hemisections with a scalpel through the third ventricle. Slices were incubated in artificial cerebrospinal fluid (aCSF, 126mM NaCl, 2.5mM KCl, 2.5mM CaCl₂•2H₂O, 1.5mM MgCL₂•6H₂O, 1.25mM NaH₂PO₄•H₂O, 26mM NaHCO₃, 10mM glucose) at 32.5°C saturated with 95% O₂ and 5% CO₂ for at least 60 minutes. Slices were laid on a layer of wire mesh glued to a plastic rack to prevent overlap and ensure equal saturation with oxygen and aCSF. Slices were incubated in aCSF until recording.

2.7 Electrophysiology

Following the incubation period, hypothalamic slices were placed in a recording chamber containing a constant flow of oxygenated aCSF and maintained at 32.5°C. An Olympus upright microscope (Olympus, Center Valley, PA) fitted with an Infinity 2 camera (Lumenera, Ottawa,

ON) was used to visualize neurons in the DMH compact zone, found between the third ventricle and fornix. A P-2000 micropipette puller (Sutter Instruments, Novato, CA) was used to pull borosilicate glass into microelectrodes with a tip resistance of 4.0-8.0 M Ω . To conduct whole cell electrophysiology recordings, the recording microelectrode was filled with internal solution (108mM potassium gluconate, 8mM KCl, 8mM sodium gluconate, 1mM EGTA (1M NaOH), 10mM HEPES, 2mM MgCl₂, 4mM potassium ATP, 0.3mM sodium GTP and corrected to pH 7.4 with NaOH and an osmolality of 285 mmol/kg with deionized water). The stimulating electrode contained picrotoxin (100 μ M; Tocris, Ellisville, MO) dissolved in aCSF to block GABA_A receptors and inhibit GABAergic synaptic transmission and activity. Amplification of electrophysiological signals was accomplished using the Multiclamp700B amplifier (Molecular Devices, Union City, CA), low-pass filtered at 1 kHz, digitized at 10 kHz using the Digidata 1322 (Molecular Devices) and stored offline for analysis.

2.7.1 Electrophysiological Protocols

The function of DMH neurons in response to acute exposure to *L*-arginine was assessed for excitability through current-clamp recordings, and synaptic transmission through voltage-clamp mode in glutamate-mediated currents. Current-clamp mode used ten sequential hyperpolarizing and depolarizing steps beginning at -100mV and ending at -10mV. The firing properties of the action potentials recorded in current-clamp mode were assessed using Clampfit 10 (Molecular Devices). The action potentials used to determine the firing properties of a neuron occurred in the first step at which action potentials were fired. The threshold of action potential firing was recorded 5ms before the depolarizing phase. The amplitude of the action potential (mV) and the amplitude of the after-hyperpolarization (mV) were determined as the amplitude relative to a baseline value that was kept consistently as 20 milliseconds before the peak of the action potential. The duration of the action potential (ms) was measured as the width of the action potential at half amplitude.

Voltage-clamp mode was used to assess synaptic transmission at glutamate synapses. Cells which had more than a 20% change in access resistance were not accepted for analysis. Each neuron was clamped at a resting membrane potential of -70mV and glutamate activity was assessed by evoked excitatory postsynaptic currents (EPSCs). EPSCs were evoked at a rate of 0.2Hz and paired-pulse responses were obtained by applying a pair of synaptic stimuli 50ms

apart. For HFS, afferent neurons were stimulated at 100Hz for 4s, this was repeated twice 20s apart. Evoked currents were analyzed using Clampfit 10 (Molecular Devices). HFS provides the ability to study the strength of synapses. This is a method that has previously been used to study synaptic strength in the DMH (Crosby *et al.*, 2011; Crosby *et al.*, 2018). The amplitude of the evoked currents was calculated from the baseline established before the evoked response to the peak of evoked response. Stimulus artifacts were digitally removed from the traces using Clampfit for clarity in the figures.

2.8 Statistical Analysis

Results are expressed as the mean \pm standard error of the mean (SEM). Statistical tests were performed using Prism 8 and SPSS. Homogeneity of variances and normality was assessed for each test to ensure assumptions were met. Unpaired and paired t-tests were performed to determine statistical significance. Action potential frequency was analyzed by an unpaired t-test at each step and the Benjamini-Hochberg procedure with a false discovery rate of 0.05 was used to control for false discovery. A significance value of $\alpha=0.05$ was used to evaluate all tests.

Chapter 3: Results

The overall objective of the current study was to begin to elucidate the mechanisms by which NO is required for eCB signaling. To determine the impact of *L*-arginine on CNR1 expression I measured the expression of the CNR1 gene in response to acute and prolonged treatment with NO precursor, *L*-arginine. RT-PCR experiments were used to determine if NO alters the expression of CB1R. I then analyzed the excitability and synaptic transmission at glutamate synapses in the DMH, to determine whether CNR1 mRNA translated to changes in neuronal properties.

3.1 RT-PCR Results

RT-PCR was used to quantify mRNA levels of CNR1 from 3 different brain regions in 24 animals. To determine the effects of a robust acute treatment of *L*-arginine, a total of 6 rats were treated with *L*-arginine injections and 6 with vehicle saline injections. Two animals given vehicle injections were excluded from analysis due to abnormal size and weight gain for their age, leaving 4 animals in the vehicle saline injection group for analysis. To determine the effects of a prolonged modest treatment with *L*-arginine, 6 animals were treated with *L*-arginine water, and 6 with vehicle water over 24-hours.

First, to determine whether a single robust dose of *L*-arginine had an effect on CB1R expression, animals were treated with a one-time acute injection of NO precursor *L*-arginine and sacrificed one hour later for tissue analysis. Animals that received an acute injection of *L*-arginine showed a significantly higher relative quantification of CNR1 mRNA in the hypothalamus, in comparison to those receiving a vehicle injection (Vehicle: 1.011 ± 0.08613 ; $n=4$; *L*-Arginine: 1.342 ± 0.06015 ; $n=6$; $p=0.0114$; Figure 3.1A). To determine if CNR1 mRNA increased in brain regions other than the hypothalamus, relative quantification of CNR1 mRNA in the cortex and hippocampus were also obtained. There was no difference observed in CNR1 mRNA relative quantification in the hippocampus (Vehicle: 1.007 ± 0.1374 ; $n=4$; *L*-Arginine: 0.9655 ± 0.08586 ; $n=6$; $p=0.5730$; Figure 3.1B) or the cortex (Vehicle: 1.004 ± 0.1106 ; $n=4$; *L*-Arginine: 1.000 ± 0.1991 ; $n=6$; $p=0.9736$; Figure 3.1C) in animals treated with *L*-arginine in comparison to those receiving a vehicle injection.

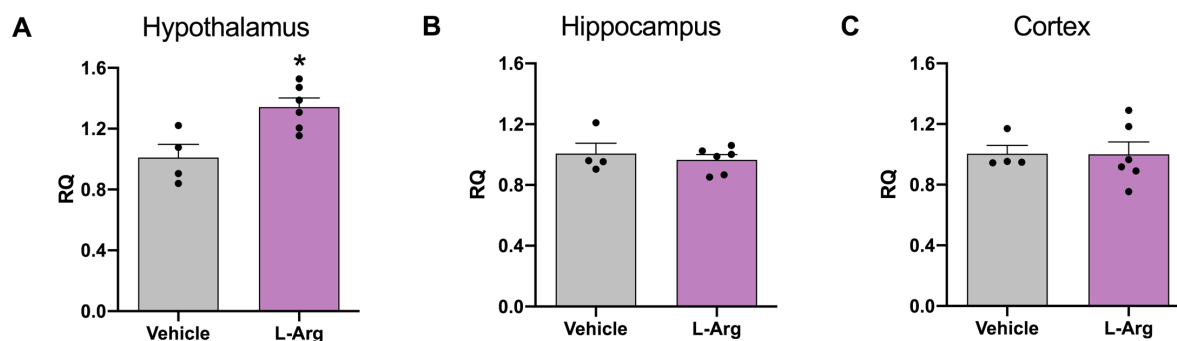


Figure 3.1. RT-PCR relative quantification of acute exposure to *L*-arginine. Relative quantification of the CNR1 mRNA by RT-PCR using the TaqMan system for brain tissue samples of male Sprague Dawley rats given an acute vehicle (saline, n=4) or *L*-arginine (100mg/kg, n=6) injection. A) Relative quantification of CNR1 mRNA in the hypothalamus. B) Relative quantification of CNR1 mRNA in the hippocampus. C) Relative quantification of CNR1 mRNA in the cortex. All values are mean \pm SEM. *= $p < 0.05$.

A prolonged exposure to *L*-arginine was achieved by dissolving *L*-arginine in animal's drinking water (2.25%) and administering the water to the animal for twenty-four hours before tissue was removed. In comparison to vehicle treated animals, no change was seen in the relative quantification of CNR1 mRNA in the hypothalamus of animals receiving *L*-arginine supplemented water (Vehicle: 1.008 ± 0.05573 ; n=6; *L*-Arginine: 0.8756 ± 0.09002 ; n=6; $p = 0.2391$; Figure 3.2A). The CNR1 mRNA of the hippocampus (Vehicle: 1.019 ± 0.09076 ; n=6; *L*-Arginine: 0.8934 ± 0.03208 ; n=6; $p = 0.2202$; Figure 3.2B) and cortex (Vehicle: 1.035 ± 0.1260 ; n=6; *L*-Arginine: 0.9976 ± 0.08380 ; n=6; $p = 0.8099$; Figure 3.2C) of animals with a prolonged exposure to *L*-arginine were also assessed and no significant difference occurred between those treated with *L*-arginine and vehicle.

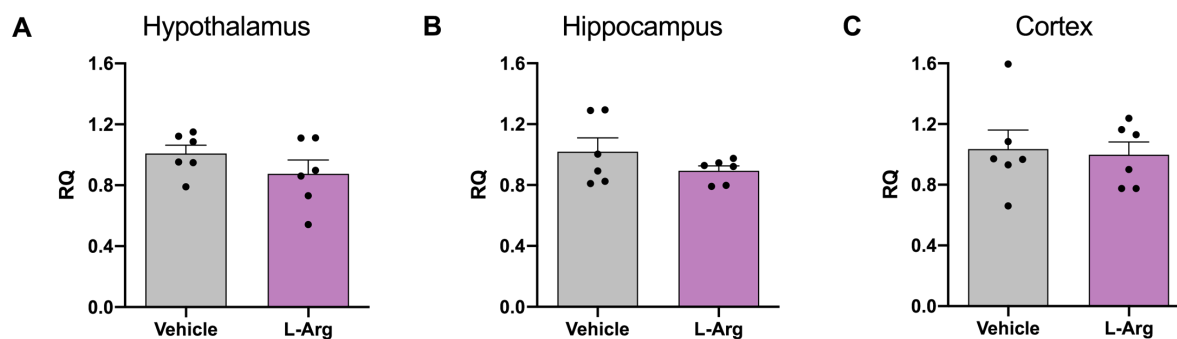


Figure 3.2. RT-PCR relative quantification of prolonged exposure to *L*-arginine. Relative quantification of the CNR1 mRNA by RT-PCR using the TaqMan system for brain tissue samples of male Sprague Dawley rats given vehicle (n=6) or *L*-arginine supplemented water

(2.25%, n=6) injection. Relative quantification of CNR1 mRNA in the A) hypothalamus, B) hippocampus, C) cortex. All values are mean \pm SEM.

3.2 Food Intake Results

Food intake over twenty-four hours was recorded in animals with a prolonged exposure to *L*-arginine. Food intake was measured at set intervals of one, two, four, six, twelve, and twenty-four hours after the start of water supplementation to calculate the cumulative food intake over 24hrs. There was no significant difference seen in food intake of animals treated with *L*-arginine in comparison to those given standard vehicle water (Vehicle: 168.4 ± 5.698 ; n=6; *L*-Arginine: 160.2 ± 9.163 ; p=0.4670; Figure 3.3A). Food intake was also recorded for the one-hour incubation period after acute injection. There was no significant difference seen between animals receiving an acute injection of *L*-arginine and those receiving a vehicle injection (Vehicle: 0.100 ± 0.200 ; n=4; *L*-Arginine: 0.3540 ± 0.1455 ; n=5; p=0.2162; Figure 3.3B).

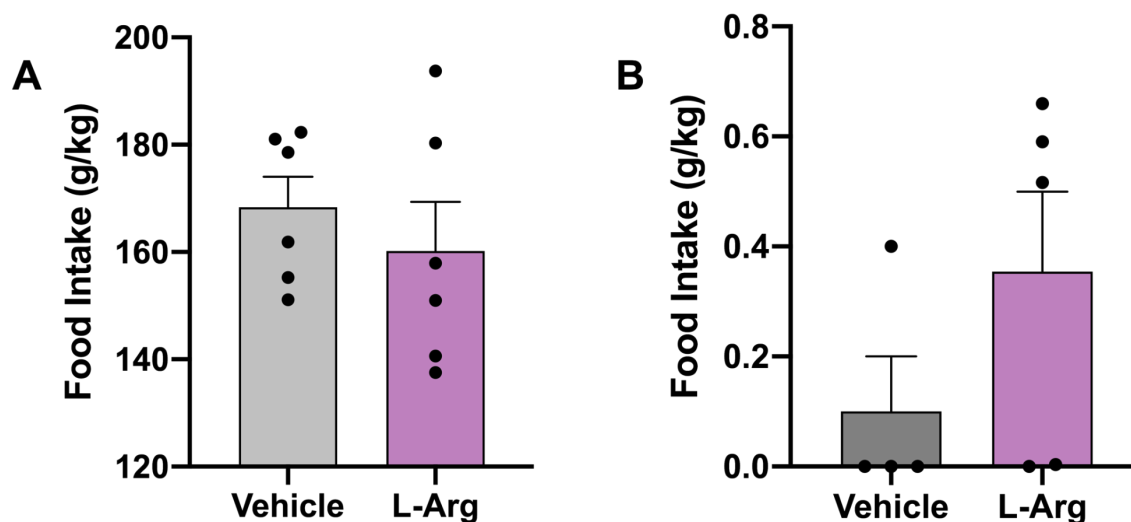


Figure 3.3. Cumulative food intake over treatment with *L*-arginine. Mean cumulative food intake in g/kg. A) Over 24-hour period of either vehicle or *L*-arginine (2.25%) supplemented water B) Over 1-hour period post injection of vehicle(saline) or *L*-arginine(100mg/kg). All values are mean \pm SEM.

3.3 Electrophysiology Recordings

Based on the increase in CNR1 mRNA in the hypothalamus, it was then necessary to investigate the effects that an acute dose of *L*-arginine has on the excitability and synaptic

transmission of neurons. As an interaction between NO and eCBs has previously been shown in the DMH, this region was chosen for electrophysiology recordings (Crosby *et al.*, 2011). Voltage and current clamp recordings at glutamate synapses from cells meeting the previously outlined criteria, were analyzed. Current clamp analysis was performed on a total of 29 cells from 11 animals, 10 cells from 6 vehicle animals and 9 cells from 5 *L*-arginine treated animals. Voltage clamp analysis was performed on a total of 18 cells from 10 animals, with 7 cells from 5 vehicle animals and 11 cells from 5 *L*-arginine animals.

3.2.1 Current Clamp Recording Results

To assess the excitability of neurons, current clamp recordings were performed. Current clamp recordings were used to control the amount of current a cell receives in 10pA stepwise intervals; from this the changes in membrane potential are recorded and excitability of the neuron can be determined. A current clamp recording was obtained after gaining access to a cell. A representative current clamp trace from each treatment group is shown in Figure 3.4. The lower black lines of the trace represent the lowest step (-100mV), the grey and purple lines indicate the first trace in which action potentials were observed, and the upper black lines indicates action potential firing at the tenth step (-10mV).

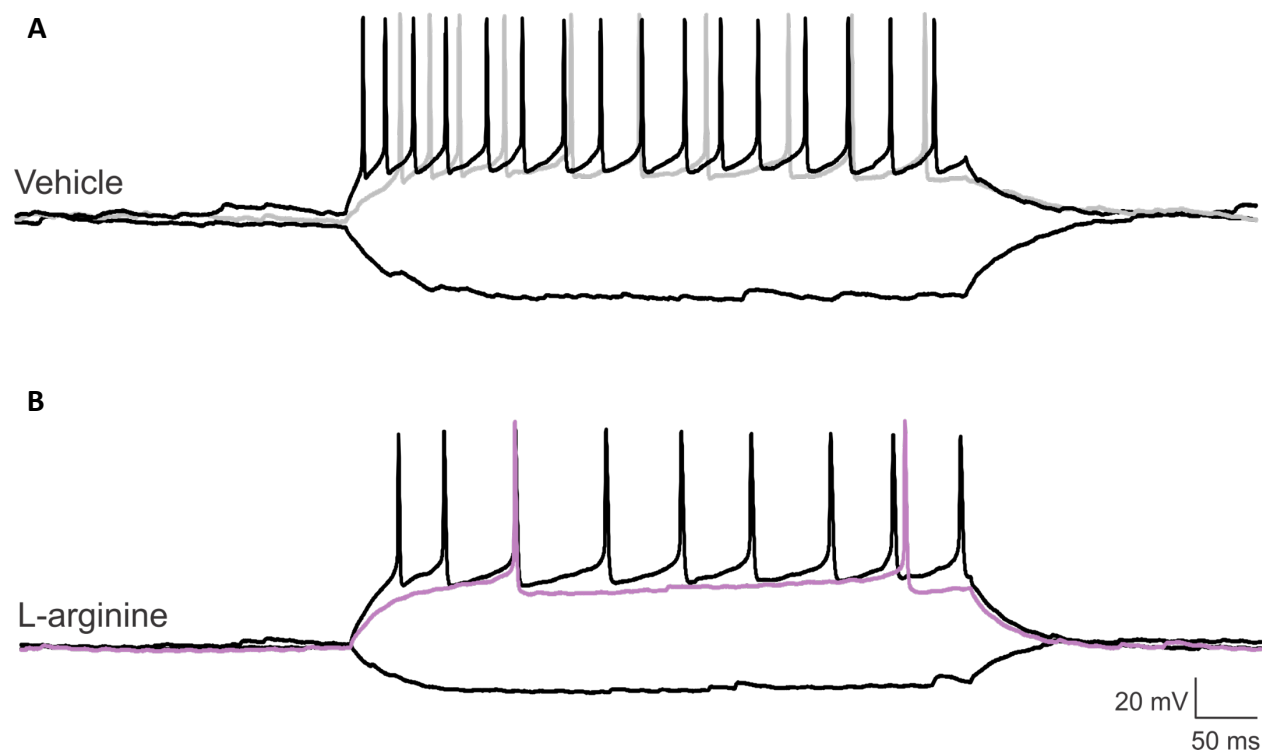


Figure 3.4. Representative current clamp traces. Representative traces of current-clamp recordings at -100mV (lower black line), first action potential fired at -60mV (red line), and -40mV (upper black line) of each treatment group: A) vehicle, B) *L*-arginine.

To assess the excitability of neurons in the DMH, action potential frequency was recorded at each current step. The action potential latency (ms), threshold (mV), amplitude (mV), half width (ms), and after hyperpolarization amplitude (mV) were determined from the current clamp recordings (Figure 3.4). The frequency of action potentials in each neuron increased with each current step as the cell became more depolarized. A t-test of each step with the Benjamini-Hochberg procedure with a false discovery rate of 0.05 used to control for false discovery, showed a statistically significant decrease in action potential frequency in the neurons of animals treated with *L*-arginine at steps 7-10 (Figure 3.5A; $p < 0.05$). Action potential latency was recorded, as it is another way to determine the excitability of a neuron. Action potential latency is the time it takes from the beginning of a depolarizing step to the firing of the first action potential. Action potential latency was recorded on the first step that action potentials were fired (-70 to -30mV). A significant increase in action potential latency was seen in animals treated with *L*-arginine (Vehicle: 96.32 ± 18.57 ms; $n=9$; *L*-arginine: 182.9 ± 33.70 ms; $n=9$; $p=0.0389$; Figure 3.5B). There was no significant difference in the membrane potential (mV) to

reach threshold depolarization for firing action potentials between the neurons from animals receiving *L*-arginine and vehicle-treated animals (Vehicle: -46.53 ± 3.542 mV; $n=10$; *L*-arginine: -40.22 ± 2.467 mV; $n=9$; $p=0.1711$; Figure 3.5C). While not significant, animals treated with *L*-arginine required slightly more depolarization (-40.22 ± 2.467 mV) to reach threshold than the control group (-46.53 ± 3.542 mV). This indicates that the majority of neurons fired their first action potential between -50 and -40 mV.

Specific properties of the action potentials were also analyzed from the current clamp recordings. *L*-arginine did not have a significant effect on the amplitude or duration at which an action potential fires. A t-test of both the amplitude (mV) (Vehicle: 79.10 ± 2.002 mV; $n=10$; *L*-arginine: 74.45 ± 4.897 mV; $n=9$; $p=0.3736$; Figure 3.5D) and half width (ms) (Vehicle: 1.398 ± 0.1425 ms; $n=10$; *L*-arginine: 1.662 ± 0.09320 ms; $n=9$; $p=0.1496$; Figure 3.5E) of action potentials did not show a significant difference between groups. The after-hyperpolarization amplitude was also recorded (Vehicle: -5.813 ± 1.911 ; $n=10$; *L*-arginine: -8.150 ± 2.905 ; $n=9$; $p=0.5024$; Figure 3.5F). There was no significant difference seen in after-hyperpolarization amplitude between vehicle and *L*-arginine treated animals. However, animals receiving *L*-arginine showed a trend towards greater after hyperpolarization amplitude (-8.150 ± 2.905 mV) in comparison to vehicle treated animals (-5.813 ± 1.911 mV).

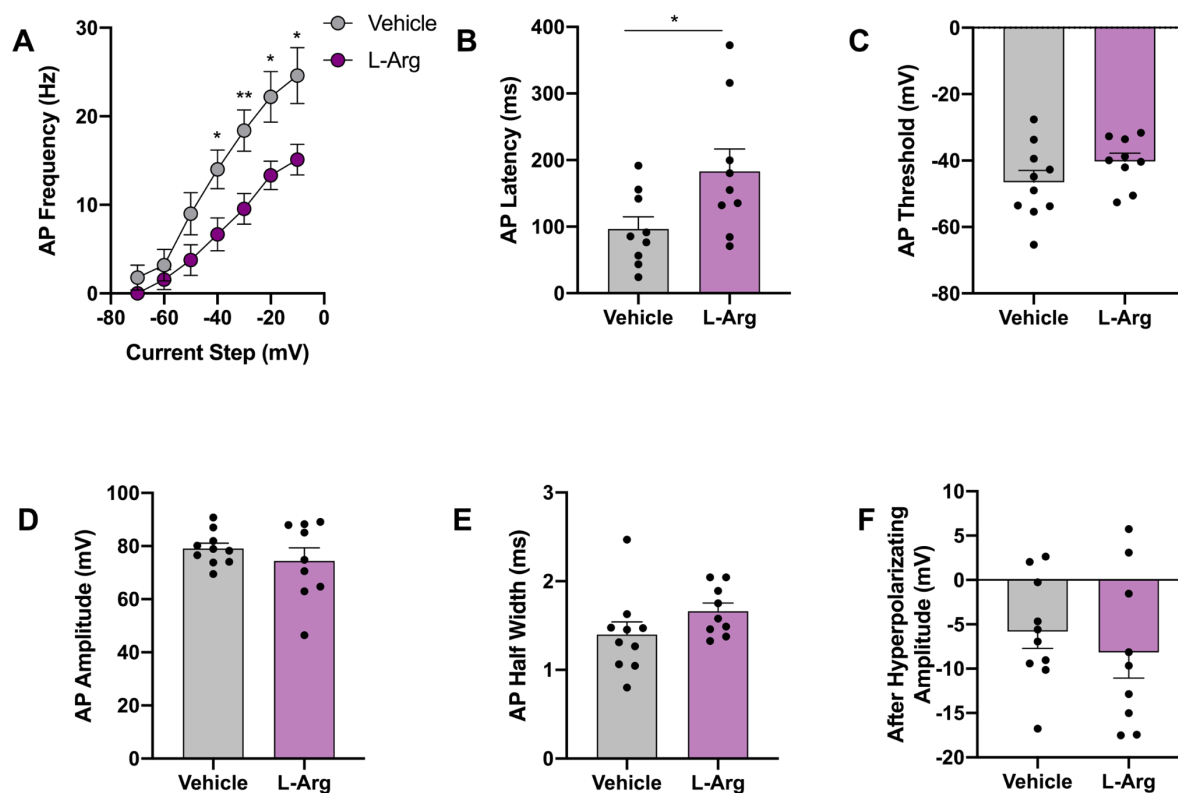


Figure 3.5. Summary of current clamp data. Current clamp recording data of the two treatment groups, vehicle (n=10) and *L*-arginine (n=9). A) Action potential frequency at each current clamp step. B) Action potential latency to fire (ms). C) Threshold of first action potential firing (mV). D) Action potential amplitude. E) Action potential half width (ms). F) After hyperpolarization amplitude. All values are mean \pm SEM. *= $p<0.05$.

3.2.2 Voltage Clamp Recordings Results

Voltage clamp recordings were analyzed to assess the impact of *L*-arginine on synaptic strength. Voltage clamp recordings were completed in five to ten-minute intervals to monitor access to the cell ($M\Omega$). A minimum of five minutes of stable baseline currents were recorded prior to high frequency stimulation (HFS). HFS was used to create an influx of neurotransmitters onto the neuron being recorded, to elicit an increase in neuronal activity. Currents were then analyzed for a maximum of twenty-five minutes post-HFS. Recordings were not included in analysis if the cell did not last more than ten minutes post-HFS or if access was not maintained. Representative voltage clamp traces of glutamate mediated currents in vehicle and *L*-arginine cells can be seen in figure 3.6A.

Evoked current amplitudes were recorded from vehicle and *L*-arginine cells to establish a baseline from which changes post-HFS can be seen. Due to the variability in the number of synapses which may be activated, a second current was evoked 50ms after the first. There was no significant difference in current amplitude between the treatment groups (Vehicle: -92.96 ± 22.72 pA; $n=9$; *L*-arginine; -66.72 ± 18.03 pA; $n=11$; $p=0.3711$). While not significant, vehicle cells tended to have larger current amplitudes (-92.96 ± 22.72 pA), than *L*-arginine cells (-66.72 ± 18.03 pA). Paired pulse ratio (PPR) is the ratio of the first evoked current in comparison to the second. The amplitude of the second current depends on the probability of synaptic vesicle release. PPR is used to measure the probability of neurotransmitter release. The PPR of the baseline currents of vehicle and *L*-arginine cells was calculated to determine whether the probability of neurotransmitter release onto DMH neurons was different in vehicle-treated vs *L*-arginine-treated animals. No significant difference was observed between baseline PPR in vehicle (1.156 ± 0.09179) and *L*-arginine (1.284 ± 0.1356) cells (Figure 3.6D, E; $p=0.4711$). PPR of vehicle and *L*-arginine cells had no significant change between pre and post HFS ($p>0.05$).

Currents were analyzed in 5-minute intervals immediately following HFS in vehicle- and *L*-arginine-treated animals. A t-test indicated a significant increase in evoked EPSC amplitude in vehicle-treated animals 0-5min post-HFS (Figure 3.6C i); $p=0.0285$). This potentiation was short-term as the amplitude of currents returned to baseline and no significant difference was noted 5-10 minutes post-HFS. Cells from animals treated with *L*-arginine did not show a short-term potentiation in the first five minutes post-HFS (Figure 3.6C ii); $p=0.3055$). Representative recordings of individual cells can be seen in figure 3.6B. Currents in representative cells are represented as negative numbers as inward currents cause downward deflections. Neither vehicle nor *L*-arginine cells were significantly different from baseline from five to ten minutes post-HFS (Figure 3.6C). No subsequent time intervals post-HFS were analyzed as changes in potentiation are unlikely to occur after returning to baseline.

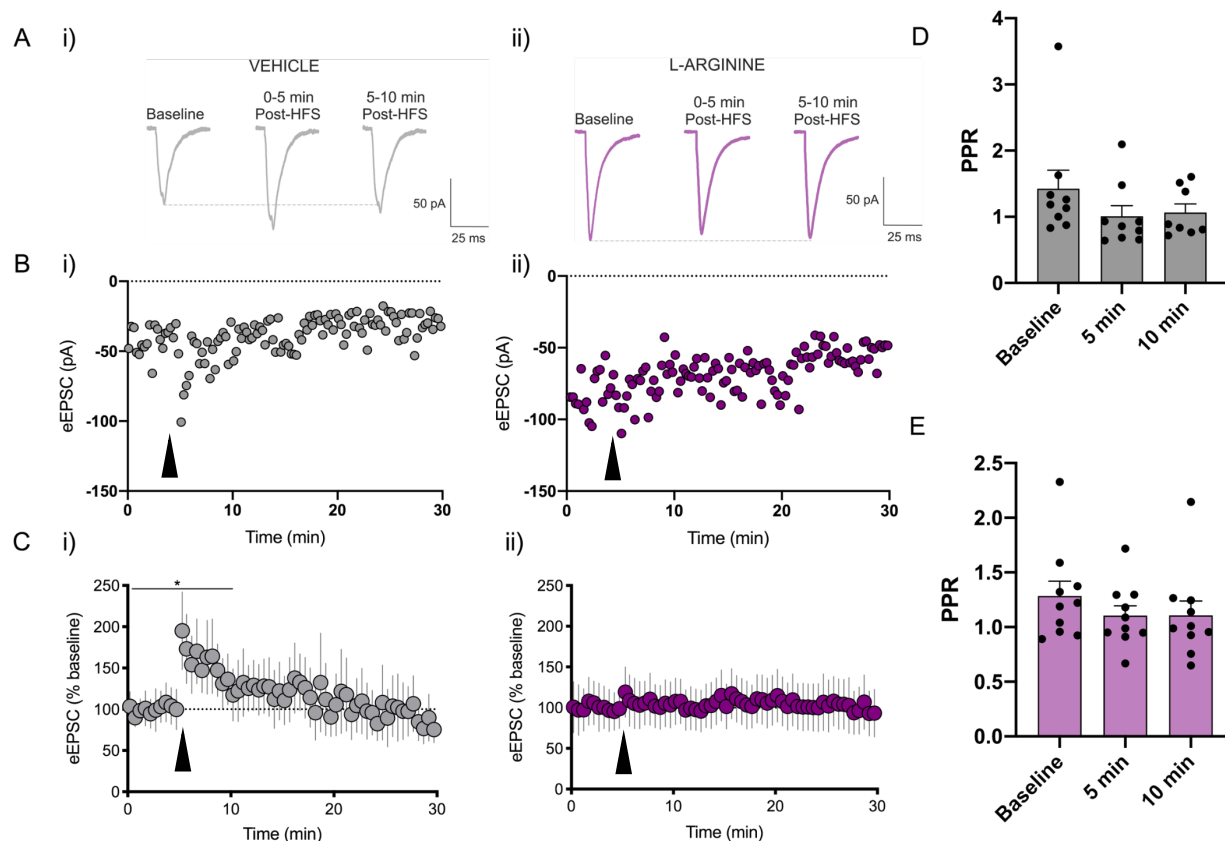


Figure 3.6. Summary of voltage clamp data. *L*-arginine diminishes short-term potentiation and has no effect on PPR. A) Representative voltage clamp traces at baseline, 0-5min post-HFS, and 5-10 min post-HFS of i) vehicle and ii) *L*-arginine cells. B) Representative cells evoked excitatory post synaptic current (eEPSC) amplitude (pA), data is averaged, arrow depicts HFS i) vehicle ii) *L*-arginine. C) Voltage clamp summary data of averaged cells, with high frequency stimulation occurring at 5 min (arrow). I) Vehicle, ii) *L*-arginine. D and E) Paired pulse ratio at baseline, 0-5 min post-HFS, and 5-10 min post-HFS. D) Vehicle and E) *L*-arginine. All values are mean \pm SEM. *= $p < 0.05$.

Overall my data suggests that an acute dose of *L*-arginine increases CNR1 mRNA levels in the hypothalamus, but not the hippocampus or cortex. In the DMH, where NO and eCBs have been shown to interact, *L*-arginine decreases neuronal excitability and prevents the short-term potentiation at glutamate synapses that is observed following HFS in vehicle-treated animals.

Chapter 4: Discussion

The goal of this study was to uncover a piece of the mechanism by which NO is required for eCB signaling. This study investigated the effect of acute and prolonged *L*-arginine exposure on CB1R expression. This was accomplished by quantifying CNR1 mRNA expression through RT-PCR. I hypothesized that exposure to *L*-arginine would alter CB1R expression, specifically in the hypothalamus. It was demonstrated by RT-PCR that animals receiving an acute injection of *L*-arginine had a significant increase in CNR1 mRNA in the hypothalamus, but not in the hippocampus or cortex, in comparison to vehicle treated animals. RT-PCR also showed no significant difference in CNR1 mRNA expression in any brain region of animals receiving a more prolonged exposure to *L*-arginine. This suggests more CB1R may be present in the hypothalamus of animals given acute injections of *L*-arginine, but not in those receiving a more prolonged exposure. From this finding, I then investigated the excitability and synaptic transmission of glutamate mediated currents in the DMH of animals receiving an acute exposure to *L*-arginine. The DMH was chosen as interactions between NO and eCBs have previously been reported in this specific region of the hypothalamus (Crosby *et al.*, 2011). I predicted that if an increase in CNR1 mRNA translated to an increase in CB1R, neuronal excitability and synaptic transmission in response to HFS would be altered. The results of electrophysiological recordings demonstrated that the neurons from animals treated with *L*-arginine were less excitable and lacked the short-term potentiation that occurred post-HFS in vehicle-treated animals. This indicates that the increase in CNR1 mRNA in the hypothalamus caused by acute exposure to *L*-arginine may have translated to an increase in CB1R expression in the DMH, as it is accompanied by neurons of the DMH becoming less likely to fire.

4.1 Acute Exposure to *L*-Arginine Increases CNR1 mRNA in the Hypothalamus

Expression of CB1Rs was measured through RT-PCR relative quantification of CNR1 mRNA from the hypothalamus, hippocampus, and cortex. I found animals receiving an acute *L*-arginine injection to have increased levels of CNR1 mRNA, specifically in the hypothalamus. There is evidence that the CB1R is up and downregulated by a variety of environmental factors such as stress, feeding state, and in the presence of drugs like THC (Hillard, 2014; Hirvonen *et al.*, 2012; Crosby *et al.*, 2011). Several studies have suggested that stress hormones can negatively impact CB1R expression through transcriptional regulation (Hillard, 2014). This same

effect has been shown through electrophysiology studies in which the stress of food deprivation downregulates CB1Rs in the DMH (Crosby *et al.*, 2011). Also in the hypothalamus water deprivation causes an increase in CNR1 mRNA (Ruginik *et al.*, 2015). These studies show that the expression of CB1Rs is subject to variation, as seen in this study through the increase of CNR1 mRNA in response to *L*-arginine injection.

The mechanisms by which CB1R expression is altered are not well understood. In the ventromedial hypothalamus steroidogenic factor 1 (SF-1) is a regulator of CB1R expression (Kim *et al.*, 2008). In SF-1 knock out mice, agonists and antagonists of CB1R lose their effect on food intake (Kim *et al.*, 2008). As SF-1 is only present in the ventromedial hypothalamus, it is possible that NO produced from the *L*-arginine injection may in some way be acting with SF-1 and causing an increase in CB1R expression. If NO was interacting with SF-1 to increase CB1R expression, this would explain why only the hypothalamus showed a significant increase in CNR1 mRNA. Further research is required to determine the pathway NO is acting on to increase the transcription of CNR1 mRNA in the hypothalamus.

4.2 Prolonged Exposure to *L*-Arginine Has No Effect on CNR1 mRNA in the Hypothalamus, Hippocampus, or Cortex

In contrast to the acute treatment, hypothalamic levels of CNR1 mRNA did not show a change in any brain area of animals which received a more prolonged exposure to *L*-arginine. There are several potential explanations for this. Animals received a dose of 2.25% *L*-arginine as this dose was seen to have sufficient effects on arginase enzyme in rats (Moretto *et al.*, 2017). However, the one-time high concentration of *L*-arginine administered by IP injection would cause a robust immediate increase in concentration of *L*-arginine in the animal versus a more modest and prolonged influx. It is possible that there was not a great enough increase in NO to elicit a change in CNR1 mRNA expression as was seen in animals that received an acute injection. Many studies have been conducted using oral *L*-arginine supplementation for endothelial effects (Khalaf *et al.*, 2019). Little research has been conducted on oral *L*-arginine supplementation and effects on the brain, so it is unknown if oral supplementation of *L*-arginine is able to increase NO in the brain. It is also possible that CB1R expression increased and returned to normal within the 24hrs of treatment. This would require future experiments where

tissue sampling occurs at varying times post treatment with *L*-arginine dosed water, to see if and when CNR1 expression may increase.

4.3 Neither Prolonged, nor Acute Exposure to *L*-Arginine have an Effect on Food Intake

Food intake was not affected by *L*-arginine in animals given acute and prolonged *L*-arginine treatments. This is consistent with previous findings from the Crosby laboratory which demonstrated that *L*-arginine injections onto the DMH do not cause an increase in food intake of standard diet (McGavin *et al.*, 2019). However, the data on food intake with *L*-arginine is variable and an effect on appetite with *L*-arginine supplementation has been shown in mice (Morely & Flood, 1991).

With the increase in CNR1 mRNA found in the hypothalamus, one might expect to see an increase in food intake. Increased activation of CB1R has been shown to stimulate food intake (Hillard, 2018). In support of this, CB1R knockout mice are leaner than their wild type counterparts (Cardinal *et al.*, 2012). This suggests that food intake and body weight may fluctuate with the expression of CB1R. In our study, animals receiving an acute injection of *L*-arginine exhibited an increase in CB1R mRNA, but no change in food intake. This may be attributed to injections being conducted during the first four hours of the light period when animals are satiated. There is evidence that CB1R mRNA fluctuates throughout the day, emphasizing the importance in this research of maintaining constant schedules of tissue extraction (Bazwinsky-Wutschke *et al.*, 2017). A change in food intake may have been observed if the animals were not satiated or if the injection occurred during the dark period when animals are most actively feeding. For this study, it was most important to maintain a constant schedule to analyze the interaction of NO and eCB signalling; in future studies it may be beneficial to look at the effect that differing incubation time post-injection has on food intake.

4.4 Acute Exposure to *L*-Arginine Decreases Neuronal Excitability in the DMH

Due to the increase in CNR1 mRNA found in the hypothalamus of animals receiving an acute injection of *L*-arginine, the next step was to look at the excitability and synaptic transmission of an area of the hypothalamus already known to exhibit an interaction between NO and eCBs: the DMH. Neuronal excitability was measured through current clamp recording

steps. DMH neurons from animals receiving an *L*-arginine injection were less excitable than those from vehicle-treated animals. NO has been found to have differential effects on neuronal excitability based on concentration (Park *et al.*, 2024). In the spinal cord, NO donors in high concentrations have been found to have an inhibitory effect while in low concentrations NO had an excitatory effect (Park *et al.*, 2014). In the cortex and hippocampus, reduced NO levels increase excitatory transmission due to the over activity of NMDA receptors in the absence of NO-mediated modulation (Ferraro *et al.*, 1999). The effects of NO on neuronal excitability are variable. One possible explanation of the decreased excitability seen in our research may be due to NO modulation of NMDA receptors. NMDA receptors have previously been found to be blocked by NOS conversion of *L*-arginine to NO (Manzoni & Bockaert, 1993). However, it is unknown how much NO is still remaining at the time of current clamp recording, as such future research would be necessary to see if NO donors decrease neuronal excitability in the DMH to ensure the same effects are seen and they are NO mediated.

The decreased neuronal excitability in *L*-arginine-treated animals may be mediated by the increased expression of CB1R. CB1R have been known to have anti-epileptic effects, as some patients using THC experience a cessation of seizures (Mechoulam, 1986). A study on mice found that the eCB anandamide has powerful anticonvulsant properties which are erased in the presence of a CB1R agonist (Wallace *et al.*, 2002). From these findings, it can be hypothesized that a greater expression of CB1R may lead to decreased excitability of neurons.

Neurons also have a property known as intrinsic excitability. This is determined by the quantity and localization of receptors and ion channels related to the depolarization of a neuron. This involves mechanisms such as changes in resting membrane potential and subthreshold excitability (D'Angelo, 2010). The homeostasis of intrinsic excitability has been shown to be mediated by voltage dependent calcium channels (D'Angelo, 2010). The *L*-arginine-induced effects on neuronal excitability may be mediated by intrinsic excitability mechanisms specific to the neuron recorded rather than external mechanisms from the surrounding neurons. In the neocortex, eCBs have been found to mediate self-inhibition in glutamatergic circuits. In CB1R knock out mice, self-inhibition in response to eCBs was not observed, suggesting that the inhibition seen was CB1R mediated (Marinelli *et al.*, 2009). These changes in self-inhibition in the neocortex were determined to be mediated by potassium channels on the receptors (Marinelli *et al.*, 2009). The changes seen in the neocortex by Marinelli *et al.* (2009) are in accordance with

those seen in the DMH in my study. This suggests that the effects seen in the decreased excitability of DMH neurons in response to *L*-arginine may be a result of intrinsic excitability related to the increased expression of CB1R.

4.5 High Frequency Stimulation Causes Short-Term Potentiation in Vehicle Treated Animals

To determine the synaptic effects of *L*-arginine in the DMH, excitatory postsynaptic currents were evoked in the DMH with paired stimuli and after a sufficient baseline was recorded, a robust high frequency stimulation of incoming neurons was elicited. In animals treated with a vehicle saline injection, short-term potentiation was observed in the first five minutes post-HFS. This is, to the best of my knowledge, the first report of the response of glutamate synapses in the DMH to HFS. Elsewhere in the hypothalamus, researchers have shown NMDA receptor-dependent long-term potentiation of glutamate synapses in the supraoptic nucleus (Panatier et al., 2006). Other research has shown that a single stress induces short term potentiation after HFS at glutamate synapses in the paraventricular nucleus (Kuzmiski *et al.*, 2010). The paired pulse ratio was determined pre and post-HFS to assess whether the short-term potentiation might be due to an increase in glutamate release from presynaptic neurons or to an increase in receptor activity or expression on the postsynaptic neurons. The short-term potentiation observed in vehicle-treated animals was not associated with a change in PPR. This suggests that the short-term potentiation is due to changes in the postsynaptic neuron. The exact mechanisms of short-term potentiation are currently unknown (Lisman, 2017). HFS may cause receptors, such as NMDA and AMPA receptors, to be phosphorylated or to open longer, thus making the receptors more active. Short-term potentiation occurs rapidly after stimulation, and because of this, it is likely that the changes in synaptic transmission may be mediated by phosphorylating a receptor such as AMPA receptors which are already present in the membrane (Lisman, 2017).

4.6 Acute Exposure to *L*-Arginine Removes Short-Term Potentiation Seen in Vehicle Cells Post-High Frequency Stimulation

Our results show that in animals treated with *L*-arginine, the short-term potentiation is abolished. One possible explanation of this that would be consistent with the RT-PCR data is that an increase in CB1R expression on the axon terminals of neurons synapsing onto the DMH

are decreasing the amount of glutamate released onto DMH neurons and thereby preventing short-term potentiation. CB1R are known to inhibit the release of glutamate when activated (Alger, 2002). HFS causes a flood of neurotransmitters, including eCBs to be released. If more CB1R are present on the membrane this may cause the abolishing of short-term potentiation that is seen.

Previous studies have shown that depression of synaptic transmission occurs at glutamate synapses in the hippocampus in the presence of CB1R agonists (Ameri *et al.*, 1999). Similar effects have been shown in GABA mediated currents in the DMH (Crosby *et al.*, 2011). If there are more CB1R receptors to be activated on the membranes, we may have expected to see a depression of currents. However, the effects of CB1R agonists at glutamate synapses in the DMH have not yet been recorded in the literature. The difference in the effects seen in the hippocampus in comparison to those seen in our experiment may be attributed to the greater number of CB1R normally present in the hippocampus (Mackie, 2005). If the effects seen in the DMH are CB1R mediated, the increased expression of CB1R seen from *L*-arginine injection, may be enough to remove the short-term potentiation seen in vehicle cells, but not great enough to show a depression of glutamate currents. The short-term potentiation seen in vehicle cells was found by PPR to occur in the postsynaptic neuron. If more CB1R are present on the presynaptic membrane and are more activated, there will be less glutamate release from the presynaptic membrane. As such, even if the receptors on the postsynaptic cell are more active due to HFS, they will still not be activated by neurotransmitters to exhibit short term potentiation.

4.7 Future Directions

Overall, I have demonstrated that an acute injection of *L*-arginine increases CNR1 mRNA in the hypothalamus, decreases neuronal excitability in the DMH, and abolishes short-term potentiation post-HFS in the DMH. From RT-PCR experiments conducted in this project, the next step would be to determine if the increase seen in CNR1 mRNA levels translates to CB1R protein expression. As CB1R antibodies have been found to be notoriously non-specific, a Western Blot or other immunodetection protocol would not be suitable (Grimsey *et al.*, 2008; Morozov *et al.*, 2013). A radioligand binding assay may be a more appropriate way of looking at receptor expression in response to *L*-arginine as it does not rely on antibodies (Bowles *et al.*, 2012).

Future electrophysiology experiments are required to determine whether the *L*-arginine-induced effects on neuronal excitability and synaptic transmission of glutamate currents require CB1R activation. To test this, an antagonist of CB1R can be applied to the slice during electrophysiology experiments or given to animals prior to sacrifice in addition to acute treatment with *L*-arginine. If the masking of short-term potentiation post-HFS is CB1R mediated, then in the presence of a CB1R antagonist this effect should be revealed. The role of CB1Rs can also be assessed by placing a CB1R agonist, such as WIN-55,212-2, on the brain slice to show any CB1R mediated changes in synaptic transmission. In animals exposed to *L*-arginine, if the effects seen are mediated but CB1R expression, a CB1R agonist should activate more CB1R and have greater inhibitory effects on glutamate signalling than those seen in vehicle-treated animals.

4.8 Conclusions

This study provides evidence that in male Sprague Dawley rats, an acute injection of *L*-arginine increases the expression of CNR1 mRNA in the hypothalamus. This led to electrophysiology studies which found that the neurons of animals receiving an *L*-arginine injection were less excitable than those receiving a vehicle injection. Animals receiving a vehicle injection showed short-term potentiation in the first five minutes post-HFS mediated by the post synaptic neuron. Animals receiving acute *L*-arginine injections showed no short-term potentiation post-HFS. This research could contribute to uncovering the mechanism by which NO is required for eCB signaling. Research in this area could potentially lead to therapeutic treatments for cannabinoid system mediated illnesses.

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References

- Al-Goblan AS, Al-Alfi MA, Khan MZ. Mechanism linking diabetes mellitus and obesity. *Diabetes Metab Syndr Obes.* 2014 Dec 4;7:587-91.
- Alger BE. Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. *Prog Neurobiol.* 2002;68(4):247–286.
- Alger BE. Endocannabinoid identification in the brain: studies of breakdown lead to breakthrough, and there may be NO hope. *Sci STKE.* 2005;2005(309):pe51.
- Ameri A, Wilhelm A, Simmet T. Effects of the endogenous cannabinoid, anandamide, on neuronal activity in rat hippocampal slices. *Br J Pharmacol.* 1999;126(8):1831–1839.
- Bazwinsky-Wutschke I, Zipprich A, Dehghani F. Daytime-Dependent Changes of Cannabinoid Receptor Type 1 and Type 2 Expression in Rat Liver. *Int J Mol Sci.* 2017;18(9):1844.
- Bellinger LL, Bernardis LL. The dorsomedial hypothalamic nucleus and its role in ingestive behavior and body weight regulation: lessons learned from lesioning studies. *Physiol Behav.* 2002;76(3):431-42.
- Bénard G, Massa F, Puente N, et al. Mitochondrial CB₁ receptors regulate neuronal energy metabolism. *Nat Neurosci.* 2012;15(4):558–564.
- Bowles NP, Hill MN, Bhagat SM, Karatsoreos IN, Hillard CJ, McEwen BS. Chronic, noninvasive glucocorticoid administration suppresses limbic endocannabinoid signaling in mice. *Neuroscience.* 2012;204:83–89.
- Cardinal P, Bellocchio L, Clark S, et al. Hypothalamic CB1 cannabinoid receptors regulate energy balance in mice. *Endocrinology.* 2012;153(9):4136–4143.
- Crosby KM, Inoue W, Pittman QJ, Bains JS. Endocannabinoids gate state-dependent plasticity of synaptic inhibition in feeding circuits. *Neuron.* 2011;71(3):529–541.
- Crosby KM, Murphy-Royal C, Wilson SA, Gordon GR, Bains JS, Pittman QJ. Cholecystokinin Switches the Plasticity of GABA Synapses in the Dorsomedial Hypothalamus via Astrocytic ATP Release. *J Neurosci.* 2018;38(40):8515–8525.
- D'Angelo E. Homeostasis of intrinsic excitability: making the point. *J Physiol.* 2010;588(Pt 6):901–902.
- Devane WA, Hanus L, Breuer A, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science.* 1992;258(5090):1946–1949.

- Di Marzo V. Endocannabinoids: synthesis and degradation. *Rev Physiol Biochem Pharmacol.* 2008;160:1–24.
- Engin A. The Definition and Prevalence of Obesity and Metabolic Syndrome. *Adv Exp Med Biol.* 2017;960:1-17.
- Feil R, Kleppisch T. NO/cGMP-dependent modulation of synaptic transmission. *Handb Exp Pharmacol.* 2008;(184):529-60.
- Ferraro G, Montalbano ME, La Grutta V. Nitric oxide and glutamate interaction in the control of cortical and hippocampal excitability. *Epilepsia.* 1999;40(7):830–836.
- Grimsey NL, Goodfellow CE, Scotter EL, Dowie MJ, Glass M, Graham ES. Specific detection of CB1 receptors; cannabinoid CB1 receptor antibodies are not all created equal!. *J Neurosci Methods.* 2008;171(1):78–86.
- Hervera A, Negrete R, Leánez S, Martín-Campos J, Pol O. The role of nitric oxide in the local antiallodynic and antihyperalgesic effects and expression of delta-opioid and cannabinoid-2 receptors during neuropathic pain in mice. *J Pharmacol Exp Ther.* 2010;334(3):887-96.
- Hillard CJ. Stress regulates endocannabinoid-CB1 receptor signaling. *Semin Immunol.* 2014;26(5):380–388.
- Hillard CJ. Circulating Endocannabinoids: From Whence Do They Come and Where are They Going? *Neuropsychopharmacology.* 2018;43(1):155-172.
- Hirvonen J, Goodwin RS, Li CT, Terry GE, Zoghbi SS, Morse C, Pike VW, Volkow ND, Huestis MA, Innis RB. Reversible and regionally selective downregulation of brain cannabinoid CB1 receptors in chronic daily cannabis smokers. *Mol Psychiatry.* 2012;(6):642-9.
- Khalaf D, Krüger M, Wehland M, Infanger M, Grimm D. The Effects of Oral *L*-Arginine and *L*-Citrulline Supplementation on Blood Pressure. *Nutrients.* 2019;11(7):1679.
- Kim KW, Jo YH, Zhao L, Stallings NR, Chua SC Jr, Parker KL. Steroidogenic factor 1 regulates expression of the cannabinoid receptor 1 in the ventromedial hypothalamic nucleus. *Mol Endocrinol.* 2008;22(8):1950–1961.
- Kuzmiski JB, Marty V, Baimoukhametova DV, Bains JS. Stress-induced priming of glutamate synapses unmasks associative short-term plasticity. *Nat Neurosci.* 2010;13(10):1257–1264.

- Lipina C, Hundal HS. The endocannabinoid system: 'NO' longer anonymous in the control of nitrenergic signalling? *J Mol Cell Biol*. 2017;9(2):91-103.
- Lisman J. Glutamatergic synapses are structurally and biochemically complex because of multiple plasticity processes: long-term potentiation, long-term depression, short-term potentiation and scaling. *Philos Trans R Soc Lond B Biol Sci*. 2017;372(1715):20160260.
- Mackie K. Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb Exp Pharmacol*. 2005;(168):299–325.
- Makara JK, Katona I, Nyíri G, Németh B, Ledent C, Watanabe M, de Vente J, Freund TF, Hájos N. Involvement of nitric oxide in depolarization-induced suppression of inhibition in hippocampal pyramidal cells during activation of cholinergic receptors. *J Neurosci*. 2007 Sep 19;27(38):10211-22.
- Marinelli S, Pacioni S, Cannich A, Marsicano G, Bacci A. Self-modulation of neocortical pyramidal neurons by endocannabinoids. *Nat Neurosci*. 2009;12(12):1488–1490.
- Martin BR, Mechoulam R, Razdan RK. Discovery and characterization of endogenous cannabinoids. *Life Sci*. 1999;65(6-7):573-95.
- McGavin JJ, Cochkanoff NL, Poole EI, Crosby KM. 2-arachidonylglycerol interacts with nitric oxide in the dorsomedial hypothalamus to increase food intake and body weight in young male rats. *Neurosci Lett*. 2019;698:27-32.
- Mechoulam R. The pharmacohistory of Cannabis sativa Cannabinoids as Therapeutic Agents. *Mechoulam, R*. 1986: 1–19.
- Mechoulam R, Ben-Shabat S, Hanus L, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol*. 1995;50(1):83–90.
- Meng XD, Wei D, Li J, et al. Astrocytic expression of cannabinoid type 1 receptor in rat and human sclerotic hippocampi. *Int J Clin Exp Pathol*. 2014;7(6):2825–2837.
- Moretto J, Guglielmetti AS, Tournier-Nappey M, et al. Effects of a chronic *L*-arginine supplementation on the arginase pathway in aged rats. *Exp Gerontol*. 2017;90:52–60.
- Morley JE, Flood JF. Evidence that nitric oxide modulates food intake in mice. *Life Sci*. 1991;49(10):707–711.

- Morozov YM, Dominguez MH, Varela L, et al. Antibodies to cannabinoid type 1 receptor co-react with stomatin-like protein 2 in mouse brain mitochondria. *Eur J Neurosci*. 2013;38(3):2341–2348.
- Myers B, Mark Dolgas C, Kasckow J, Cullinan WE, Herman JP. Central stress-integrative circuits: forebrain glutamatergic and GABAergic projections to the dorsomedial hypothalamus, medial preoptic area, and bed nucleus of the stria terminalis. *Brain Struct Funct*. 2014;219(4):1287–1303.
- Otgon-Uul Z, Suyama S, Onodera H, Yada T. Optogenetic activation of leptin- and glucose-regulated GABAergic neurons in dorsomedial hypothalamus promotes food intake via inhibitory synaptic transmission to paraventricular nucleus of hypothalamus. *Mol Metab*. 2016;5(8):709–715.
- Pacher P, Bátkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev*. 2006;58(3):389–462.
- Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from *L*-arginine. *Nature*. 1988;333(6174):664–666.
- Panatier A, Gentles SJ, Bourque CW, Oliet SH. Activity-dependent synaptic plasticity in the supraoptic nucleus of the rat hypothalamus. *J Physiol*. 2006;573(Pt 3):711–721.
- Park AR, Lee HI, Semjid D, Kim DK, Chun SW. Dual effect of exogenous nitric oxide on neuronal excitability in rat substantia gelatinosa neurons. *Neural Plast*. 2014;2014:628531.
- Pertwee RG. Endocannabinoids and Their Pharmacological Actions. *Handb Exp Pharmacol*. 2015;231:1-37.
- Ruginsk SG, Vechiato FM, Uchoa ET, Elias LL, Antunes-Rodrigues J. Type 1 cannabinoid receptor modulates water deprivation-induced homeostatic responses. *Am J Physiol Regul Integr Comp Physiol*. 2015;309(11):R1358–R1368.
- Safo PK, Cravatt BF, Regehr WG. Retrograde endocannabinoid signaling in the cerebellar cortex. *Cerebellum*. 2006;5(2):134-45.
- Schurman LD, Lu D, Kendall DA, Howlett AC, Lichtman AH. Molecular Mechanism and Cannabinoid Pharmacology [published online ahead of print, 2020 Apr 1]. *Handb Exp Pharmacol*. 2020;10.1007/164_2019_298.

- Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature*. 2000 Apr 6;404(6778):661-71.
- Stellar, E. The physiology of motivation. *Psychol. Rev.* 61, 5 (1954).
- Svízenská I, Dubový P, Sulcová A. Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures--a short review. *Pharmacol Biochem Behav.* 2008;90(4):501-11.
- Vincent SR. Nitric oxide neurons and neurotransmission. *Prog Neurobiol.* 2010;90(2):246-55.
- Wallace MJ, Martin BR, DeLorenzo RJ. Evidence for a physiological role of endocannabinoids in the modulation of seizure threshold and severity. *Eur J Pharmacol.* 2002;452(3):295-301.
- Xin X, Storlien LH, Huang XF. Hypothalamic c-fos-like immunoreactivity in high-fat diet-induced obese and resistant mice. *Brain Res Bull.* 2000;52(4):235-242.
- Zhang PW, Ishiguro H, Ohtsuki T, Hess J, Carillo F, Walther D, Onaivi ES, Arinami T, Uhl GR. Human cannabinoid receptor 1: 5' exons, candidate regulatory regions, polymorphisms, haplotypes and association with polysubstance abuse. *Mol Psychiatry.* 2004;9(10):916-31.