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The Influence of Sustained CB1 Blockade During Adolescence on Breakpoints in a Progressive-ratio Paradigm

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Table of Contents

List of Tables

List of Figures

Abbreviations

2-AG – 2-arachidonoylglycerol
α-MSH – α-melanocyte stimulating hormone
AEA – anandamide
AGRP – agouti-related peptide
ANOVA – analysis of variance
CART – cocaine and amphetamine related transcript
CB_1 – type-1 cannabinoid receptor
CB_2 – type-2 cannabinoid receptor
CCK - cholecystokinin
Δ^8-THC – Δ^8-tetrahydrocannabinol
Δ^9-THC – Δ^9-tetrahydrocannabinol
FAAH – fatty acid amide hydrolase
FR – fixed-ratio
FST – forced-swim test
GABA – γ aminobutyric acid
GPCR – G-protein coupled receptor
MAGL – monoglyceride hydrolase
NPY – neuropeptide Y
PD – postnatal day
PLD – phospholipase D
PPI – prepulse inhibition
PR – progressive-ratio
TST – tail-suspension test

Abstract

THE INFLUENCE OF SUSTAINED CB1 BLOCKADE DURING ADOLESCENCE ON BREAKPOINTS IN A PROGRESSIVE-RATIO PARADIGM

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The developmental psychopharmacology of cannabinoids is poorly understood and little is known about the developmental consequences of repeated exposure to cannabinoid antagonists. In these experiments, male Long-Evans rats were treated with SR141716A, a cannabinoid antagonist, throughout adolescence and allowed unrestricted access to food. Control groups were treated with vehicle during the same developmental period and allowed either unrestricted access to food or were pair-fed with a member of the SR-treated group. Motivation to work for food was measured in progressive-ratio sessions at varying levels of food deprivation. For rats that consumed fewer calories throughout adolescence, whether because of pharmacological intervention or food-restriction, motivation was not significantly related to the level of food deprivation. Additionally, the SR-treated group ate more of a novel, palatable food than the vehicle-treated group.

Finally, the SR-treated group was generally more motivated to work for food than the pair-fed group, irrespective of the level of deprivation.

Introduction

Experimental Rationale

The purpose of these experiments was to determine how chronic adolescent exposure to the CB_1 antagonist SR141716A affects the motivation to consume food in adulthood. The major hypothesis of these studies was that sustained CB_1 blockade during adolescence will result in higher breakpoints in a progressive-ratio session when compared to animals treated with the vehicle (1 ethanol:1 emulphor:18 saline). If noted, these differences in breakpoints would suggest a greater motivation for a food reward in rats chronically treated with SR141716A. The rationale for this hypothesis is derived from the recent finding that chronic pubertal exposure (PD 40-65) to a CB_1 agonist (WIN 55,212-2) was associated with a long-lasting reductions in the motivation for a food reward as indexed by lower breakpoints in a progressive-ratio schedule (Schneider & Koch, 2003).

The Endogenous Cannabinoid System

The endogenous cannabinoid, or endocannabinoid, system is a widely-distributed lipid signaling system that modulates the intensity of adjacent neural impulses (DiMarzo et al, 1998). When endocannabinoids are released, they suppress inhibitory signals from GABAergic neurons and excitatory signals from glutamatergic neurons (Iverson, 2003; Rodriguez de Fonseca, et al, 2005). Endocannabinoids also modulate signal transmission by interacting with other neurotransmitter systems (e.g., dopamine; Giuffrida et al, 1999).

In the brain, endocannabinoids modulate movement, basal metabolic functions, learning and memory and the brain's reward system (Herkenham, et al, 1990). Acute administration of high doses of anandamide can produce catalepsy and ataxia (Rodriguez

de Fonseca et al, 1998). The high density of CB_1 receptors in the cerebellum and parts of the substantia nigra and globus pallidus also supports the existence of an endocannabinoid system regulating motor activity. In the hippocampus, endocannabinoids influence memory consolidation and extinction (Castellano et al, 2003; Varvel & Lichtman, 2002). Endocannabinoids stimulate two distinct types of feeding behavior; hedonic and homeostatic. Hedonic feeding seems to be driven by the reward value of food and is may be mediated by the brain reward system (NAcc, MFB, etc.). Though CB_1 receptor densities are relatively low in the hypothalamus, endocannabinoids seem to play an important role in basal metabolic functions like energy balance, including homeostatic feeding, and thermoregulation (Wenger & Moldrich, 2002; Harrold and Williams, 2003). In the dorsal horn of the spinal cord, endocannabinoids modulate the transmission of painful stimuli from sensory neurons. Cannabinoid receptors are also found in pain pathways in brain and on the peripheral terminals of primary afferent neurons. Together, these receptors and their endogenous ligands are thought mediate cannabinoid-induced analgesia (Pertwee, 2001; Pertwee & Ross, 2002).

The discovery of the endogenous cannabinoid system can be traced to the isolation of the primary psychoactive constituent of marijuana (Cannabis sativa), (-)Δ^9-tetrahydrocannabinol (Δ^9-THC; Gaoni & Mechoulam, 1964). Yechiel Gaoni and Raphael Mechoulam isolated dozens of structurally similar compounds from Cannabis plants and systematically tested each of them in behavioral assays with monkeys. In the end, (-)Δ^9-THC was shown to act as a sedative in monkeys and, in dogs, produced a form of locomotor inhibition known as static ataxia (Gaoni & Mechoulam, 1964).

For nearly the next quarter century, researchers labored to understand the structure-activity relationship of cannabinoids and to explore the burgeoning field of cannabinoid pharmacology. While the biological activity of Δ^9-THC was clear, no distinct physiological mechanism had yet been discovered. Some thought that since cannabinoids were so lipophilic, the in vivo effects of Δ^9-THC could be explained without postulating the existence of a receptor (Leuschner et al., 1986). Others believed that the complete pharmacologic profile of cannabinoids could only be explained by action at some receptor or on some enzyme (Harris et al., 1978; Mechoulam, 2006).

By the mid-1980's, there was evidence that at least some cannabinoids (i.e., Δ^8-THC) displayed high-affinity for a binding site in the brain and other organs (Nye et al., 1985). In 1988, Bill Devane and Allyn Howlett published the first pharmacological evidence of a cannabinoid receptor in the brain. Using cannabidiol and cannabigerol, two naturally occurring plant cannabinoids, and a radiolabeled form of CP-55,940, a synthetic cannabinoid, Devane and Howlett were able to identify a stereoselective, pharmacologically distinct receptor in the brain that had high-affinity for cannabinoids (DeVane et al., 1988). Once the existence of a cannabinoid receptor, later called CB_1, was established, it seemed logical to infer the presence an endogenous cannabinoid receptor ligand.

By the early 1990's, the first known endocannabinoid had been identified and the associated structure elucidated (Devane et al., 1992). This lipid-based compound, technically known as arachdonylethanolamine, was named "anandamide" by Devane and his team. Devane coined the term by combining the ancient Sanskrit word for "bliss" (ananda) with the central chemical moiety of the structure (an amide). Anandamide was

quickly found to act as an agonist at CB_1 receptors (Mechoulam and Fride, 1993) and to have a pharmacological profile very similar to that of Δ^9-THC (Crawley et al., 1993; Weidenfeld et al., 1994; Williams & Kirkham, 1999).

Shortly after anandamide was identified, a second endocannabinoid was isolated in the canine small intestine (Mechoulam et al., 1995). Once isolated, the structure was elucidated and the molecule was named 2-arachidonoylglycerol, or 2-AG. Mechoulam's group found that the newly discovered endocannabinoid was an agonist at CB_1 receptors, but with a potency that was about half that of anandamide. Though 2-AG is a relatively low-potency agonist, the concentrations of 2-AG in the brain are nearly 200 times higher than those of anandamide (Sugiura et al., 1995; Stella et al., 1997). While there is a fair amount of structural homology between 2-AG and anandamide, they are synthesized by distinct pathways. The endocannabinoid 2-AG is a metabolic intermediate produce in lipid metabolism, while anandamide is the cleaved from phospholipid precursors found in cellular membranes (Rodriguez de Fonseca et al., 2005). To date, three other endocannabinoids have been identified: noladin-ether, virodhamine and N-arachidonoyldopamine (NADA). While each of these compounds appears to be active at cannabinoid receptors, their physiological significance is still under investigation.

Within the brain, CB_1 receptors are very common in the cerebral cortex and hippocampus, where they are thought to be involved with cognition and short-term memory (Herkenham et al., 1990; Bliss & Collingridge, 1993; Collins et al., 1994), in the basal ganglia (especially in the striatum, globus pallidus and substantia nigra; Miller & Walker, 1995; Herkenham et al., 1991) and cerebellum, where they influence motor function and movement (Herkenham et al., 1990; Rodriguez de Fonseca; 1998). Though

CB_1 receptor densities are relatively low in the hypothalamus, endocannabinoids seem to play an important role in basal metabolic functions like thermoregulation and food intake (Fitton & Pertwee, 1982; Mattes et al., 1994). Outside of the brain, CB_1 receptors are commonly found in peripheral neurons (Ishac et al., 1996), the enteric neurons of the gastrointestinal tract (Izzo et al., 2001) and even in the liver where they are thought to regulate lipogenesis (Osei-Hyiaman et al., 2005).

Within a few years of the discovery of the CB_1 receptor, a second cannabinoid receptor was found on macrophages in the spleen (Munro et al, 1993). The new receptor, CB_2, is now thought to modulate immune function (Cabral, 2001) and inflammation that produce chronic and acute pain (Rice et al, 2002). Recent evidence suggests that CB_2 receptors may also be present in the brain, but it is unclear whether they are expressed in neurons or in microglia (Van Sickle et al., 2005; Aston et al., 2006).

In 1994, a team of scientists working at Sanofi Research Labs in Montpelier, France discovered the first selective and orally active CB_1 antagonist (Rinaldi-Carmona et al., 1994). The compound, originally called SR141716A and now known as rimonabant, has very a high affinity for CB_1 receptor, but is not active at the CB_2 receptor. In early in vitro experiments, SR141716A blocked the inhibitory effects of CB_1 agonists on both mouse vas deferens contractions and adenylyl cyclase activity in rat brain membranes. In later tests, SR141716A proved to be an effective antagonist of the physiological and behavioral effects of Δ^9-THC and other cannabinoid agonists. Rimonabant is now being marketed as Accomplia® in several European countries, where Sanofi is promoting it as a treatment for obesity and obesity-related metabolic

syndromes. Sanofi has also petitioned the Food and Drug Administration for a license to sell rimonabant United States, perhaps under the trade name Zimulti® .

Within a few years, the same group at Sanofi announced the discovery of SR144528, the first selective and orally active CB_2 antagonist (Rinaldi-Carmona et al., 1998). Recent evidence suggests that the endocannabinoid 2-AG plays an important role in stimulating some inflammatory processes and that SR144528 may be able to block the resulting inflammation (Suguira et al, 2004).

From the earliest experiments with anandamide, there was evidence that the duration of action was limited because the molecule was rapidly degraded by some membrane-bound enzyme (Deutsch & Chin, 1993). In the mid-1990's, a membrane-bound enzyme that hydrolyzed a whole class of fatty-acid amides, including anandamide, was discovered in the rat liver (Cravatt et al, 1996). The name of this enzyme evolved as the understanding of the function expanded; today the enzyme is known as fatty-acid amidohydrolase or FAAH. The proposed role of FAAH in the degradation of anandamide was bolstered when FAAH and CB_1 receptors were found to be co-localized in the brain (Egerton et al., 1998) and the pharmacological inhibition of FAAH resulted in enhanced anandamide levels in the brain (de Lago et al., 2005).

Eventually, a second route of enzymatic inactivation was discovered for some endocannabinoids. A serine hydrolase enzyme that was found in high concentrations in the hippocampus, cortex, anterior thalamus and cerebellum proved to be the primary mechanism for hydrolyzing 2-AG (Dinh et al., 2002). Unlike FAAH, the newly discovered enzyme, monoacylglycerol lipase or MAGL, turned out to display a high degree of substrate specificity.

While FAAH is the enzyme responsible for degrading anandamide, it also degrades 2-AG and whole classes of both long-chain acylethanolamines and primary amides. There is also some evidence that FAAH can inactivate noladin-ether and virodhamine (Steffens et al, 2005). In contrast, MAGL can only hydrolyze a subset of monoacylglycerols (Matias et al., 2006). Finally, FAAH seems to function as a postsynaptic enzyme, while MAGL seems to function presynaptically (Gulyas et al., 2004). While these enzymes both serve to inactivate endocannabinoids, the compartmentalization of the respective biosynthetic pathways and segregation of the resultant enzymes has led some to suggest that anandamide and 2-AG have distinct physiological functions (Chevaleyre et al., 2003).

While the mechanisms by which anandamide and 2-AG are degraded seem to have been discovered, there is some controversy about how anandamide and FAAH come in contact with each other. Evidence suggests that FAAH is primarily found on the membranes of cytoplasmic organelles (i.e., mitochondria, smooth endoplasmic reticulum) and the intracellular face of the plasma membrane in postsynaptic neurons (Gulyas et al., 2004). In contrast, anandamide is released into the extracellular space after being cleaved from phospholipid-precursors by an enzyme (or enzymes) related to phospholipase D (Di Marzo et al., 1994; Leung et al., 2006). Because no known mechanism will permit an intracellular enzyme to degrade an extracellular substrate, logic dictates the existence of a reuptake mechanism.

Though the enzymes that degrade endocannabinoids are well-characterized, the mechanisms of endocannabinoid reuptake are still controversial (Beltramo et al, 1997; Glaser et al, 2005). Some suggest that a membrane-bound transport protein facilitates

diffusion of anandamide across the postsynaptic membrane. However, this elusive transporter has yet to be cloned. Supporters of the "anandamide transporter" theory point out that the hydrolysis of anandamide is a saturable, heat-dependent process. Those factors, combined with data that suggest that transport can be inhibited, would tend support the existence of a transport protein. However, many of the drugs that are thought to act as transport inhibitors also serve to inhibit FAAH (Jarrahian et al., 2000; Fowler et al., 2004) or compete for intracellular binding sites (Deutsch et al., 2001; Glaser et al., 2003).

Opponents of the "anandamide transporter" theory point out that that the hydrolysis by FAAH is concentration-dependent in at least some cell lines (Hillard et al., 1997; Fasia et al., 2003). Indeed, some evidence can be viewed to support the notion that anandamide uptake is dependent upon simple diffusion (Piomelli et al., 1999; Deutsch et al., 2001). Such concentration-dependent, or "first-order", kinetics would not support the existence of a transport protein (Glaser et al., 2005). If a transport protein were responsible for the uptake of anandamide, then the rate at which anandamide could be degraded would limited by the number of transporters on the surface of the neuron. The saturable nature of the transporter would provide an upper limit on the rate of hydrolysis and produce zero-order kinetics.

Other evidence suggests that the hydrolysis of anandamide may appear to be saturable only after enough time has elapsed for other mechanisms (i.e., intracellular sequestration) to begin (Glaser et al., 2003; Fowler et al., 2004). Finally, some have questioned why a transporter protein would have evolved to transport an uncharged, hydrophobic molecule across a phospholipid membrane.

The Behavioral Impact of Cannabinoid Antagonism

Therapeutic uses for marijuana, including appetite stimulation, are described in writings that date back nearly two-thousand years (Mechoulam, 1986). Not surprisingly, when the CB_1 agonist Δ^9-THC was isolated in the 1960's, researchers quickly found that it influenced appetite (Paton & Pertwee, 1973). Since then, a variety of cannabinoid receptor antagonists have been shown to suppress feeding and preferentially reduce the consumption of highly-palatable food. While there is substantial evidence of a reduction in motivation to eat, some studies suggest that nausea or malaise may also be involved. On the whole, these experiments support the idea that feeding is mediated by the endocannabinoid system and the current consensus among most researchers is that cannabinoid antagonists are viable targets for drug development (McLaughlin et al., 2003; Kirkham & Williams, 2004).

In 1998, the effect of the cannabinoid CB1 receptor antagonist, SR 141716A, on feeding and body weight was assessed in adult, non-obese Wistar rats (Colombo et al., 1998). The daily administration of SR 141716A dose-dependently reduced both food intake and body weight. While tolerance to the anorectic effect developed within 5 days, body weight in SR 141716-treated rats remained markedly below that of vehicle-treated rats throughout the entire treatment period. The results of this early study suggested that brain cannabinoid receptors were involved in the regulation of appetite and body weight.

Further studies investigated the possible behavioral mechanisms underlying the anorectic effect of SR141716A. In one such study, male and female rats were food-restricted and trained to respond for food-reinforcers on an FR10 schedule (DeVry et al., 2004). Under those conditions, as well as under free-feeding conditions, SR141716A

dose-dependently attenuated responding. Interestingly, SR141716A did not affect activity which suggested that the observed inhibition of operant behavior was not a consequence locomotor inhibition. In the same type of operant procedure, SR141716A suppressed intracranial self-stimulation, though at a diminished potency (Arnold, et al., 2001).

Some studies have shown that SR141716A preferentially reduces the intake of sweet foods (Simiand et al, 1998) as well as the motivation to work for a sweet reinforcer (Ward & Dykstra, 2005). A similar study found that SR 141716A dose-dependently reduced bland food-reinforced responding in adult, male Sprague-Dawley rats. Pretreament with the exogenous cannabinoid agonist WIN 55,212-2 significantly attenuated the rate-suppressing effects of SR141716A, suggesting a principal role of CB_1 receptors in mediating these behavioral effects. These data suggest that high palatability is always not necessary to observe an anorectic effect of SR141716A (Freedland, Poston & Porrino, 2000). Importantly, SR141716A also attenuated saccharin-preference in a conditioned taste aversion paradigm. Those results introduced the possibility of drug-induced aversion or malaise.

Very recently, a comprehensive study examined the impact of variety of compounds on feeding behavior in wild-type and CB_1 knockout mice. All mice were food-restricted for 24 hours, administered a randomized drug/dose combination, and then allowed to feed on pre-weighed quantities of their regular rodent chow for 1 hour. Low doses of Δ^9-THC stimulated feeding and did not inhibit locomotion. Two structural analogs of SR141716A, O-3259 and O-3257, dose-dependently decreased food consumption at doses that did not inhibit locomotion. Diazepam, a CNS depressant, also

decreased food consumption, but only at doses that decreased locomotor activity. The selective CB_2 antagonist SR144528 and cannabidiol, a cannabinoid compound that is not psychoactive, affected neither food intake nor locomotor activity.

In contrast, the CB_1 antagonist SR141716A dose-dependently decreased food consumption at doses that did not inhibit locomotion in wild-type mice, but lacked pharmacological activity in CB_1 knockout mice. Amphetamine decreased feeding in both mouse genotypes. These results suggest that CB_1 receptors may play a role in regulation of feeding behavior, though the exact pharmacological mechanism remains unknown (Wiley et al., 2005).

The effects of AM 251, anandamide, the putative anandamide membrane transporter inhibitor VDM 11, and a stable derivative of anandamide, methanandamide on food intake were examined. A single dose of AM 251 significantly reduced food intake and reduced weight gain for up to 6 days. Contrary to expectations, neither anandamide nor VDM 11 served to increase food. However methanandamide, a more stable agonist derivative of anandamide, significantly increased food intake for up to 3 hours. Though endocannabinoids are notoriously short lived, these results suggest that AM 251 may remain biologically active long fairly long periods of time (Chambers, Sharkey & Koopmans, 2004).

Considering the known antiemetic and motor-suppressive effects of CB_1 agonists, and largely in response to an ongoing controversy about nausea and malaise, a series of experiments were conducted to determine if the reductions in food intake induced by the CB_1 antagonist AM 251 could result from nausea or impairments in intake-related motor control, rather than solely from appetite suppression.

In the first experiment, acute administration of AM 251 dose-dependently decreased food intake. Despite this change, the mass of food consumed per time spent eating (the feeding rate) and the amount of time spent food handling were unaffected. These data suggest that food intake was not reduced because of substantial motor impairments. In the second experiment, AM 251 dose-dependently reduced intake of a vanilla-flavored solution with which it had previously been associated. Those results are consistent with the development of conditioned taste avoidance. Again, it is impossible to rule out nausea or malaise as the cause of the observed reduction in feeding (McLaughlin et al., 2005).

In a study designed to evaluate cannabinergic action in animal models of depression and on feeding behavior. Acute doses of AM251 were administered before execution of the tail-suspension test (TST) and the forced-swim test (FST), both which have been used widely as test for antidepressant activity. AM251 significantly reduced immobility in both the TST and the FST. The co-administration of the cannabinoid agonist CP-55940 reversed effects of AM251 in the TST and the effects of AM251 in the FST were not noted in CB_1 knockout mice. In addition to an antidepressant-like effect, AM251 reduced hyperphagia in food restricted mice. Taken together, these data suggest that regulation of mood and food intake might be influenced by CB_1 receptor activity (Shearman et al., 2003).

It has also been demonstrated that the porcine-derived cannabinoid agonist Noladin (2-arachidonylglyceryl-ether) dose-dependently produces hyperphagia while simultaneously inhibiting weight gain. Because the hyperphagic effects of were attenuated by SR141716A, it seems that Noladin exerts its consumptive and metabolic

influences through the cannabinoid system. While the mechanism of Noladin is unclear, these results indicate the low doses of Noladin may allow an increase in food intake without a gain in weight after dieting (Avraham et al., 2005).

Effects of Repeated Exposure to Cannabinergic Agents

In vitro studies show that chronic treatment with cannabinoid agonists produces CB_1 downregulation (i.e., loss of binding sites) and desensitization (i.e., loss of G-protein effector activity; Sim-Selley, 2003). When cannabinoid agonists are administered chronically during early adulthood, the reward value of food diminishes and recognition memory is impaired (Schneider & Koch, 2003).

There is also evidence that repeated exposure to cannabinoids during late adolescence and early adulthood (PD day 40-65) is associated with behavioral alterations in adult rats. Prepulse inhibition (PPI) was significantly disrupted by repeated adolescent cannabinoid exposure, but the PPI deficit was reversed by the acute administration of haloperidol. More to our point, the animals repeatedly exposed to a cannabinoid agonist during late adolescence and early adulthood demonstrated lower break points in a progressive-ratio schedule, whereas food preference and locomotion were not affected (Schneider & Koch, 2003).

In contrast to what is known about the effects of chronic exposure to cannabinoid agonists, very little is known about chronic exposure to cannabinoid antagonists. In naïve mice the cannabinoid antagonist SR141716A dose-dependently produced wet dog and head shakes, forepaw fluttering, grooming and facial rubbing. These types of behavior resemble precipitated cannabinoid withdrawal. With repeated exposure to

moderately high doses (5 mg/kg), those behavioral signs diminished (Rubino et al., 2000).

Neuroanatomical and Behavioral Changes during Adolescence

Adolescence and puberty are temporally-related events, though each has a distinct definition. Puberty refers to the attainment of sexual maturity (Graber & Brooks-Gunn, 1998) while adolescence is the gradual period of transition from childhood to adulthood (Pickles et al, 1998). Because the beginning of adolescence cannot be defined by any discrete milestone, it is difficult to characterize the precise timing and duration of adolescence (Rosenblum, 1990).

Instead, adolescence can be characterized by age-specific behavior such as increased risk-taking and social interaction. The behavior of adolescent animals is also highly-peer directed and is characterized by novelty-seeking. In rats, adolescent-typical behavior is commonly observed between postnatal day 28 and postnatal day 42 (PD28-42; Spear, 2000).

These behavioral changes do not seem to be significantly related to changes in gonadal hormonal levels (Brooks-Gunn et al., 1994). Instead the maturational changes in the brain that occur around PD28-42 contribute to the age-specific behavioral characteristics of adolescence (Spear, 2000). During adolescence many brain regions (i.e., prefontal cortex) and brain systems (i.e., dopaminergic system) are undergoing important changes (Van Eden et al., 1990). Another of the systems undergoing such changes is the endocannabinoid system.

During gestation, endocannabinoids play an important role in neurological development (Fernandez-Ruiz et al., 1999). After birth, cannabinoid receptor densities

increase until about postnatal day 30 (PD30) in female rats and PD40 in males rats (Belue et al., 1995, McLaughlin et al., 1994, Rodriguez de Fonseca et al., 1993). Some receptor pruning occurs during later adolescence, and by PD60, both male and female rats express adult levels of cannabinoid receptors (Belue et al., 1995).

While CB_1 receptors mature slowly during the postnatal period, behavioral data suggest that the endocannabinoid system may reach functional maturity during adolescence. Both anandamide and Δ^9 - THC decreased locomotion and induced antinociception in rodents examined shortly after the adolescent period (PD45) and in adulthood. While these changes, which are characteristic features of cannabinoid pharmacology, were present in post-adolescent animals, neither very young rodents (PD6–20) nor weanlings (PD23) had the same response ([A]Fride & Mechoulam,1996; [B]Fride & Mechoulam,1996).

Perhaps related to the slow maturation of the endocannabinoid system, there seems to be a vulnerable developmental period in rodents during which cannabinoid exposure produces long-lasting behavioral alterations, especially in behavior involving cognitive tasks (Stiglick & Kalant, 1985). Other work in rodents shows that chronic pubertal treatment with cannabinoids produces long-lasting reductions in the motivation to work for a food reward, but similar treatment during adulthood does not produce the same effect (Schneider & Koch, 2003). There is complimentary evidence from humans that long-lasting attentional deficits may be related to cannabis abuse that began before age sixteen (Ehrenreich et al., 1999).

Stimulation of CB_1 receptors activates the brain's reward system. The primary psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC) binds to CB_1

receptors in many brain areas (Herkenham et al, 1990). When Δ^9-THC binds to CB_1 receptors in the nucleus accumbens, the brain's dopaminergic reward system is indirectly activated (Chen et al, 1990). In rats, drugs that block CB_1 receptors also reduce food intake (Colombo et al, 1998) as well as the self-administration of heroin (Fattore et al, 2005) and alcohol (Economidou et al, 2005). In summary, the endocannabinoid system influences the hedonic value of reinforcing stimuli by modulating the brain's reward system.

Appetite Regulation

Food intake and energy balance are regulated by the complex interplay of a series of neurotransmitters, neuromodulators, and neuropeptides. The complexity of this highly-evolved system may well reflect the physiological importance of feeding behavior. It goes without saying that organisms that cannot control food intake and regulate metabolism do not persist long enough to reproduce and therefore represent evolutionary dead ends.

Food intake and energy balance is regulated in the hypothalamus. Some neurons in the hypothalamus induce food-intake (orexigenic neurons) and synthesize the orexigenic neuropeptides like neuropeptide Y (NPY) and agouti-related peptide (AGRP). Other neurons inhibit food-intake (anorexigenic neurons) and synthesize the anorexigenic neuropeptides α-melanocyte-stimulating hormone (α-MSH) and cocaine and amphetamine regulated transcript (CART). Peripheral neuropeptides such as cholecystokinin (CCK) and ghrelin regulate important gastrointestinal functions such as motility and absorption. As such, peripheral neuropeptides may influence food intake by

providing information to the central nervous system about nutrient availability (Wilding, 2002).

Production of the hypothalamic neuropeptides is influenced by plasma levels of a hormone called leptin. Leptin is secreted by fat cells and acts in rodents via hypothalamic receptors to inhibit feeding and increase energy-expenditure. When animals are well-fed, adipose tissue mass increases and leptin levels rise. Leptin receptors in the hypothalamus integrate the intensity of the leptin signal. Energy intake and energy expenditure are regulated by suppressing the production of orexigenic neuropeptides (NPY & AGRP) and inducing the production of anorexgenic neuropeptides (α-MSH & CART). As such, the leptin system is classic example of a feedback regulatory loop (Jequier, 2002).

Leptin levels are also inversely related to endocannabinoid levels in the hypothalamus. This inverse relationship between leptin and endocannabinoids is important given the orexigenic properties of anandamide and 2-AG (Di Marzo et al., 2001). Anandamide has been shown to increase Fos expression in the paraventricular nucleus of the rat hypothalamus (Wenger et al. 1997; Patel et al. 1998) and administration of anandamide into the ventromedial hypothalamic nucleus of satiated rats induces hyperphagia (Jamshidi & Taylor, 2001).

Though CB_1 receptor densities are relatively low in the hypothalamus, it appears that receptor coupling to G-proteins is more efficient in the hypothalamus than in other areas of the brain with higher CB_1 receptor density (Breivogel et al., 1997). CB_1 receptors are very dense in brain areas such as the nucleus accumbens, the hippocampus and the entopeduncular nucleus. These areas are either directly involved in hedonic

aspects of eating or are connected to reward-related brain areas (Finkelstein et al. 1996; Gorbachevskaia, 1999; Pecina & Berridge, 2000).

In summary, endocannabinoids play a significant role in the complex cascade of events that regulate food intake and energy balance. Endocannabinoids are implicated in both hedonic feeding, which is reflective of the reinforcing efficacy of food, and homestatic feeding, which is reflective of the energy balance (Harrold & Williams, 2003).

Progressive-ratio Schedules and the Motivation to Eat

Progressive ratio schedules are used to quantify the motivation to work for a given reward (i.e., food; Hodos, 1961). Unlike fixed-ratio schedules of reinforcement, in which the same number of responses are required for every reward received during the experimental session, progressive-ratio schedules incrementally increase the number of responses required for successive rewards. As such, subjects in progressive-ratio paradigms are required to work harder and harder to receive subsequent rewards. The theoretical basis for progressive-ratio schedules is that at some point the amount of work required to receive a given reward will exceed the reinforcing efficacy of that reward (Ferguson & Paule, 1997).

Progressive ratio schedules can be traced to a series of experiments published by F.A. Moss in 1924. These studies purported to quantify various types of motivation. Various objects (e.g. food, prospective mates, offspring) were placed behind a charged metal grid. The grid was wide enough that it could not be cleared in a single bound and required the subjects to cross while making contact with the grid surface. The voltage across the grid could be modulated to produce a shock of varying intensity. After 72-

hours of food deprivation, rats would endure shocks up to 28-volts in order to obtain food. Using the same strategy, Moss found that the "maternal drive" was weaker than the "sex drive". Moss concluded that "Any animal drive may be measured in terms of the resistance overcome or the resistance may be measured in terms of the drives, depending on which is known" (Moss, 1924). For Moss, all behavior could be explained as a probabilistic interaction between drives and resistances. The implications of these findings were that differential motivation for various rewards can be quantified. However, Moss did not control for the fact that repeated handling during the session can interfere with motivation and that repeated use of electric shocks produced highly variable within-subject data which made interpretation very difficult (Moss, 1924).

In 1958, Jack Findley published the results of a series of experiments involving pigeons that were trained to peck an illuminated key for grain. The color of the key was either red or green, and a specific reinforcement schedule was associated with each color. Pecking on a second key permitted the birds to switch the color appearing on the first key. The general behavior resulting from this type of procedure suggested an operant chain in which pecking on the second key was maintained by its consequences for reinforcement on the first key. Preferences for a given color and rate of switching colors were found to be a function of the particular schedules and switching contingencies imposed. These findings imply that pigeons can recognize the effort required to obtain a given reward and can express preferences for a less demanding reinforcement schedule (Findley, 1958).

The first documented use of a progressive-ratio schedule of reinforcement was published by William Hodos in 1961. Rats were first trained to lever press for a sweet

milk reward. Later, those rats were placed in experimental conditions that required more and more responses for each subsequent reward (i.e., 2, 4, 6, 8, etc.). Eventually, the response requirement became so large that the subject failed to respond for an arbitrarily defined period of 15 minutes and the session was terminated. Hodos defined this point as the "breaking point" of the subject's performance. Hodos found that in free-feeding animals, the number of responses emitted in the final completed ratio (i.e., those that resulted in a reward) was directly related to the concentration of the reward. Hodos also found that, for any given reward concentration and volume, the number of responses in the final completed ratio was a function of the level of food deprivation. One of the problems with this study was the slow progression of the ratios used. Slowly increasing ratios increase the likelihood that the onset of satiety interferes with determining reward value (Hodos, 1961).

Two years later, Hodos replicated the original work, but he modified the procedure to include a more aggressive ratio progression (i.e., 5, 10, 15, etc.). Hodos found that the number of responses in the final completed ratio was a function of the incremental size of the reinforcer. However, Hodos again noted that for any given reward volume, the number of responses in the final completed ratio was a function of the level of food deprivation. While Hodos and Kalman used a more aggressive ratio progression in these experiments, the largest reward volumes were associated with a lower number of responses in the final completed ratio. This observation can best be explained by the onset of satiety and points to the importance of a sufficiently aggressive ratio progression (Hodos & Kalman, 1963).

Subsequently, it was determined that progressive-ratio breakpoints vary closely with daily percentage changes in bodyweight (Gilbert, 1967). It also became apparent that the level of food deprivation has a greater impact on performance in a progressive-ratio schedule than do feeding schedules. In fact, regardless of whether weight loss is controlled by feeding the subjects only when they dropped below a given bodyweight or by feeding closely controlled amounts of food every day, performance in the progressive-ratio was equivalent (Allen, 1968). Pre-feeding does not significantly alter either response rates or breakpoints under the progressive-ratio schedule. In studies using food-deprived rats that were allowed brief access to small amounts of food before an experimental session, it was determined that the duration of food-deprivation did not significantly alter the motivation to eat (Ferguson & Paule, 1995).

It also seems that response rates are well-correlated with level of food deprivation and are not substantially affected by diet. Rats were trained to run on a wheel to receive a food reward and successive food rewards required longer and longer running sessions. The rats tested in multiple sessions as they were then food-deprived to until they reached 75% of their free-feeding weight. The results demonstrate that, regardless of initial weight or adiposity, body weight must fall to some relatively fixed level (i.e., diet independent) before activity increases during a fast (Sclafani & Rendel, 1978). Others have assessed progressive ratio performance of male rats in varying states of food deprivation down to 75% of their free-feeding body weight. The results of those experiments also demonstrate that, in progressive-ratio schedules, response rates and post-reinforcement pauses vary significantly as a function of the level of deprivation Ferguson & Paule, 1997).

In summary, when progressive-ratio schedules are combined with food rewards, the motivation to consume food can be quantified. Evidence suggests that breakpoints and response rates are primarily affected by levels of food-deprivation. As the level of food-deprivation increases, breakpoints and response rates typically increase. Hence, it seems logical to suggest that performance under progressive-ratio schedules is largely related to energy balance and the drive to engage in homeostatic feeding.

Though levels of deprivation are of paramount importance to performance in progressive-ratio schedules, the significance of reward size and palatability should not be dismissed. As the reinforcing efficacy of the reward increases, breakpoints typically increase. Hence, it seems logical to suggest that progressive-ratio breakpoints can be affected by hedonic feeding. It is important to reiterate, however, that the reinforcing efficacy of food rewards does not reliably predict breakpoints under progressive-ratio schedules.

Because the endocannabinoid system is believed to be involved in both hedonic and homeostatic feeding behavior, changes in the endocannabinoid system may well be reflected in performance under progressive-ratio schedules. This possibility was the major rationale for using a progressive-ratio paradigm in this proposal.

Method

Species

All of the experiments described herein were conducted on male Long-Evans rats that were bred in-house using the same dams (n=20) repeatedly and pairing 10 sires randomly with the dams. During these experiments, each animal received daily intraperitoneal injections of SR141716A (3 mg/kg) beginning PD28 and ending on PD68. In order to adhere to standard scientific practices in the developmental psychopharmacology field, only one male pup was used from each litter for a particular testing condition (e.g., drug and diet combination). In order to complete the studies, we used rat pups from 30 matings (3 drug and diet combinations X 10 pups from different litters). Only male rats were used in these experiments because the time constraints of my academic program prevented an adequate evaluation of the influence of estrous cycle on feeding and on endocannabinoids in female rats. Determination of sex differences represents a possible future direction of this research.

After weaning, all rats were single-housed in 11" X 17" X 8" Makrolon cages in a temperature-controlled (20–22°C) environment with 12-hour light and dark cycle (lights on at 7:00 am). The rats used in these experiments were single-housed because it individual food-intake was measured every day. While single-housing increased the precision of food-intake measurements, it also created a methodological concern. Unlike behavior in adult rats, adolescent rat behavior is highly peer-directed (Spear, 2000). One of the weaknesses of this experimental design is the possibility that the absence of the

social interaction that is a characteristic part of rat adolescence might significantly affect motivation and eating patterns in adulthood.

Water and standard rodent chow were freely available in the home cages unless otherwise mandated by an experimental condition (see below). In all cases, water was available *ad libitum*. Each of these studies was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Virginia Commonwealth University is also an AAALAC accredited institution and adheres to the strictest practical animal care and use guidelines.

Groups

Three groups of adolescent rats were required to determine the developmental impact of chronic CB_1 blockade on performance in a progressive-ratio schedule. Adolescent Group 1 was treated with SR141716A (3 mg/kg) each day throughout adolescence. Even though Adolescent Group 1 was allowed *ad libitum* access to both food and water, they it was expected that they would be smaller than other Long-Evans rats of comparable ages. This is because the dose of SR141716A used in these experiments is sufficient to reduce food intake and slow body weight gains in young animals.

Adolescent Group 2 was treated with treated with vehicle (1 ethanol:1 emulphor:18 saline) each day throughout adolescence. Adolescent Group 2 also had *ad libitum* access to both food and water. This group provided a baseline measure of the breakpoints in the progressive-ratio test.

Adolescent Group 3 was also treated with vehicle (1 ethanol:1 emulphor:18 saline) each day throughout adolescence, but they were pair-fed such that each member received the same number of calories as a corresponding member of SR-treated group (Adolescent Group 1).

The animals in Adolescent Group 3 were pair-fed with a corresponding member of the SR-treated group instead of feeding them the mean caloric intake for the SR-treated group in order to minimize potential seasonal differences between the treatment groups. For any given postnatal day, the mean caloric intake for the SR-treated group could not be determined until all members had matured beyond that point. Given the vagaries of rodent husbandry and the uncertainty of the composition of the resulting litter, it is likely that it would have taken upwards of 12 weeks to fill the SR-treated group. As such, the data points for the pair-fed group (Adolescent Group 3) would be separated from the data points from the SR-treated group by about 3 months. By pair-feeding each member of Adolescent Group 3 with a corresponding member of SR-treated group, the data points for the pair-fed group would be separated from the corresponding data points from the SR-treated group by about one day.

Because their daily caloric intake was similar, it was thought that the size of the rats in Adolescent Group 1 and Adolescent Group 3 would be indistinguishable, though both groups were likely to be smaller than the animals that are treated with vehicle and given free access to food. The data obtained from the pair-fed group were used to control for changes in progressive-ratio breakpoints that may develop after sustained periods of diminished caloric intake.

Sample Size

Sample size for each experiment was determined with SigmaStat (ver. 2.0; Jaundel Corporation, San Raphael, CA). Parameters entered in this program included effect size, standard deviation of the residuals, number of groups, desired power, and alpha level. Cohen (2002) recommends values of 0.10 for a small effect size, 0.25 for a medium effect size, and 0.40 for a large effect size and these values have been found to be useful for most behavioral science experiments (Howell, 1997). For these studies, I chose a medium effect size for power analysis. The standard deviation for the residuals was estimated to be about 0.125 (one-half of the effect size). This size of standard deviation was reasonable, given standard deviations that we obtained in previous studies using these procedures. By convention, the desired power level was set at 0.80 and the alpha level was set at 0.05 (Howell, 1997). SigmaStat calculated that a sample size of 10 per group is necessary in order to detect differences based on these parameters. This sample size was consistent with sizes we used previously in similar studies and was used for all studies in this protocol.

Drugs

SR141716A was obtained from the National Institute on Drug Abuse (Rockville, MD, U.S.A.). It was mixed at a concentration of 3 mg/ml and dissolved in a vehicle of absolute ethanol, Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ, U.S.A.), and saline in a ratio of 1:1:18. This solution was administered to the rats intraperitoneally at a volume of 0.1 milliliters per 100 grams of body weight.

Apparatus

The weight of food pellets was measured with a Mettler AT261 Delta Range scale (Toledo, OH, U.S.A.) with precision set to 0.1 gram. All operant training and each progressive-ratio session was conducted in a standard two-lever modular test chamber (12" X 9.5" X 8.25"; Model ENV-008, Med-Associates, Inc., St. Albans, VT, USA) running MED-PC for Windows software. Spontaneous locomotor activity was assessed in clear plastic home cages (11"X11"x17") equipped with a Photobeam Activity System (San Diego Instruments, San Diego, CA, USA) that is capable of recording the degree of movement (fine vs. ambulatory) and the location of movement (central vs. peripheral).

Statistical analyses

All statistical analyses were conducted using SPSS statistical software. Bodyweights and consumption of a novel, palatable food were analyzed with a one-way ANOVA. Growth rates were compared by linear regression analysis. The acquisition of the operant procedure as well progressive-ratio breakpoints and response rates were analyzed with a factorial ANOVA (split-plot). The relationship between the various levels of deprivation on breakpoints and response rates was subsequently analyzed using a one-way ANOVA. Final completed ratios and locomotor data were also analyzed with a factorial ANOVA as well as a one-way ANOVA. The total percentage of subjects that reached a breakpoint was also analyzed with a one-way ANOVA. Tukey post hoc tests ($\alpha = 0.05$) were used to specify differences revealed by significant ANOVAs.

Procedures

SR-treated group (Adolescent Group 1)

A schematic overview of the treatment and testing timeline can be found in Table 1. Male Long-Evans rats (n = 10) were injected once daily with SR141716A (3 mg/kg, i.p.) for 41 consecutive days. For the adolescent animals, the treatment regimen began on PD28 and ended on PD68. The duration of this treatment regimen was chosen because it encompassed a behaviorally-defined period of adolescence and the transition into early adulthood (Spear, 2000).

Table 1.

Treatment and testing timeline.

Treatment Phase	Magazine Training	FR1 Training	1st Progressive-ratio	Locomotion Test	2nd Progressive-ratio	3rd Progressive-ratio	4th Progressive-ratio
PD28-PD68	PD71	PD72-PD74	PD75	PD76	PD82	PD89	PD96

While the animals were being treated, they will be allowed *ad libitum* access to both food and water. The dose of SR141716A used in these experiments has been shown to reduce food intake and body weight (Colombo et al., 1998). The animals were permitted a minimum of a 7-day wash-out period before the first progressive-ratio session to allow for the metabolism and elimination of any residual SR141716A. During the

treatment period, each animal was weighed every day and daily food consumption was measured.

Twenty-four hours after the final injection of SR141716A (PD69), each animal was weighed again and began period of food restriction. The purpose of food restriction was to reduce the bodyweight to a series of predetermined levels before the each of the progressive-ratio sessions. Previous research showed that motivation for a food-reward, as indexed by progressive ratio breakpoints and response rates, is related to the extent of food deprivation (Hodos, 1961; Gilbert, 1967; Ferguson & Paule, 1997) and is relatively independent of feeding schedule (Allen, 1968) or duration of food deprivation (Ferguson & Paule, 1995). A repeated measures design was used to gain a full understanding of group differences in breakpoints and response rates. By employing such a design, it was possible to run progressive ratio sessions when the subjects are at 85%, 100%, 95% and 115% of their pre-testing body weight. The weight recorded on PD69 was deemed to be the pre-testing weight (100% body weight) and all levels of deprivation were set in relation to that weight.

One area of concern involved use of this arbitrarily defined pre-testing weight. A weakness of these experiments was the repeated manipulation of body weight in animals that are continuing to grow. While it is true that male Long-Evans rats at PD69 are still growing, it is important to remember that male Long-Evans rats typically grow continuously throughout their lifetime (personal observations). In behavioral pharmacology research, food-restriction to some predetermined percentage of a free-feeding body weight is common. While the notion of a single free-feeding weight is

widely accepted, if male Long-Evans rats are allowed continuous access to free-feed, they will frequently exceed 600 grams by the time they are 2 years old (personal observations). In a sense, all pre-testing weights (or reference weights) must be viewed as artificial because, in rats, body weight continues to change as a function of age.

What is different about 69-day-old male Long-Evans rats is the rate at which they are growing. Our pilot studies indicate that, when given access to free-feed, these rats increase their body weight from 1.0% to 1.5% each day. In an attempt gain a more accurate measure of the motivation to work for a food reward, it might be important to allow some period of unrestricted weight gain after the initial period of deprivation. In doing so, we could set a new pre-testing weight that would be more reflective of the "natural" body weight for a young adult male Long-Evans rat.

Allowing unrestricted weight gain to set a new pre-testing weight, however, might produce more problems than it solves. Establishing a new pre-testing weight might invalidate the comparison of breakpoints at 85% of "old" pre-testing weight and breakpoints at any percentage of the "new" pre-testing weight. To determine whether breakpoints observed at the "old" and "new" pre-testing weights could be compared, we would need to have breakpoint data from 100% of the PD69 body weight. Since it is not possible to test breakpoints in rats without training them to lever press for a food reward, and since rats are notoriously bait shy, food-restriction is a necessary component of this paradigm. However, food restriction changes body weight and the time required for training makes the pre-testing weight less representative of the natural weight. In the

end, it seems unlikely that data derived from multiple pre-testing weights could be compared reliably.

After reaching a desired percentage of the pre-testing body weight, the subjects were maintained at that point for 48-72 hours before being tested in a progressive-ratio session. Rats used in these trials were not be allowed to drop below 85% of their pre-testing weight and were never be maintained at 85% of their pre-testing weight for more 3 days.

Probe trials using early adult male Long-Evans rats have shown that daily intake of 0.07-0.11 kcal/g body weight was sufficient to reduce body weights by 15% within 5 days. Those same trials indicated that weights can be maintained at a given level of deprivation (down to 85% of pre-testing weight) with a daily intake of 0.20-0.25 kcal/g body weight. Male Long-Evans rats near PD75 typically eat 0.29-0.35 kcal/g body weight each day if allowed free access to their regular chow (unpublished data). While a substantial degree of food-restriction to required to reach 85% of pre-testing weight in 5 days, it is important to remember that the duration of this deprivation is brief (96-120 hours), each animal is weighed at least once a day and animals are always permitted free access to water.

Forty-eight hours after the initiation of food-deprivation and seventy-two hours after the last dose of SR141716A (PD71), the animals underwent a single 30-minute session of magazine training in an operant chamber (Med-Associates, St. Albans, VT) during which one non-contingent 45 mg dextrose/sucrose pellet (3.68 kcal/g; Bio-Serv Corp., Frenchtown, NJ) will be delivered every 30 seconds. Sweet pellets were chosen

because SR141716A has been shown to differentially reduce the consumption of sweet foods in food-restricted animals (Arnone et al., 1997) and the goal of these experiments is quantify changes in motivation to work for a food reward after chronic CB_1 blockade. During the magazine training session, the chamber was illuminated by a house light, but no levers will be present. The purpose of the magazine training session was to habituate the animals to the operant chamber and to allow the animals to orient themselves to the position of the tray into which the sucrose pellets are dispensed. After the magazine session, the caloric value the pellets consumed during the session was deducted from the total daily caloric allowance.

On each of the following 3 days (PD72-74), the animals underwent a single 30-minute session in the same operant chamber during which a 45 mg sucrose pellet will be delivered for each lever response (fixed ratio 1: FR1). During these sessions, a house light was be illuminated and a single lever was extended. The purpose of these sessions was to allow the animals to learn the association between responses on the lever and the presentation of a food reward. While the session length was limited to 30 minutes, there was no externally-imposed limit to the number of food rewards that an animal can earn during the session. After each of the FR1 training sessions, the caloric value the pellets consumed during the session was deducted from the total daily caloric allowance.

Upon reaching the 85% body weight criterion, the animals underwent a single 30-minute session in a single-lever operant chamber during which one sucrose pellet was delivered in an exponentially-progressive schedule (e.g., 1, 4, 9, etc.). In exponentially-progressive schedules, n^2 responses are required for n^{th} pellet during the session. The

animals were allowed to continue responding until the 30-minute session has expired or until the animal stops responding for 2 minutes. A 2-minute response hiatus was deemed to constitute a breakpoint for the session. After an animal reached a breakpoint, the house light was extinguished and the lever was retracted. All animals remained in the boxes for thirty minutes, regardless of whether they reached a response hiatus or continued working. Breakpoints are construed to be reflective of the motivation to work for a food reward (Hodos, 1961). The last ratio that was completed before the 2-minute response hiatus or the end of the session was the primary dependent variable in these experiments

It is thought that a 30-minute progressive ratio session was appropriate because the majority of rats used in probe trials (~80%) reached a breakpoint within 30 minutes. An exponential ratio progression, which increases the work required for successive rewards at a fairly aggressive rate, was chosen to increase the likelihood that the subjects would reach a breakpoint before the end of the 30-minute session. Sweet rewards, like the dextrose/sucrose pellets used in these experiments, are known to be more reinforcing than bland rewards (Baron et al., 1992) and breakpoints are known to increase as a function of the reinforcement magnitude of the resulting reward (Hodos, 1961; Hodos & Kalman, 1963). When these facts were combined with the hypothesis that chronic CB_1 blockade will increase breakpoints, it seemed prudent to use an aggressive ratio progression.

While the anorectic effects of SR141716A are fairly well established, there is less agreement regarding the impact of CB_1 blockade on locomotion. Some studies indicate

that acute doses of SR141716A can stimulate motor activity (Costa & Colleoni, 1999), while other studies have shown only minor changes in locomotion (Wiley et al., 2005). More directly, chronic perinatal exposure to SR141716A has been shown to reduce immobility without affecting overall measures of locomotion (Moreno et al., 2005). To determine whether differences in breakpoints are associated with differences in motor activity, locomotor inhibition will be assessed in these same rats during a 20-minute session in a photobeam-equipped locomotor chamber (San Diego Instruments, San Diego CA). The measure of locomotor activity will be performed the day after the first progressive-ratio session (PD76).

While consistent levels of deprivation are vital to this research design, test points must occur at regular intervals beyond the last exposure to SR141716A. Repeated treatment with SR141716A has been shown to produce transient reductions in food intake and sustained reductions on body weight in normal size rats (Colombo et al., 1998) and obese mice (Ravinet et al., 2003). However, when chronically dosed obese mice were taken off AM251, a structural analog of SR141716A, they became transiently hyperphagic and demonstrated gradual increases in body weight (Hildebrandt et al., 2003). Because treatment with CB_1 antagonists produces changes in appetite that are transient in nature, the possibility that chronic CB_1 blockade induces compensatory mechanisms cannot be discounted. The transient hyperphagia that accompanies the cessation of CB_1 blockade is of particular interest in formulating this research design. It is expected that the motivation for a food reward will change not only as a consequence of the varying levels of food deprivation, but as appetite moves toward the level exhibited

by vehicle-treated animals. Accordingly, caloric intake must be managed such that each animal reaches the exact percentage of pre-testing body weight on exactly the same day. The levels of deprivation and testing schedule are outlined in Table 2.

Vehicle-treated, free-fed group (Adolescent Group 2)

The animals in Adolescent Group 2 (n = 10) received daily vehicle injections (i.p., 1 ethanol: 1 emulphor: 18 saline) on PD28-68. These animals were allowed *ad libitum* access to both food and water until PD68. At that point, these animals began a training, treatment and testing regimen that was identical to the one described for the experimental group. This group provided a baseline measure of the reward value of food.

Table 2.

Levels of deprivation and testing schedule for progressive-ratio tests.

Developmental stage when treated	Progressive-ratio at 85% of pre-testing body weight	Progressive-ratio at 100% of pre-testing body weight	Progressive-ratio at 95% of pre-testing body weight	Progressive-ratio at 115% of pre-testing body weight
	PD75	**PD82**	**PD89**	**PD96**
Adolescent	(7 days after last treatment)	(14 days after last treatment)	(21 days after last treatment)	(28 days after last treatment)

Vehicle-treated, pair-fed group (Adolescent Group 3)

The animals in Adolescent Group 3 (n = 10) were pair-fed such that each member received the same number of calories as a corresponding member of Adolescent Group 1. Though pair-fed, each of these animals had *ad libitum* access to water. This group was

injected with vehicle (1 ethanol: 1 emulphor: 18 saline; i.p.) once a day on PD28-68. At that point, these animals began a training, treatment and testing regimen that is identical to the one described for the experimental group. The data obtained from the pair-fed group was used to control for changes in the reward-value of food that might develop after sustained periods of diminished caloric intake.

Results

A one-way analysis of variance revealed that by the end of the treatment period (PD69), both the SR-treated group and the pair-fed group had significantly lower bodyweights than the vehicle-treated group (Fig.1), $F(2, 27) = 6.407$, $p < 0.05$. Interestingly, the bodyweights of the pair-fed animals were indistinguishable from the animals chronically treated with SR141716A. A linear regression analysis revealed that

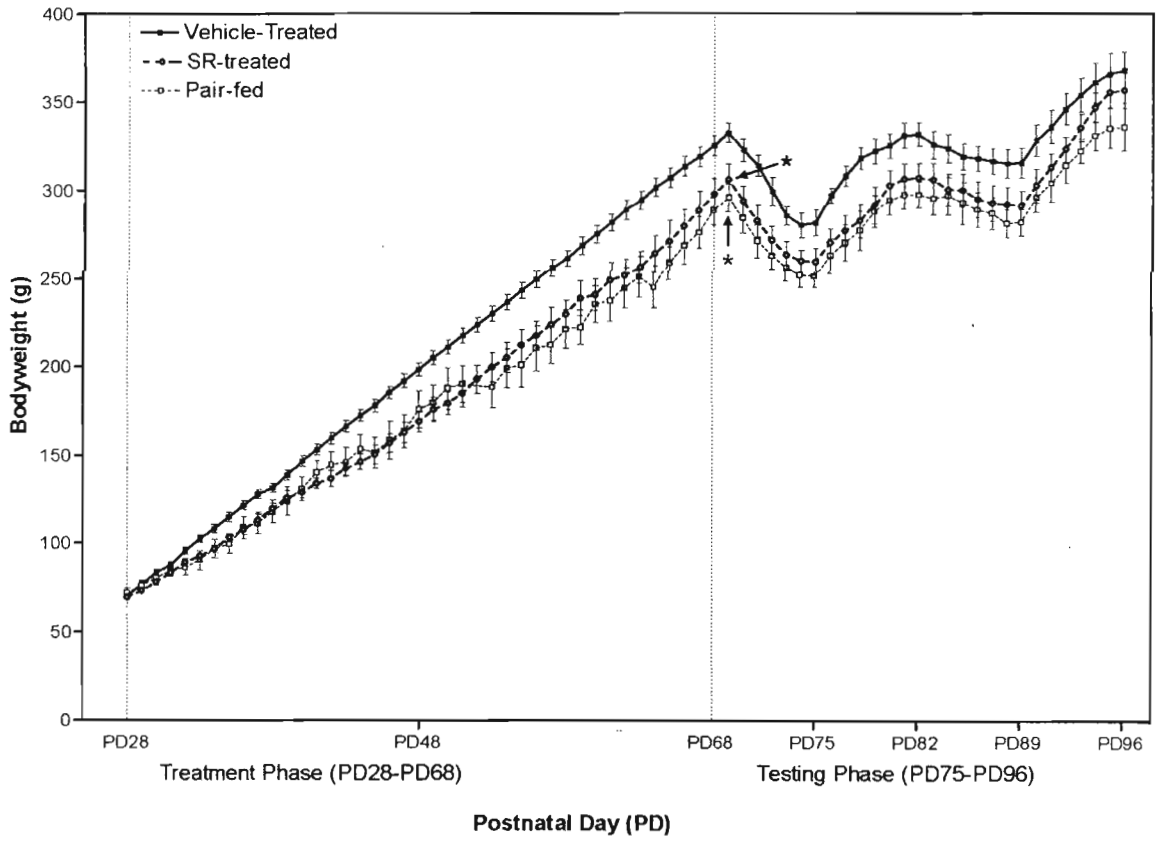

Figure 1 – Mean bodyweights (grams) for each treatment group (+/- SEM) from PD28 through PD96. One-way ANOVA revealed that the SR-treated and pair-fed groups had significantly lower bodyweights than vehicle-treated groups at the end of the treatment phase PD69, $F(2, 27) = 6.407$, $p < 0.05$. The SR-treated and pair-fed groups were also significantly lighter at nearly all points between PD45 and PD69, but asterisks are omitted for visual clarity.

while both the SR-treated and pair-fed groups were lighter than the vehicle-treated group, the growth rates for these groups were not significantly different, $F(2, 200) = 31.829$, $p > 0.05$.

On the magazine training day (PD71), which was 72 hours after the final drug treatment, each animal was given the opportunity to consume up to 60 dextrose/sucrose pellets during a 30-minute session. The animals had not been exposed to these pellets, or any similarly sweetened food, prior to this session. The results of this experiment were evaluated with a one-way analysis of variance. When given access to these novel and presumably palatable pellets (as compared to normal chow), the SR-treated animals ate significantly more pellets than did the vehicle-treated animals (Fig. 2), $F(2,27) = 5.134$, $p < 0.05$. Though the pair-fed group ate more pellets (M = 55.10, SEM = 2.47) than the

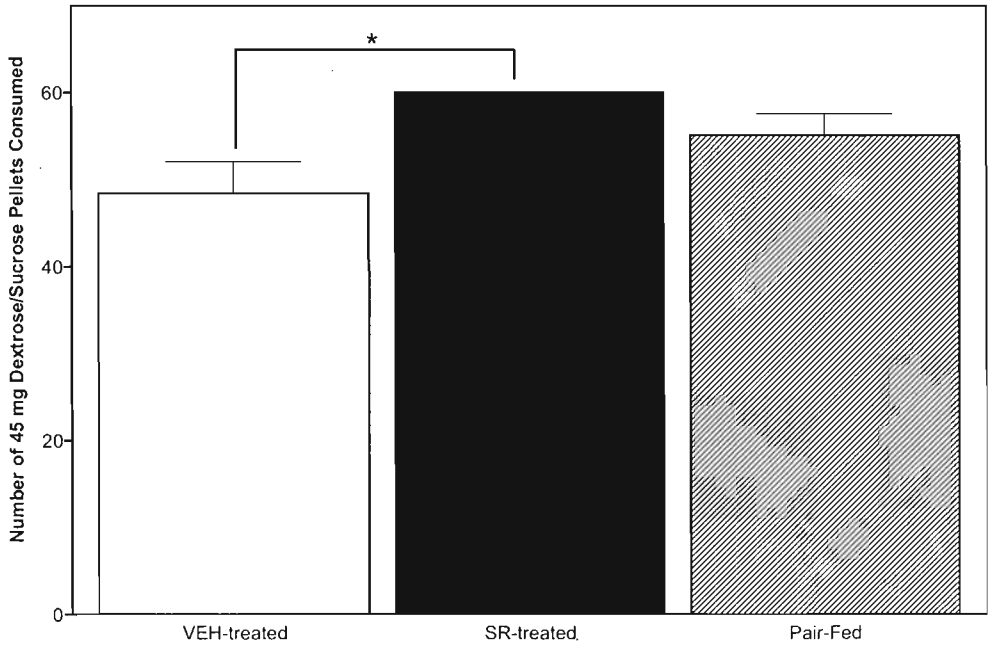

Figure 2 – Mean number of dextrose/sucrose pellets consumed (+/- SEM) during magazine training session. The SR-treated group ate significantly more of the novel, palatable food than did the vehicle-treated group, $F(2,27) = 5.134$, $p < 0.05$.

vehicle-treated group (M = 48.40, SEM = 3.71), those differences were not statistically significant. The mean number of pellets eaten by the pair-fed group and the SR-treated group was likewise indistinguishable.

All of the groups learned to lever-press at the same rate and to the same degree (Fig. 3). Each of the groups exceeded the threshold response rate of 0.1 responses per second by the third training session.

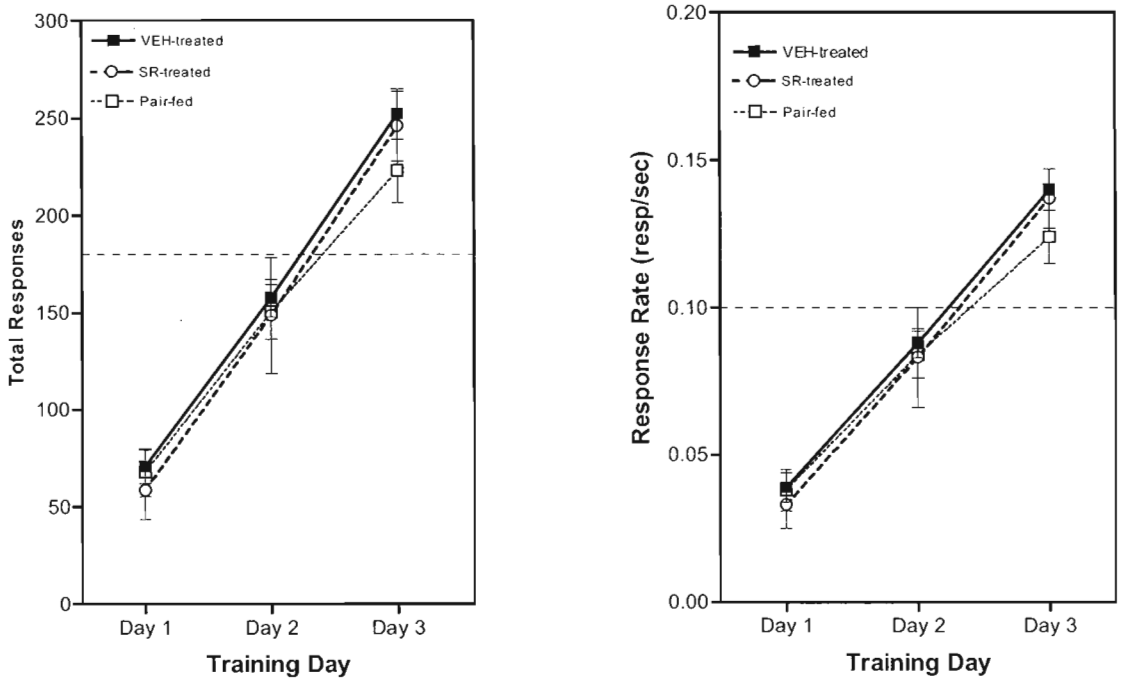

Figure 3 - Acquisition of operant procedure during three successive 30-minute FR1 training sessions. All the groups learned to lever press for a food reward at the same rate and to the same degree (mean number of responses/session, +/- SEM). Each animal responded at the threshold level (0.1 responses/second) by the end of the third training session.

The effect of the varying levels of deprivation on progressive-ratio breakpoints was initially explored with a factorial analysis of variance. The results of this analysis

revealed a linear main effect that was manifested as elevated breakpoints at the highest levels of deprivation (Fig. 4), $F(3, 30) = 3.677$, $p > 0.05$. The effect size was very large, $\eta^2 = 0.561$. Despite the presence of a main effect, there was no interaction between the respective treatments and levels of deprivation and none of the groups differed

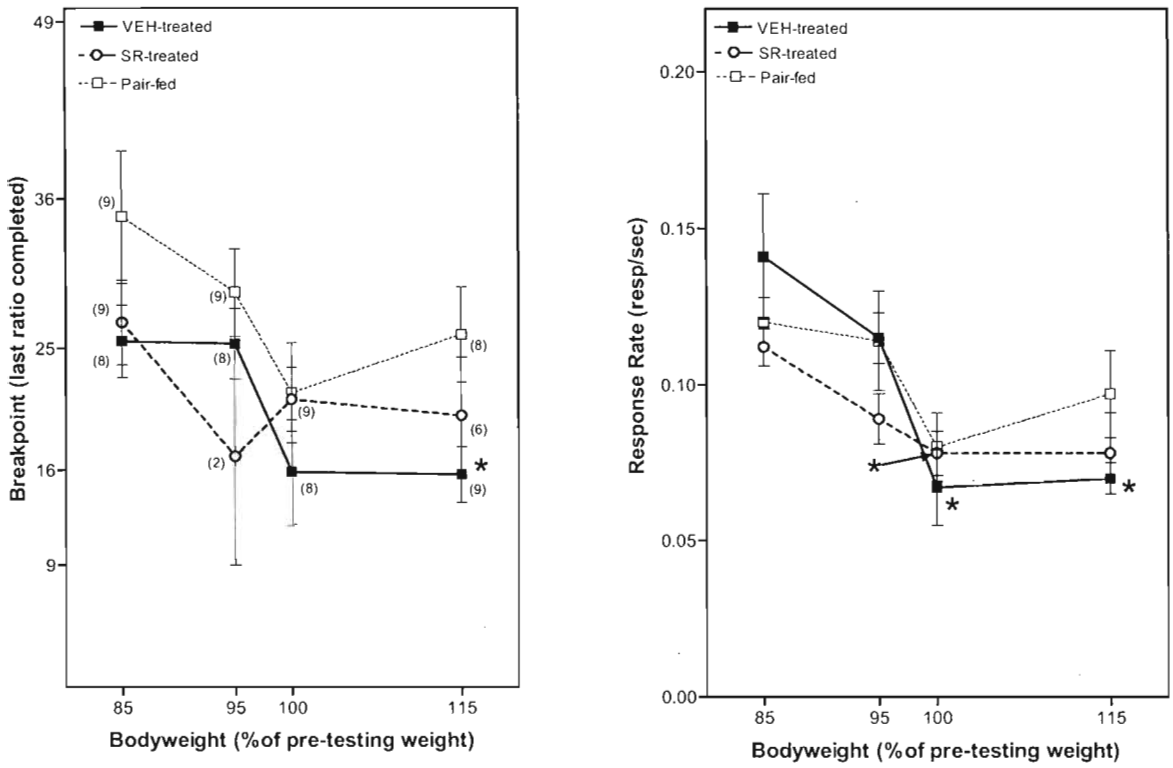

Figure 4 – Progressive-ratio breakpoints and response rates varied significantly as a function of the level of deprivation for the vehicle-treated group. For the vehicle-treated group, breakpoints were significantly lower at 115% of the pre-testing bodyweight than they were at 95% of the pre-testing bodyweight. Response rates were significantly lower at 100% and 115% of the pre-testing bodyweight than they were at 85% of the pre-testing bodyweight for the vehicle-treated group. For the SR-treated group, response rates were lower at 100% of the pre-testing weight than they were at 85% of the pre-testing bodyweight ($p < 0.05$). Numbers in parentheses indicate number that reached a breakpoint during the progressive ratio session, when different from 10. The final completed ratio was excluded from the breakpoint data for animals that did not reach a breakpoint. Response rate data includes all animals.

significantly at any of the points tested. It is important to note that some animals did not exhibit a response hiatus during in various progressive-ratio sessions. The failure to exhibit a response hiatus precludes the determination of a breakpoint. The numbers in parentheses in the breakpoint graph (Fig. 4) indicate number that reached a breakpoint during the progressive ratio session, when different from ten.

The effect of the varying levels of deprivation on progressive-ratio breakpoints for each group was subsequently explored with a repeated measures analysis of variance followed by multiple pairwise comparisons incorporating a Bonferroni adjustment. For the vehicle-treated group, breakpoints varied significantly as a function of the level of deprivation (Fig. 4), $F(3, 9) = 10.993$, $p < 0.01$. Pairwise comparisons revealed that breakpoints at 95% of the pre-testing bodyweight were significantly higher than breakpoints at 115% of the pre-testing bodyweight. A measure of the strength of the relationship between the varying levels of deprivation and the corresponding progressive-ratio breakpoints (the effect size) was very large, $\eta^2 = 0.944$.

For the pair-fed group, the omnibus F-test did not reveal a significant effect of the varying levels of deprivation on progressive-ratio breakpoints, $F(3,18) = 3.118$, $p = 0.052$. Despite this, multivariate analysis revealed a very large effect size, $\eta^2 = 0.683$. Likewise, varying levels of deprivation were not significantly associated with changes in progressive-ratio breakpoints for the SR-treated of animals (Fig. 4).

A factorial analysis of variance revealed a significant interaction between treatment and the number of subjects that of reached a breakpoint during the progressive-ratio sessions (Fig. 5), $F(6, 81) = 2.630$, $p < 0.05$. When data from all the progressive-

ratio sessions are combined, significantly lower percentage of SR-treated animals reached a breakpoint (M = 65.0%, SEM = 7.0%) when compared to the pair-fed group (M = 87.5%, SEM = 7.0%).

The effect of the varying levels of deprivation on progressive-ratio response rates was initially explored with a one-way factorial analysis of variance. The results of this analysis revealed a linear main effect that was manifested as elevated response rates at the highest levels of deprivation (Fig. 4), $F(3, 81) = 15.836$, $p > 0.001$. The effect size was very large, $\eta^2 = 0.645$. Despite the presence of a main effect and a very large effect size, there was no interaction between the levels of deprivation and progressive-ratio response rates and none of the groups differed significantly at any of the points tested.

The effect of the varying levels of deprivation on progressive-ratio response rates for each group was subsequently explored with a repeated measures analysis of variance followed by multiple pairwise comparisons incorporating a Bonferroni adjustment. For the vehicle-treated group, response rates varied significantly as a function of the level of deprivation (Fig. 4), $F(3, 27) = 10.671$, $p < 0.001$. Pairwise comparisons revealed that response rates at both 100% and 115% of the pre-testing bodyweight were significantly lower than response rates at either 85% or 95% of the pre-testing bodyweight. A measure of the strength of the relationship between the varying levels of deprivation and the corresponding progressive-ratio response rates was very large, $\eta^2 = 0.907$.

For the SR-treated group, response rates also varied significantly as a function of the level of deprivation (Fig. 4), $F(3, 27) = 4.886$, $p < 0.01$, $\eta^2 = 0.673$. Pairwise comparisons revealed that response rates at 100% of the pre-testing bodyweight were

significantly lower than response rates at 85% of the pre-testing bodyweight. For the pair-fed group, levels of deprivation were not significantly related to progressive-ratio response rates.

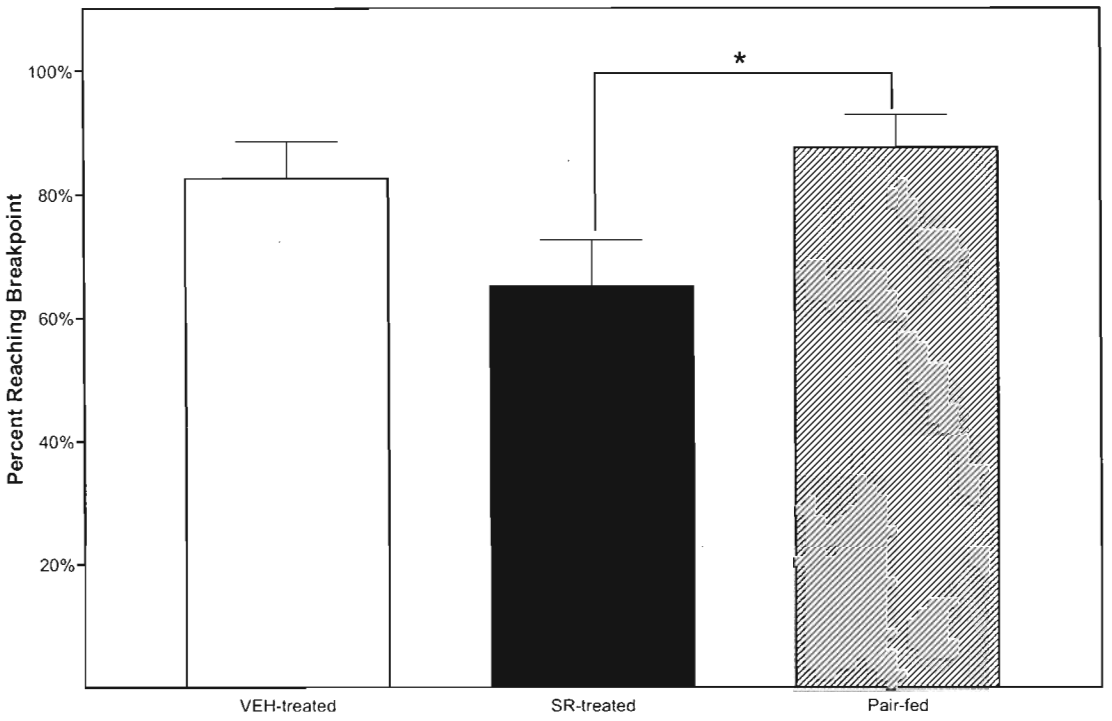

Figure 5 – Percentage of subjects from each group that reached a breakpoint the progressive-ratio sessions. When the data from all the trials were combined (10 animals X 4 trials each), a significantly lower percentage of the SR-treated group reached a breakpoint as when compared to the pair fed the pair-fed group, $p < 0.05$

A factorial analysis of variance was used to investigate the influence of treatment on the final completed ratio, regardless of whether or not a breakpoint was reached during the session. These analyses revealed a main effect of the level of deprivation on the final completed ratio, $F(3,81) = 6.259$, $p = 0.001$. Despite the presence of a main

effect, there was no interaction between the respective treatments and final completed ratio and none of the groups differed significantly at any of the points tested (Fig. 6).

A repeated measures analysis of variance revealed a significant positive relationship between the level of deprivation and the final completed ratio for the vehicle-treated group, $F(3, 27) = 5.111$, $p < 0.01$. The effect of the varying levels of deprivation on progressive-ratio breakpoints for each group was subsequently explored with a one-way repeated measures analysis of variance followed by multiple pairwise comparisons incorporating a Bonferroni adjustment.

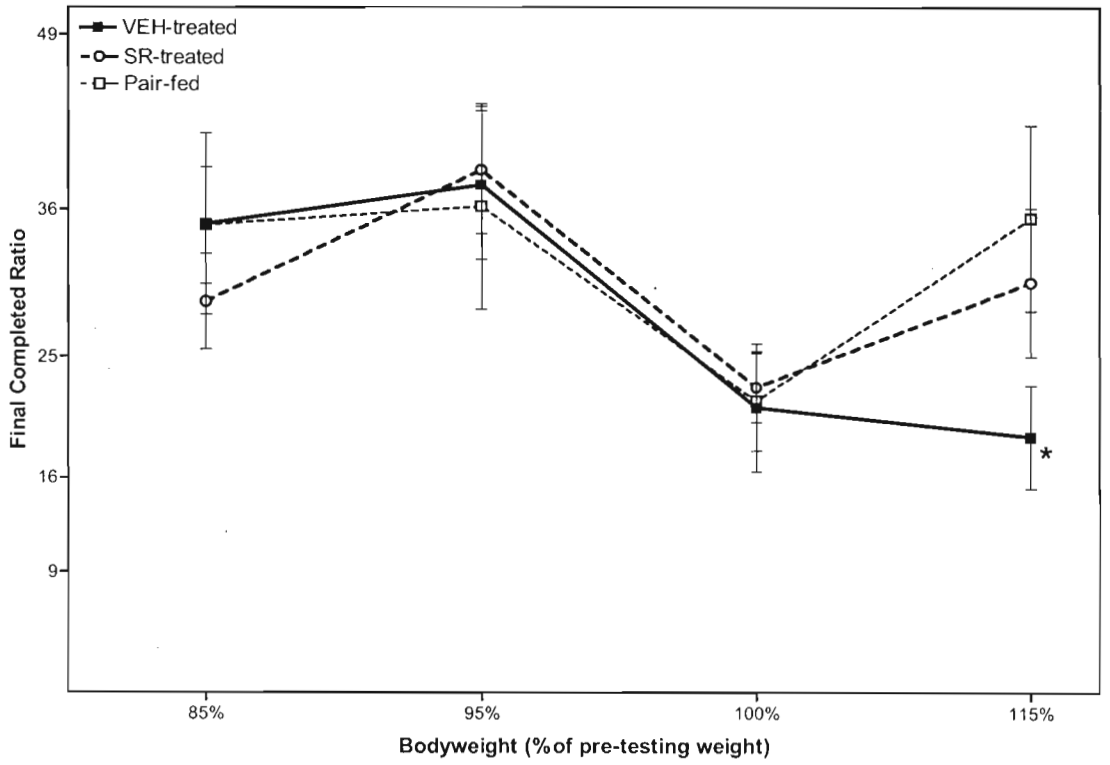

Figure 6 - The influence of treatment on the final completed ratio, regardless of whether or not a breakpoint was reached during the session. The mean of value of the final completed ratio at 95% of the pre-testing bodyweight were significantly higher than final completed ratio at 115% of the pre-testing bodyweight for the vehicle-treated group, $p < 0.01$. No significant differences were noted between any of the groups at any of the points tested.

For the vehicle-treated group, final completed ratios varied significantly as a function of the level of deprivation (Fig. 6), $F(3, 9) = 10.993$, $p < 0.01$. Pairwise comparisons revealed that the final completed ratio at 95% of the pre-testing bodyweight were significantly higher than final completed ratio at 115% of the pre-testing bodyweight. A measure of the strength of the relationship between the varying levels of deprivation and the corresponding progressive-ratio breakpoints (the effect size) was very large, $\eta^2 = 0.875$. Similar analyses on the data collected from the SR-treated and pair-fed groups revealed that none of the final completed ratios differed significantly from any other final completed ratios.

Finally, no significant differences were noted in the mean number of beams broken or the patterns of movement during the locomotion experiments (Fig. 7).

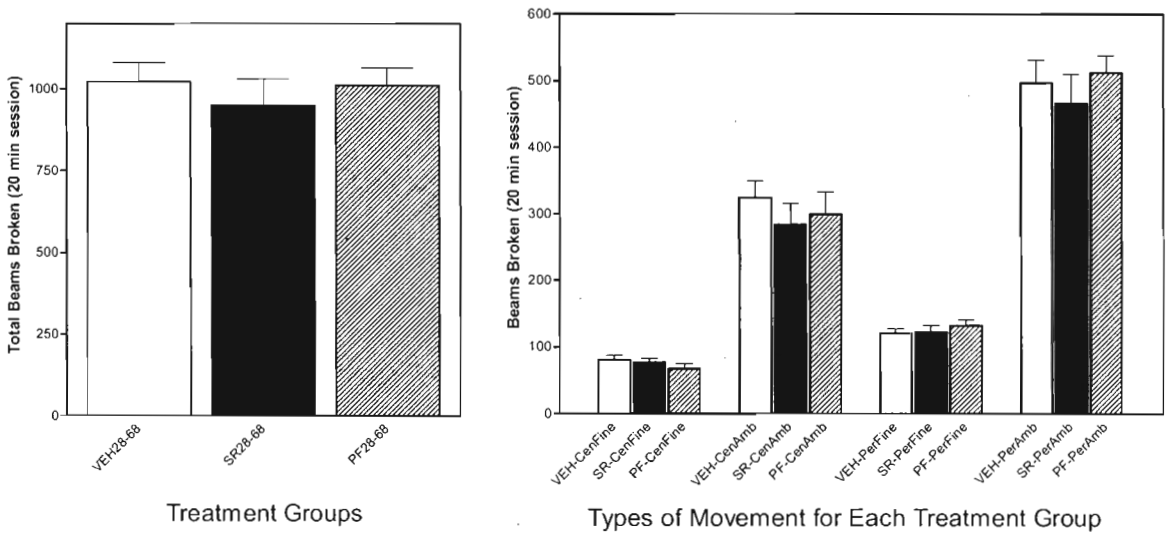

Figure 7 – The number of beams broken during the locomotion sessions and the type of movement did not vary significantly between the treatment groups. Types of movement are either small (Fine) or large (Amb) and take place around either the periphery of the chamber (Per) or in the center of the chamber (Cen). For example, "CenAmb" movements are large movements that occur near the center of the chamber and "PerFine" movements are small movements that take place around the periphery of the chamber.

Discussion

The central finding of these experiments is that the long-term consequences of maintaining adolescent rats on reduced calorie diets include uncoupling the motivation to work for a food reward from the level of food deprivation. For rats that were allowed ad libitum access to standard rodent chow during adolescence, the motivation to work for a food reward (e.g., progressive-ratio breakpoint) was significantly and positively related to their level of food deprivation. For these animals, progressive-ratio breakpoints were significantly higher when they were deprived to 85% and 95% of their pre-testing weight than breakpoints when they weighed 115% of their pre-testing bodyweight (Fig. 4).

In contrast, rats that consistently consumed fewer calories throughout adolescence displayed similar levels of motivation to work for a food reward regardless of the whether they were highly food-deprived or well-satiated. Interestingly, these differences appear regardless of whether the reduction in caloric intake was accomplished by pharmacologic manipulation of food intake (e.g., chronic CB_1 blockade) or by maintenance on restricted-calorie diets (e.g., pair-feeding).

While relationship between level of deprivation and motivation appears to have been disturbed in both the pair-fed group and the SR-treated group, this perturbation appears to be less pronounced in the pair-fed animals (Fig. 4). A repeated measures analysis of variance conducted on the breakpoint data collected from the pair-fed animals resulted in a nearly significant p-value of 0.052. Though a significant main effect of levels of deprivation on progressive-ratio breakpoints was not detected for pair-fed group, the mean progressive-ratio values clearly trended in that direction.

In contrast, the progressive-ratio breakpoints for SR-treated group changed very little as levels of deprivation increased. If progressive ratio breakpoints are accurate measures of motivation, then it can be concluded that the SR-treated group exhibited very similar levels of motivation, regardless of whether they were substantially food deprived or well-fed.

While the progressive-ratio data suggest that motivation to work for a food reward was not significantly related to the level of deprivation for the SR-treated group, other data suggest that this group may have a greater appetite for palatable food than the vehicle-treated group. When presented with a novel, palatable food, the SR-treated group ate significantly more than the vehicle treated group (Fig. 2). It is important to note that the differences between these groups may be understated by this observation because each of the SR-treated animals ate every pellet presented to them. Because the number of pellets was capped at 60, it seems possible that the experimental design may have imposed an artificial ceiling and thereby minimized the differences between these groups.

Interestingly, the SR-treated group's increased appetite for palatable food is probably not related to the hyperphagia that frequently follows dietary restriction (Ogawa *et al*, 2005). The history of caloric intake of the SR-treated group was identical to the pair-fed group and their weights were indistinguishable at the time of testing, but the pair-fed group did not eat significantly more of the palatable food than the vehicle-treated group.

The SR-treated group may also have been generally more motivated to work for a food reward, irrespective of the level of deprivation, than the pair-fed group. When data

from all the progressive-ratio sessions were combined, significantly fewer members of the SR-treated group reached a breakpoint when compared to the pair-fed group (Fig. 3). Though the response rates for the treatment groups were not significantly different, about one-third of the SR-treated group was still responding when the 30-minute progressive-ratio session ended.

Once again, it appears that the experimental conditions may have minimized the differences between the groups. If the progressive-ratio session had been longer, it seems likely that more members of the SR-treated group would have reached a breakpoint. By terminating the session before many of the SR-treated animals reached a breakpoint, the impact of the level of deprivation on progressive-ratio breakpoints may have been underestimated for this group

Because the progressive-ratio session terminated before many of the animals reached a breakpoint, it seemed logical to examine the relationship between the level of deprivation and the final completed ratio, irrespective of whether a breakpoint was achieved. For the vehicle-treated animals, the final completed ratio at 95% of the pre-testing bodyweight was significantly higher than the final completed ratio at 115% of the pre-testing bodyweight. The final completed ratios for the SR-treated and pair-fed groups were indistinguishable from each other at each of the levels of deprivation. These findings further support the conclusion that that the long-term consequences of maintaining adolescent rats on reduced calorie diets include uncoupling the motivation to work for a food reward from the level of food deprivation.

For both the SR-treated and vehicle-treated groups, response rates were significantly and positively related to the level of deprivation (Fig. 4). For those groups, response rates were highest at the greatest levels of deprivation and declined as bodyweights increased. While a main effect of level of deprivation on progressive-ratio response rates was noted, the strength of that relationship was larger for the vehicle-treated group ($\eta^2 = 0.907$) than for the SR-treated group ($\eta^2 = 0.673$). In contrast, response rates were not significantly related to the level of deprivation for the pair-fed group.

Other studies have shown that the length of time between responses in a progressive-ratio schedule declines as the reinforcing efficacy of the food reward increases (Baron *et al*, 1992). Of course, the length of time that separates responses affects the final response rate. As such, progressive-ratio response rates would be expected to be higher if the animals were working for a palatable food reward. Unfortunately, this experimental design could not exclude the possibility that chronic food-restriction preferentially alters the reinforcing efficacy of sweet foods. As such, it might be valuable to conduct a similar series of experiments that varied the reinforcing efficacy of the food reward.

Finally, it can be concluded that the differences observed in breakpoints and response rates are not likely attributable to treatment-related cognitive impairment or changes in motor activity. The possibility of such deficits or changes could not have been categorically excluded because the adolescent brain is particularly susceptible to events that produce long-term behavioral alterations. Despite this possibility, each of the

subjects used in these trials learned to lever press for a food reward at essentially the same rate and to the same degree (Fig. 3). This observation demonstrated that the treatments administered to the various groups did not differentially create a cognitive deficit that interfered with the acquisition of an operant procedure. Differential changes in motor activity were also ruled out by the data collected from the 20-minute locomotion session that followed the first progressive-ratio session (Fig. 8). In these trials, each of the groups demonstrated indistinguishable numbers of beam breaks and virtually identical types of movement (i.e., large movements around the periphery of the chamber, fine movements near center of chamber, etc.).

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Vita

Jerry Wright was born in Orlando, Florida and grew up in nearby Lakeland, Florida. Jerry's father, Mayo, was a lifelong resident of central Florida. Jerry's mother, Phyllis, was raised in Charleston, WV and moved to central Florida after high school. Jerry has one sister, Lisa, who graduated from Kathleen High School and lives in Lakeland, Florida with her husband Tracy. After attending Kathleen High School, Jerry earned an Associate of Arts degree from Polk Community College and a Bachelor of Science degree from the University of South Florida. Jerry is a member of the American Society of Pharmacology and Experimental Therapeutics as well as the International Cannabinoid Research Society. In 2005, Jerry presented his research findings at the ASPET Conference in San Diego, California and, more recently, at the 16th Annual Conference on the Cannabinoids in Tihany, Hungary. Recent publications include "Comparative effects of dextromethorphan and dextrorphan on nicotine discrimination in rats" published in *Pharmacology, Biochemistry and Behavior* and "Pharmacological effects of acute and repeated administration of Δ^9-tetrahydrocannabinol in adolescent and adult rats" published in *The Journal of Pharmacology and Experimental Therapeutics*.