

ENDOCANNABINOIDS AND NITRIC OXIDE: EFFECTS ON HIGH-FAT
FOOD CONSUMPTION IN YOUNG RATS

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Abstract

Childhood obesity has become an increasingly prevalent disease worldwide, predisposing children and adolescents to physiological and psychological health complications, extending into adult years. Generally, weight gain is a result of a prolonged imbalance of food intake and energy expenditure. Further investigation of pathways affecting appetite regulation is required to develop safer, effective treatments for obesity. Two signals of interest are endocannabinoids (eCBs) and nitric oxide (NO), which have been shown to regulate food intake in adult animals. Research in young animals is limited, but it has been suggested that these neurotransmitters have different age-dependent effects on appetite in rats fed a standard diet. The current investigation was undertaken in fasted and non-fasted rats fed a high-fat diet, to gain insight into the effect of inhibiting or exciting eCB and NO pathways on palatable food consumption. In the first condition, young Sprague-Dawley rats were fasted for 24 hours, assigned randomly to a drug treatment or control group, and were re-fed for a 2-hour period, after which they were sacrificed and perfused. Food consumption data and accompanying body weight data were collected from each trial. In fasted animals, inhibiting the production of nitric oxide was shown to significantly reduce food intake and body weight, and blocking cannabinoid receptors also resulted in a significant decrease in food consumption compared to a control group receiving saline injections. In subsequent trials in non-fasted animals, blocking cannabinoid receptors resulted in a significant decrease in body weight compared to control animals. Results demonstrated that the manipulation of eCB and NO pathways in young rats fed a high-fat diet differed in their effects when compared to standard diet fed animals. These results confirm that further investigation is needed in non-fasted animals, as well as animals chronically exposed to a high-fat diet, to understand the physiological response of these pathways to drug interference.

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List of Abbreviations

ANOVA	Analysis of variance
BAT	Brown adipose tissue
CB1R	Type 1 cannabinoid receptor
CB2R	Type 2 cannabinoid receptor
CNS	Central nervous system
DMH	Dorsomedial hypothalamus
DMSO	Dimethyl sulfoxide
eCBs	Endocannabinoids
eNOS	Endothelial nitric oxide synthase
HFC	High-fat chow
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
L-NAME	L-nitroarginine methyl ester
L-NO Arg	L-N ^G -nitro arginine
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
POMC	Pro-opiomelanocortin
SC	Standard chow
THC	Delta 9-tetrahydrocannabinol

Chapter 1: Introduction

1.1: Obesity

The Centers for Disease Control and Prevention define obesity as a “weight that is higher than what is considered as a healthy weight for a given height” (2016). Childhood obesity is most prevalent in Europe and the Western world, however children from families of higher socioeconomic status in underdeveloped countries are also found to be at a disproportionately higher risk (Abarca-Gómez et al., 2017). The etiology for childhood obesity can be the result of a genetic predisposition or early-life factors. Most commonly, however, it is correlated with an imbalance in energy intake and expenditure over time, as children live increasingly sedentary lifestyles with high-fat and high-sugar diets (Bircan, 2009; Ebbeling et al., 2002; Hills et al., 2011). Obesity in children is a major health issue requiring remediation to prevent long-term effects into adult years. Childhood and adolescent obesity increases life-long health risk factors for developing chronic conditions including hypertension, type 2 diabetes mellitus, metabolic syndrome, and psychosocial problems (Janicke et al., 2007; Seth & Sharma, 2013; Vos & Welsh, 2011). Evidence has suggested that as early as age three to four, children begin to respond to environmental cues in lieu of biological satiety cues, in terms of food intake, potentially leading to the development of obesity (Rolls et al., 2000).

1.2: The Brain and Food Intake

Hunger is a product of the interaction between sensory and metabolic signaling in the central nervous system (CNS) and hormonal release from the gastrointestinal tract (Wren & Bloom, 2007). Qualities such as a food’s palatability and nutritional usability are established prior to ingestion. Eventually, through energy homeostasis, the initial desire to eat is decreased (a process known as “satiety”) as an individual begins to consume food. Energy derived from this food is either metabolized or stored as fat (Morton et al., 2006). In mammals, a hormone known as leptin, which originates in adipose tissue, acts as a signaling device to communicate the size of a fat mass to the brain, and induces satiety. Insulin, the main glucose regulating hormone of the body, also has dense populations of receptors in the brain, and acts as an appetite-suppressing signal (Pliquett et al., 2006). Regulating metabolic balance against these anorexigenic signals is

ghrelin, a hormone in the gastrointestinal tract which increases appetite upon activation (Holst & Schwarz, 2004). These hormones, as well as many others, communicate along a gut-brain axis, beginning in the brainstem and continuing through to the hypothalamus and on to higher brain centers.

Episodic feedback is a regulatory mechanism driven by an organism's consumption of a single meal. It includes biological responses throughout the body in response to the stimulus, such as the anticipation of intake, gut distention and chemo-detection of nutrients once digestion has occurred. These signals are delivered to the CNS and subsequently, the hypothalamus, where they interact with long-term (tonic) feedback signals in the brain regions' regulatory circuits. Significant abnormalities in this feedback loop can lead to extreme hyperphagia (over-consumption of food), which is of interest in obesity research (Harrold & Halford, 2006).

1.3: The Hypothalamus

The hypothalamus acts as a food intake and energy expenditure modulator. This brain region receives direct feedback on food intake via the brain stem from the gut. In turn, the hypothalamus produces an appropriate bodily response through stimulating further food consumption, which can occur through activation of NPY neurons, or satiety through activation of POMC neurons (Camiilleri, 2015), among other mechanisms. Specific nuclei within the hypothalamus, including the arcuate nucleus, paraventricular nucleus, and dorsomedial hypothalamus (DMH) regulate energy balance (Kontureket al., 2004; Suzuki et al., 2012). This regulatory signaling was discovered through lesioning studies in the mid-1900's, allowing researchers to better understand the complexity of pathways in these distinct regions (Reichenbach et al., 2012).

1.4: Nitric Oxide

Many regulatory signals in the hypothalamus are involved in appetite regulation (Kalra, 1997). Research has proposed nitric oxide (NO) to be a central signal in this process, as it has been shown to regulate levels of such central signals as NPY and ghrelin in mice (Morley et al., 2011). NO is a gaseous molecule derived from its precursor L-arginine with the addition of NADPH and oxygen (O₂) by the enzyme nitric

oxide synthase (NOS) (Aktan, 2004), which also produces L-citrulline (Andrew & Mayer, 1999; Moncada & Higgs, 1991). Three distinct synthases produce NO: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Bredt, 1999). While activation of eNOS and nNOS are dependent on the presence of calcium (Ca^{2+}) to facilitate the binding of calmodulin (Nathan & Xie, 1994), iNOS operates independently of Ca^{2+} levels and is activated instead by cytokines and other inducers of the immune response (Aktan, 2004).

Although it is known through studies in mice and humans that NO levels are downregulated after the onset of obesity, the direct impact of manipulating these levels on food intake is still poorly understood (Wellhauser et al., 2016). Some past research has indicated that inducing the production of NO using its precursor, L-arginine, results in increased food intake in mice (Czech, 1996; Morley & Flood, 1991). Inversely, blocking NO production with the antagonist L-N^G-nitro arginine (L-NO Arg) inhibited food intake in the same sample (Morley & Flood, 1991), with subsequent delivery of L-arginine partially reversing the antagonistic effect. Other research in non-fasted adult animals demonstrates L-arginine shows the opposite effect (Alamshah et al., 2016; Hazut et al., 2018), with injection of the drug resulting in a decrease in food intake. Research from the Crosby laboratory (Thebeau, 2015) shows evidence of NO significantly decreasing food intake in the fasted, young rat. This may indicate a difference between regulatory pathways in young versus adult animals. In addition, reduced food intake also occurred after simultaneously inhibiting NO while exciting the endogenous endocannabinoid pathway, suggesting an interaction between these pathways in appetite regulation.

1.5: Endocannabinoids

Endogenous cannabinoids (endocannabinoids; eCBs) are neurotransmitters that activate the same cannabinoid receptors, type I and type II cannabinoid receptors (CB1Rs and CB2Rs, respectively), as the exogenous psychotropic cannabinoid, delta 9-tetrahydrocannabinol (THC). These signals act to regulate energy intake, including food consumption (Matias & Di Marzo, 2007). Endocannabinoids, while located postsynaptically, act presynaptically to decrease levels of intracellular Ca^{2+} release by binding to CB1Rs, while also exciting G-protein coupled receptors (Kano et al., 2009).

This information, supported with empirical findings, suggest CB1Rs operate along a retrograde signaling pathway (Kano et al., 2009). CB2Rs are expressed primarily in the peripheral nervous system, and are highly concentrated in microglial cells. They have been shown to be inducible in response to inflammation or injury (Maresz et al., 2005). This thesis will focus on CB1Rs and how they are involved in the regulation of food intake (Cristino et al., 2014). CB1Rs are highly concentrated in the hypothalamus (Zou & Kumar, 2018). For the most part, activation of CB1Rs has been shown to increase food intake (Bellocchio et al, 2010). In mice, when CB1R agonists are delivered at low to moderate doses, the resulting increase in appetite is accomplished through the receptor's suppression of glutamatergic signaling (Bellocchio et al., 2010).

Alternately, blocking CB1Rs results in decreases in food intake and body weight, even in diet-induced obese animals (Ravinet Trillou et al., 2002). Previously in the Crosby laboratory, blocking CB1Rs in young, fasted animals resulted in a significant decrease in body weight, while inducing eCB production had no significant effect on food intake or body weight (Thebeau, 2015). In non-fasted adult rats, dose-dependent delivery of the CB1R antagonist SR141716A has been shown to suppress appetite when animals are fed a highly palatable, "cafeteria" diet (Carai et al., 2006). The current study aims to investigate whether this finding can be replicated in young animals in both fasted and non-fasted conditions.

1.6: Interactions Between eCB and NO Pathways

Previous research has demonstrated interactions between NO and eCB pathways in many physiological processes, including neuronal plasticity (Rafalovich et al., 2015), immunological response (Vannacci et al., 2004), and food intake (Crosby et al., 2011; Lipina & Hundal, 2017). Although the exact mechanism of these interactions remains unknown, research has been performed to investigate how blocking NO pathways and exciting eCB pathways would affect food intake and body weight change in fasted, young rats fed a standard lab diet. The results of this study demonstrate the significant effects of simultaneously exciting the eCB and blocking the NO pathway on food intake (Figure 1.1). Namely, injecting rats with L-NAME, a NOS antagonist, followed by WIN 55,212-2 (referred to in Figures 1.1 and 1.2 as WIN), a CB1R agonist, results in a significant

decrease in food intake (measured in grams) when compared to other drug treatments, including WIN 55,212-2 and L-NAME administered alone, and a vehicle (DMSO (1% v/v) in 0.9% saline solution) control (Thebeau, 2015). This shows that in the absence of NO signaling, eCBs act in the young, male rat to reduce appetite (Thebeau, 2015). This presents a key finding for the development of future treatments for childhood obesity.

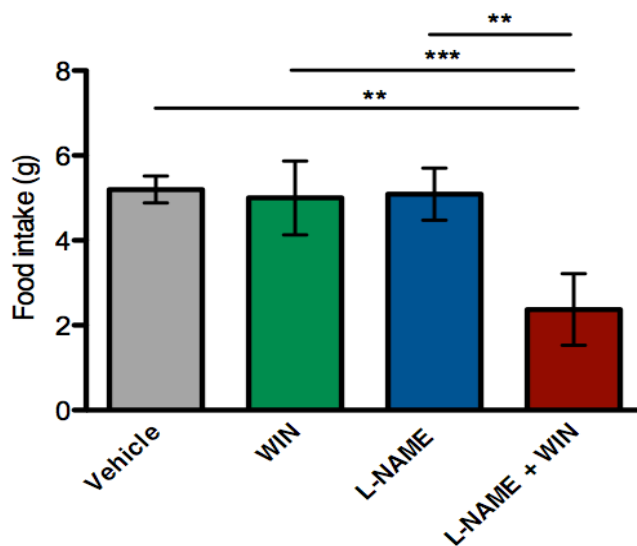


Figure 1.1: Mean food intake (\pm SE) in rats during a 2-hour period following 24 hours of food deprivation. The treatment groups used are as follows: Vehicle (drug control; saline), NO (NOS inhibitor: L-NAME), and +eCBs (CB1R agonist: WIN 55,212-2). All data are \pm SEM. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ (Thebeau, 2015).

The same results were replicated in terms of body weight; when delivered the combination of both L-NAME and WIN 55,212-2, animals weighed significantly less after having consumed standard diet for two hours following a 24-hour food-deprivation period (Figure 1.2).

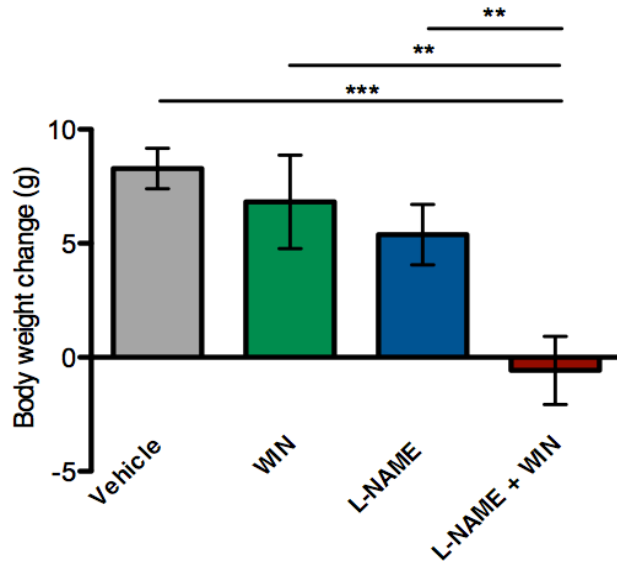


Figure 1.2: Change in body weight (\pm SE) after 2 hours of re-feeding in fasted rats. The treatment groups used are as follows: Vehicle (drug control: saline), -NO (NOS inhibitor: L-NAME), and +eCB's (CB1R agonist: WIN 55,212-2). All data are \pm SEM. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. (Thebeau, 2015)

1.7: Current Study

Based on previous findings, I propose to investigate eCB and NO pathway interactions in young rats fed a high-fat diet, and how this affects food intake and body weight in both fasted and non-fasted animals. Past results indicate conflicting conclusions between the effect of NO on food intake in young animals (Thebeau, 2015) and mature animals (Morley & Flood, 1991). In order to progress towards solutions in obesity research, high-fat diets have been implicated in studies (Carai et al., 2006) with adult animals. However, more research is required in the area of eCB and NO pathway interactions in young animals. This will hopefully provide a clearer picture of how these neurotransmitters can affect appetite to propose treatments for childhood obesity.

My hypotheses were that: 1) eCBs and NO would interact to decrease appetite in fasted animals fed a high-fat diet, and 2) In non-fasted animals, exciting NO pathways would result in an increase in appetite. To investigate these questions, rats were fasted for a 24-hour period, and received injections via the intraperitoneal route which would either activate or inhibit eCB or NO pathways, or a combination of both. By providing animals with a palatable food source this experiment modeled the high-fat, high-sugar diet

contributing to the human obesity epidemic. In order to investigate the effects of pathway manipulation without food deprivation, a sub-set of animals were allowed *ad-libitum* access to standard food prior to receiving injections.

Chapter 2: Materials and Methods

2.1: Experimental Animals

Eighty-three male Sprague Dawley rats were purchased from Charles River Laboratories (Sherbrooke Quebec), Canada. Rats arrived at a postnatal age range of 21-23 days. They were housed in polycarbonate cages (65 × 25 × 15 cm) with metal lids in a holding room at Mount Allison University maintained at a temperature of $22 \pm 0.5^\circ \text{C}$, and relative humidity of 30-50%. Photoperiod was set at a 12:12 hour light-darkness cycle, with the light cycle initiated at 0730. To provide environmental enrichment, cages were supplied with wood shavings and paper towel for nesting materials, a wooden block, a nyla bone and a piece of PVC tubing. In efforts to minimize stress, the young rats were housed together in groups of 3-4 upon arrival to the laboratory and handled daily for familiarization to human contact and manipulation during experimentation. During the one-week period of acclimatization, animals were fed a standard lab chow diet and reverse osmosis water *ad libitum* until they reached a target body weight of 80-120g. Once the desired weight range was reached, each animal was transferred to an individual cage using the same form of environmental enrichment beginning 24 hours prior to experimentation. All procedures were approved by the Animal Care and Use Committee at Mount Allison University and performed in accordance with the Canadian Council for Animal Care guidelines.

2.2: Experimental Procedure

Drug Preparation and Dosage

By means of an online random number generator at random.org, animals were randomly assigned to one of four experimental conditions: Vehicle, WIN 55,212-2, L-nitroarginine methyl ester (L-NAME), or WIN 55,212-2/L-NAME. Vehicle consisted of DMSO (1% v/v) in 0.9% saline solution and 100 μL was administered to vehicle treated animals. WIN 55,212-2 was dissolved in DMSO, added to 0.9% saline solution and

administered at 2mg/kg, and L-NAME was dissolved in vehicle (DMSO (1% v/v) in 0.9% saline solution) and delivered at 100 mg/kg.

Fasted Animals: The Effects of WIN 55,212-2 and L-NAME

Animals were weighed, then fasted for 24 hours. Reverse osmosis water was accessible *ad libitum* during the fasting period. All animals received two injections (these were scheduled before noon) via the intraperitoneal (IP) administration method, using a 1 mL syringe and a 26 x 1/2 gauge needle. Animals in the combination treatment group received an L-NAME injection 50 minutes prior to re-feeding, and a WIN 55,212-2 injection 20 minutes later. To keep injection schedules consistent, animals in other drug groups were delivered a vehicle injection, followed by either WIN 55,212-2 or L-NAME. Control animals received two vehicle injections. At the end of the fasting period, rats were reweighed, and given a pre-weighed amount of high-fat diet lab chow. Intake was monitored over the next two hours. Animals were re-weighed at the end of this period, then anesthetized using a sodium pentobarbital injection at a dosage of 130 mg/kg via the intraperitoneal route. Cages were inspected for pellets to ensure food was being consumed and not stored. Following the 2 hours of refeeding, all injected rats were euthanized, perfused, and brown adipose tissue (BAT), blood, and brain tissues were removed and preserved for future studies.

Fasted Animals: The Effects of SR141716A and L-arginine

Animals were randomly assigned to one of three experimental conditions: SR141716A (n=6), L-arginine (n=6), or SR141716A/L-arginine (n=5). SR141716A was dissolved in DMSO (1% v/v) and administered at 2 mg/kg. L-arginine was dissolved in DMSO (1% v/v) and delivered at 100 mg/kg. Experiments were carried out as for the WIN 55,212-2/L-NAME trials.

Non-Fasted Animals: The Effects of SR141716A and L-arginine

Further experiments were carried out in non-fasted animals. These animals were allowed *ad libitum* access to a standard laboratory chow diet prior to experimentation. Each animal was weighed prior to beginning experimentation, and was administered one

of the following drugs: vehicle (100 μ L), SR141617A (2 mg/kg), L-arginine (100 mg/kg) or SR141716A/L-arginine via the IP injection route. All injections were scheduled for delivery before noon. Regular food was substituted for a pre-weighed amount of high-fat diet chow, and intake was monitored along with body weight after 2 hours. Animals were sacrificed thereafter by means of a sodium pentobarbital injection at a dosage of 130 mg/kg via the intraperitoneal route. Cage was inspected for any stored food. Blood and BAT tissues were collected prior to perfusing. Brain tissues were also collected at the end of each perfusion.

2.3: Perfusion

Rats were deeply anesthetized using a 130mg/kg dose sodium pentobarbital injection delivered via the IP route using a 1mL syringe and a 21x1/2 gauge needle. After the animal appeared sedated, a toe pinch was performed to check for a reflex. If a reflex was present, subsequent delivery of sodium pentobarbital continued (no more than 100 μ L at a time) until a toe pinch no longer elicited a reflex response. An incision was made using surgical scissors between the scapulae, cutting along the back of the neck until enough internal tissue was exposed to collect BAT using forceps. A second incision was made ventrally in the abdomen, and the diaphragm and thoracic cavity were cut to expose the heart. A 21x1/2 gauge needle and a 1 mL syringe was used to collect a blood sample (approximately 0.4 mL) from the right ventricle of the heart, before clamping the descending aorta to promote blood flow to the brain. Both BAT and blood samples were then immediately placed in chilled cryogenic tubes and stored in a small dewer until transportation to a -80°C freezer was possible. An 18x1/2 gauge needle was then inserted into the left ventricle of the heart and the right atrium was cut to allow the perfusate to drain. Perfusion was performed using 50 mL of 1 x PBS solution followed by 50 mL of 4% paraformaldehyde (PFA) in PBS to fix the tissue. Both solutions were kept on an ice bath prior to injection. Following perfusion, rats were decapitated using a guillotine and brain tissue was subsequently removed. Brains were stored in 4% PFA at 4°C for approximately 24 hours and were then transferred to 30% (w/v) sucrose solution in 1 x PBS for long term storage at 4°C.

2.4: Statistical Analyses

Statistical analysis was carried out using R Studio. A Levene test was first run on raw data to compare homogeneity of variances between groups for all data sets. Once this assumption was met, a one-way ANOVA was run to analyze the difference between means for each condition. In the case of $p < 0.05$, residuals were calculated and the Shapiro test was run to test for their normality. If data failed to meet this assumption, it was log transformed, and tested for normality again. If assumptions were not met upon transformation, a non-parametric test (Dunn's) was run to determine which drug treatments held significant differences when being compared to the vehicle control. If assumptions of normality were met, Dunnett's test was run to assess differences between vehicle and drug treatment groups. Tukey's test was run to assess significant differences between means of drug treatments. To compare animals fed a high-fat diet to those fed a standard diet, t-tests were run between groups to check for the presence of a significant difference.

Chapter 3: Results

Experiments were carried out to measure both food intake of a high-fat diet and body weight in rats which were either fasted, or non-fasted. For fasting, a 24-hour food deprivation protocol was followed. Past studies have shown this period of time allows for physiological adjustment of fluid and energy losses, and an increase in the desired motivational food-seeking behavior (Rowland, 2007). During this 24-hour period, fasted animals ($n = 27$) consistently lost weight (Mean = -12.52 %, SEM = ± 0.49 %), compared to non-fasted animals ($n = 18$), who, on average, gained weight (Mean = 7.93 %, SEM = ± 0.71 %) over the same time frame when allowed *ad libitum* access to food.

Animals have been shown to consume a larger quantity of palatable food when compared with standard food (Hume et al., 2016). High-fat nutritional intake has been demonstrated to alter activity in multiple signaling pathways (Reyes, 2012; Valladolid-Acebes, 2012). Consistent with this, when comparing animals consuming a high-fat diet with those consuming a standard diet (the latter was taken from Thebeau, 2015), it was found that high-fat-fed rats ate significantly more ($p < 0.0001$; Figure 3.1). Body weight

gain from food consumption was consistently lower in the standard diet group, though these differences were not statistically significant ($p = 0.0874$; Figure 3.2).

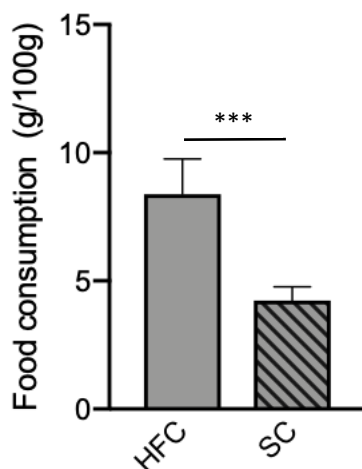


Figure 3.1: Food consumption of vehicle-treated animals (grams of food per 100 grams of body weight) during a 2-hour refeeding period following a 24-hour fast. Animals were given either high-fat chow (HFC, $n = 7$) or standard chow (SC, $n = 6$). All data are \pm SEM. *** = $p < 0.001$.

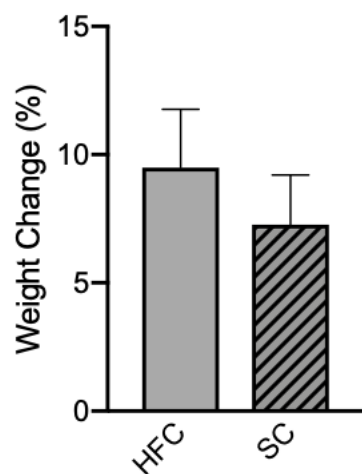


Figure 3.2: Mean body weight change (\pm SEM) of vehicle-treated animals during a 2-hour refeeding period following a 24-hour fast. Animals were given either high-fat chow (HFC, $n = 7$) or standard chow (SC, $n = 6$).

A previous study demonstrated eCBs decreased appetite in the absence of NO in young rats fed a standard diet. To investigate whether these effects of eCB and NO pathway manipulation would occur in young rats fed a high-fat diet, we first examined food intake and body weight change during a 2-hour refeeding period. Animals were given pre-weighed high-fat diet lab chow, following 24 hours of food deprivation after which rats were injected with either a CB1R agonist, which excites eCB pathways, a NOS inhibitor, inhibiting the production of nitric oxide, or a combination treatment of both the CB1R agonist and NOS inhibitor. Control animals were delivered a vehicle injection, consisting of saline dissolved in DMSO. Subsequently, two more studies were carried out using a different group of drugs: a CB1R antagonist, a NO precursor, or a combination of both the CB1R antagonist and NO precursor. Control animals again received a vehicle injection. Once data from all aforementioned pathway manipulations were gathered in fasted animals, non-fasted animals were treated with a CB1R antagonist, NO precursor, a combination treatment or vehicle. This was done after animals were allowed *ad libitum* access to a standard diet before receiving high-fat diet for a 2-hour feeding period.

3.1: Blockade of NO synthesis decreases food intake and body weight gain in young fasted rats.

Food intake of young male rats was measured after the 2-hour refeeding period following a 24-hour fast. Food consumption data was plotted in grams per 100 grams of body weight, and analyzed to determine the effects of blocking production of NO (L-NAME: NOS inhibitor) or activating CB1Rs (WIN 55,212-2: CB1R agonist) on intake of high-fat diet. A one-way ANOVA (Mean = 6.732 ± 2.17 g/100g, $n = 29$; $p = 0.0017$; Figure 3.3) followed by a Dunn's test indicated animals receiving L-NAME ($n = 8$) consumed significantly less food when compared to vehicle ($n = 7$, $p = 0.0022$) and WIN 55,212-2 alone ($n = 6$, $p = 0.0188$). When exciting CB1Rs with WIN 55,212-2, no change in food intake was noted ($p > 0.9999$). No difference resulted from treating high-fat chow-fed animals with L-NAME followed by WIN 55,212-2 ($p = 0.1684$).

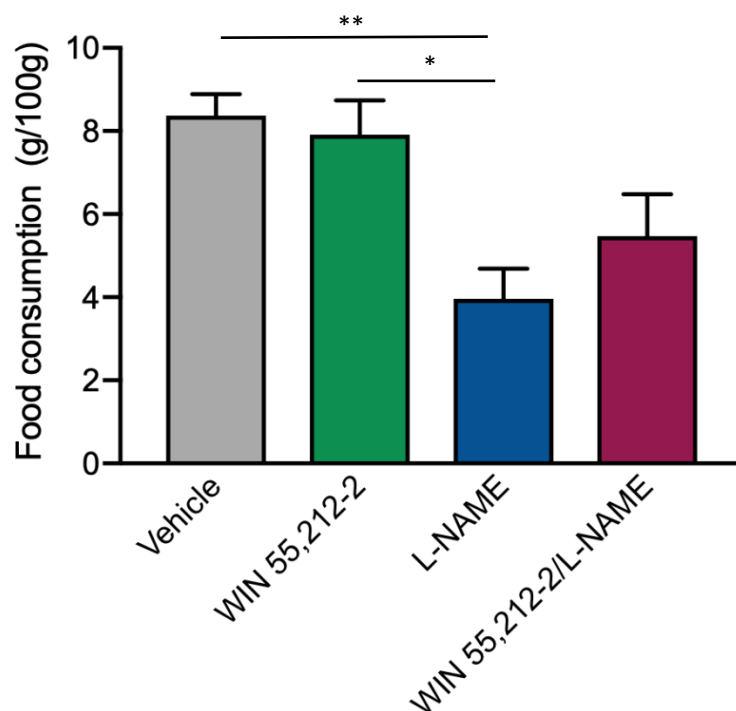


Figure 3.3: Food consumption (measured in grams of food consumed per 100 grams of body weight) after 2 hours of re-feeding with high-fat diet in fasted rats. Treatment groups were as follows: Vehicle (n = 7), WIN 55,212-2: CB1R agonist (n = 6), L-NAME: NOS inhibitor (n = 8), and combination treatment of WIN 55, 212-2 and L-NAME (n = 8). All data are \pm SEM.

* = $p < 0.05$, ** = $p < 0.01$.

Consistent with food consumption data, body weight data (measured in % change) of juvenile male rats (Figure 3.4) demonstrates a significant difference between vehicle and L-NAME treated animals ($p = 0.0217$) as well as vehicle and WIN 55,212-2-treated animals ($p = 0.0212$).

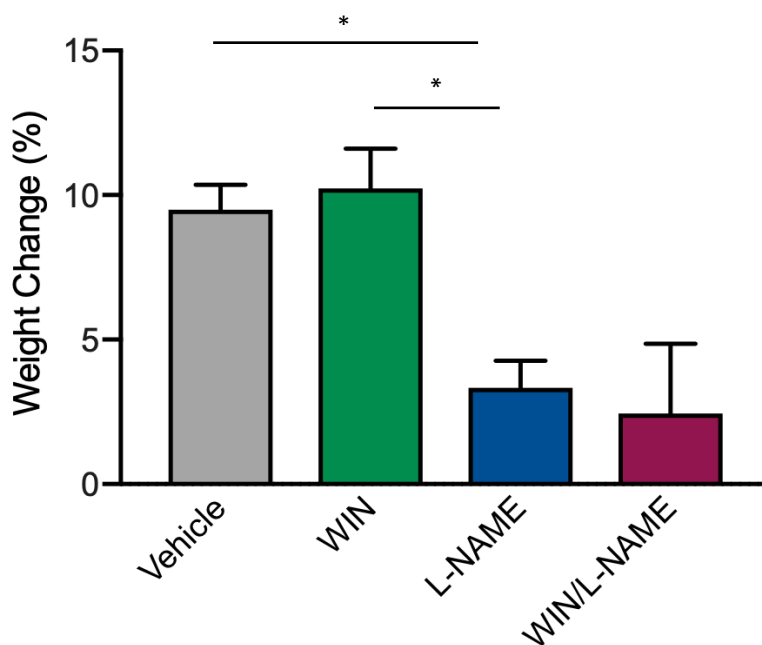


Figure 3.4: Percent body weight change (%) after 2 hours of re-feeding with high-fat diet in fasted rats. Treatment groups were as follows: Vehicle (n = 7), WIN 55,212-2: CB1R agonist (n = 6), L-NAME: NOS inhibitor (n = 8), and combination treatment of WIN 55,212-2 and L-NAME (n = 8). All data are \pm SEM. * = $p < 0.05$.

3.2: Blockade of type 1 cannabinoid receptors alone, or with subsequent delivery of a nitric oxide precursor, decreases food intake in young, fasted rats.

As blocking NOS was shown to reduce appetite in young animals, further investigation was undertaken to verify whether enhancing NO production would significantly increase appetite in the presence or absence of endocannabinoids. Further pathway manipulation involved three drug treatments (SR141716A, a CB1R antagonist, L-Arginine, a NO precursor, and the combination treatment of SR141716A/L-Arginine) and a vehicle control. Food intake of animals was measured after a 2-hour refeeding period with high-fat diet following a 24-hour fast (Figure 3.5). Food consumption data was analyzed to determine the effects of blocking CB1Rs or inducing NOS on appetite.

A one-way ANOVA indicated a significant difference between groups (Mean = 4.965 ± 1.94 g/100g, n = 22; $p = 0.0110$; Figure 3.5). Subsequently, a Dunnett's test showed animals treated with L-arginine alone showed no significant changes in food intake (n = 5, $p = 0.9949$), while those delivered SR141716A/L-Arginine consumed

significantly less when compared with vehicle ($n = 4$, $p = 0.0232$). The same effect was found when comparing food consumption in rats delivered SR141716A alone to vehicle, suggesting the effect of appetite reduction observed is solely due to blocking cannabinoid receptors ($n = 6$, $p = 0.0293$), with L-arginine showing no effect.

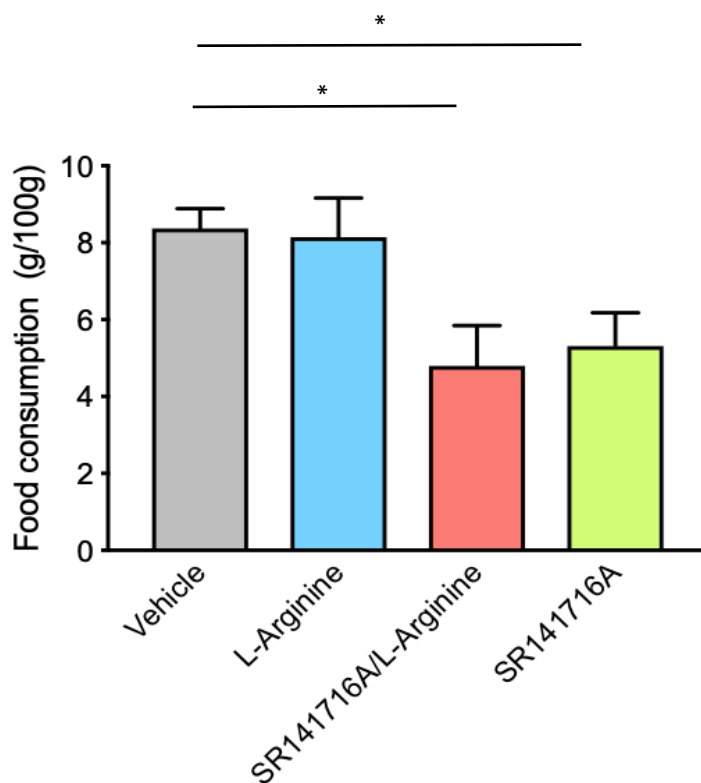


Figure 3.5: Food consumption (measured in grams of food consumed per 100 grams of body weight) after 2 hours of re-feeding with high-fat diet in fasted rats. Treatment groups were as follows: Vehicle ($n = 7$), SR141716A: CB1R antagonist ($n = 6$), L-Arginine: NO precursor ($n = 5$), and combination treatment of SR141716A and L-Arginine ($n = 4$). All data are \pm SEM. * = $p < 0.05$.

Body weight change was also measured following the 2-hour re-feeding period. A one-way ANOVA indicated a significant difference between means (Mean = 3.942 ± 2.81 %, $n = 22$; $p = 0.0253$; Figure 3.6). When a Dunnett's test was run, it showed no significant differences between any treatment groups and the control group. Subsequently, a Tukey's test indicated animals treated with L-Arginine in combination with SR141716A gained

significantly less weight when compared with those receiving L-Arginine alone ($p = 0.0307$).

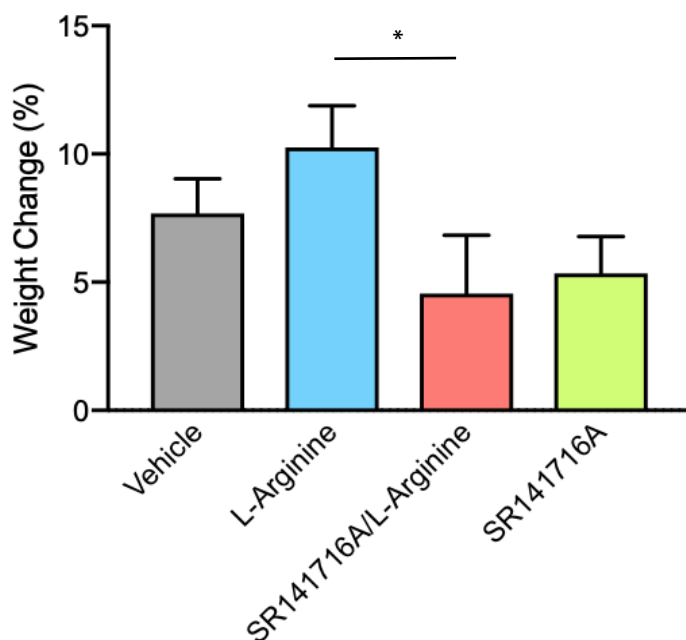


Figure 3.6: Percent body weight change (%) after 2 hours of re-feeding with high-fat diet in fasted rats. Treatment groups were as follows: Vehicle ($n = 7$), SR141716A: CB1R antagonist ($n = 6$), L-Arginine: NO precursor ($n = 5$), and combination treatment of SR141716A and L-Arginine ($n = 4$). All data are \pm SEM. * = $p < 0.05$.

3.3: Blockade of type 1 cannabinoid receptors alone, or with subsequent delivery of a nitric oxide precursor, decreases body weight in young, non-fasted rats.

Research suggests that when satiated rats are exposed to a high-fat diet, they will increase meal size but decrease meal frequency compared to a standard diet (Melhorn et al., 2010). When compared to a previous study in vehicle-treated, non-fasted rats fed a standard diet (Thebeau, 2015), food intake did not vary significantly between the two groups ($p = 0.7864$; Figure 3.7). Vehicle-treated animals who were non-fasted gained significantly more weight ($p = 0.0367$; Figure 3.8) in the 2-hour feeding period compared to their counterparts.

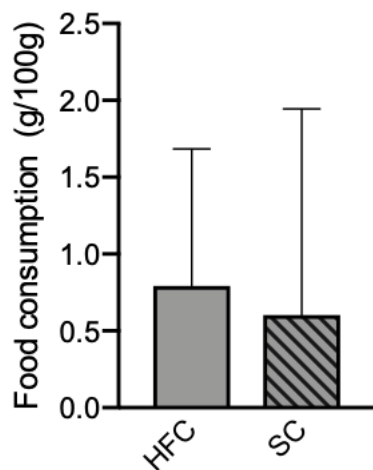


Figure 3.7: Food consumption of vehicle-treated animals (grams of food consumed per 100 grams of body weight, \pm SEM) during a 2-hour feeding period. Animals were given either high-fat chow (HFC, $n = 6$) or standard chow (SC, $n = 5$).

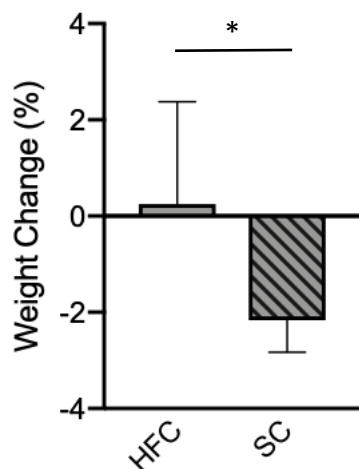


Figure 3.8: Weight change (%) of non-fasted, vehicle-treated animals during a 2-hour feeding period. Animals were given either high-fat chow (HFC, $n = 6$) or standard chow (SC, $n = 5$). All data are \pm SEM. * = $p < 0.05$.

Previous research investigating effects of L-arginine on non-fasted, adult animals shows varying results in regards to food intake. Some studies propose the NO precursor

results in an increase in appetite (Ayaso et al., 2014; Czech, 1996; Morley & Flood, 1991), while others propose a decrease (Alamshah et al., 2016; Hazut et al., 2018). Although minimal research has been conducted in young animals as of yet, it appears that central administration of L-arginine into the DMH of young rats increases appetite. An investigation was carried out in non-fasted animals to examine whether L-arginine would also display this appetite-inducing effect in young animals when delivered peripherally.

After conducting a one-way ANOVA, no significant differences were found between mean food intake for any treatment groups (SR141716A, L-arginine, or SR141716A/L-arginine) in the satiated trial (Mean = 0.2267 ± 10.80 g/100g, n = 22; p = 0.8765; Figure 3.9).

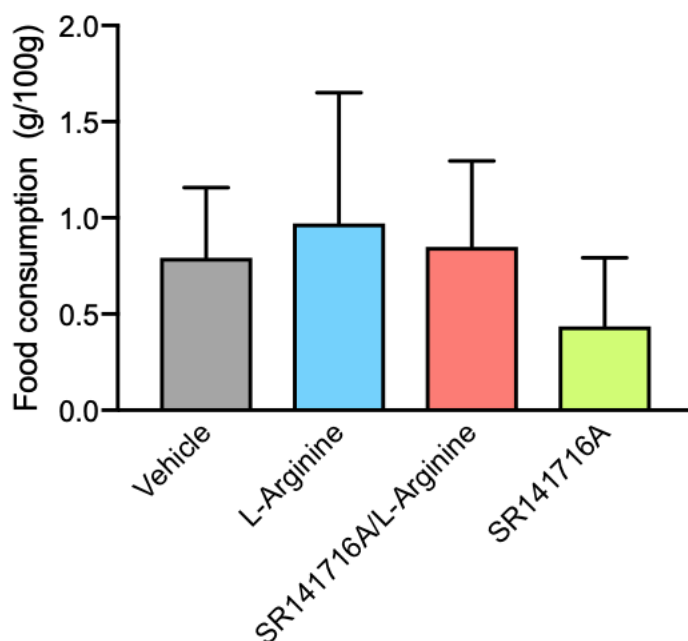


Figure 3.9: Food consumption (measured in grams of food consumed per 100 grams of body weight) after 2 hours of feeding with high-fat diet in non-fasted rats. Treatment groups were as follows: Vehicle (n = 6), SR141716A: CB1R antagonist (n = 5), L-Arginine: NO precursor (n = 5), and combination treatment of SR141716A and L-Arginine (n = 5). All data are \pm SEM.

A one-way ANOVA confirmed a significant difference between mean body weight change of treatment groups (Mean = $4.418 \pm 1.91\%$, n = 22; p = 0.018; Figure

3.10) Subsequently, a Dunnett's test was run, revealing those animals receiving an L-arginine injection (n = 5) showed no significant difference in weight change (p = 0.0872). Animals receiving a combination treatment of both SR141716A and L-Arginine demonstrated a significant decrease in body weight compared to the control (p = 0.0072). As animals treated with the SR141716A injection (n = 5) also lost a significant amount of weight when compared to vehicle (p = 0.0100), the possibility of the addition of L-arginine as a cause of weight loss was eliminated; blocking CB1Rs alone is sufficient to cause this effect.

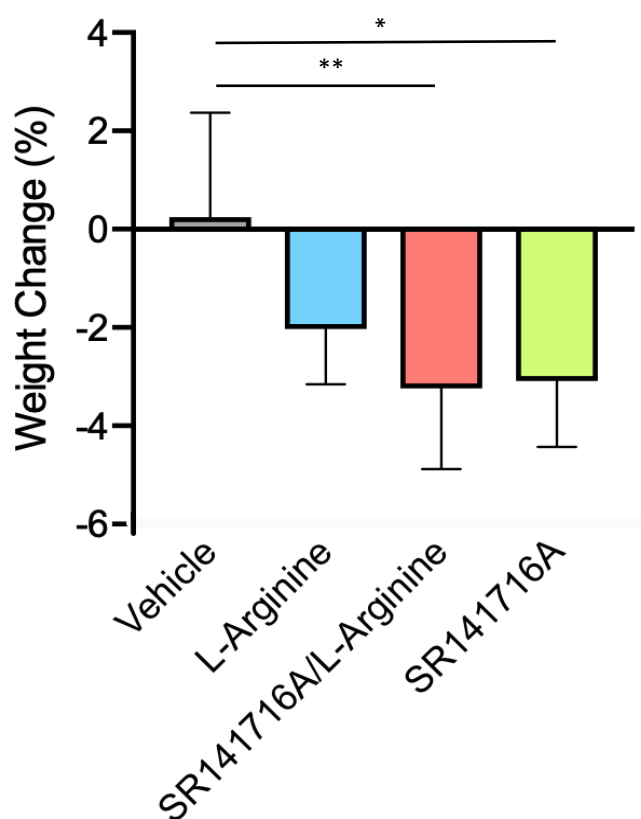


Figure 3.10: Percent body weight change (%) after 2 hours of feeding with high-fat diet in non-fasted rats. Treatment groups were as follows: Vehicle (n = 6), SR141716A: CB1R antagonist (n = 5), L-Arginine: NO precursor (n = 5), and combination treatment of SR141716A and L-Arginine (n = 5). All data are \pm SEM. * = p < 0.05, ** = p < 0.01.

Chapter 4: Discussion

Results of the current investigation demonstrated that the effects seen on appetite regulation by manipulation of eCB and NO pathways differ in young animals, depending on whether they are fed with a high-fat or standard diet. When fasted, rats receiving high-fat chow upon refeeding show a decrease in both food intake and body weight in absence of NO pathways. No effect was seen when treating animals with a CB1R agonist, or when exciting eCB pathways in the absence of NO. Upon further investigation into NO and eCB pathway manipulation, it was found that inducing the production of NO does not affect food intake or body weight in rats fed a high-fat diet. However, when a CB1R antagonist is injected, followed by a NO precursor, animals demonstrate a decrease in food intake. This same effect is noted when delivering the CB1R antagonist alone. When examining food intake and body weight effects in a non-fasted sample, no significant effects were found on food intake between the control treatment and any of the drug treatments, however delivery of a CB1R antagonist followed by a NO precursor significantly reduced body weight. Administering the CB1R antagonist alone had the same effect on body weight. Overall, these results demonstrate that the manipulation of eCB and NO pathways show different effects on food intake and body weight in young animals when fed a high-fat diet, as compared to those fed a standard diet.

This study was carried out to verify whether eCB and NO pathway manipulations would affect food intake in young rats fed a high-fat diet. Previous literature has modeled a palatable diet in mature animals by feeding them a “cafeteria” meal (Carai et al., 2006). Following drug treatment, blocking CB1Rs was shown to alter food intake in these animals in a dose dependent manner. As very few comparable investigations have been performed using young animals, I hypothesized that eCB and NO pathways would interact to affect food intake in this population.

Preliminary comparisons between fasted animals fed a high-fat diet and a standard diet were carried out (Figures 3.1, 3.2) to show animals would consume more food when presented with a high-fat diet compared to a standard diet. Animals in the high-fat diet condition consumed significantly more food in the 2-hour refeeding period than their standard fed counterparts, which has been supported in the literature (La Fleur et al., 2014; Swithers et al., 2011). Although the animals in the high-fat condition

consistently gained more weight in the 2-hour timeframe, the difference observed was not significant. This may be due to the brevity of the food exposure period for both groups, as well as suggestions from previous studies that body weight change may not be significantly altered by the consumption of a single high-fat meal in lean rats (Loh et al., 1998).

When compared to control animals, enhancing CB1R signaling, with the CB1R agonist, WIN 55-212,2 had no significant effect on food intake. Unlike what was observed in animals fed a standard diet, following an injection of a NOS inhibitor with a CB1R agonist, injection did not result in any significant alteration in food intake. This may suggest eCB and NO pathways operate independently in rats fed a high-fat diet to regulate appetite, as opposed to their suggested interactions in rats fed a standard diet (Thebeau, 2015). Additionally, when NOS was inhibited in isolation (by delivery of an L-NAME injection), rats experienced a significant decrease in both food intake and body weight. It is possible that the differences seen between standard diet and high-fat diet-fed animals may be influenced by the key role of eCBs and their interactions with dopaminergic signaling associated with the consumption of a highly-palatable diet (Coccorello & Maccarrone, 2018). I suggest these pathways may be more active in young rats fed a high-fat diet when compared to those fed a standard diet. The influence of high-fat diet on NOS has been previously investigated in adult, female animals (Huang et al., 2011), demonstrating that even after a short exposure, a decrease in eNOS is noted in the liver. This manipulation leads to a subsequent decrease in NOS activity, and thus NO production. If this acute exposure has initiated a reduction of NOS levels in young, male animals, this may be an explanation as to why this drug manipulation demonstrated a significant effect in this sample, as the already-reduced pathway activity would be further inhibited.

Based on the finding that animals fed a high-fat diet experienced a reduced food intake and body weight response to a drug inhibiting the production of NO, further investigations were carried out to determine what effect increasing NO production (with an injection of NO precursor L-arginine) would have on appetite. Manipulation of eCB pathways with CB1R antagonist, SR141716A was also performed to test for pathway interactions. Results showed that the delivery of L-arginine alone had no impact on food

consumption or body weight in young, high-fat diet-fed rats. However, when animals were injected with CB1R antagonist SR131716A, followed by L-arginine, food intake was significantly reduced. A subsequent treatment group receiving SR141716A in isolation also demonstrated this significant decrease in appetite, demonstrating the effects seen are due to the blockade of CB1Rs, which has also been supported in the literature (Son et al., 2010; Ward et al., 2009). Surprisingly, the NO pathway-inducing drug L-arginine exerted no significant effect in fasted young animals, as literature suggests either a resulting increase (Morley & Flood, 1991; Czech, 1996) or decrease (Alamshah et al., 2016; Fu et al., 2005) on subsequent food intake. L-arginine has been shown to regulate eNOS activity (Cernadas et al, 1998) and in a previous study, aging rats were shown to have an enhanced sensitivity to eNOS pathway manipulation (Cernadas et al, 1998). Thus, I propose the findings from my study may be a result of this age-dependent response, with young animals showing no response to L-arginine administration regarding food intake. Examining body weight, there were no significant differences between control and drug-treated groups. There was a significant difference between the L-arginine-treated group and those receiving the combination treatment of SR14716A and L-arginine, however this is likely attributed to animals in the L-arginine group drinking more water and retaining water weight, as thirst has been previously reported as a side effect of exciting NO pathways in young animals in the Crosby laboratory (Smithers, 2016).

The existing literature proves inconclusive in regards to appetite regulation via the manipulation of NO pathways in non-fasted, adult animals. Some findings suggest delivery of L-arginine increases appetite (Ayaso et al., 2014; Czech, 1996; Morley & Flood, 1991), while others propose it decreases food intake (Alamshah et al., 2016; Hazut et al., 2018). From a current study in the Crosby laboratory (Poole, 2019), it appears as though the central delivery of L-arginine into the brain results in an increase in high-fat diet consumption in young, male rats. In order to investigate the effects of peripheral drug delivery on appetite, experiments were run in *ad-libitum* fed animals maintained on a standard diet, prior to a 2-hour feeding period using high-fat diet chow. Preliminary comparisons were run demonstrating that non-fasted animals fed a standard diet showed a

significant decrease in body weight when compared to their high-fat diet-fed counterparts. Food intake did not vary significantly between groups (Thebeau, 2015). Food intake data from non-fasted animals in this study demonstrates no significant differences between any treatment groups. A proposed rationale for this was the nocturnal nature of rats, with most of their food consumption occurring during the dark cycle (Johnson & Johnson, 1990). As high-fat food was presented to the animals in the late morning hours of the light cycle, it is possible a lack of drive to consume the food may have been due to the animals being presumably satiated from dark-cycle consumption of the standard diet.

In regard to body weight, animals in the control group were the only individuals to gain weight as a result of the amount of food consumed during the two-hour period. Those treated with L-arginine lost weight, though not significantly in comparison to control. Animals being injected with SR141716A followed by L-arginine showed a significant decrease in body weight, as did those treated with SR141716A alone. This expands on the earlier finding that blocking CB1Rs resulted in a significant decrease in food intake in rats fed a high-fat diet. Evidence would suggest blocking CB1Rs also significantly reduces body weight, but only when animals are non-fasted. Cani et al (2004) published a similar finding in adult, non-fasted animals, suggesting the effects of SR141716A modulate ghrelin to influence appetite and body mass. Contrastingly to what was found in the current study, the adult, fasted animals in Cani et al. (2004) also lost a significant amount of weight when treated with the CB1R antagonist. Thus, I propose the difference observed is age-dependent, with metabolic response in young animals being more resistant to the drug's anorectic effects, as supported by literature (Lipina et al., 2016).

Chapter 5: Conclusions and Future Directions

Due to the lack of research regarding the effects of modulating eCB and NO pathways in young animals, and how they affect appetite, this study provides a foundational model for how these pathways might be affected by the consumption of a high-fat diet. Results of this study demonstrated clear discrepancies between the interactions in fasted versus non-fasted animals, as well as those fed a standard versus a

high-fat diet. These experiments demonstrated that when NO pathways are inhibited, or CB1Rs are blocked, fasted animals display a significant reduction in food intake of a high-fat diet. Additionally, this effect of blocking CB1Rs on food intake is eliminated by allowing *ad libitum* food consumption prior to the introduction of a high-fat diet. As this investigation has raised many questions about how high-fat foods might affect satiety signals in young animals, I propose future research could expand on this study by performing chronic trials in rats fed a high-fat diet, to examine whether modulation of these pathways would affect appetite regulation differently after the onset of obesity. This, in conjunction with a non-fasted trial performed in animals treated with WIN 55,212-2 and L-NAME would help in providing a better understanding of how these pathways operate in young animals fed palatable foods.

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