NEURAL AND BEHAVIORAL MECHANISMS UNDERLYING CANNABINOID-INDUCED HYPERPHAGIA

by

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(Under the Direction of Emily E. Noble)

ABSTRACT

Cannabinoids are lipophilic signaling molecules produced endogenously in the body, in the Cannabis plant, and synthetically in the laboratory. Decades ago, researchers confirmed in the laboratory what Cannabis users have known for centuries: cannabinoids augment eating behavior. Since then, the field has made great strides in understanding how cannabinoids confer their hyperphagic effect, which is namely through the broad expression of cannabinoid type-1 (CB1) receptor in the central nervous system. Despite the advances, there are still many unknowns, and this work seeks to fill several gaps. The preclinical study of cannabinoids is vital to determining their utility, e.g. in hypophagic disorders, but its paramount that preclinical studies implement translationally relevant methodology to achieve this goal. For this reason, we developed a model of edible consumption for rodents. We show that gelatin-based edibles increase food intake in rodents, importantly, through distinct behavioral mechanisms between the sexes. Males acutely increase their chow intake via an increase in meal frequency while females do so via an increase in meal size. There is a general dearth of behavioral data in females following cannabinoid administration; therefore, we explored the effects of cannabinoid

receptor agonism on several behaviors of interest in the context of psychoactive drug use: impulsivity, motivation, and anxiety-like behavior. Females given a hyperphagic dose of the cannabinoid receptor agonist display elevated impulsive behavior toward a sucrose reinforcer, but this did not coincide with changes in motivation or anxiety-like behavior. Males displayed an acute increase in meal frequency following cannabinoid receptor agonist consumption, which suggests an increase in appetitive behavior. Known for its role in appetitive behavior, the neuropeptide orexin/hypocretin (OH) is produced in the lateral hypothalamus and has anatomical and physiological interactions with the endocannabinoid system. We therefore investigated the necessity of the OX1 receptor, responsible for the food-seeking properties of OH, in cannabinoid-induced hyperphagia in males and found that OX1 receptor signaling is required for the hyperphagic properties of cannabinoids. Taken together, these data further our understanding of the neural and behavioral mechanisms that underscore the hyperphagic effect of cannabinoid signaling.

INDEX WORDS: lateral hypothalamus, meal, microstructure, orexin, hypocretin, edible, impulsivity, motivation, anxiety, sex differences, CB1

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DEDICATION

This dissertation is dedicated to my family. Thank you all for making this possible.

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CHAPTER 1

HYPOTHALAMIC CANNABINOID SIGNALING: CONSEQUENCES FOR EATING BEHAVIOR[1]

1. Lord M.N. and Noble E.E. Hypothalamic cannabinoid signaling: consequences for eating behavior. Pharmacology Research & Perspectives. (2024). Reprinted here with permission of the publisher.

Abstract

In parallel to the legalization of cannabis for both medicinal and recreational purposes, cannabinoid use has steadily increased over the last decade in the United States. Cannabinoids, such as tetrahydrocannabinol and anandamide, bind to the central cannabinoid-1 (CB1) receptor to impact several physiological processes relevant for body weight regulation, including appetite and energy expenditure. The hypothalamus integrates peripheral signals related to energy balance, houses several nuclei that orchestrate eating, and expresses the CB1 receptor. Herein we review literature to date concerning cannabinergic action in the hypothalamus with a specific focus on eating behaviors. We highlight hypothalamic areas wherein researchers have focused their attention, including the lateral, arcuate, paraventricular, and ventromedial hypothalamic nuclei, and interactions with the hormone leptin. This review serves as a comprehensive analysis of what is known about cannabinoid signaling in the hypothalamus, highlights gaps in the literature, and suggests future directions.

1. Introduction

Endogenous cannabinoid compounds (endocannabinoids) are lipid-based signaling molecules produced throughout the body, including in the central nervous system (1-3). Endocannabinoids exert their physiological effects, including modulating pain processes (4), sleep (5), anxiety (6), appetite regulation, and energy homeostasis (7) via binding to at least two known cannabinoid receptors: CB1 and CB2 (though additional putative receptors are under investigation) (8). Cannabinoids are so named because the first known ligands for the CB1 receptor were isolated from the *Cannabis* plant, also known as marijuana (9). While the phenomenon of "the munchies," i.e., augmentation of normal

food intake following marijuana use, has been described in popular culture for decades, cannabinoid research on food intake has only surfaced in the last 30 years – coinciding with the discovery of two endogenous ligands for the CB1 receptor: anandamide (AEA) (1) and 2-arachidonyl glycerol (2AG) (10). Cannabinoid signaling molecules have since been clinically shown to both increase food intake and regulate overall energy balance. The hypothalamus is a brain region that orchestrates energy balance regulation (11), expresses CB1 receptors (12), and locally produces endocannabinoids (13). The hypothalamus has received notable attention for its contribution to cannabinoid-induced feeding. The purpose of this review is to synthesize what is known to date about cannabinoid action in the hypothalamus with a focus on orexigenic behaviors.

CB1 receptor is a G-protein-coupled receptor that is ubiquitously expressed in the brain (12, 14), with the highest density of expression in the basal ganglia, hippocampal CA3 and dentate gyrus regions, and the cerebellar molecular layer (12). Early evidence of the role for CB1 receptor in the endogenous control of food intake comes from transgenic knockout (KO) mice. CB1 receptor KO male mice have reduced energy intake without changes in energy expenditure when compared with WT mice, and this is associated with increased lean mass, decreased fat mass, and overall reduced body weight gain starting from 5 weeks of age into adulthood (15). Lage and colleagues similarly report a reduction in body weight gain in whole body CB1 KO mice starting at 12 weeks of age (16). Interestingly, resistance to weight gain persists in whole body CB1 KO mice of both sexes following chronic high-fat diet (HFD) feeding (16), despite isocaloric intake (17). Resistance to HFD-induced weight gain in CB1 KO mice is coupled with blunted

adiposity and lack of hyperinsulinemia compared to HFD-fed controls, but CB1 KO mice remain susceptible to increased blood triglycerides (17).

Evidence links the hypothalamic endocannabinoid system with energy status. For example, the endocannabinoid 2AG markedly increases in the hypothalamus of male rats after a 24-hour fast, and levels decrease below ad libitum fed controls upon refeeding (18). Hypothalamic AEA is unaffected by feeding, satiation, deprivation and food restriction (18) (19). However, food restriction reduces expression of the fatty acid amide hydrolase gene in the hypothalamus, the enzyme responsible for degrading AEA (19). This suggests that while there may not be acute changes in AEA production, long-term food restriction may lead to elevated AEA over time, but this was not directly tested. Conversely, female rats exposed to a binge-eating paradigm with limited intermittent access to margarine display reduced endocannabinoid tone in the hypothalamus, suggesting that binge-eating behavior may lower endocannabinoid tone in the hypothalamus (20). Some evidence also suggests that hypothalamic CB1 receptor transcription may be affected by palatable food consumption (21). While CB1 receptor is only moderately expressed in feeding-relevant hypothalamic nuclei (12), the receptor exhibits high G-protein coupling activity in the hypothalamus in response CB1 receptor agonism (22). In our survey of the literature, we found several areas of focus regarding consequences of cannabinoid action in hypothalamic regions governing appetite and energy homeostasis, including the lateral, arcuate, paraventricular, and ventromedial nuclei, as well as the interplay with the adipocyte-derived hormone leptin, which are subsequently discussed. Several endogenous, synthetic, and phyto-cannabinoid agonists and antagonists that have been used to investigate the function of the CB1 receptor will

be mentioned and abbreviated throughout the text (see Table 1.1). We will first highlight the established orexigenic effect of peripheral CB1 receptor agonists and the anorexigenic effect of CB1 receptor antagonist administration.

Table 1.1: Cannabinoid compounds that bind to the cannabinoid 1 (CB1) receptor, their origin, and binding capacity [8]. 'Selective' indicates selective for the CB1 receptor.

Cannabinoid	**Origin**	**Binding at CB1**
Delta^{9}-tetrahydrocannabinol (d9THC)	Cannabis	Non-selective partial agonist
CP55940	Synthetic	Non-selective full agonist
WIN55,212-2 mesylate (WIN)	Synthetic	Selective full agonist
AM251 / AM281	Synthetic	Selective inverse agonist
HU210	Synthetic	Non-selective full agonist
Arachidonyl-2'-chloroethylamide (ACEA)	Synthetic	Selective full agonist
Rimonabant/SR141716(A)	Synthetic	Selective inverse agonist/antagonist
2-arachidonyl glycerol (2AG)	Endogenous	Non-selective full agonist
Anandamide (AEA)	Endogenous	Non-selective partial agonist

Pharmacological manipulation of CB1 receptor signaling and the orexigenic response

Several studies have demonstrated central and peripheral cannabinoid administration increases food intake, and peripheral application of CB1 receptor antagonists reliably reduce feeding (23-28) (29) (30, 31). In the case of agonists ACEA and CP55940, while lower doses of the drug given via intraperitoneal injection increase food intake, high doses of these CB1 receptor agonists diminish eating behavior (29), suggesting a bell-shaped dose-response curve with respect to cannabinoid-induced food intake, and these findings may be due to hypolocomotion or lethargy, which are observed at higher doses of these drugs (30). In addition to intraperitoneal dosing, oral delivery of THC also substantially increases food intake in sated male and female rats (25, 32). Moreover, intraperitoneal CB1 receptor blockade counters food intake in conditions when hyperphagia usually occurs, such as with hyperpalatable diets or following food restriction. For example, intraperitoneal injection of AM281 reduces acute HFD consumption in male mice (16), and peripheral administration of rimonabant decreases food intake in 24-hour fasted mice compared to controls (33). While peripheral injection of CB1 receptor agonists and antagonists have been extensively shown to increase and decrease eating behavior, respectively, the degree to which cannabinoids are acting in the central nervous system directly to modulate food intake is less clear. Indeed, lateral ventricular injection of the endocannabinoid 2AG had no impact on chow intake in free feeding male rats at any dose applied (0-160 µg icv) (18), and lateral ventricular injection of AM251 (0-160 µg icv) to male rats had no effect on food intake nor motivated responding for food (34), suggesting that mediators of cannabinoid-induced feeding are not accessible via cerebrospinal fluid (CSF) transmission in the lateral ventricle.

However, WIN (3 µg icv) into the third ventricle greatly upregulates food intake in female guinea pigs compared to vehicle (35), and fourth ventricular injection of the CB1 agonist CP55940 is sufficient to increase palatable food intake in both male and female rats (23). While several factors may account for the differences observed with CSF injection of cannabinoids in the aforementioned studies, such as drug dosage and half-life, it is also possible that the mid- and hindbrain injection of CB1 receptor agonists are closer to the site of action and, therefore, more accessible for cannabinoid-mediated modulation of food intake control. Controlled studies comparing ventricular injection sites and standardized doses of CB1 receptor agonists would be revealing.

The hypothalamus as a mediator of cannabinoid-induced eating behavior

While the pathway from exogenous cannabinoid administration to initiation of food intake is still under investigation, it is noteworthy that several feeding-relevant regions of the hypothalamus are engaged by a hyperphagic dose of intraperitoneally administered CP55940 to sated male rats. This CB1 receptor agonist evoked immediate early gene expression (c-FOS) in the lateral hypothalamus (LHA), ventromedial hypothalamus (VMH), and the paraventricular hypothalamus (PVH), as well as extra hypothalamic regions such as the nucleus accumbens shell and core (30). Dodd and colleagues further complemented c-FOS gene expression data with blood-oxygen level dependent (BOLD) imaging. Upon CP55940 administration, BOLD imaging revealed increased activity of the arcuate nucleus of the hypothalamus (ARH), and the VMH, as well as the nucleus accumbens shell and core while decreased BOLD signal was detected in the LHA and PVH (30). The authors note that areas showing an increase in gene

expression and a decrease in BOLD signal may reflect the phenomenon of disinhibition (30), i.e., decreased GABA input to surrounding neurons. Endocannabinoids are produced throughout the brain and released on demand to bind CB1 receptor in retrograde fashion and acutely regulate neuronal transmission (36, 37). Thus, while it is not clear precisely where, how, nor in what order the relevant brain regions respond to peripheral cannabinoids to elevate food intake, regions with regulating capacity can be identified by conducting well-controlled pharmacological studies.

2. Lateral hypothalamus

The lateral hypothalamus (LHA) represents a key region modulating both homeostatic and hedonic regulation of food intake (38). Two populations of LHA neuropeptides are heavily involved in promoting food consumption and modulating energy expenditure: orexin/hypocretin (O/H) neurons (39) and melanin-concentrating hormone (MCH) neurons (40). Specifically, O/H neurons increase food anticipatory behavior by promoting motivation and arousal (41-43), and MCH signaling promotes impulsive eating (44) and appetition (40) as well as overall increased food intake (45, 46), which is dependent on the estrous cycle in females (47). Previous work has demonstrated that O/H neurons are depolarized in the immediate pursuit of food, but once food is being consumed, O/H neurons are silent and MCH neurons are activated (48). Interestingly, 2AG injected into the LHA has no effect on food intake alone but does increase food intake when co-administered with the metabotropic glutamate receptor mGluR1/5 agonist (49). This evidence suggests an interaction between the endocannabinoid system and the O/H and MCH systems of the LHA, but glutamatergic input may also be requisite for initiating food intake, which is discussed below.

While CB1 receptors have been reported on O/H and MCH neurons (15), cannabinoids are also synthesized by O/H and MCH neurons. Evidence suggests these cannabinoids can act presynaptically in retrograde fashion to adjust activity in the neuron by which they were produced. This phenomenon has been termed depolarization-induced suppression of inhibition or excitation (DSI/DSE) (37, 50-52), and has been succinctly demonstrated in electrophysiological studies. Using slice electrophysiology, it was shown that direct application of the endocannabinoid AEA to MCH neurons in the perifornical LHA decreases inhibitory input from GABA neurons (50). Thus, endocannabinoids may bind to presynaptic CB1 receptors on GABA neurons to disinhibit MCH neurons. Furthermore, application of the CB1 agonist WIN and $GABA_A$ receptor antagonist bicuculline depolarizes MCH neurons and inhibitory currents to MCH neurons are blocked by WIN; both effects are absent in the presence of AM251. In parallel, WIN also inhibits excitatory input to MCH neurons, suggesting that CB1 may also be present on presynaptic glutamate terminals that modulate MCH neuron activity (53). Taken together, these data suggest that cannabinoids in the LHA are adjusting fast neurotransmitter input to MCH neurons, but in vivo studies are required to understand the cumulative effect on eating behaviors.

Concerning O/H neurons, orexigenic action may be implicit in cannabinoid-induced feeding, as antagonism of the CB1 receptor via AM251 injection into the third ventricle decreases orexin A expression (54) and central administration of orexin A attenuates peripheral rimonabant-induced decreases in food intake (55). However, in contrast to the depolarizing effect of CB1 receptor agonists in the LHA on MCH neurons, the CB1 receptor agonists WIN and AEA hyperpolarize O/H neurons and glutamate

receptor antagonists attenuate the hyperpolarizing effect of WIN, suggesting that cannabinoids suppress excitatory glutamatergic input to O/H neurons (53, 56). This notion is supported by evidence showing O/H neurons synthesize 2AG in the soma and dendrites, while CB1 receptors and the enzyme responsible for the degradation of 2AG are predominantly located on presynaptic glutamate terminals when mice are fed a standard diet (57). Shockingly, when mice are fed a HFD, the input to O/H neurons shifts to predominantly GABAergic, as shown by triple immunolabeling (57), and this also the case in the *ob/ob* genetically obese mouse who is deficient in leptin (56). This is significant because CB1 receptors colocalize in these terminals, which may imply a switch from DSE to DSI (57), potentially removing the brakes on O/H neurons once depolarized. While sample sizes in these studies were small, overall, these observations are suggestive that consumption of a HFD and/or obesity may induce synaptic remodeling of the O/H system, potentially impacting cannabinoid-dependent eating behavior long-term.

Taken together, evidence suggests that cannabinoids have an important role in modulating the excitability of O/H and MCH neurons in the LHA and may even aid in the switch from O/H activity during food seeking behavior to MCH activity during consummatory behavior. However, future research is needed to understand when and how cannabinoids impact food intake control in the LHA. The balance between these neuronal populations may be disrupted with chronic HFD consumption and/or obesity, and the involvement of CB1 receptors in this phenomenon requires further investigation. Interestingly, LHA neurons may act in concert with cannabinoids in the ARH to modulate food intake. For example, hyperphagia induced by intra-ARH orexin is

attenuated by intra-ARH injection of AM251 (58). Orexin signaling induces release of 2AG and CB1 receptor activation in the ARH, which subsequently blunts alpha-melanocyte-stimulating hormone (aMSH) release in the PVH (59). Thus, cannabinoid signaling may be an important component of how orexin elevates food intake via modulating signaling from the ARH.

3. Arcuate hypothalamus

The ARH is a hypothalamic nucleus that is responsive to signals from the periphery regarding energy status and modulates food intake through neuropeptidergic transmission. In brief, neuropeptide Y (NPY) and Agouti-related peptide (AgRP) neurons generally elevate food intake (60, 61), while aMSH synthesized by proopiomelanocortin (POMC) neurons and cocaine- and amphetamine-related transcript (CART) neurons reduces food intake (62-64). Local injection of CB1 receptor agonist ACEA to the ARH increases food intake, and intra-ARH rimonabant injection blocks peripheral ACEA increases in food intake (29), suggesting the net effect of cannabinoids in the ARH is hyperphagic and that ARH CB1 signaling is required for the hyperphagic effects of peripheral cannabinoid administration.

CB1 receptor is present predominantly in presynaptic GABAergic neurons in the ARH located in apposition to NPY/AgRP neurons that express diacylglycerol lipase (enzyme catalyzing 2AG synthesis) (65), suggesting that endocannabinoid signaling in the ARH may regulate the activity of NPY/AgRP neurons. CB1 receptor mRNA and protein expression is amplified both in rats with diet-induced obesity and, paradoxically, also in obesity resistant rats (58). In normal weight, overweight, and lean rats, orexin-induced hyperphagia is attenuated by intra-ARH injection of CB1 antagonist AM251

(58), suggesting that ARH CB1 receptor agonism mediates orexin-induced increases in food intake. Indeed, in rat hypothalamic explants, AEA and CP55940 increase the release of the potent orexigenic peptide NPY, and this is blocked by pretreatment with the CB1 receptor antagonist AM251 (66). However, it is unclear if the release of NPY is via direct depolarization of NPY neurons or an intermediate mechanism (66). On the contrary, others have shown that bath application of ACEA in ex vivo explants reduces the activity of NPY/AgRP neurons (29). However, CB1 agonist WIN injected into the lateral ventricle did not affect NPY protein expression, while AM251 decreases NPY expression in the ARH (67). Additional in vivo evidence shows that intraperitoneal injection of the CB1 receptor antagonist rimonabant evokes similar reductions in food intake in both wild-type and NPY-deficient mice after 24-hour food restriction (33). Bringing these data together, CB1 receptor activation in the ARH increases food intake and mediates cannabinergic modulation of orexin signaling. This is likely independent of NPY signaling and may involve modulation of aMSH release in the PVH. However, more research is required to tease apart the contrary evidence presented here concerning the relevance of NPY to the cannabinergic control of food intake, including well-designed behavioral studies to understand the impact of these interactions on eating behaviors.

In the ARH, CART and POMC are produced in the same cells and are anorexigenic (63, 64, 68). Acute blockade of CB1 receptor by intraperitoneal AM281 increases CART and POMC expression and reduces HFD intake (16). Early electrophysiological evidence suggested that CB1 receptor agonism by WIN decreases evoked excitatory currents to POMC neurons (69), in line with the notion that POMC neuronal activation reduces food intake. Contrary to the consensus that POMC neurons

promote reductions in food intake, Koch et al. show that a hyperphagic peripheral dose of the CB1 receptor agonists ACEA or WIN induces immediate early gene expression along with increased excitatory activity in POMC neurons(29) (29). However, at a high dose of ACEA showing no effect on food intake, immediate early gene expression in POMC neurons is unchanged, and this high dose of ACEA hyperpolarizes POMC neurons (29). The *pomc* gene transcript is a precursor for several signaling molecules, such as aMSH and beta-endorphin (b-End), and it has been shown that intracerebroventricular administration of WIN increases b-End expression and immediate early gene activity in b-End neurons (67). Additionally, b-End itself injected to the lateral ventricle and directly to the hypothalamus acutely increases food intake (70, 71) Rather than decreasing the release of the anorexigenic neuropeptide aMSH from POMC neurons (72), intra-ARH administration of ACEA induces b-End release from ARH projections (29). Moreover, peripheral injection of the mu-opioid receptor antagonist naloxone blocks the hyperphagic effect of intra-ARH ACEA and WIN (29). Hyperphagia induced by intra-ARH injection of cannabinoid agonists is blocked by naloxone injected directly to the PVH, where ARH POMC neurons have terminals, suggesting that b-End release to the PVH may be an essential component of the hyperphagic effect of CB1 receptor agonists in the ARH and that CB1 receptor agonists act through a mu-opioid pathway to impact feeding. More work is necessary to fully understand the mechanism behind the selective release of peptides from multi-peptidergic neurons, but this work suggests that cannabinoids may control the shift from release of anorexigenic to orexigenic signals in POMC neurons. Mazier and colleagues show that glutamatergic activity from POMC neurons of the ARH is dependent on 2-AG release from PVH neurons binding at CB1. As

the authors state, a decrease in energy status most likely triggers 2-AG production and release from PVH neurons, and 2-AG retrogradely binds to CB1, decreasing glutamatergic input to the PVH (73), discussed in the following section. The implications of this are that endocannabinoids may promote quick changes in excitability of appetitive signaling based on energy status.

It is clear the endocannabinoid system in the ARH modulates energy balance. Cannabinoid signaling in the ARH is required for orexin-induced hyperphagia and may act through increasing b-End production and decreasing aMSH release from in POMC neurons. Cannabinoids likely elevate NPY secretion from the ARH, potentially not via DSE/DSI, but rather an indirect mechanism. The impact of peripheral cannabinoid administration to directly impact ARH signaling as well as interactions with the opioid system should be more deeply considered in future studies. Endocannabinoid signaling in the PVH is discussed in more detail in the subsequent section.

4. Paraventricular hypothalamus

The paraventricular hypothalamus (PVH) houses several populations of neurosecretory cells, playing an indispensable role in energy homeostasis. The phytocannabinoid delta^{9}-THC, endocannabinoid AEA, and synthetic cannabinoid ACEA injected directly into the PVH of male rats increases regular chow intake, and this effect is blocked by CB1 receptor antagonists (74-76). While rimonabant alone into the PVH does not affect food intake in rats (74), AM251 in the PVH in ad libitum fed rats produces conflicting results (76, 77), which may be due to experimental differences (see Table 2). This pharmacological evidence points to the PVH as a site where cannabinoids are sufficient to increase eating behavior. Future studies are needed to better understand conflicting

results from antagonist injections to determine if cannabinoid signaling is necessary to increase food intake in the PVH. Conflicting results may be in part explained by the mid-range affinity both antagonists have for the mu-opioid receptor (MOR), as AM251 has a stronger affinity for MOR than rimonabant (78), and MOR is expressed in the PVH (79). Further pharmacological and behavioral evidence discussed below reveals the interactions between the cannabinoid system and serotonin and ghrelin in the PVH.

Serotonin (5'-hydroxytryptamine; 5HT) is a monoamine neuromodulator largely released from the dorsal raphe nucleus (80), lesions of which induce hyperphagia (81). Subsequent work demonstrated the ability of 5HT signaling to reliably reduce food intake (82, 83). Evidence suggests that cannabinoids oppose the actions of 5HT in the PVH in relation to eating behavior. 5HT neurons terminate in the PVH, and 5HT acts as a satiation signal via 5HT1 and 5HT2 receptors (84-86). As mentioned above, ACEA, when delivered directly to the PVH, increases food intake, but when co-administered with 5HT, food intake increases are attenuated when compared to ACEA alone (75) suggesting a potential interaction between these two systems. In support of this, in rat PVH explants, ACEA decreases 5HT release and increases GABA release with respect to control, whereas AM251 application blocks these effects. Of note, GABAergic projections originating from the LH and terminating in the PVH have been shown to promote eating behavior, as optogenetic stimulation of these neurons initiates feeding (87). Like the effect of AM251, co-administration of 5HT, a 5HT1A agonist, and a 5HT1B agonist also blocked the increase in GABA release in the PVH (75). These data suggest that CB1 receptor activation may be promoting GABA release in the PVH to increase food intake and that serotonin receptor signaling attenuates the cannabinergic

effect of increased GABAergic signaling. The data show that 5HT signaling attenuates cannabinoid-induced hyperphagia, likely via 5HT1A and 1B receptors in the PVH. In a freely behaving animal, this suggests that endocannabinoid release in the PVH promotes the release of GABA, promoting eating behavior, and serotonin release may promote satiation and meal termination.

Ghrelin is a potent orexigenic signal originating from the gastrointestinal tract that regulates eating behavior via the PVH (88). Research indicates that ghrelin systems may require endocannabinoid systems to elevate food intake. Of note, CB1 receptor KO mice do not respond to the orexigenic effects of a peripheral injection of ghrelin (89). Furthermore, intraperitoneal ghrelin increases hypothalamic 2AG and AEA content in wild-type mice, which is blocked by rimonabant and absent in CB1 KO mice (89). Additionally, a subthreshold subcutaneous injection of rimonabant that has no effect on food intake alone prevents intra-PVH ghrelin-induced hyperphagia (90). These data taken together suggest that ghrelin signaling in the PVH works in concert with the cannabinoid system to promote food intake. As it has been iterated several times in this review, CB1 receptor antagonists generally decrease food intake or show no effect. Evidence from Soria-Gomez and colleagues contradicts this working knowledge of CB1 receptor antagonists, as intra-PVH AM251 injected at the end of a 24-hour fast *increases* fasting-induced hyperphagia (77). Specifically, AM251 given in conjunction with ghrelin directly into the PVH *potentiates* the hyperphagic effect of ghrelin in free-fed rats, but not until 4 hours post treatment (77). The possibility that the acute effect of AM251 is expired after 4 hours, however, should not be excluded. Much of the evidence points to

an inverse relationship between circulating ghrelin and hypothalamic endocannabinoids, and CB1 receptor being required for ghrelin-induced increases in food intake.

Cannabinoid action in the PVH likely has an overall potentiating effect on food intake working in conjunction with ghrelin and in opposition to 5HT. Ghrelin likely acts to increase food intake partially by elevating hypothalamic endocannabinoid content, and cannabinoids may promote food intake in the PVH via increasing GABA release and suppressing 5HT release. Work from Rorato and colleagues shows that acute and prolonged (one week) treatment with rimonabant increases CB1 receptor mRNA in the PVH (91), but there is a notable lack of evidence of the location of CB1 receptors in the diverse PVH region (15). Future study should shed light on production and local signaling of endocannabinoids as well as receptor localization in this region to better understand the perplexing behavioral data gathered from these few studies. Relevant to overall energy balance, AEA administration to the PVH increases the respiratory quotient (76), indicating potential changes in energy expenditure. Interestingly, when the CB1 receptor is knocked out of Sim1 neurons (expressed by most neurons of the PVH), no changes in energy expenditure could be detected until mice were maintained on a HFD when animals lacking Sim1 neuronal CB1 receptor showed increased energy expenditure and adrenergic receptor gene expression (92), suggesting CB1 receptor in Sim1 neurons is responsive to the metabolic effects of a HFD.

5. Ventromedial Hypothalamus

Like other regions discussed, the ventromedial hypothalamus (VMH) has moderate expression of CB1 receptor in both GABAergic and glutamatergic synapses (93). Steroidogenic factor 1 (SF1), a transcription factor exclusively produced in the VMH

(94) that has been implicated in energy balance regulation via leptin (95) and glucose (96) sensing, is necessary for the expression of CB1 receptor in the VMH (97). Kim and colleagues found that SF1 can directly stimulate CB1 receptor transcription activity via two potential binding sites in the promoter region of the gene, and agonism of the CB1 receptor by WIN decreases the firing rate of SF1 neurons (97). This suggests that SF1 is regulating the expression of CB1 receptor, which, in turn, regulates the activity of SF1 neurons (97). Two groups have contributed data to the field examining the metabolism of mice wherein the CB1 receptor has been specifically knocked out of SF1-positive neurons. Neither group found differences in body weight or lean mass in male SF1-CB1-KO mice on standard chow (98, 99). However, Cardinal and colleagues found reduced fat mass and modest improvements in glucose and insulin tolerance, while Castorena et al., found no differences in fat mass nor plasma insulin (98, 99). Cardinal et al., further examined markers of sympathetic activity due to exhibition of a reduced respiratory quotient in male SF1-CB1-KO mice indicating a preference for fat as energy substrate compared to carbohydrates (98). Male SF1-CB1-KO mice on chow showed increased adrenergic receptor expression and increased phosphorylated hormone sensitive lipase expression in white adipose tissue relative to WT mice (98). Interestingly, when SF1-CB1-KO mice were put on a HFD for eight weeks, these measures inverted, i.e., modest deterioration of glucose tolerance, increased respiratory quotient, decreased adrenergic receptor expression, and decreased phosphorylated hormone sensitive lipase expression in white adipose tissue (98). SF1-CB1-KO mice further displayed increased total HFD intake, total body weight, and fat mass on HFD, with no differences in lean mass nor insulin tolerance (98). Castorena et al., contradict this evidence showing male SF1-CB1-

KO mice measures of weight, body composition, nor food intake do not change when placed on a HFD (99). However, SF1-CB1-KO males did display decreased plasma glucagon, decreased hepatic glucose production, and increased glycogen synthase (99), in line with decreased sympathetic activity when placed on HFD. Female SF1-CB1-KO mice on standard show or a HFD did not show differences in weight, lean or fat mass, plasma insulin, glucose or insulin tolerance (99). A separate study by Cardinal and colleagues showed virally-mediated CB1 knockdown (60% reduction) specifically in the VMH reduces weight gain compared to control littermates driven by increased energy expenditure in both phases of the diurnal cycle, while total locomotor activity and food intake are not different from wild-type mice (100). They replicate the elevation in adrenergic receptor mentioned above and further show elevated uncoupling protein-1 mRNA gene expression (100). Inconsistencies between studies may be due to differences in KO model generation, housing conditions, or diet composition, as noted by the authors (99). Overall, these data demonstrate that CB1 receptor in the VMH, specifically in SF1 neurons, regulate sympathetic activity and contribute to adipose tissue maintenance, and these functions are dysregulated in the presence of HFD.

There is limited pharmacological evidence investigating how the body responds to exogenous cannabinoids in the VMH. Early investigations by Jamshidi and Taylor showed that AEA injected into the VMH of sated male rats increases standard food intake with a bell-shaped dose response (101). Additionally, intraperitoneal injection of CB1 agonists (methanadamide and ACEA) to 24h fasted male and female mice augments standard chow intake in WT mice, but this effect is absent in SF1-KO mice (97), suggesting that CB1 receptor and/or SF1 is required for the hyperphagic effects of

peripheral CB1 agonists. Similarly, AM251 blunted refeeding in WT mice, but not in SF1-KO mice (97). Together these data suggest that SF1 in the VMH is required for cannabinoid-induced hyperphagia and given that SF1 is required for CB1 receptor expression it is possible that these drugs work directly on CB1 receptors in the VMH, but this possibility has not yet been directly tested.

6. Hypothalamic leptin signaling and the endocannabinoid system

Leptin is an adipocyte-derived hormone that increases proportionally to body fat, and leptin is one of the hormones that relays to the hypothalamus information regarding the body's energy status (102). There is ample evidence for an interaction between hypothalamic leptin signaling and CB1 receptor signaling in the regulation of energy balance. CB1 receptor null mice have decreased plasma leptin, coinciding with decreased fat mass (15), and this is true even for CB1 receptor null mice that are fed a HFD (16). Furthermore, the hypophagic effect of peripheral leptin injection is abolished in mice with hypothalamic specific CB1 receptor knock out (100), but the mechanism remains unclear. In line, exogenous leptin given to diet-induced obese male rats in combination with rimonabant results in elevated weight loss compared to either treatment alone and vehicle (103). Interestingly, intravenous delivery of leptin reduces hypothalamic levels of 2AG and AEA in male rats (33). Further, animals lacking the leptin receptor display increased hypothalamic levels of endocannabinoids, and mice lacking leptin itself also present with increased 2AG in the hypothalamus, which returns to control levels upon leptin administration (33). Conversely, AEA levels in the *ob/ob* mouse, which lacks the ability to produce functional leptin, are not different from control mice, but leptin administration significantly decreases AEA below control values in these animals (33).

Taken together, leptin signaling reduces hypothalamic endocannabinoid levels and endocannabinoid signaling may be necessary for the normal effects leptin.

There are several lines of evidence examining CB1 receptor and leptin receptor expression in the hypothalamus (104), specifically in the VMH and PVH. In the PVH of male Zucker rats that lack the leptin receptor, no differences could be detected in CB1 receptor expression in lean or obese rats regardless of prandial state (105). However, authors do note changes in CB1 receptor expression in the VMH of obese leptin receptor deficient rats in that ad libitum fed rats have elevated CB1 receptor expression, while fasted rats have diminished CB1 expression compared to lean controls (105). Specific to the VMH, deletion of CB1 receptor from SF1-positive neurons has direct consequences on leptin signaling in the VMH (98). While WT and SF1-CB1-KO have indistinguishable leptin receptor expression, SF1-CB1-KO mice are more sensitive to leptin as shown by a 20% decrease in 24h food intake and greater phosphorylated-STAT3 expression in the VMH (98). These changes were coupled with elevated adrenergic receptor and hormone sensitive lipase gene expression and a reduced respiratory quotient (98). When SF1-CB1-KO mice were placed on a HFD for only two weeks, the sensitivity SF1-CB1-KO mice displayed on chow was abolished with markedly decreased phosphorylated-STAT3 expression (98). These data suggest that CB1 receptor in the VMH reduces leptin receptor sensitivity in the VMH with consequent susceptibility to weight gain.

7. Conclusions

Endocannabinoid signaling plays an essential role in eating behaviors coordinated by the hypothalamus. Evidence reviewed here suggests that cannabinoid signaling is critical to the integration of peripheral signals, as lack of the CB1 receptor negates the hypo- and

hyperphagic effects of leptin (100) and ghrelin (89), respectively. Additionally, hypothalamic endocannabinoid tone may be dependent on energy status. Fasting and feeding have inverse effects on endocannabinoid production (18), but there is little data to attest to how these energy states affect CB1 receptor expression (105) or membrane localization, and sex differences in endocannabinoid regulation may be present (19) but are understudied. There is consensus that CB1 KO mice weigh less, but whether this is due to caloric deficit or increased energy expenditure is still debated. Knocking down the CB1 receptor in the VMH increases energy expenditure in both phases of the diurnal cycle, generating supporting evidence for the latter hypothesis. While CB1 receptor is expressed in SF1 neurons of the VMH, CB1 receptor expression in the PVH has been shown to colocalize with CRH (15) and is expressed in Sim1 neurons (92), and further characterization of CB1 expression on the various neurons of the PVH controlling endocrine function may shed light on cannabinoid interactions with energy expenditure.

Strong evidence suggests that CB1 receptor signaling in the PVH is critical for CB1-mediated orexigenic effects, but more data are needed (site-specific antagonist injections/receptor knockdown) to pinpoint the roles of other hypothalamic regions in orchestrating cannabinoid-induced eating behavior. Indeed, there are many outstanding questions regarding CB1 signaling in the hypothalamus. Further research is needed to investigate how cannabinoids may be increasing GABA release in the PVH (75), as this data point is incongruent with the hypothesis of disinhibition. In the arcuate nucleus, there are several lines of conflicting evidence attempting to elucidate how cannabinoids are interacting with NPY/AgRP neurons. It stands to reason that the cannabinoid system may regulate NPY neuron activity, but more in-depth analysis of this potential interaction

is needed to make sense of the existing literature. Other outstanding questions in the arcuate nucleus include the potential role of cannabinoids in regulating or participating in the selective release of peptides from multipeptidergic neurons, such as POMC neurons. Solving one such mystery of this nature may additionally give us clues into how cannabinoids may be regulating other multipeptidergic neurons involved in food intake control, such as MCH neurons. Finally, evidence suggests that endocannabinoid signaling is modulating the excitability of lateral hypothalamic MCH and O/H neurons, but the effect on intake behavior, if any, remains to be determined.

Regarding the relationship between HFD feeding and the endocannabinoid system, the evidence indicates that lack of cannabinoid signaling is protective against the negative metabolic effects of a HFD (17), and antagonism of the CB1 receptor reduces HFD consumption (16). However, the long-term effects of HFD feeding on the endocannabinoid system are unclear. HFD feeding may alter leptin sensitivity in the VMH through a CB1 receptor-mediated mechanism (98), but the endogenous relevance of the CB1 receptor to VMH-controlled energy balance is still under investigation (98, 99). Most interestingly, limited evidence demonstrates that chronic HFD consumption potentially induces synaptic remodeling in O/H neurons, increasing the density of O/H innervation to target regions (57). In the acquisition of a meal, it has been shown that O/H neurons are active until meal initiation, and, subsequently, previously silent MCH neurons are depolarized during meal consumption (48). Given the impact of HFD feeding on O/H neurons, it may be suggested that HFD feeding perturbs this balance between lateral hypothalamic neurons and should be a topic of further investigation.

The scientific debate these studies conjure up can sometimes be simply attributed to differences in experimental design. Experimental conditions likely contribute to differences noted in pharmacological manipulations of the cannabinoid system. In particular, ventricular injections have yielded highly inconsistent results, and this may be due to dosing and distance from injection to the site of action. Controlled study comparing lateral, third, and fourth ventricular injection sites with standardized dosing of CB1 receptor ligands would aid in alleviating the existing discrepancies in the literature. Concerning intrahypothalamic injections of cannabinoids, the differing experimental conditions and resulting behavior are noted in Table 1.2.

While the intersection of cannabinoid and neuroscience research has made great strides in the past few decades, the field has many questions yet to be answered. There is still a dearth of research focusing on the endocannabinoid system and cannabinoid-induced eating in female subjects. To have a complete picture of how the endocannabinoid system is orchestrating energy intake, female subjects must be included in all future endeavors.

Table 1.2: Differing experimental conditions in studies that injected a cannabinoid directly to the hypothalamus. Lowercase x indicates that the experimental detail could not be found. *hyperphagia was not observed until four hours post injection

Species	**Sex**	**CB1R agonist**	**Route**	**Energy status**	**Dose**	**Feed**	**Circadian**	**Outcome**	**Reference**
rats	male	2AG	LHA	ad lib	1.2 µg	Rodent Laboratory Chow	dark cycle	no effect	[49]Sanchez Fuentes et al., 2016

rats	male	d9-THC	PVH	ad lib	5 µg	Standard chow (Rat and mouse chow, Ridley AgriProducts, Australia)	dark cycle	hyperphagia	[74]Verty et al., 2005
rats	male	ACEA	PVH	21h fasted	0.25 µg	Standard rodent chow (LabDiet #5008)	dark cycle	hyperphagia	[75]Cruz Martinez et al., 2018
rats	male	AEA	VMH	sated	50 ng	Standard rodent chow (ARM pellets)	mid-light cycle	hyperphagia	[101]Jamshidi & Taylor, 2001
mice	x	ACEA	ARH	x	x	Standard rodent chow	mid-light cycle	hyperphagia	[29]Koch et al., 2015
rats	female	d9-THC (l isomer)	VMH	ad lib	0.25 µg	Purina Lab Chow	x	hyperphagia	[32]Anderson-Baker et al., 1979
rats	female	d9-THC (l isomer)	LHA	ad lib	0.125, 0.25 µg	Purina Lab Chow	x	no effect	[32]Anderson-Baker et al., 1979
rats	female	d9-THC (d isomer)	VMH	ad lib	0.25 µg	Purina Lab Chow	x	reduced intake	[32]Anderson-Baker et al., 1979
rats	female	d9-THC (d isomer)	LHA	ad lib	0.25 µg	Purina Lab Chow	x	hyperphagia	[32]Anderson-Baker et al., 1979
rats	male	AEA	PVH	ad lib	100, 400	Standard rodent chow	dark cycle	hyperphagia	[76]Chapman et

					pmol				al., 2012
Species	**Sex**	**CB1R antagonist**	**Route**	**Energy status**	**Dose**	**Feed**	**Circadian**	**Outcome**	**Reference**
rats	male	RIM	PVH	ad lib	0.03, 0.3, 3.0 µg	Standard chow (Rat and mouse chow, Ridley AgriProducts, Australia)	dark cycle	no effect	[74]Verty et al., 2005
rats	male	AM251	PVH	ad lib	1.6 µg	Standard rodent chow	mid-light cycle	reduced intake	[77]Soria Gomez et al., 2014
rats	male	AM251	PVH	24h fasted	1.6 µg	Standard rodent chow	dark cycle	hyperphagia*	[77]Soria Gomez et al., 2014
rats	male	RIM	VMH	sated	30 µg	Standard rodent chow (ARM pellets)	mid-light cycle	no effect	[101]Jamshidi & Taylor, 2001
mice	x	RIM	ARH	fasted overnight	3 mg/kg	Standard rodent chow	mid-light cycle	reduced intake	[29]Koch et al., 2015
rats	male	AM251	PVH	ad lib	5, 10, 20 µg	Standard rodent chow	dark cycle	no effect	[76]Chapman et al., 2012

CHAPTER 2

EDIBLE CANNABINOIDS IMPACT MEAL STRUCTURE AND FOOD IMPULSIVITY IN FEMALE RATS[1]

1. Lord M.N., Spaulding M.O., Hoffman J.R., Basma R. B., and Noble, E.E. Edible cannabinoids impact meal structure and food impulsivity in female rats. Accepted by iScience. Reprinted here with permission of publisher, March 12, 2025.

Abstract

Cannabinoid receptor agonists increase eating in a dose-dependent manner. However, the behavioral mechanisms by which cannabinoids modulate food intake control aren't clear, particularly in females. We utilized a rodent model of cannabinoid administration modeling a common route of cannabinoid consumption in humans: edibles. Herein we administered the dual cannabinoid receptor agonist CP55940 in edible form to female rats and observed acute increases in standard chow intake due to an increase in meal size with no change in meal number. We further observed that the hyperphagic dose of edible CP55940 increases impulsive responding for sucrose, but this didn't coincide with changes in motivation for sucrose. Finally, cannabinoids can affect anxiety-like behavior, but the acutely hyperphagic dose used in our studies had no effect on anxiety-like behavior. We conclude that edible cannabinoid administration delays satiation and increases impulsive eating behavior without impacting food motivation, potentially by reducing inhibitory control.

1. Introduction

Cannabis (marijuana) use in America has increased in the past decade, with nearly 30% of young adults (aged 19-30) and over 19% of mid-life adults (aged 35-50) self-reporting having used cannabis in the past thirty days (106). Changing legality has largely shifted the cultural opinion of cannabis use, with some being misled to believe use has little consequence (107, 108). However, compounds in cannabis act on cannabinoid receptors to modulate a host of physiological functions including modulation of pain processes, sleep, anxiety, energy homeostasis, and appetite regulation, see reviews: (4-7). This wide

array of functions modulated by cannabinoids is due to the vast central and peripheral distribution of cannabinoid receptors (109), conferring equally vast behavioral effects.

One of the known behavioral effects of cannabinoids is an upregulation in food intake, colloquially coined as “the munchies”. Marijuana’s effect on food intake in healthy individuals was reported as early as 1986 when a Johns Hopkins group observed increased snacking behavior in adult males following marijuana cigarettes (110). Laboratory rodents also get the munchies as has been demonstrated using various cannabinoids of differing dosages (24, 25, 29, 30). The munchies as an observed behavior can be the result of multiple potential neurobiological changes, such as motivated appetitive drive, reduced satiation, or impaired inhibitory control. Therefore, we set out to determine the impact of voluntary edible cannabinoid consumption on eating behavior in female Wistar rats, with a focus on meal patterns, food impulsivity, and food motivated behavior.

Cannabinoids have been shown to be anxiogenic in some individuals (111). Another potential contributor to the enhanced ingestive behavior associated with cannabinoid usage is eating for anxiety reduction and mood regulation. In women, anxiety is associated with increased calorie consumption at a buffet and reduced activation of brain regions associated with satiety when visualizing high fat palatable food cues (112). Therefore, we further investigated whether a dose of cannabinoids sufficient to increase food intake also affects anxiety-like behavior in female rodents.

Basic mechanisms underlying how cannabinoids modulate behavior and metabolic processes in preclinical models have generally been studied using injection of cannabinoid receptor ligands as the route of administration. One benefit of injectable

cannabinoid administration is that the dose in the blood can be controlled. However, cannabinoid drugs are not typically injected during recreational or clinical cannabinoid use; rather, cannabinoids are generally smoked or taken orally. Oral ingestion of cannabinoids is of particular importance to investigate due to its greater clinical utility over smoking. For example, the only FDA-approved cannabinoid therapeutics currently available are in oral or oromucosal preparations (113). Within the last decade, it has become of interest to develop models of cannabinoid consumption that more closely model humans' administration methods, i.e., inhalation and ingestion of edibles in rodents. Self-administration of THC-containing gelatin or cookies provided the first models of voluntary oral ingestion of THC edibles in rodents (114-117). Subsequently, others built upon the self-administration model by incorporating palatable agents, such as Ensure (Abbott), to further encourage voluntary consumption of THC (118). These revolutionary models of voluntary oral consumption of cannabinoids are credited with initiating this vital area of study. However, the self-administration model produces a variety of THC concentrations and volumes of gelatin consumed, and cannabimimetic responses can only be categorized based on dose averages. To circumvent this dose and ingested volume range that self-administration confers, Amissah and colleagues utilized the hyperpalatable vehicle Nutella (Ferrero) to dissolve the precise dose each rodent needs based on their weight (119). The study of eating behavior following consumption of an edible requires the volume ingested to remain consistent. Therefore, utilizing a combination of these techniques, we have developed a model of edible cannabinoid consumption wherein the synthetic CP55940, a THC-like cannabinoid, is dissolved in coconut oil and incorporated into a gelatin-based edible with enhanced palatability from

Jello (Kraft-Heinz). The precise dose for each subject is then portioned according to the weight of the animal. All data were collected using edible cannabinoid administration wherein we examined female rats' behavioral responses to a cannabinoid-containing edible or its vehicle preparation.

2. Results

Edible cannabinoid receptor agonist acutely promotes eating behavior via an increase in meal size

Female rats given the CP55940-containing edible increased their intake of chow compared to rats given vehicle edible over the first two hours of the dark cycle ($t=2.230$, $df=10.67$, $p=0.048$; Figure 1A). Meal number and meal size were analyzed within this two-hour window, and results revealed that in response to edible CP55940, female rats elevated their meal size ($t=2.467$, $df=7.047$, $p=0.043$; Figure 1B) without changing the number of meals eaten ($t=0$, $df=12.40$, $p>0.99$; Figure 1C) compared to rats given a vehicle edible. These results were acute, as over the twenty-four-hour period, chow intake ($t=0.1446$, $df=12.90$, $p=0.89$; Figure 1D) and meal size ($t=1.541$, $df=13$, $p=0.15$; Figure 1E) were not different between groups, and there was a notable decrease in meal number in the edible CP55940 group compared to the vehicle edible group ($t=3.548$, $df=11.71$, $p=0.0042$; Figure 1F). Taken together, these data indicate CP55940 acutely elevates chow intake in female rats via an increase in meal size, with compensatory reductions in meal number over the course of 24 hours.

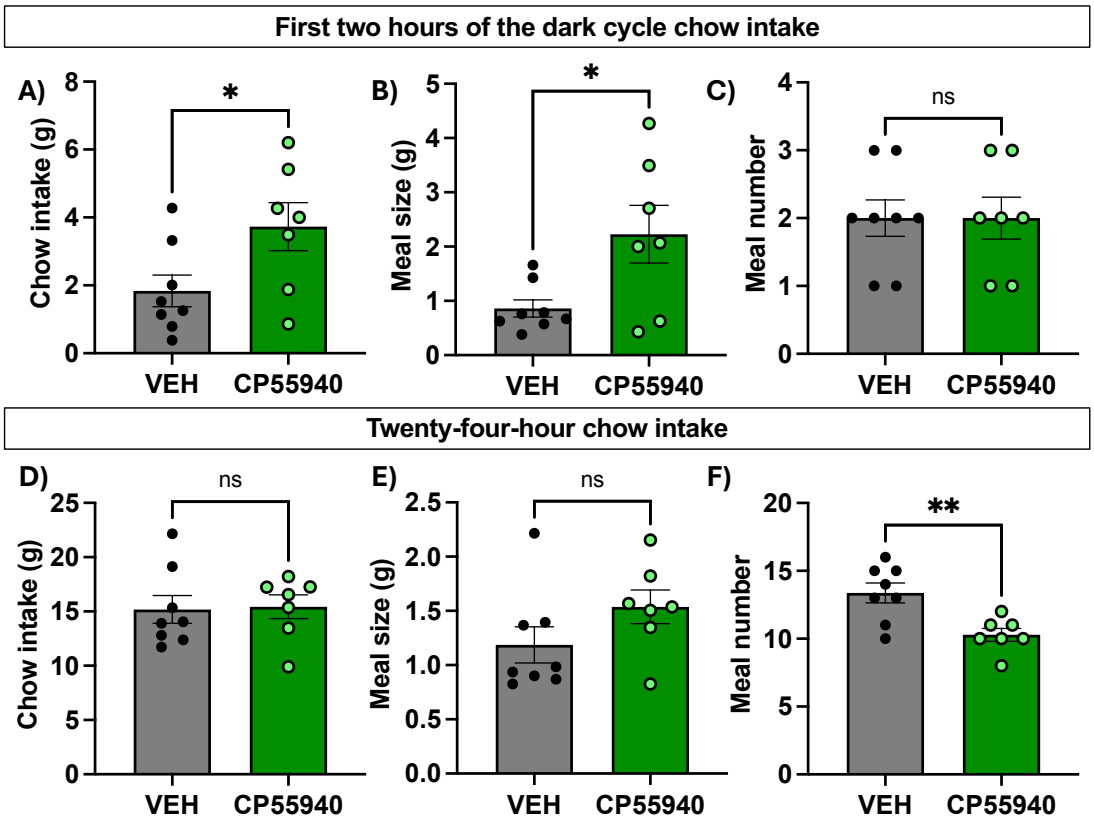


Figure 2.1: Edible cannabinoid receptor agonist acutely promotes eating behavior via an increase in meal size. Female rats (n=15) increased standard chow intake over the first two hours of the dark cycle (A) via an increase in meal size (B) with no changes in meal number (C). However, over the course of 24 hours, there was no difference in overall chow intake (D), nor meal size (E), and a decrease in meal number (F). Data are means +/- SEM; *p < 0.05, **p < 0.01

Edible cannabinoid receptor agonist increases impulsive action for sucrose

Female rats were trained in the DRL task prior to testing the effects of the cannabinoid-containing edible on impulsive action for sucrose (visual representation shown in Figure 2A). Rats given edible CP55940 prior to testing in DRL 20 pressed the active lever more

(t=2.766, df=7, p=0.028; Figure 2B) yet earned fewer pellets when compared to responses following a vehicle edible (t=2.908, df=7, p=0.021; Figure 2C). This increase in lever pressing from the CP55940 group was not due to increased nonspecific activity as there was no difference in lever pressing on the nonreinforced, inactive lever (t=1.34, df=7, p=0.22; Supplemental Figure 2A). An efficiency score was calculated by dividing the number of pellets earned by the number of active lever presses (Figure 2D), which revealed that the edible CP55940 group was less efficient in obtaining sucrose than the vehicle group (t=2.491, df=7, p=0.042; Figure 2E). Taken together, these data indicate that animals given edible CP55940 exhibit greater impulsive action for sucrose when compared within-subjects to responses given the vehicle edible.

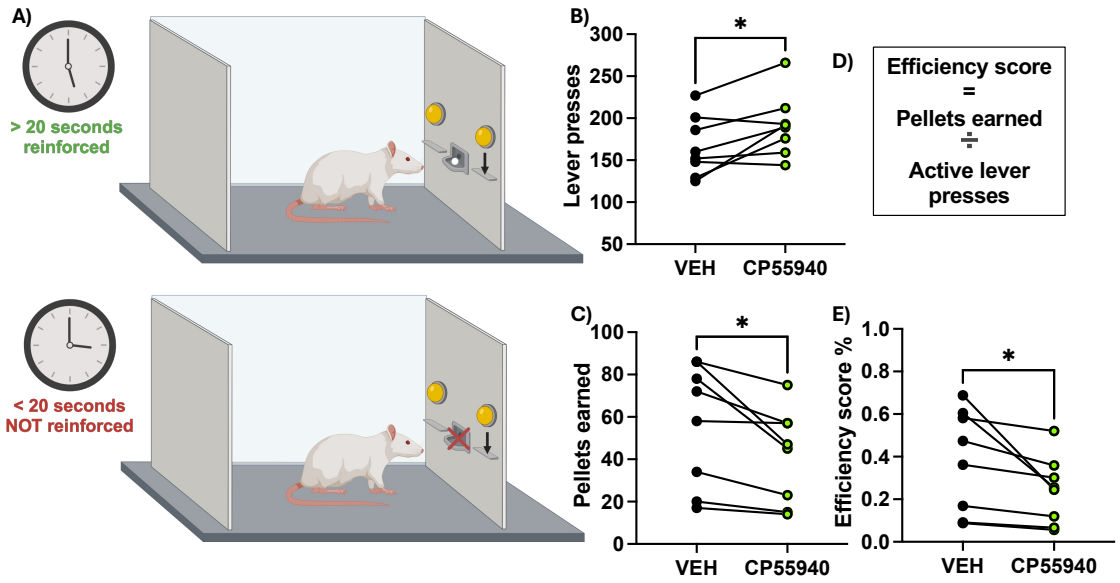


Figure 2.2: Edible cannabinoid receptor agonist increases impulsive action for sucrose in female rats (n=8). The differential reinforcement of low rates of responding task was utilized to measure impulsive behavior; a schematic of the task shows the binary outcome of either pressing the lever after the 20-second interval or prematurely pressing the lever

(A). Compared to vehicle, cannabinoid-treated females pressed the active lever more (B), and earned fewer pellets (C). An efficiency score is calculated by the equation in (D). Cannabinoid-treated females were less efficacious in obtaining sucrose in the task (E) indicating that edible CP55940 elevates impulsive action for sucrose in female rats. All behavior was examined during the dark cycle. Data are means +/- SEM; *$p < 0.05$

Edible cannabinoid receptor agonist does not affect motivation for sucrose

Rats were tested in the PR task to determine if the acute, hyperphagic dose of edible CP55940 that increases impulsive responding for sucrose also increases motivation to obtain sucrose. In an exponential PR schedule, (Figure 3A), there were no differences between vehicle and CP55940 groups in number of active lever presses ($t=0.2364$, $df=14$, $p=0.82$; Figure 3B) nor pellets earned ($t=0.314$, $df=14$, $p=0.76$; Figure 3C). We reasoned that the exponential progression may have advanced too quickly to detect differences between groups, and thus we tested a separate cohort of rats using a more gradual PR schedule wherein the number of lever presses that it took to obtain one sucrose pellet increased linearly by three (Figure 3D). Again, no differences were detected between vehicle and CP55940 groups in number of active lever presses ($t=1.308$, $df=13$, $p=0.21$; Figure 3E) nor pellets earned ($t=1.452$, $df=13$, $p=0.17$; Figure 3F) on the linear PR schedule. Neither PR schedule produced differences in pressing on the inactive, nonreinforced lever (PR schedule 1: $t=0.3537$, $df=14$, $p=0.73$; PR schedule 2: $t=0.5953$, $df=13$, $p=0.56$; Supplemental Figure 2B, C). Taken together, we conclude that an acute, hyperphagic dose of edible CP55940 does not affect motivated responding for sucrose.

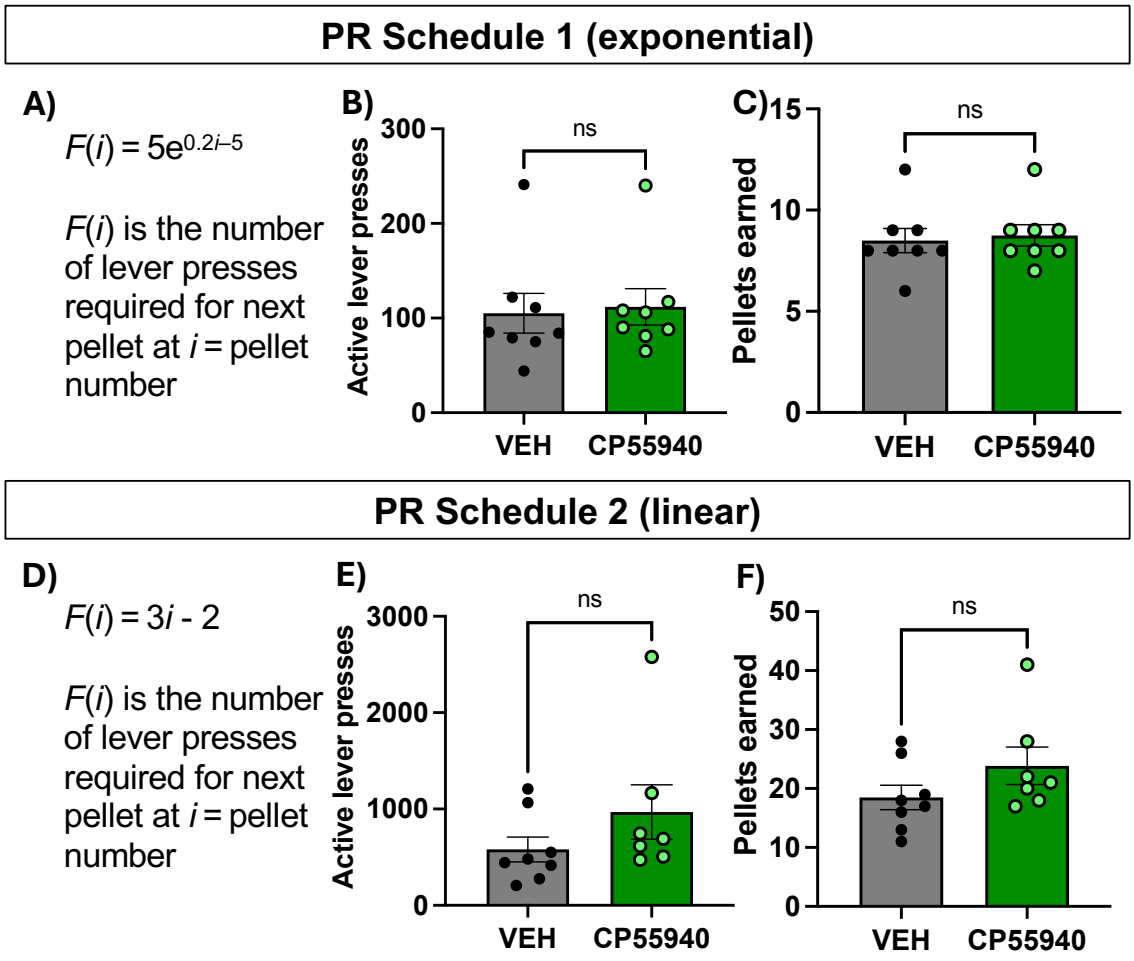


Figure 2.3: Edible cannabinoid receptor agonist does not affect motivation for sucrose. Two progressive ratio (PR) tasks were used to examine motivated responding for sucrose with two separate cohorts (n=16 in each cohort). In PR, rats must work progressively harder (more lever presses) to obtain a single sucrose pellet. In schedule 1, the number of lever presses needed for a single pellet increased exponentially by the equation shown in (A). In schedule 2, the number of lever presses needed for a single pellet increased linearly by the equation shown in (D). No differences were detected in lever presses (B, E) nor pellets earned (C, F) using either schedule. All behavior was examined during the dark cycle. Data are means +/- SEM.

Edible cannabinoid receptor agonist does not affect anxiety-like behavior in the elevated plus maze

Rats were tested in the EPM to determine if the acute, hyperphagic dose of edible CP55940 increases anxiety-like behavior. Given five minutes in the EPM, no differences were observed between groups in total distance travelled (t=0.9031, df=14, p=0.38; Figure 4A), mean speed in the maze (t=0.8978, df=14, p=0.38; Figure 4B), nor time spent immobile (t=0.08136, df=14, p=0.94; Figure 4C). Furthermore, no differences were observed in time spent in the closed arms (t=0.2918, df=14, p=0.77; Figure 4D), the center of the maze (t=0.9630, df=14, p=0.36; Figure 4E), nor the open arms (t=0.02406, df=14, p=0.98; Figure 4F). Taken together, these data indicate that an acute, hyperphagic dose of edible CP55940 does not affect anxiety-like behavior in female Wistar rats.

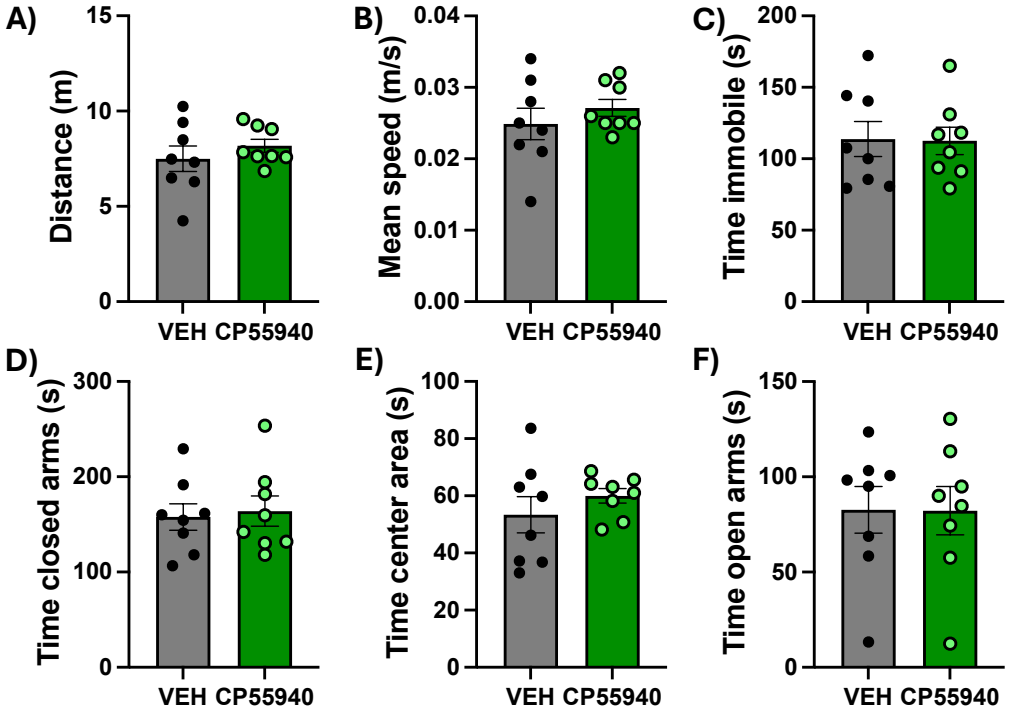


Figure 2.4: Edible cannabinoid receptor agonist does not affect anxiety-like behavior in the elevated plus maze. In female rats (n=16), no differences were detected between groups in total distance travelled in the maze (A), speed in the maze (B), time spent immobile in the maze (C), time spent in the closed arms (D), time spent in the center area (E), nor time spent in the open arms (F). Data are means +/- SEM.

3. Discussion

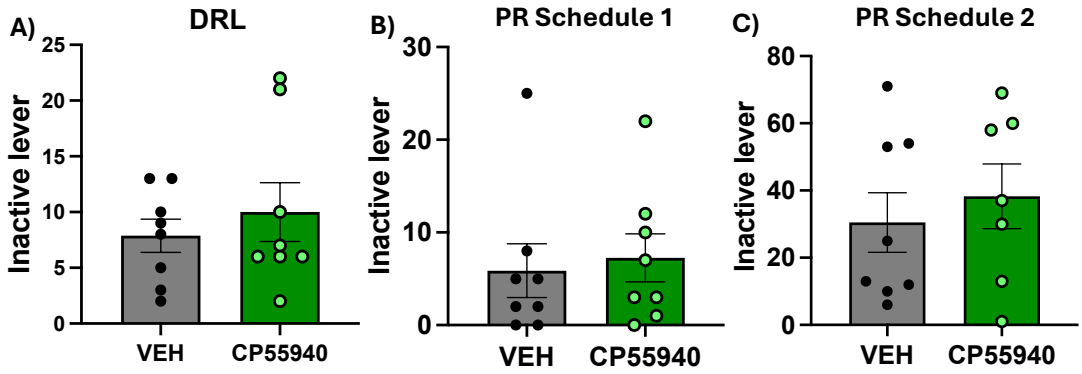
The route of administration of cannabinoids is critically important for the interpretation of behavioral outcomes. Orally ingested cannabinoids have access to a vast distribution of cannabinoid receptors in the body, especially in the enteric nervous system (120-122). Gelatin-based edibles more closely model human cannabinoid use when compared with injection, and injection as the route of administration may bypass putative sites of cannabinoid action via gut to brain communication, which likely contributes to the hyperphagic effect of cannabis-containing edibles (123-125). Furthermore, the pharmacokinetics of cannabinoids differ between injection and oral administration. Using THC as the model cannabinoid, it's been shown that orally administered cannabinoids are slowly and erratically absorbed (126), and surprisingly result in the highest cannabinoid concentrations in the brain as compared to other routes of administration (127). Oral gavage is an existing method of non-voluntary oral cannabinoid administration (25), albeit rarely used, but has been shown to induce stress in rodents (128), which is not ideal when measuring many physiological and behavioral parameters, such as eating behaviors. Our results show that precisely dosed gelatin-based edibles are well-accepted by rats and

induce hyperphagia when they contain a cannabinoid receptor agonist. To the best of our knowledge, this is the first evidence showing that oral administration of a cannabinoid-containing gelatin increases meal size in female rodents.

While it is well established that CB1 receptor agonists increase food intake, there is a relative paucity of data into the effects of cannabinoids on food intake control in females (23). Furthermore, it is not clear at which point(s) in the sequence of a meal these ligands have their impact when cannabinoids are administered orally. Blundell and McArthur identified three stages to the "feeding cycle": appetitive, consummatory, and satiety phases (129). Pharmacological agents, e.g. cannabinoids, modulate one or more of these feeding cycle phases to induce their largely hyperphagic effects, and understanding how pharmacotherapies impact the feeding cycle is crucial to determining their clinical utility (130). We show here that the hyperphagic effects of oral cannabinoid administration in female Wistar rats is due to increases in meal size and not meal number. Our data are consistent with prior research conducted by Ogden and colleagues in intact female rats where the CB1 receptor agonist AM11101 injected intraperitoneally produced hyperphagia via an increase in meal size with no changes in meal frequency, a meal as defined by intake bouts greater than 0.3 grams spaced no more than 15 minutes apart (131). Importantly, these data further elucidate how cannabinoid receptor agonists are modulating food intake control in females, suggesting that acute CB1 receptor agonism may delay satiation within the consummatory phase of the feeding cycle.

Our findings demonstrate that cannabinoids not only increase food intake by elevating meal size, but also increase impulsive responding for a palatable reinforcer. Impulsivity is a complex behavioral trait of particular interest in the context of

psychoactive drug use, defined as acting without consideration of the consequences (132). Previous reports support a role for the endocannabinoid system in modulating impulsivity; for example, systemically administered CB1 receptor antagonist rimonabant diminishes stimulant-induced impulsivity in male rats (133, 134). The direct effect of CB1 receptor agonism on impulsive behavior may be dependent on dosing or duration of use. One group found that three weeks of chronic intraperitoneal injection of THC reduced impulsive responding in adult male rats (135), while another group found that chronic exposure during adolescence also reduced impulsive responding for sucrose in young males, but only in the lower-dose group (136). Further evidence suggests CB1 receptor agonism impairs response inhibition and CB1 receptor antagonism improves inhibitory control in male rats (137-139), with one study showing no effect of THC on response inhibition in female rats (139). In the acute study herein, we utilized the DRL task to investigate whether a hyperphagic dose of CP55940 modulates impulsive action in female rats. Cannabinoid-treated females showed greater impulsive lever pressing for obtaining the palatable sucrose reinforcer compared to themselves when given vehicle. This is not due to a general increase in nonspecific lever pressing, as there was no increase in lever activity on the inactive lever (Supplementary Figure 2). These data indicate that an edible cannabinoid receptor agonist that acutely increases meal size also elevates impulsive responding for sucrose in female rats.



Supplementary Figure 2.2: Pressing on the inactive lever in each operant chamber behavioral task. In the differential reinforcement of low rates of responding task (DRL), presses on the inactive lever were not different between groups (A). In schedule 1 of the Progressive Ratio (PR) task, presses on the inactive lever were not different between groups (B). In schedule 2 of the PR task, presses on the inactive lever were not different between groups (C). Data are means +/- SEM.

Given this finding, we further investigated what could be driving elevated impulsivity in females in response to an oral cannabinoid receptor agonist. Increases in impulsive behavior in the DRL task may be due to increased motivation to seek sucrose (140). A substantial body of literature shows that antagonizing the endocannabinoid system leads to decreased motivation to work for a food reward in male rats (28, 141-146). It's also been shown in male rats that CB1 receptor agonists THC and anandamide further enhance sucrose palatability (147, 148). However, few studies have directly interrogated the effect of CB1 receptor agonism on motivated responding (149, 150). While one study in male mice shows that an acute dose of intraperitoneal CP55940 increases responding for palatable reinforcers (151), others show a biphasic response to

THC vapor wherein lower dose THC vapor increases and higher dose THC vapor decreases PR breakpoint (152). Similarly, Wheeler and colleagues show a transient increase in operant responding for a sucrose reinforcer in male rats following whole-plant cannabis vapor exposure (153). In our study, we utilized two schedules of reinforcement to investigate motivation to obtain a palatable sucrose reinforcer in females. Using an exponential formula that has previously been used to evaluate willingness to work for drugs of abuse (154), we found no differences in willingness to work for sucrose. We questioned whether an exponential progression with this PR schedule may have advanced too quickly to detect differences between groups using sucrose as the reinforcer. Therefore, a more conservative, linear progression that is used to evaluate food motivation was adapted from (155) and utilized as the second schedule of reinforcement. We found no differences in motivation to work for the sucrose reinforcer in CP55940 vs. vehicle treated animals in either version of the PR task, indicating that this dose of oral CP55940 that elevates impulsive responding for sucrose does not affect motivation for sucrose in female rats. While the limited existing literature suggests increased motivation for palatable rewards in response to CB1 receptor agonism, we highlight that this low dose of CP55940 was used to investigate the behavioral underpinnings of elevated impulsivity observed with this dose in female rats.

Finally, given that cannabinoids can induce anxiolytic or anxiogenic effects depending on dose (156), we further investigated whether this model of edible cannabinoid administration affected anxiety-like behavior. Our findings suggest that this acutely hyperphagic dose of edible CP55940 has no effect on anxiety-like behavior in

female rats, demonstrating that cannabinoid-induced hyperphagia is not secondary to changes in anxiety-like behavior.

4. Limitations of the study

Voluntary consumption of a cannabinoid receptor agonist-containing edible by female rats produced acute hyperphagia of standard chow via an increase in meal size. While our study demonstrates that an acutely hyperphagic dose of a cannabinoid receptor agonist increases impulsive responding for food without impacting food motivated behavior or anxiety-like behavior, further study should investigate how varying the dosage of cannabinoid and how chronic dosing may influence eating behavior over the long term. Furthermore, the model cannabinoid CP55940 has been used extensively in the literature to investigate preclinical outcomes, but to further enhance the translatability of future findings, investigators may consider the use of whole-plant cannabis extract.

Impulsive eating may have several underlying causes. Increases in food motivation is one potential factor driving impulsive eating, but in our studies, we did not observe increases in motivated responding for sucrose. Emotional eating to alleviate anxiety may be another underlying cause of impulsive eating, but we did not observe changes in anxiety-like behavior. Future study may investigate an augmented sense of internal timing as a contributing factor; when an animal's internal sense of time is expanded, they may be more likely to prematurely respond on the lever.

One final limitation is that these studies were only conducted in female rodents. The exclusion of male conspecifics from our study may affect the generalizability of these results; however, there have been several studies investigating the effects of cannabinoids on motivation and impulsivity in male rodents, and there is considerably

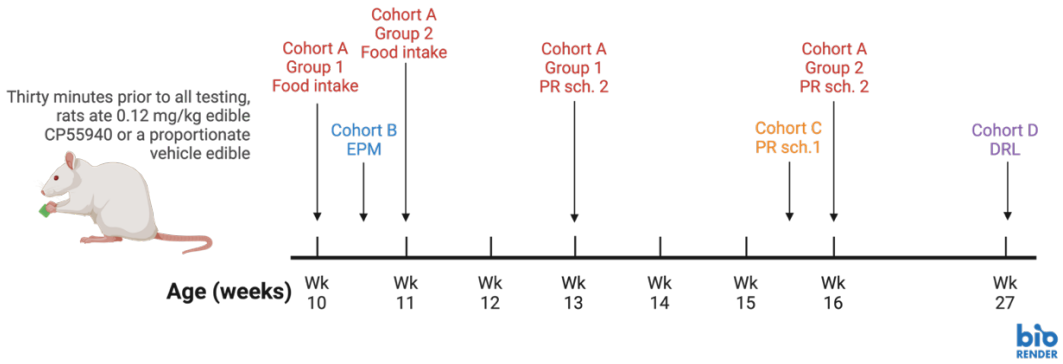
less work conducted in females. Our contribution to the field aims to alleviate this discrepancy.

Overall, adopting this preclinical gelatin-based edible model of cannabinoid consumption simulates the human experience of edible consumption with a high degree of integrity and enhances translational relevance in the mechanistic study of cannabinoids.

5. STAR Methods

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Fifty-six female Wistar rats (Envigo, Indianapolis, IN, USA) were singly housed in standard shoebox cages on a 12:12 reverse light/dark cycle in a temperature-controlled vivarium (22°C) with ad libitum access to standard chow (LabDiet 5053, LabDiet, St. Louis, MO, USA) and water except when noted below. All animals arrived between eight to ten weeks of age as determined by the supplier with the exception of the n=8 females utilized in the differential reinforcement of low rates of responding task, which arrived at three and a half weeks of age. A timeline of experiments is provided in Supplementary Figure 1 relative to the age of the rat at the time of testing. Rats were handled and weighed daily except during three U.S. national holidays. All procedures were approved by the Institute of Animal Care and Use Committee at the University of Georgia (Athens, GA, USA) (protocol number A2022 06-035-A12).



Supplementary Figure 2.1: A timeline of experiments relative to the age of the cohort at the time of testing. Cohort A (color coded in red) was utilized in the food intake and meal patterning (FI/MP) measurements, as well as in the progressive ratio (PR) schedule 2 testing. Cohort A was drug-naïve for the FI/MP measurements. Cohort A was then trained in PR, and on the day of PR schedule 2 testing, animals that received vehicle in the FI/MP experiment were kept drug-naïve for PR schedule 2 testing. Animals that were treated with CP55940 in the FI/MP experiment were receiving their second dosing upon PR schedule 2 testing. Cohort B (color coded in blue) was only utilized in elevated plus maze (EPM) testing of anxiety-like behavior, and they were drug-naïve at the time of testing. Cohort C (color coded in orange), prior to PR schedule 1 testing, was involved in a pilot test of the FI/MP experiment, meaning that half of the females in PR schedule 1 were previously exposed to CP55940 three weeks before testing, allowing ample time for washout. Cohort D (color coded in purple) was only utilized in differential reinforcement of low rates of responding (DRL) testing of impulsive behavior, and they were drug-naïve at the time of testing.

METHOD DETAILS

Cannabinoid administration via gelatin-based edibles:

Edibles were made in the laboratory by combining coconut oil and lecithin; Jello and potassium sorbate; and set gelatin in a 1:1:1 ratio. The lipophilic nature of cannabinoids requires the use of a lipophilic solvent, coconut oil. The nonselective dual cannabinoid receptor agonist CP55940 (Item No. 13608; Cayman Chemical, Ann Arbor, MI, USA) was first dissolved in 14 grams of coconut oil (Simple Truth Organic) and ¾ tsp lecithin (Earthfare, Athens, GA, USA) at the concentration of 0.18 mg/mL. In a separate container, 11.3 grams Jello and ¼ tsp potassium sorbate were added to 18 mL boiling deionized water after removal from the heat. The use of Jello allowed for multiple flavors to be employed and enhanced palatability, while potassium sorbate was used as a mold inhibitor. In a third vessel, 5 grams of gelatin was combined with 10 mL of water and allowed to set. Next, equal parts Jello and coconut oil mixtures were combined on a stir plate with constant stirring while a third equal part of set gelatin was added. The mixture was constantly stirred on a hot plate at 35-45°C. After five minutes of constant stirring, the homogenous mixture was portioned into two-milliliter molds using a syringe and allowed to set overnight at 4°C. Vehicle edibles were made in the exact same manner, absent of drug. This recipe makes a total of 12 2-mL edibles.

CP55940 was selected for these studies because, like THC, CP55940 is an agonist at both cannabinoid receptors, with stronger activation of downstream effectors at the CB1 receptor compared to the CB2 receptor (157), making it an ideal THC-like model cannabinoid. A pilot study was performed to determine a hyperphagic dose of orally administered CP55940. Edibles were delivered to subjects at 0.12 mg/kg by portioning

the edible according to the weight of the animal. The two-milliliter molded edible contains 0.06 mg/mL (0.12 mg/2mL). Vehicle edibles were portioned and administered based on 2 mL/kg to match the size of the drug edible. Once administered, edibles were voluntarily consumed by the rodents in two minutes or less. Bedding was meticulously scoured for any unconsumed edible, and rodents with leftover edible were excluded from analyses (n=1 rat utilized in the progressive ratio schedule 2 experiments was excluded).

Chow intake and meal patterns:

To analyze meal patterns with minimal disturbance to the animal, food intake measurements were automatically recorded with Sable Systems Food Intake Monitoring Cages (4826 Rat cage; Sable Systems International, North Las Vegas, NV, USA). Data were recorded over 24-hours, with a food bout being recorded each time the animal removed food from the food hopper. With eight cages and sixteen females, rats were habituated and tested in two groups. Each group was first acclimated to the food intake monitoring cages for seven days, with testing occurring on the eighth day in the specialty cages. On three of the seven acclimation days, rats were habituated to the vehicle edible by giving them one-fourth of a whole 2-mL edible (containing no drug) to consume to reduce neophobia on the day of testing. The eighth day in the specialty cages was test day. Subjects were aged ten to eleven weeks old on the day of testing. Each test day ran as follows: chow was removed 1.5 hours before the start of the dark cycle. Thirty minutes prior to the start of the dark cycle, rats received either CP55940-containg edible at 0.12 mg/kg or vehicle edible. At the immediate beginning of the dark cycle, standard chow was replaced in the food hopper, and data were collected from the monitoring software (Promethion Live Software Platform; Macro Interpreter) 24 hours after the animals were

given food back. Total food intake was calculated by the hour, and meals were defined as single food bouts in an amount greater than 0.2 grams spaced no more than 15 minutes apart. One rat was excluded from analysis due to not eating.

Differential reinforcement of low rates of responding (DRL):

DRL is an operant chamber task designed to test impulsive action in rodents (protocol modified from (44)). Rats were placed in an operant chamber with two retractable levers and one food receptacle and trained to associate one of the levers with earning a sucrose reinforcer (45 mg sucrose pellets, F0023, Bio-Serv, Flemington, NJ). During the first week of training, animals were given a fixed ratio 1 schedule (FR1), where a lever press results in a pellet delivery to the food receptable. A pellet was automatically dispensed if a rat did not press the lever for 600 seconds (autoshaping). The subsequent week a five second delay was enforced, wherein rats must wait for 5 seconds after each lever press before pressing again or the reinforced lever would not deliver a sucrose pellet. For the subsequent two weeks a ten second period elapsed between presses for a reinforcer to be earned. Finally, a delay of twenty seconds was enforced for the last two weeks of training. Training sessions lasted for 45 minutes (one training session per day). In each session, the light above the active lever flashed on for presses that resulted in a reward. For the final week of training, a tone accompanied active presses that resulted in a reward. Rats were considered trained in the task when they achieved a steady efficiency score over several days. The efficiency score was calculated by dividing the number of pellets earned by the number of lever presses on the reinforced (active) lever. All training and testing occurred during the dark cycle.

A group of drug naïve female rats (n=8) were trained in DRL for five days per week over seven weeks at the start of the dark cycle. Animals were initially trained in this task during early adulthood (postnatal day 73-119) and then retrained in the task periodically prior to testing (for 5 days at PND 138 and at PND 178 they were retrained a final time on the twenty second delay with the tone for six days). Rats were aged 27 weeks at the time of testing. Animals were tested using a within-subjects design with test days separated by a three-day washout period. On each test day, animals received a CP55940-containing edible or a vehicle edible thirty minutes in a counterbalanced manner prior to being placed in the operant chambers. The number of active and inactive presses, as well as the number of pellets earned were measured, and efficiency scores were calculated.

Progressive ratio (PR):

The PR task was conducted in a cohort of females (PR schedule 1, n=16), and subsequently repeated in a separate cohort of females utilizing a different progressive ratio schedule (PR schedule 2, n=16). Training and testing occurred in eight Med Associates operant conditioning chambers (Med Associates; Fairfax, VT, USA). With eight operant chambers and sixteen females in each cohort, each cohort was split into two groups. In every session regardless of schedule, there was an active lever which when pressed dispensed sucrose pellets (45 mg sucrose pellets, F0023, Bio-Serv, Flemington, NJ) to the food cup and an inactive lever that provided no reinforcement. All training sessions lasted one hour, and PR test sessions lasted for two hours maximum. All testing occurred early in the dark cycle.

For all training and testing sessions, home cage chow is pulled two hours before the start of the task and returned after the task. Animals were habituated to vehicle edibles (containing no drug) before the training sessions to reduce neophobia on the day of testing. During PR schedule 1, rats were trained in the operant chambers in fixed ratio-1 (FR1; one press delivers one pellet) sessions for four to six days until they reached a passing threshold of > 50 presses on the active lever. Rats were then switched to the fixed ratio-3 (FR3; three presses deliver one pellet) schedule for two days. After two days off from training, rats were tested using an exponential PR schedule where an exponential increase in the number of presses was required for the rat to receive a reinforcer, according to the equation: $F(i) = 5e0.2i–5$, where F(i) is the number of lever presses required for next pellet at i = pellet number. On test day, subjects received either edible CP55940 or vehicle edible thirty minutes prior to the start of PR schedule 1. Animals were aged fifteen and a half weeks at the time of testing with all subjects being tested the same day, one group of eight immediately following the other.

For PR schedule 2, female rats were the same cohort utilized in the meal patterning experiment. Rats were trained and tested in the PR task following the conclusion of the meal patterning experiment in order to keep animals that received the vehicle edible in that experiment drug-naïve for PR testing. During PR schedule 2 training, home cage chow was removed two hours before the start of the dark cycle. Rats were trained on a FR1 schedule for six days with autoshaping (pellet automatically dispensed if a rat did not press the lever for 600 seconds). For the first four days of FR1, the task began immediately after the start of the dark cycle. On the fifth day of FR1, the task began one hour after the start of the dark cycle. On the final day of FR1, the task

began four hours into the dark cycle. This was done to gradually increase fasting time prior to the start of the task. Rats were then trained on a FR3 schedule for six days starting four hours into the dark cycle. Rats were given one day of rest prior to testing in a linear PR schedule, which required an additional three presses for each additional pellet after the first initial press modeled by $F(i) = 3i-2$, where $F(i)$ is the number of lever presses required for next pellet at i = pellet number. Rats were tested four hours into the dark cycle (same time as training) and food was pulled two hours prior to the start of the task (two hours into the dark cycle, to alleviate the influence of hunger on motivation). Subjects received either edible CP55940 or vehicle edible thirty minutes prior to the start of PR schedule 2. As mentioned above, these cohorts of sixteen were split into two groups for training and testing in the eight operant chambers. For schedule 2 testing, the first group was tested at thirteen weeks of age, and the second group was tested at sixteen weeks of age. One female was excluded from analysis due to nonconsumption of the edible.

Elevated Plus Maze (EPM):

The EPM task is used to measure anxiety-like behavior in rodents by comparing the time they spend in the "closed" arms of the apparatus to the amount of time they spend in the "open" arms of the apparatus (158). The EPM is made of treated wood and consists of two opposing open arms (8.75 × 45 cm) and two opposing closed arms (10.3 × 45 cm) with walls that are 40 cm high. The arms of the EPM were elevated 56 cm off the floor. Lux was equal over the two open (60 lux) and the two closed (10 lux) arms. White noise was played while the animal was in the maze. The open arms were kept brighter than the closed arms and white noise was played to simulate an anxiogenic environment. A closed

arm entry was counted when the rat's center was registered as being in a closed arm. An open arm entry was recorded when the rat's center was registered as being in an open arm. The apparatus was cleaned with 10% ethanol between animals to eliminate olfactory cues.

Female rats (n=16) aged ten and a half weeks were tested using a between-subjects experimental design. One week prior to testing, rats were habituated to the vehicle edible by giving them one-fourth of a whole 2-mL edible (containing no drug) to consume to reduce neophobia on the day of testing. Animals were tested one at a time with timing balanced by alternating the order in which vehicle- and cannabinoid-treated animals were tested. Animals always had access to food and water except when in the apparatus. The task began two hours into the dark cycle to ensure animals were satiated upon the start of the task, with the last animal being tested approximately five hours into the dark cycle. Animals were placed in the maze 30 minutes post-edible administration facing away from the researcher and toward the open arm of the maze. A video camera overhead was used to record the behavior of the rat over a 5-minute period. Testing was recorded and analyzed using ANYmaze software (Stoelting, CO, USA). Comparisons were made between animals' time spent in the closed vs. the open arms of the maze, as well as the speed with which they explored the maze and total distance travelled.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed with GraphPad Prism (Version 10.3.0). DRL data were analyzed using a two-tailed paired t-test. Food intake and meal pattern data were analyzed with

two-tailed unpaired t-test with Welch's correction. PR and EPM data were analyzed with a two-tailed unpaired t-test.

CHAPTER 3

CANNABINOID-INDUCED HYPERPHAGIA IS MEDIATED BY INCREASED MEAL FREQUENCY AND THE OREXIN-1 RECEPTOR IN MALE RATS[1]

1. Lord, M.N., Madu G.C., Loera-Lopez A.L., Aaron A.P., Lin J., and Noble E.E. Cannabinoid-induced hyperphagia is mediated by increased meal frequency and the orexin-1 receptor in male rats. To be submitted to Appetite.

Abstract

Exogenous cannabinoids have long been known to promote eating. However, the underlying mechanisms have not been completely elucidated, which is critical to understanding their utility. The orexin/hypocretin (OH) system of the lateral hypothalamus (LHA) has known anatomical, biochemical, and physiological interactions with the endocannabinoid system, and has an established role in promoting appetitive behavior, yet it's still unknown if the OH system mediates food intake following cannabinoid administration. Herein we validated an oral method of cannabinoid receptor agonist administration via gelatin-based edibles, showing that voluntarily consumed cannabinoid-containing edibles produce acute hyperphagia via an increase in meal number in male rats. Following cannabinoid administration, rats displayed an upregulation in the immediate early gene c-Fos in OH neurons compared to vehicle-treated animals. We further employed a within-subjects design to investigate whether orexin-1 (OX1) receptor signaling was necessary for cannabinoid-induced hyperphagia by co-administering a subeffective dose of a OX1 receptor antagonist with the cannabinoid-containing edible. Data were collected from metabolic monitoring cages, simultaneously capturing chow intake, locomotor activity, and metabolic variables. Results showed that the OX1 receptor antagonist blocked cannabinoid-induced hyperphagia and the transient increase in locomotor activity following cannabinoid administration. Furthermore, both the edible cannabinoid receptor agonist and the OX1 receptor antagonist individually reduced energy expenditure for several hours following administration. Taken together, we conclude that the OX1 receptor is required for the hyperphagic response to exogenous cannabinoid administration.

1. Introduction

Cannabinoid-based therapeutics are an emerging market, but despite the well-known hyperphagic effects associated with cannabinoid use, we still do not fully understand how exogenous cannabinoids work in the brain to promote hyperphagia. In order to fully realize their medicinal value, a deeper understanding of how isolated cannabinoids have their intended effects and their unintended side effects is warranted. Cannabinoids act via a vast distribution of cannabinoid receptors throughout the body, with the majority of cannabinoid type-1 (CB1) receptors located in the central nervous system (CNS) (109). CB1 receptors are the most widely distributed G protein-coupled receptor in the CNS, and relevant to this work, in the reward-related limbic structures and the hypothalamus (12).

One of the established effects of CB1 receptor agonists is an increase in eating behavior (24, 25, 29, 30, 159), and the hypothalamus is chief among several brain regions that orchestrate eating behavior (62). CB1 receptors distributed amongst subpopulations in the hypothalamus are known to contribute to eating behavior regulation (160), but some populations have been better studied than others. In the lateral hypothalamus (LHA) CB1 receptors are expressed on orexin/hypocretin (OH) neurons and on the glutamatergic inputs to OH neurons (53). The orexin/hypocretin (OH) system consists of two peptides, orexin-A and orexin-B, and two receptors, orexin type-1 (OX1) receptor and orexin type-2 receptor (161, 162). Each receptor has been implicated in distinct behavioral processes, with the effects of orexin-A on the OX1 receptor largely responsible for the food-seeking properties of the OH peptides (163). The OH system promotes reward-seeking behavior, such as palatable food intake, with projections to

reward-related limbic structures as well as other regions of food intake control (164, 165). Additionally, these projections terminate where the OX1 receptor and the CB1 receptor are co-expressed (166). Prior research has shown that both a peripherally administered subeffective dose of rimonabant, a CB1 receptor antagonist that crosses the blood-brain barrier, and CB1 receptor antagonist AM251 administered to the arcuate hypothalamus block the hyperphagic effect of centrally administered orexin-A (55). These findings suggest that CB1 receptor signaling is necessary for orexin-A induced feeding. However, whether cannabinoid-induced feeding requires orexin A signaling has not yet been determined. Therefore, this study investigated the necessity of OX1 receptor signaling in cannabinoid-induced hyperphagia. The OH system plays a well-known role in food anticipatory activity, spontaneous physical activity, and increased energy expenditure (165, 167). Therefore, we further analyzed whether the OH system mediates the effects of cannabinoids on activity and energy metabolism. These data are critical to understanding the involvement of the OH system in upregulating food intake following oral cannabinoid ingestion.

2. Methods

Animals

Twenty-four male Wistar rats (Envigo, Indianapolis, IN, USA) were singly housed on a 12:12 reverse light/dark cycle in a temperature-controlled vivarium (22°C) with ad libitum access to standard chow (LabDiet 5053, LabDiet, St. Louis, MO, USA) and water except when noted below. Rats were eight to ten weeks of age upon arrival as determined by the supplier. Rats were handled and weighed daily. All procedures were approved by

the Institute of Animal Care and Use Committee at the University of Georgia (Athens, GA, USA; protocol number A2022 06-035-A12).

Drugs

The cannabinoid receptor agonist CP55940 (Item No. 13608) and the OX1 receptor antagonist SB334867 (Item No. 19145) were obtained from Cayman Chemical (Cayman Chemical, Ann Arbor, MI, USA). CP55940 was administered as a gelatin-based edible as previously described (Chapter 2). Briefly, edibles are composed of coconut oil, lecithin, Jello, potassium sorbate, gelatin, and distilled water. The cannabinoid was dissolved in coconut oil and lecithin at 0.18 mg/mL and diluted with Jello, potassium sorbate, and gelatin to 0.06 mg/mL. Vehicle edibles are of identical composition without the addition of the cannabinoid drug. Edible molds are 2 mL, and therefore, all edibles were delivered at 2 mL/kg to deliver 0.12 mg/kg according to their weight. This dose was selected based on pilot studies to determine a hyperphagic dose of CP55940.

SB334867 was first dissolved in 100% DMSO, then diluted in cyclodextrin in distilled water that was slightly heated (37C) to 1 mg/mL. The final concentrations were 10% cyclodextrin and 4% DMSO in distilled water, and this was the composition of the vehicle. Three mg/kg SB334867 and its vehicle were delivered at 3 mL/kg intraperitoneally (i.p.).

Experiment 1: Standard chow intake and meal patterns following edible CP55940 or vehicle

Sable Systems Promethion Core metabolic and phenotyping system were used (4826 Rat cage; Sable Systems International, North Las Vegas, NV, USA) to measure male rodents' standard chow intake and meal patterns following edible CP55940 or vehicle, validating

this method of cannabinoid adimistration as a model of cannabinoid-induced hyperphagia in male rats. Sable Systems automatic recording of behavioral events allows for minimal disturbance to the animal enabling more accurate data with high temporal resolution. Data were recorded over twenty-four hours, with a measurement being recorded each time the animal displaced food from the food hopper.

Male rats (n=8) were first habituated to the food intake monitoring cages for seven days. On three of the seven days, rats were habituated to the vehicle edible (containing no cannabinoid) to alleviate the influence of food neophobia during testing. On the eighth day, chow was removed 1.5 hours before the start of the dark cycle. Thirty minutes prior to the start of the dark cycle, rats received either CP55940-containg edible at 0.12 mg/kg or vehicle edible. At the immediate beginning of the dark cycle, food access was returned to the rats and food intake data were collected from the monitoring software (Promethion Live Software Platform; Macro Interpreter). After a seventy-two-hour washout period, treatment groups were switched, and the experiment was repeated for a within-subjects, counterbalanced design.

Animals with more than three grams of unconsumed chow remaining in the bedding were excluded from analysis (n=1 male). Total food intake was calculated by the hour, and meals were defined as food removal from the hopper greater than 0.2 grams no more than 15 minutes apart. Data analyzed from this first experiment assessed whether an edible formulation of CP55940 affects food intake and if and how meal patterning changes in response to edible CP55940.

Experiment 2: c-Fos activity in orexin/hypocretin neurons of the LHA

We assessed c-Fos expression in orexin neurons following administration of the edible cannabinoid via transcardial perfusion and immunofluorescence staining. Rats (two subsets from cohorts utilized in prior food intake experiments; n=10) received 0.12 mg/kg of edible CP55940 ninety minutes prior to sacrifice to capture the maximum amount of c-Fos protein expression (30). Prior to perfusion for immunofluorescence, food was removed two hours before the start of the dark cycle. Rats were sacrificed during the first hour of the dark cycle and remained fasted prior to sacrifice. Rats were deeply anesthetized with isofluorane and perfused first with ice-cold 0.9% saline then 4% paraformaldehyde (PFA). Brains were rapidly extracted and allowed to post-fix in 12% sucrose-PFA for twenty-four hours after which they were frozen with isopentane cooled in dry ice and stored at -80°C until sectioning. Brains were sectioned at 30 μm and stored in cryoprotectant at -20°C until immunofluorescence analyses. LHA sections of the perifornical region were chosen according to the Paxinos and Watson rat brain atlas (levels 56-62). Sections were incubated in rabbit anti-orexin-A (Cat No: H-003-30, Phoenix Pharmaceuticals, Burlingame, CA, USA) and mouse anti-c-Fos (Cat No: AB208942, Abcam, USA) primary antibodies and donkey anti-rabbit 647 (Cat No: 711-605-152, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and donkey anti-mouse 488 (Cat No: 705-545-147, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) secondary antibodies tissues were stained as in (46). Sections were mounted using Prolong Glass anti-fade mountant (Cat No: P36984, Invitrogen, Eugene, OR, USA) and stored at 4°C to reduce signal loss prior to imaging. Sections were imaged with the LSM 900 confocal microscope at 10x in Zeiss ZEN software and individual

images were stitched to visualize the entire LHA of each section. OH+ cells and double labeled c-Fos+ OH+ cells were counted using ImageJ by undergraduate researchers blinded to experimental conditions. A representative matched sample from levels 56-62 of seven total sections from the LHA of each male were counted. We calculated the percentage of c-FOS+ OH cells by dividing the number of c-FOS+ OH+ cells by the individual rodent's total OH cell count to account for individual differences in total OH cells counted.

Experiment 3: Standard chow intake and metabolism in response to edible CP55940 and IP SB334867 or vehicles

Sable Systems Promethion Core metabolic and phenotyping system was used to simultaneously collect standard chow intake, activity levels, energy expenditure (EE), and respiratory exchange ratios (RER) following exposure to edible CP55940 and the OX1 receptor antagonist SB334867 or their vehicles (n=16). We selected a subeffective dose of SB334867 that was previously shown to have no effect on food intake alone (168). We employed a within-subjects, counterbalanced design. Each of the four treatment days were as follows: food was removed one hour prior to the start of the dark cycle, and an i.p.injection of SB334867 (3 mg/kg) or vehicle were delivered at 3 mL/kg fifteen minutes after the start of the dark cycle. Following i.p. injection of the OX1 receptor antagonist, animals were given edible CP55940 (0.12 mg/kg) or a vehicle edible. Thirty minutes after edible delivery and one hour after the start of the dark cycle, food was replaced in each cage and data were collected from the monitoring software (Promethion Live Software Platform; Macro Interpreter) for twenty-three hours. This experiment was designed to test if the OX1 receptor is necessary for cannabinoid-induced

increases in food intake, and to understand the impact of these drugs on locomotor activity, energy expenditure, and RER.

Statistical analyses

Data were analyzed with Graphpad Prism (Version 10.3.0). Food intake and meal pattern data from experiment 1 were analyzed using a paired two-tailed Student's t-test. Immunohistochemical data were analyzed with unpaired two-tailed Student's t-test with Welch's correction applied only to the number of OH+ neuron counts due to unequal standard error of the mean. Food intake, activity, EE, and RER from experiment 3 were analyzed with two-way ANOVA. Outliers were identified with Grubb's test and excluded.

3. Results

Experiment 1: Standard chow intake and meal patterns following edible CP55940 or vehicle

Over the first two hours of the dark cycle, male rats given the CP55940-containing edible increased their intake of chow compared to those given a vehicle edible (Figure 3.1A; $t = 3.765$, $df = 6$, $p = 0.009$). Meal number and meal size were analyzed within this two-hour window, and we detected that in response to edible CP55940, male rats' meal size remains unaffected (Figure 3.1B; $t = 1.276$, $df = 6$, $p = 0.25$), while meal number increased (Figure 3.1C; $t = 2.5$, $df = 6$, $p = 0.047$) compared to rats given vehicle. However, over the twenty-four-hour period, chow intake (Figure 3.1D), meal size (Figure 3.1E), and meal number (Figure 3.1F) were not significantly different. One rat was excluded due to excess food spillage in the cage bedding, disallowing accurate meal patterning. These

data indicate that edible CP55940 acutely increases chow intake via an increase in meal number, specifically influencing appetitive behavior in male rats.

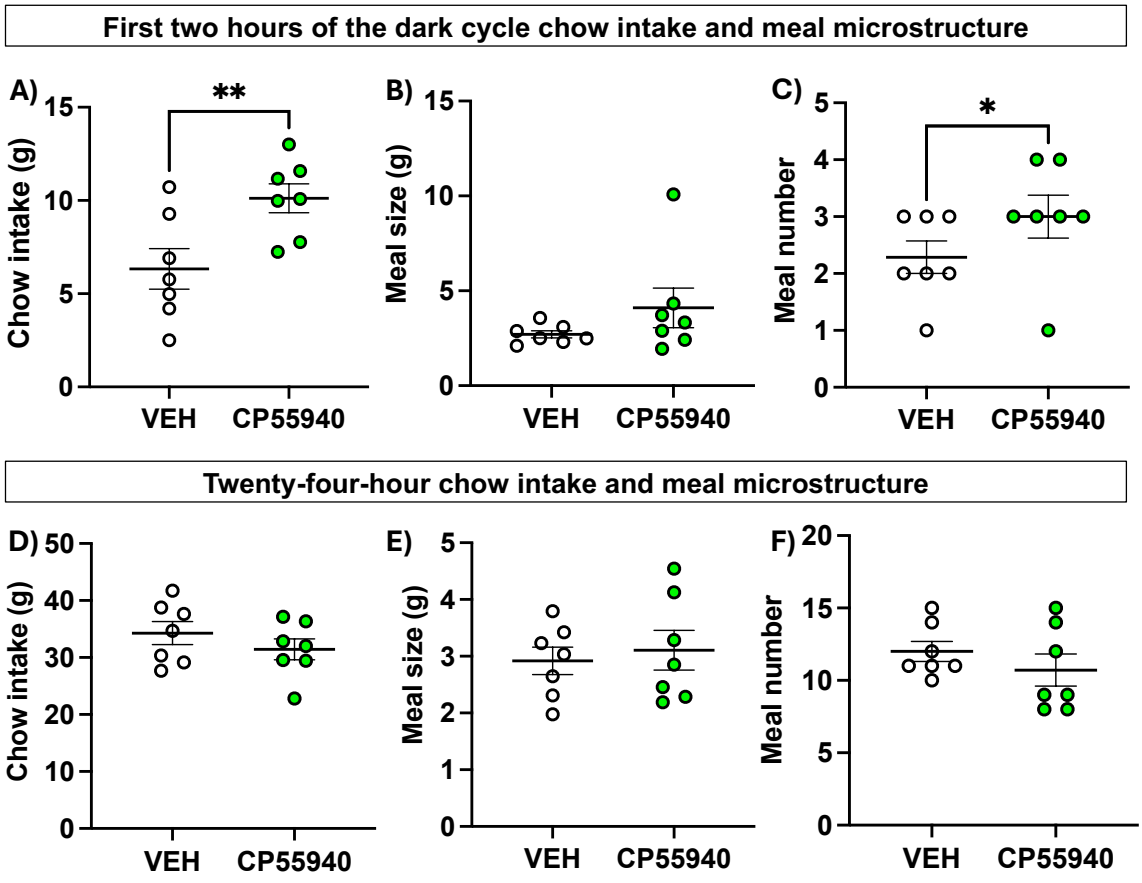


Figure 3.1: Edible cannabinoid receptor agonist CP55940 acutely promotes eating behavior via an increase in meal number. Male rats increased standard chow intake over the first two hours of the dark cycle (A). Meal pattern analyses revealed hyperphagia was mediated not by an increase in meal size (B) but by an increase in meal number (C). However, over the course of twenty-four hours, there were no differences in overall chow intake (D), meal size (E), or meal number (F). Data are means +/- SEM; *$p < 0.05$, **$p < 0.01$.

Experiment 2: c-FOS activity in OH neurons of the LHA

Tissue sections from the perifornical region of the LHA of animals that received edible CP55940 or vehicle ninety minutes prior to sacrifice were stained for OH and the c-Fos protein, which is commonly utilized to measure the neuronal response to external stimuli (169). Representative images are shown in Figure 3.2A. Semi-automatic quantification of OH+ and c-FOS+ orexin cells via ImageJ reveal no differences in the total number of OH+ cells (Figure 3.2B; $t = 1.111$, $df = 7.015$, $p = 0.3$), but a greater number of doubly labeled c-Fos+ OH+ cells in the cannabinoid-treated group, compared to the vehicle-treated group (Figure 3.2C; $t = 3.306$, $df = 8$, $p = 0.011$). While there were no statistical differences in OH+ cell number, we can account for individual differences in total OH neurons by calculating the percentage of total OH+ cells that are doubly labeled. Accounting for individual differences in OH neuron expression, the percentage of orexin cells expressing the c-Fos protein remained higher in the cannabinoid-treated group compared to the vehicle group (Figure 3.2D; $t = 2.332$, $df = 8$, $p = 0.048$).

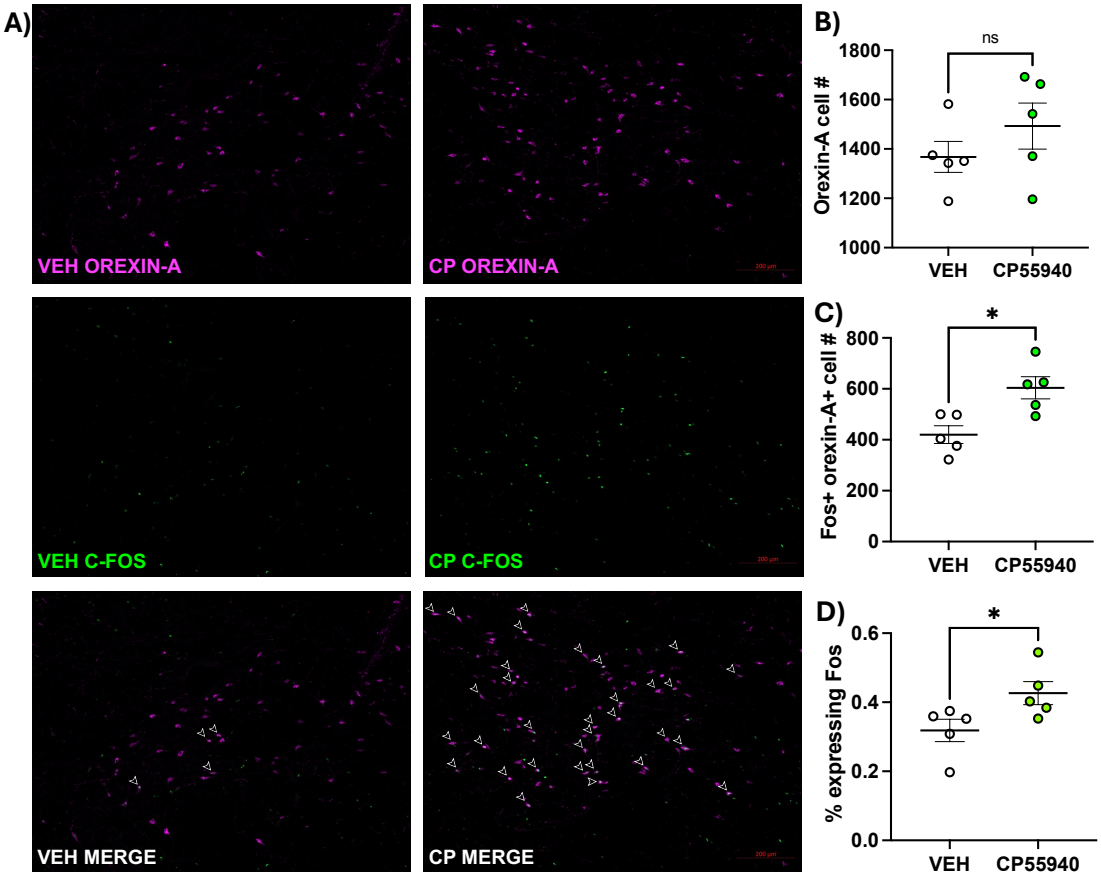


Figure 3.2: Edible cannabinoid receptor agonist CP55940 increases c-Fos expression in orexin neurons. Representative images, cropped for ease of viewing, from each treatment are shown in (A) with vehicle-treated in the left column and CP55940-treated in the right column. There were no differences in total orexin-A+ cell number (B), but there was an increase in doubly labeled c-Fos+ orexin-A+ cells in the cannabinoid-treated group (C). The percentage of c-Fos+ orexin-A neurons was calculated for each animal showing that the cannabinoid-treated group had a higher percentage of orexin-A neurons expressing c-Fos compared to vehicle-treated animals (D). Data are means +/- SEM; *$p < 0.05$

Experiment 3: Standard chow intake and metabolism in response to edible CP55940 and IP SB334867 or vehicles

Following i.p. injection of the OX1 receptor antagonist SB334867 or i.p. vehicle and administration of edible CP55940 or the edible vehicle, food intake, energy expenditure, RER, and activity levels were simultaneously monitored for twenty-three hours. Six rats were excluded due to nonconsumption of the full dose of the edible and two rats were excluded as outliers. The first six hours of chow intake is shown in Figure 3.3A. Two hours after food replacement (wherein changes in meal patterning were observed in experiment 1 above), two-way ANOVA revealed a main effect of CP55940 on food intake (Figure 3.3B; $F(1, 14) = 7.261$, $p = 0.017$). Post-hoc analyses with uncorrected Fisher's LSD showed that given i.p. vehicle, the cannabinoid-containing edible increased chow intake compared to vehicle edible-treated animals ($p = 0.01$). Additionally, SB334867 had no effect on food intake when co-administered with the vehicle edible ($p = 0.37$), while SB334867 co-administered with CP55940 blunted food intake compared to CP55940 co-administration with i.p. vehicle ($p = 0.029$). At the two-hour mark, meal size remained unaffected by both drug treatments (Figure 3.3C), but two-way ANOVA revealed a main effect of CP55940 on meal number (Figure 3.3D; $F(1, 14) = 21$; $p = 0.0004$). Post-hoc analyses with uncorrected Fisher's LSD showed that edible CP55940 when co-administered with the i.p. vehicle increased meal number ($p = 0.047$), as expected. However, meal number was also increased when CP55940 was co-administered with SB334867 ($p = 0.001$). There was a trend toward SB334867 co-administered with the vehicle edible in decreasing meal number ($p=0.08$), but this did not reach significance.

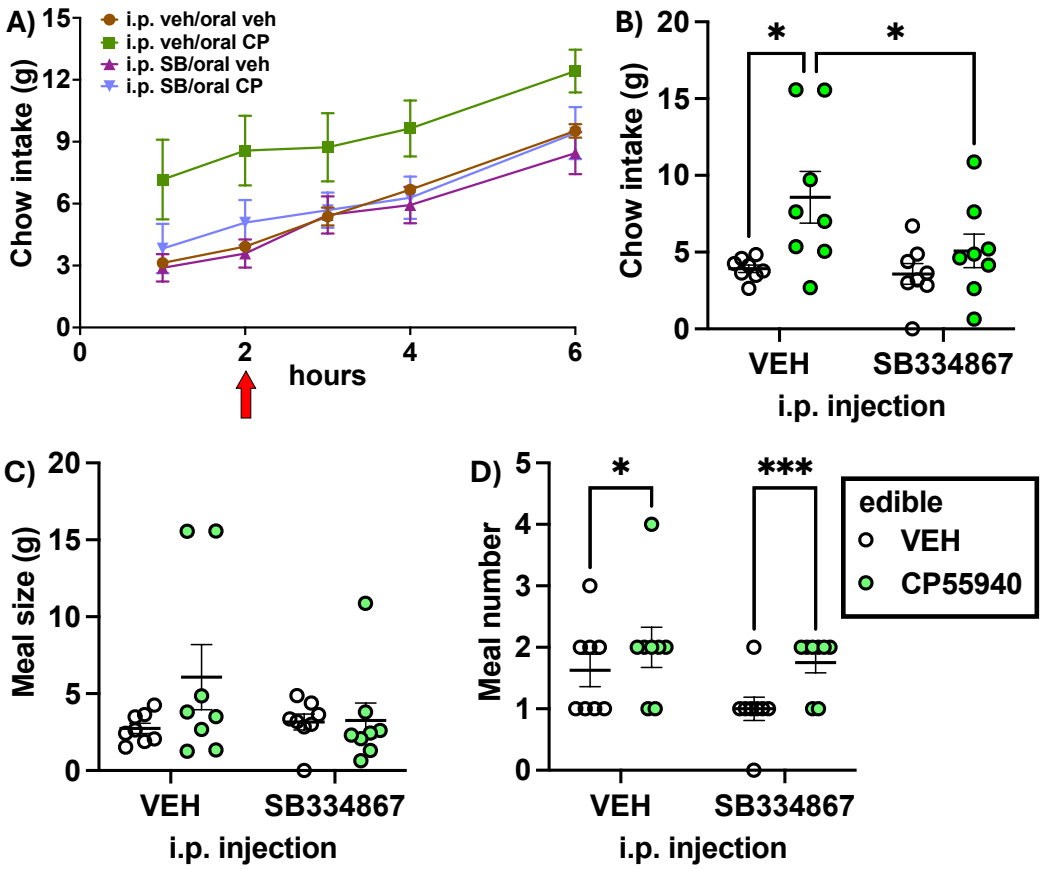
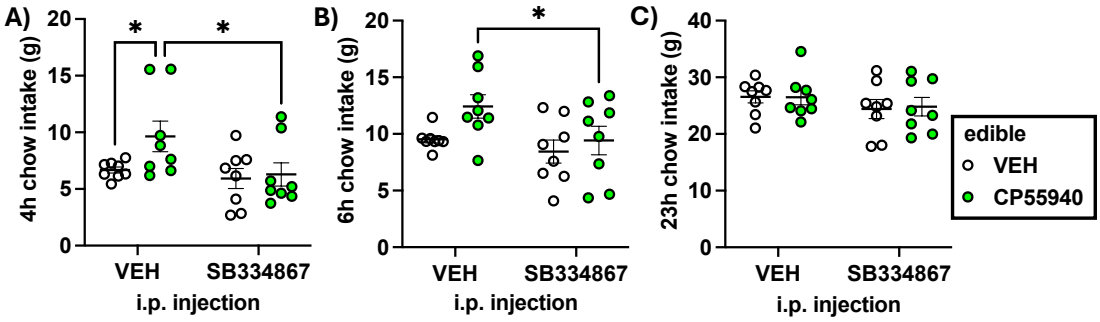


Figure 3.3: OX1 receptor antagonist SB334867 blocks cannabinoid-induced hyperphagia. Chow intake by the hour is shown in (A) up to six hours following the return of food access. Two-way ANOVA and post-hoc analyses of food intake two hours following food access revealed that CP55940 increased chow intake that was blocked by SB334867 (B). Microstructural meal analysis at the two hours following food access showed no differences in meal size (C), but increased meal number in both cannabinoid-treated conditions (D). There was a trend toward SB334867 (co-administered with the vehicle edible) decreasing meal number, but this did not reach significance. Data are means +/- SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Food intake following edible CP55940-i.p. vehicle remained elevated for up to four hours (Supplemental Figure 3.1A; p =0.047), while edible CP55940-i.p. SB334867 group food intake remained below that of edible CP55940-i.p. vehicle for up to six hours (Supplemental Figure 3.1B; p =0.038). These differences in food intake and meal microstructure were transient, as over the course of twenty-three hours there were no differences in chow intake between groups (Supplementary Figure 3.1C).



Supplementary Figure 3.1: Differences in chow intake following cannabinoid receptor agonist CP55940 and OX1 receptor antagonist SB334867. Two-way ANOVA and post-hoc analyses of food intake revealed that chow intake was increased following CP55940 alone and CP55940-induced hyperphagia was blocked by SB334867 up to four hours after food access was returned (A). Cannabinoid-induced hyperphagia was diminished by six hours after food access was returned, but food intake in the SB334867—CP55940 group was still below that of CP55940 alone (B). These changes were transient, as there were no differences in cumulative food intake over the twenty-three-hour period.

Locomotor activity for the first three hours is shown in Figure 3.4A. Coinciding with changes in food intake and meal microstructure, there was a main effect of CP55940

on locomotor activity two hours after food access was returned (Figure 3.4B; F (1, 14) =8.157, p =0.013). Post-hoc analyses with uncorrected Fisher's LSD revealed that only CP55940 co-administered with i.p. vehicle increased locomotor activity (p =0.021). SB334867 co-administered with CP55940 attenuated the increases in activity at both time points, while having no effect when given with vehicle edible. This effect was acute, as there were no differences between groups by the third hour, and there were no differences in activity over the twenty-three-hour period (Figure 3.4C).

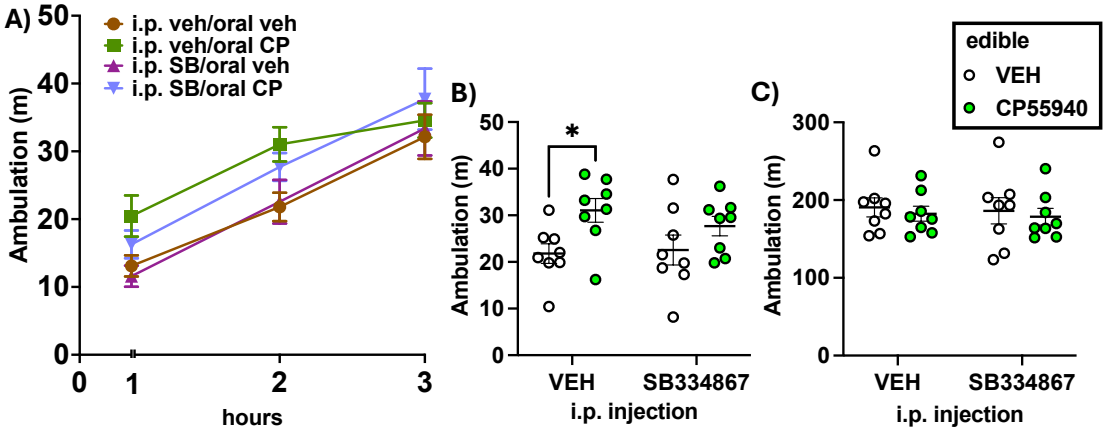


Figure 3.4: OX1 receptor antagonist SB334867 attenuates acutely elevated locomotor activity following cannabinoid receptor agonist CP55940. Locomotor activity in meters is shown over the first three hours following the return of food access in (A). Two-way ANOVA and post-hoc analyses of activity two hours following food access revealed that CP55940 increased ambulation that was attenuated, but not completely blocked, by SB334867 (B). These changes were transient, as there were no differences in activity over the twenty-three-hour measurement period (C). Data are means +/- SEM; *p < 0.05

Coinciding with the increase in chow intake and the increase in locomotor activity, two-way ANOVA showed an interaction between the cannabinoid receptor agonist and the OX1 receptor antagonist on energy expenditure during the three hours (180 minutes) following food replacement (Figure 3.5A; $F(1, 14) = 5.72$, $p = 0.032$). Post-hoc analyses with uncorrected Fisher's LSD revealed that animals given CP55940 co-administered with i.p. vehicle have an overall decrease in EE (Figure 3.5B; $p=0.018$), and animals given i.p. SB334867 co-administered with the vehicle edible also have decreased EE (Figure 3.5B; $p=0.014$) compared to the vehicle-vehicle condition. These data show that each intervention alone, CP55940 and SB334867, decreased EE to a similar degree in the three hours following food replacement. A main effect of the cannabinoid persisted for six hours (Figure 3.5C; $F(1, 14) = 9.22$, $p = 0.009$), with post-hoc analyses revealing that EE remained decreased in the edible CP55940-i.p. vehicle condition and in the i.p. SB334867-vehicle edible condition.

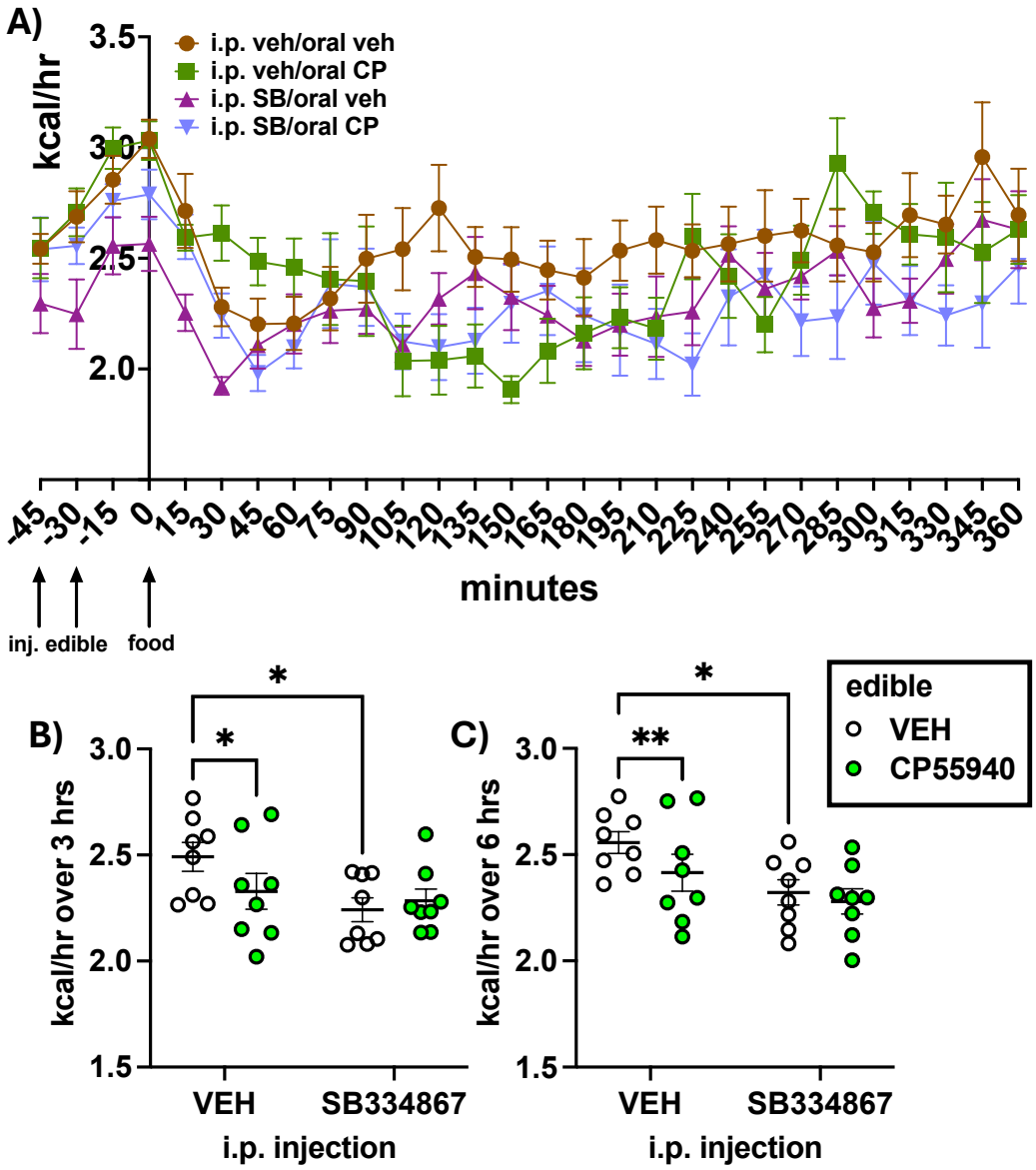
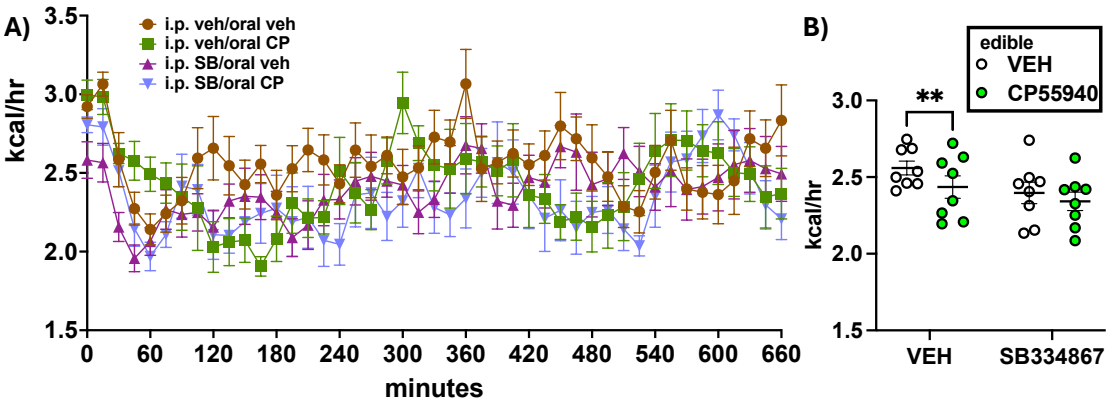


Figure 3.5: Reduced energy expenditure following cannabinoid receptor agonist CP55940 and OX1 receptor antagonist SB334867. Energy expenditure (EE) in kcal/hour is shown in (A) for up to six hours with arrows indicating the time of i.p. injection, edible administration, and the return of food access. Two-way ANOVA and post-hoc analyses of EE three hours following food access revealed that CP55940 and SB334867 both individually reduced EE, while the combination of the two interventions did not have any

additive effect (B), and these differences persisted for up to six hours (C). Data are means +/- SEM; *p < 0.05, **p < 0.01.

The main effect of CP55940 persisted over the dark period (Supplementary Figure 3.2A; F (1, 14) =11.68, p =0.004) with post-hoc analyses showing EE was suppressed over the entire dark period in the edible CP55940-i.p. vehicle condition only (p =0.005). While the i.p. vehicle-edible CP55940 group shows a collective decrease in EE over the dark period compared to the vehicle-vehicle condition, there is a transient elevation in EE in the i.p. vehicle-edible CP55940 group that only drops below that of the vehicle-vehicle groups' after the first ninety minutes of food access.



Supplementary Figure 3.2: CP55940 suppressed energy expenditure over the dark cycle. Energy expenditure over the dark period is shown in (A). Two-way ANOVA and post-hoc analyses revealed that the edible cannabinoid receptor agonist suppressed average energy expenditure over the dark period (B).

Finally, there was a main effect of cannabinoid treatment on RER during the six hours following food replacement (Figure 3.6A; F (1, 14) =15.63; p=0.001). Post-hoc analyses revealed CP55940 did not affect RER when co-administered with i.p. vehicle; however, edible CP when co-administered with i.p. SB334867 suppressed RER when compared to the SB334867 antagonist alone (Figure 3.6B; p=0.001). However, on average the range of RER between groups was 0.91 to 0.94, indicating that all groups are oxidizing a mixture of carbohydrates and fat substrates for fuel.

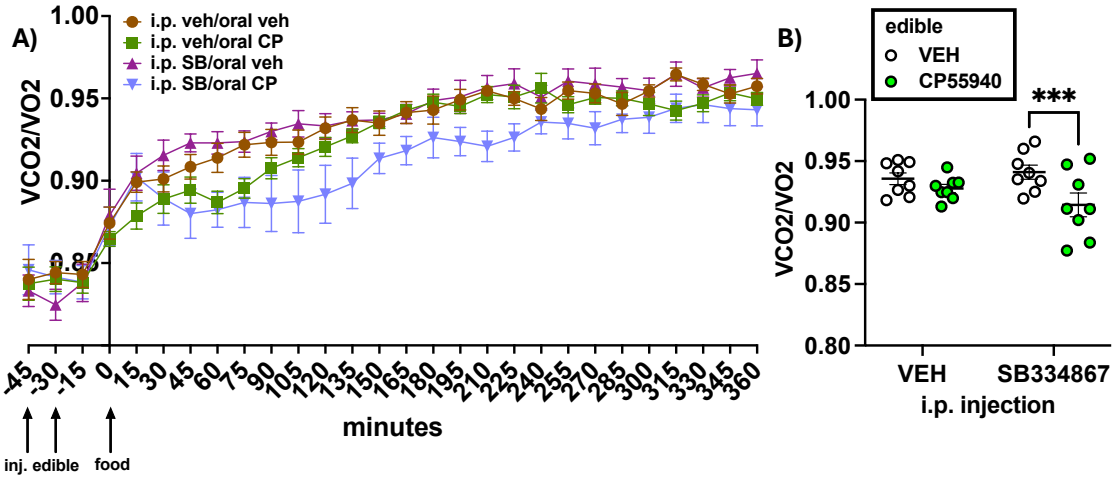


Figure 3.6: Respiratory exchange ratios following cannabinoid receptor agonist CP55940 and OX1 receptor antagonist SB334867. Respiratory exchange ratios (RER) are shown in (A) for up to six hours with arrows indicating the time of i.p. injection, edible administration, and the return of food access. Two-way ANOVA and post-hoc analyses of RER revealed that only the dual intervention of edible CP55940 and i.p. SB334867 decreased RER over the six hours following the return of food access (B). Data are means +/- SEM; ***p < 0.001

4. Discussion

Here we show that a dual cannabinoid receptor agonist CP55940 orally administered acutely increases chow intake in male rats via an increase in the number of meals consumed. Elevated cannabinoid-induced food intake coincides with increased locomotor activity and increased energy expenditure (EE), which is not long-lasting. EE of cannabinoid-treated animals is reduced below that of vehicle-treated animals from the second hour after food access was returned and up to six hours. We further show that OH neurons express more c-Fos following cannabinoid administration, suggesting a higher level of activity in OH neurons with exogenous cannabinoid receptor agonism. Moreover, our data show that OX1 receptor signaling is required for elevated food intake and the increased spontaneous activity observed following edible cannabinoid administration, as the OX1 receptor antagonist SB334867 attenuated these cannabinoid-induced changes.

The increase we observed in eating behavior following oral CP55940 administration is aligned with numerous reports showing CB1 receptor agonists increase food intake in male rats, including those orally administered (25, 29, 30, 170). Elevated food intake following a pharmacological intervention is achieved via manipulation of one or more phases of the feeding cycle: appetition, consumption, and/or satiation, (129, 130). Our microstructural analyses of the eating pattern following edible CP55940 showed that male rats increase their meal frequency rather than meal size, suggesting that the appetitive phase is mainly affected in male rats. Our findings are congruent with data from previous reports showing oral THC administration and whole plant Cannabis sativa vapor inhalation increases meal frequency (153, 170). However, Wheeler and colleagues note a compensatory decrease in meal size following whole plant Cannabis sativa vapor inhalation, which we did not observe with the edible CP55940. These differences may be

attributed to differences in route of administration, dose of THC, and/or the presence of other cannabinoids in the whole plant vs. isolated THC (153). The intake behavior observed in our study and by others mimics some of the earliest human data available showing that after marijuana cigarettes, appetitive "snacking" behavior is elevated in males. Thus, here we show that voluntarily consumed edible CP55940 is a translationally relevant way to investigate the underlying behavioral and neural mechanisms contributing to cannabinoid-induced hyperphagia. However, after receiving the edible on more than four to five occasions, animals ceased to consume the entire dose and must be excluded from analysis. This is likely due to the association of the effects of CP55940 with the taste of the edible, as others have shown that rodents will consume less of a cannabinoid containing mixture (116, 118). Therefore, future study with voluntary oral cannabinoid administration may seek to build upon this model and look to other successful models of voluntary oral cannabinoid consumption (118, 119).

These food intake data in males further reveal an interesting sex difference in cannabinoid-induced hyperphagia. Our lab previously demonstrated that, like males, female rats increase their intake of standard chow following edible CP55940 compared to vehicle-treated females (Chapter 2). However, the increase in chow intake in females was due to an increase in meal size rather than meal number, opposite the meal pattern changes in males. As shown above with the increase in meal number in males, we noted that the increase in meal size following cannabinoid administration in females was aligned with the limited literature available in females (131). Collectively, these data extend the findings of other showing sex differences in the effects of cannabinoids (171,

172) and highlight the importance of sex differences in the design of clinical investigations of how cannabinoids affect eating and other behaviors.

Since its discovery, it's been known that the OH system increases food intake behavior (161), and it later was revealed that these increases were specific to appetitive, food-seeking behavior (41, 173, 174). Based on these characteristics, we investigated whether OH neurons were impacted by the hyperphagic dose of CP55940 using the c-Fos protein as a measure of neuronal activity. Our data reflect that a hyperphagic dose of CP55940 increases c-Fos expression in the early dark cycle in OH (specifically orexin-A+) neurons. The literature suggests that differential c-Fos expression is observed in OH neurons when animals are anticipating a reward, such as a food reward (175-177). For example, Cason and colleagues demonstrated increased c-Fos expression in OH neurons during the extinction phase of a cue-induced reinstatement protocol (177). Similarly, Choi and others trained rodents to associate contextual cues with receiving a piece of chocolate, and subsequently sacrificed rats after presentation of the context in the absence of chocolate, showing increased c-Fos in rodents expecting the chocolate, while differences were absent in rodents not expecting the chocolate reward (176). In our study we capitalized on a time period when rodents are already expecting to have their largest meal of the day, the very early dark cycle, when OH neuron activity is elevated based on circadian rhythm (178, 179). We show that exogenous cannabinoid administration elevates c-Fos expression in OH neurons above that of an already elevated baseline. These data follow suit with the existing literature showing that c-Fos expression in OH neurons is selectively elevated with food reward anticipation.

However, c-Fos data alone is not enough to conclude that the OH system is necessary for cannabinoid-induced hyperphagia. The food anticipatory properties of OH signaling are largely attributed to orexin-A ligand binding at the OX1 receptor. The literature on these two closely intertwined systems shows that CB1 receptor antagonists block orexin-A-induced eating behavior (55, 58). Flores and colleagues have investigated the effects of CB1 receptor agonism on orexin type-2 receptor mediated variables such as hypothermia, antinociception, and anxiety, but not food intake (180). Furthermore, mice lacking the OX1 receptor have attenuated dopamine release in the nucleus accumbens following THC exposure and pharmacological blockade of OX1 receptors with SB334867 reduces IV self-administration of the synthetic cannabinoid WIN55,212-2, suggesting that the physiological reward associated with cannabinoids are partially mediated by the OX1 receptor (181). We are not currently aware of any studies investigating the involvement of the OX1 receptor in cannabinoid-induced food intake, preclinical data that may be of interest in the development of cannabinoid-based therapeutics for appetite regulation. Our data show that a dose of the OX1 receptor antagonist that had no effect on food intake alone blocked the acute CP55940-induced increases in standard chow intake. However, removal of OX1 receptor signaling does not completely obliterate appetitive behavior produced by cannabinoids as we found that co-administration of SB334867 with CP55940 did not block increases in meal number. Gonzáles et al. provide convincing evidence that the activity of orexin neurons is immediately suppressed upon contact with food (48), highly consistent with the hypothesis that the neuropeptide specifically promotes appetitive behavior, not within-meal consumption (41). However, with the addition of an exogenous cannabinoid that is

also promoting appetitive behavior, it stands to reason that blocking the OX1 receptor alone would not be enough to fully extinguish the appetitive drive to initiate another meal in males. Taken together, these data suggest that OX1 receptor signaling is required for the elevated food intake but only partially mediates the appetitive behavior induced by exogenous cannabinoids.

Coinciding with the increases in food intake following cannabinoid consumption, locomotor activity was also increased by CP55940, which is in alignment with an increase in appetitive drive. It is worth noting that at higher doses CB1 receptor agonists have a dampening effect on activity levels (171, 182). Similar to its impact on food intake, SB334867 also attenuated the cannabinoid-induced increases in activity, suggesting that these transient increases in activity may be related to the food anticipatory effects mediated by OH. Regarding EE, there was a transient increase in the CP55940 group coinciding with the increase in activity and chow intake. The acute increase in activity and EE is in line with a previous report showing these same effects following whole plant cannabis vapor exposure (153). The transient increase in EE in the CP55940-treated group was followed by a drop off that lasted up to six hours. Existing evidence suggests that cannabinoids modulate energy metabolism largely in the periphery of rodents by enhanced lipogenesis (15) and impaired lipolysis (183), and therefore the long-lasting effect of suppressed EE is consistent with the notion that exogenous cannabinoid administration modulates EE in favor of energy storage (184). Administration of the OX1 receptor antagonist suppressed average energy expenditure to a similar degree as CP55940 and the co-administration of both drugs. Thus, the combination of the two drugs together did not have an additive effect on EE reduction.

The reduction in EE following i.p. OX1 receptor antagonist injection is consistent with the well-established effect of the OH system in stimulating EE through increased arousal (185, 186). While this dose of SB334867 has been shown to have no effect on locomotor activity (168) as we observed, the dose delivered here effectively reduced EE. Therefore, the suppression of EE observed in cannabinoid-treated animals is likely not mediated by the central OX1 receptor. This notion is interesting when juxtaposed against our data showing that RER is reduced in animals that received both SB334867 and CP55940, as lower RER indicates increased utilization of fat substrates for energy production. However, the magnitude of this difference was small, with each group on average displaying an RER between 0.91 and 0.94, suggesting that all groups are normally oxidizing a mixture of carbohydrate and fat substrates for fuel.

5. Conclusions

Exogenous cannabinoids can enhance the rewarding properties of food, augmenting even bland chow consumption via increased meal number, and this behavior is partially mediated by OH neurons and the OX1 receptor. Concomitantly with elevated food intake, locomotor activity was increased by cannabinoid receptor agonism and attenuated by OX1 receptor blockade, which when taken together with the increases in meal number observed in the cannabinoid-treated animals suggests that the cannabinoids act via the OH system to increase meal anticipatory locomotor activity. Our data agree with others suggesting the reduced acceptability of edible cannabinoids in rats following chronic administration. In conclusion, edible cannabinoids act via the OX1 receptor to increase food intake and appetitive behavior.

CONCLUSIONS

The literature consistently suggests that cannabinoid type-1 (CB1) receptor agonists increase eating behavior in a dose-dependent manner. There have been incredible strides in the study of cannabinoids over the last few decades, but several gaps in the literature persist. Therefore, the aim of this dissertation was to fill several of those gaps by investigating sex differences in cannabinoid-induced eating behavior, alleviate the dearth of behavioral data in females, and further our understanding of how cannabinoids interact with hypothalamic neuropeptides to confer hyperphagia. The better our preclinical understanding of how cannabinoids work in the brain to modulate behavior, the better studies will be designed to investigate relevant outcomes of interest in clinical populations.

Historically, the predominant methods of cannabinoid administration in preclinical studies have been either intraperitoneal or subcutaneous injection. However, the predominant routes of administration in humans are inhalation and oral consumption. While inhalation is common, the detrimental effects of smoking and the purported detrimental effects of vaping highlight that oral administration is the preferred administration method. Therefore, we set out to develop a translationally relevant model of oral cannabinoid (CP55940) consumption in rodents. We subsequently validated that this novel route of administration in rodents upregulated food intake as the literature would suggest, and indeed, we found that edibles increased standard chow intake in male

and female rats over the first two hours of the dark cycle. In line with the literature, we found that CP55940 acutely increased chow intake in a sexually dimorphic manner through distinct meal structure. Females preferentially increased their meal size following edible CP55940, while males show an increase in meal frequency, underscoring the importance of designing clinical studies investigating how cannabinoids affect eating and other behaviors with sex differences in mind.

While the preponderance of evidence suggests that cannabinoids augment behavioral impulsivity and endogenous cannabinoid signaling increases motivation for sucrose in males, there was a dearth of female data regarding these behavioral phenomena. Therefore, we tested behavioral impulsivity using the differential reinforcement of low rates of responding task and motivation using the progressive ratio task in female rats following CP55940 consumption. Employing a within-subjects, counterbalanced design, rats given the hyperphagic dose of CP55940 were more impulsive for the sucrose reinforcer than when treated with vehicle. However, no differences in motivation to obtain the same sucrose reinforcer were observed under two schedules of reinforcement in the progressive ratio task. Given the mixed data on cannabinoids and how they may affect anxiety-like behavior depending on dose, it was also important to investigate whether the hyperphagic dose of CP55940 affected anxiety-like behavior in the elevated plus maze. However, no differences in any measure were observed, indicating no effect of this dose of cannabinoid on anxiety-like responses. Taken together, we conclude that edible cannabinoid consumption at a hyperphagic dose increases impulsive behavior toward sucrose in female rats but has no influence on motivation or anxiety-like behavior. Future research building on this work may

investigate disturbances in perception of time as an underlying influence of impulsive behavior in female rats. Indeed, the CB1 receptor has been detected in nuclei that regulate rhythmicity including the intergeniculate leaflet of the thalamus, the dorsal and median raphe nuclei, and, importantly, the suprachiasmatic nucleus (SCN). While one study demonstrated that lesioning the SCN had no effect on interval timing, others show that accurate interval timing depends on an intact circadian clock. Furthermore, electrophysiological data suggest that exogenous cannabinoid signaling may suppress GABAergic transmission in the SCN and increase the firing rate of postsynaptic SCN neurons. However, more work is necessary to definitively determine whether cannabinoid signaling in the SCN materially perturbs behavior dependent upon accurate internal timing.

In chapter 3, we demonstrated that CP55940 specifically increased appetitive behavior in males by acutely upregulating meal frequency, and preliminary staining for c-Fos in orexin/hypocretin (OH) neurons suggested that OH neurons may be engaged by the hyperphagic dose of CP55940. OH is a neuropeptide produced in the lateral hypothalamus, a region that has been proposed as a hub for crosstalk between homeostatic and hedonic eating behavior, that is hypothesized to be a driver of appetitive behavior. Thus, we then conducted a formal analysis of animals given CP55940 or vehicle by doubly labeling the c-Fos protein and orexin-A-producing neurons via fluorescent immunohistochemistry and found that the hyperphagic dose of CP55940 upregulated c-Fos expression in OH neurons compared to vehicle. While increased c-Fos expression demonstrates that OH neurons are engaged by exogenous cannabinoid administration, these data aren't sufficient to suggest that OH is necessary for

cannabinoid-induced hyperphagia. Therefore, we next designed a within-subjects experiment co-administering both the edible CP55940 and a subeffective dose of the orexin-1 (OX1) receptor antagonist, SB334867, and automatically recorded food intake over twenty-three hours in metabolic monitoring cages. Analysis of food intake and meal microstructure reaffirmed that edible CP55940 acutely increases meal number in males, and revealed that SB334867 blocked cannabinoid-induced hyperphagia, but not the increase in meal number. We simultaneously measured energy expenditure and locomotor activity in the metabolic monitoring cages, variables that both the endocannabinoid system and the OH system are involved in. We found that locomotor activity and energy expenditure were acutely elevated over the same time frame that food intake was increased, suggesting that these increases were specific to appetitive behavior. While locomotor activity normalized after two hours, energy expenditure in the CP55940 group dropped below that of vehicle-treated animals and remained suppressed for up to six hours. The OX1 receptor antagonist attenuated both transient increases in activity and energy expenditure, and in the group that received SB334867 alone, energy expenditure was similarly suppressed for up to six hours. Taken together, we conclude that the OX1 receptor is required for cannabinoid-induced hyperphagia, but cannabinoids are modulating energy expenditure through a mechanism likely independent of the OH system. Future study in this area may explore potential neural sites of action mediating the interaction of these systems to promote eating behavior, which likely involves dopaminergic signaling. Dopamine (DA) is a powerful neuromodulator of behavior with established anatomical and physiological connections to the orexinergic and cannabinergic systems. OH neurons project directly to the ventral tegmental area (VTA),

which send its dopaminergic projections to the nucleus accumbens (ACB). Specifically, orexin-A application onto dopaminergic neurons of the VTA increases the firing rate of VTA-DA cells projecting to the shell region of the ACB. Furthermore, others have shown that the CB1 receptor in the VTA is required for phasic activation of mesolimbic DA signaling, and DA release in the ACB following THC administration is diminished in OX1 receptor knockout animals. Moreover, the OX1 receptor antagonist SB334867 given peripherally, as we did here in our studies, or the CB1 receptor antagonist AM251 blocked orexin-A-induced elevated DA. Thus, it is reasonable to postulate that the hyperphagic, appetitive behavior, whether initiated by endogenous OH signaling or exogenous cannabinoid administration, recruits dopaminergic signaling to modulate behavior.

This work has limitations. The edible model of cannabinoid administration built on the work of others to create a dosing strategy that was both accurate to the weight of the animal and voluntarily consumed. The data bear out that this is a viable model of administration to study the acute effects of oral cannabinoid consumption. However, it is also critically important to measure the chronic effects of cannabinoid administration, which may not be possible with this model without modification. As mentioned in chapter 3, we found that after four to five administrations of the edible, a subset of rats developed an aversion that precluded consumption of the entire dose contained in the edible. Therefore, modification of this protocol would be necessary prior to any testing with chronic administration. Furthermore, these experiments utilized a THC-like cannabinoid, CP55940, to induce hyperphagia and measure co-occurring responses. While we've appropriately justified its use as a THC-like cannabinoid, future research

should consider using whole-plant cannabis extract or isolated phyto-cannabinoids that would further increase the translatability of future findings.

In conclusion, this work has validated a precisely dosed and voluntarily consumed edible model of cannabinoid consumption ideal for studying the acute effects of cannabinoids. We describe sex differences in the hyperphagic response to cannabinoids, partially alleviate the discrepancy of behavioral data in females describing elevated impulsive responding for sucrose without changes in motivation or anxiety-like behavior, and report that the OX1 receptor is required for cannabinoid-induced hyperphagia in males. Overall, the findings in this dissertation make a notable contribution to the field of preclinical cannabinoid research and provide a basis for future work in the area of oral cannabinoid consumption.

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