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A COMPARISION OF DELTA-9-TETRAHYDROCANNABINOL DEPENDENCE IN C57Bl/6j MICE AND FATTY ACID AMIDE HYDROLASE KNOCK OUT MICE

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

by

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List of Abbreviations

AEA	anandamide
ANOVA	analysis of variance
cAMP	cyclic AMP
CB	cannabinoid
CB_1	cannabinoid receptor, subtype 1
CB_2	cannabinoid receptor, subtype 2
CL	confidence limits
CNS	central nervous system
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
EC_{50}	Effective Concentration-50
ED_{50}	Effective Dose-50
FAA	fatty acid amides
FAAH	fatty acid amide hydrolase
FDA	Food and Drug Administration
GDP	guanosine diphosphate
G-protein	guanine nucleotide regulatory protein
GTP	guanosine triphosphate
hr	hour

i.m.	intramuscular
i.p.	intraperitoneal
i.t.	intrathecal
i.v.	intravenous
kg	kilogram
KO	knockout
mg	milligram
min	minute
MPE	maximal percent effect
PKA	protein kinase A
s.c.	subcutaneous
sec (s)	second
SR	SR141716A (Rimonabant)
$t_{1/2}$	half life
THC	delta-9-tetrahydrocannabinol
µg	microgram
µl	microliter

Abstract

Title of Thesis: A COMPARISION OF DELTA-9-TETRAHYDROCANNABINOL DEPENDENCE IN C57Bl/6j MICE AND FATTY ACID AMIDE HYDROLASE KNOCK OUT MICE

By Brittany Leigh Alice Carlson, B.S.

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The idea that humans and laboratory animals can become physically dependent on marijuana or its primary psychoactive constituent, delta-9-tetrahydrocannabinol (THC), is gaining acceptance. However, there are no currently approved pharmacotherapies to treat cannabinoid withdrawal. The objective of this thesis was to evaluate whether elevating endogenous anandamide levels using mice lacking fatty acid amide hydrolase (FAAH), the

enzyme responsible for anandamide metabolism, would ameliorate THC dependence. Mice were treated subchronically with a low or high THC dosing regimen and challenged with the CB_1 receptor antagonist, rimonabant, to precipitate withdrawal. Following subchronic THC treatment, rimonabant precipitated a significant increase in paw flutters that was dependent on THC dose. However, FAAH-/- mice displayed a similar magnitude of withdrawal responses as wild type control mice, regardless of subchronic dosing regimen. Finally, rimonabant was equipotent in precipitating withdrawal responses in both genotypes. Collectively, these results demonstrate that FAAH-/- and +/+ mice show identical THC dependence, thus arguing against the notion that elevating anandamide levels through FAAH suppression will reduce cannabinoid withdrawal.

Introduction

Cannabis sativa, the Marijuana Plant

The therapeutic and recreational use of marijuana, or *Cannabis sativa,* has been noted for many centuries. Marijuana is the most widely used illicit drug in Western cultures (Pacher et al. 2006). *Cannabis sativa* is an annual plant. It can reach a height of 15 ft. depending on the region of the world it is grown. The plant is dioecious; the male reproductive components are on one plant while the female reproductive components are on another plant (Neumeyer and Shagoury, 1971). Delta-9-tetrahydrocannabinol (THC) was discovered to be the primary active constituent and is responsible for the euphoric properties produced by the plant (Gaoni and Mechoulam, 1964). THC is classified as a cannabinoid. Cannabinoids are a class of compounds that bind to one of two subtypes of receptors, named for their affinity and activation by the active constituents of the plant *Cannabis sativa* (Howlett et al, 2002). This cannabinoid is present in the stalk, leaves, flowers and seeds of the plant and also in

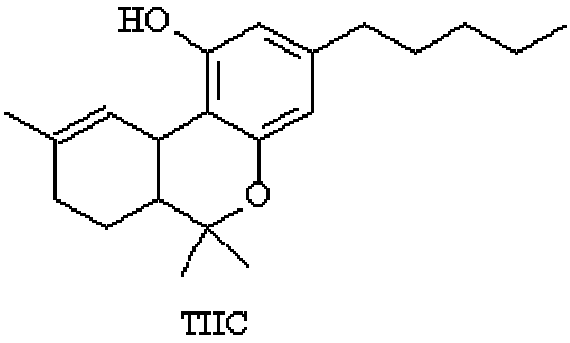

Figure 1: Chemical structure of Delta-9-tetrahydrocannabinol

the resin secreted by the female plant (Ashton, 2001). The plant contains over 420 chemical components and of those, 66 are considered cannabinoids (Turner et al., 1980).

Cannabinoid Chemical Pharmacodynamics and the Endocannabinoid System

Following the discovery of THC, some of the first evidence for the existence of cannabinoid receptors in the brain started in the 1980s with the demonstration of specific binding of CBs to a specific receptor (Devane et al., 1988). Some of this compelling evidence for a CB receptor was from structure-activity relationship studies showing that slight alterations in structure increased potency or rendered THC inactive (Little et al., 1988). The primary site of action was found to be the G protein coupled receptor CB_1 (Matsuda et al., 1990) and CB_2 receptor (Munro et al., 1993). The receptors were then cloned and research in the cannabinoid field increased (Matsuda et al. 1990). There now stands evidence of at least two difference cannabinoid receptors, CB_1 and CB_2 (Pertwee, 1999) and perhaps a third, CB_3 (Jarai et al., 1999; Breivogel et al. 2001; Zimmer et al., 2001; Zygmunt et al. 2002). There are several endogenous ligands that bind to these receptors, including anandamide (Devane et al., 1992), 2-arachidonylglycerol (2AG) (Mechoulam et al., 1995), 2-arachidonyl-glyceril-ether (Hanus et al., 2001), virodhamine (Porter et al., 2002) and N-arachidonyl-dopamine (NADA) (Huang et al. 2002). CB_1 receptors are most abundant in the brain and CB_2 receptors are found predominantly in the periphery, primarily immune and hematopoietic systems. A large amount of scientific evidence has been collected indicating that brain CB_1 receptors mediate most of the behavioral and neurochemical properties of cannabinoids. The CB_1 receptor is one of the

most abundant receptors in the CNS, having high concentrations in the cerebellum, hippocampus, striatum, globus palladum, and substantia nigra (Herkenham et al., 1991; Matsuda et al., 1993), areas which influence tolerance effects (Di Marzo et al. 2000), physical dependence (Tanda et al., 1999) and rewarding effects (Tanda et al., 1997; Tanda et al., 2000).

The endocannabinoid anandamide and its receptors reside within the neuronal lipid membranes and act as neuromodulators though intracellular G-proteins controlling cyclic adenosine monophosphate formation and Ca+ and K+ ion transport. Therefore, this system may have significant interactions with other systems and neurotransmitters including opioid and monoamines. Specifically, THC has been shown to increase the release of dopamine from the nucleus accumbens and prefrontal cortex (Tanda et al., 1997). This effect, which is common to many drugs of abuse including heroin, may contribute to reinforcing properties (Ashton 2001). Intracellular degradation of anandamide is carried out by the integral membrane protein fatty acid amide hydrolase or FAAH.

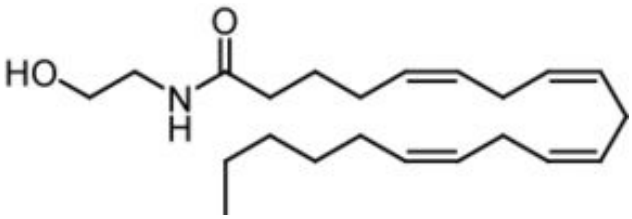

Figure 2: Chemical structure of anandamide

Pharmacokinetics of Cannabinoids

Herbal cannabis is traditionally inhaled and approximately 50% of the THC is inhaled in the smoke with the majority of this directly absorbed by the lungs (Ashton,

2001). Cannabinoids can also be taken orally, injected, transdermally or given rectally (Perlin et al., 1985; Mattes et al., 1993). THC absorption via inhalation is rapid, resulting in peak blood levels which are analogous to intravenous administration (Ohlsson et al., 1981). The bioavailability of THC is much less following oral administration than the amount absorbed from smoked marijuana. A person taking the same dose orally as one smoking will have blood concentrations of about 25-30% of the smoker, partly because of the first-pass metabolism in the liver. The onset of action is delayed to between 0.5 to 2 hours but the duration of action is prolonged because of the continuous slow absorption from the gastrointestinal tract (Ashton, 2001).

Once THC and other cannabinoids are absorbed, they distribute quickly to all other tissues depending on the blood flow. THC is exceptionally lipid soluble and can redistribute from various tissues and blood and accumulate in adipose tissue (Klausner and Dingell, 1971). In rodents, a biphasic curve has been described where blood levels decline much more quickly in the α phase and significantly slower in the β phase (Agurell et al., 1970; Lemberger et al., 1970; Lemberger et al., 1971). The half life ($t_{1/2}$) of the α phase is similar in all species studied with a $t_{1/2}$ of 30 minutes. The β phase $t_{1/2}$ is longer than the α phase and varies with different species. Human chronic marijuana users were found to have a β phase $t_{1/2}$ of 27 hours (Lemberger et al., 1970; Lemberger et al., 1971). The neocortical, limbic, sensory and motor areas reach high concentrations of THC and other cannabinoids (Ashton 2001).

Cannabinoids are metabolized in the liver. This metabolism may also contribute to some of the effects of cannabis. One of the major metabolites is 11-hydroxy-THC and

could possibly be more potent than THC. There are over 20 metabolites of THC, some are psychoactive and have very long half-lives. These metabolites are excreted in the urine (25%) but 65% are reabsorbed by the gut, prolonging their actions (Ashton 2001).

Pharmacological Effects in Laboratory Animals

Several methods have been devised to evaluate the pharmacological effects of cannabinoids on laboratory animals. Dr. Billy Martin's group was the first that routinely assessed four behavioral pharmacological indices that predict cannabinoid activity. These assays include locomotor inhibition, antinociception, ring immobility and hypothermia and have been coined the "tetrad." Locomotor inhibition examination tests motor sedation. The ring immobility assay is a method for analyzing the cataleptic effect of cannabinoids. Catalepsy is defined as the loss of voluntary motion in which the limbs remain in the position they are placed. Antinociception is measured with the tail-flick test. Changes in body temperature are measured also. While none of these behaviors is especially selective for any class of drugs, collectively these assays provide a high degree of confidence that it is in fact a cannabinoid effect.

Pharmacological Effects in Humans

In the latter part of the 19th century, many human cannabis experiments were conducted. It was only in 1967 when pure synthetic THC became available for human studies (Agurell et al., 1986). The acute pharmacological effects of marijuana in humans begins within minutes after inhaling cannabis smoke with an increase in heart rate and

relaxation and enlargment of the bronchial passages. The blood vessels in the eyes expand and make the eyes appear red (Neumeyer and Shagoury, 1971). As THC enters the brain, it acts on the reward system creating a euphoric feeling or a "high." As mentioned before, THC activates this reward system in the same way that nearly all drugs of abuse do, by stimulating brain cells to release the chemical dopamine (Chen et al., 1990; French, 1997). The cannabis user may also experience dry mouth, hunger, and drowsiness. Marijuana is also known to produce untoward effects, such as anxiety, fear, or panic.

Heavy marijuana use may inhibit a person's ability to form memories, recall events, and shift attention from one thing to another (Fletcher et al., 1996; Pope and Yurgelun-Todd, 1996). THC also binds to receptors in the cerebellum and basal ganglia, parts of the brain that regulate balance, posture, coordination of movement, and reaction time. Consequently, it is not surprising that acute marijuana intoxication upsets coordination and balance (Ameri, 1998). Individuals who have taken high doses of the drug may experience acute toxic psychosis, which includes hallucinations, delusions, and depersonalization - a loss of the sense of personal identity, or self-recognition (Graham et al. 1998; Gilman et al. 1998).

Physical Dependence in Laboratory Animals

There are two general types of dependent measures that are used to access withdrawal in laboratory animals. The first is the use of operant procedures which is described as animals that have been previously trained to emit an operant response (i.e. lever pressing) for food reinforcement will exhibit decreases in response rates during

withdrawal. The second approach is the recording the occurrence of behavioral and physiological unconditional responses. There are two types of protocols that are used to induce withdrawal in a drug-dependent animal, abstinence withdrawal and precipitated withdrawal. In the abstinence withdrawal procedure, drug administration is abruptly stopped following a long exposure to the drug. The pharmacodynamic and pharmacokinetic characteristics of the particular drug and the degree of dependence determine the intensity and onset of the specific withdrawal syndrome. The second method induces withdrawal by administration of a receptor antagonist which displaces the drug immediately causing the withdrawal effects. Again, characteristics of the particular drug and the degree of dependence play determining roles in the display of precipitated withdrawal symptoms.

The development of rimonabant and other selective CB_1 receptor antagonists has provided useful tools to investigate cannabinoid pharmacology. Rimonabant was the first selective and orally active antagonist of the brain cannabinoid receptor (Rinaldi-Carmona et al., 1994). It has been useful, not only in showing if the acute actions of an agent are mediated through a CB_1 receptor mechanism and endocannabinoid tone, but also to precipitate withdrawal symptoms following subchronic administration of cannabinoids in dogs (Lichtman et al., 1998), rats (Aceto et al., 1995; Tsou et al., 1995) and in mice (Cook et al., 1998; Rubino et al., 1998). The THC precipitated withdrawal syndrome consists of a variety of observed somatic signs mainly including front paw fluttering, “wet dog” shakes, and head shakes and some studies have included a measure of time spent hind leg

scratching. Rimonabant has been shown to precipitate THC withdrawal effects following a low dose THC regimen with only a few treatments (Cook et al., 1998).

Physical Dependence in Humans

Cannabis is not commonly viewed as an addictive drug, but there are users who find it extremely challenging to stop and there is still controversy on a withdrawal syndrome in humans. The DSM-IV does not classify cannabis withdrawal symptoms as being clinically significant and the issue of cannabis having a withdrawal syndrome is under constant controversy. Some studies have suggested that the occurrence of cannabis dependence in those individuals who have ever tried the drug varies from 10-15% (Anthony et al., 1994; Budney et al., 1999) and rates of dependence tend to increase with the frequency of use. Using DSM-IV criteria, individuals using regularly on a weekly basis over several years, have been shown to have dependence rates ranging from 57 to 92% (Swift et al., 2000). Nonetheless, the knowledge relating to the degree, formation, and consequences of dependence on cannabis is limited.

Withdrawal is a strong indicator of physiological dependence and work done by Budney et al. in 1999, Haney et al. in 1999b and Coffey et al. in 2002 show that upon cessation of prolonged cannabis use there are symptoms reported that fit the criteria of a withdrawal syndrome. Symptoms including nervousness, restlessness, sweating, and headaches have been reported. In 1999, Budney and colleagues found that people seeking treatment due to their marijuana dependence rated their withdrawal symptoms severe and reported a history of many of these symptoms during past abstinence periods. The

consistency of these detailed symptoms within the Budney et al. studies and alongside other recent studies across the field suggests that a valid marijuana withdrawal syndrome occurs in a significant number of abusers who abruptly stop using marijuana (Jones et al., 1976; Georgotas and Zeidenberg, 1979; Haney et al., 1999b). Also, the amount and magnitude of these withdrawal symptoms suggest that these effects may contribute to the development of dependence problems and may negatively manipulate attempts to stop using the drug (Budney et al., 1999). Tanda and colleagues conclude that "…marijuana has as much potential for abuse…as cocaine and heroin" (Tanda et al., 2000).

Many of the early investigations into human physical dependence on cannabis were outpatient case studies and lacked experimental controls. This made it difficult to associate the information found with cannabis use alone (Jones et al., 1981). Early experiments also revealed that cessation after frequent cannabis use showed several specific symptoms. These symptoms included autonomic symptoms, insomnia, loss of appetite and feeling "jittery" (Williams et al., 1946). When THC versus placebo was given to individuals, abstinence symptoms such as tension, anger, restlessness, insomnia and decreased appetite were observed (Bachman et al. 1979).

Latest laboratory studies have mostly supported the appearance of withdrawal symptoms from cannabis in humans. Kouri et al. (1999) showed that people who abstained from marijuana use showed more aggression and more depression over controls across a 7 day trial (Kouri et al., 1999). Haney and colleagues found that cessation after prolonged exposure to smoked THC caused a decrease in food intake, increased anxiety,

and irritability (Haney et al., 1999b). The researchers also looked at oral THC opposed to smoked and found similar results along with sleep disturbances (Haney et al., 1999a).

Biochemical Changes due to Chronic Cannabinoids

Although it is known that repeated stimulation of CB_1 receptors by THC and other cannabinoid agonists is needed to develop cannabinoid dependence, recent research is just beginning to elucidate the underlying mechanisms of action. There are many areas of interest that include analyzing changes in CB_1 receptor density, looking at the signal transduction pathway of the receptor and other neurochemical systems that have an affect on, or are affected by, these processes. Studies have used the strategy of analyzing behavioral withdrawal signs to reveal the interaction of cannabinoid dependence and neuroadaptions.

Using radioligand binding, it is generally seen that repeated treatment with a cannabinoid agonist results in a decrease in CB_1 receptor density in the brain (Romero et al., 1998; Breivogel et al., 1999). There was a significant decrease seen in the G-protein activity in many brain regions after daily injections with THC for 21 days. These regions included the hippocampus, cerebellum, caudate-putamen, globus pallidus, substantia nigra, septum and several different regions of the cortex. Not only was this region-dependent, but also this desensitization was time-dependent and looked to be specific to CB_1 receptors (Breivogel et al., 1999).

There appears to be a link between cannabinoid withdrawal and alterations in the cAMP second messenger cascade. A significant increase in basal and forskolin-stimulated

adenylyl cyclase (AC) activity in the cerebellum was seen in THC-dependent mice treated with rimonabant (Hutcheson et al., 1998). Also, higher levels of calcium-calmodulin stimulated AC were found in the cerebellum of THC-dependent rats in precipitated withdrawal than those found in non-dependent rats that were also administered rimonabant (Tzavara et al., 2000). Tzavara and colleagues also found that a cAMP analog, Sp-8Br-cAMP, actually induced these behavioral effects in vehicle treated mice. From these studies, it can be suggested that up-regulation of cAMP signal transduction in the cerebellum may represent a critical biochemical event underlying precipitated withdrawal (Lichtman and Martin, 2005).

Fatty Acid Amide Hydrolase

FAAH is a mammalian integral membrane enzyme that degrades the fatty acid family of endogenous signaling lipids including the endogenous cannabinoid anadamide. As mentioned previously, in 1992 Devane and colleagues found that anandamide was an endogenous ligand for the CB_1 cannabinoid receptor which also recognizes THC. Anandamide has also been found to bind to the CB_2 receptor (Calignano et al., 1998; Sokal et al., 2003). Anandamide can cause some of the same pharmacological effects as THC including hypothermia, analgesia and motor dysfunction (Smith et al., 1994). Other studies have shown that CB_1 (−/−) mice, as well as mice pretreated with the cannabinoid CB_1 receptor antagonist rimonabant, exhibit pharmacological effects following intravenously administered anandamide, suggesting a non-CB_1 receptor mechanism of action (Smith et al., 1994; Adams et al., 1998; Di Marzo et al., 2000). Also, a recent double knockout study

(FAAH-/- and CB_1 -/-) showed that the locomotor suppression is possibly not totally CB_1 mediated (Wise et al. 2007). The specific role that FAAH plays in the degradative process of anandamide was still unclear until the generation and characterization of a transgenic mouse model that lacked the FAAH enzyme. These FAAH-/- mice were generated by standard targeted gene disruption procedures and were viable, healthy, and fertile. Tissue samples were taken from the FAAH-/- mice and showed a 50- to 100-fold decrease in hydrolysis rates for anandamide and other fatty acid amides (FAAs), a 15-fold increase of anandamide in brain tissues (Cravatt et al., 2001), and an increase in peripheral tissues also (Weber et al. 2004). These findings indicate that FAAH is the primary enzyme responsible for the hydrolytic breakdown of these lipids in vivo. Greatly exaggerated behavioral effects were seen in the FAAH-/- mice following the administration of anandamide compared to wild-type mice. These effects included hypomotility, analgesia, hypothermia and catalepsy as shown in the tetrad assay. All of the effects of anandamide were blocked in FAAH-/- mice by rimonabant indicating that anandamide operates as a selective CB_1 ligand (Cravatt et al., 2001).

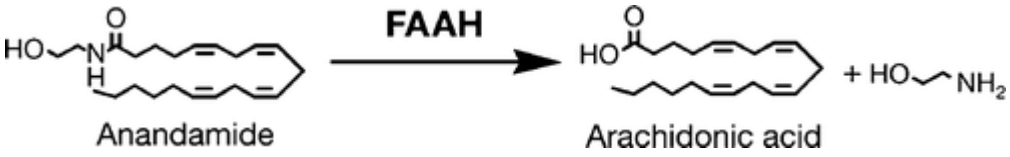

Figure 3: The breakdown of anadamide by fatty acid amide hydrolase into arachidonic acid.

Endogenous anandamide and other FAAs were found to be over 10-fold higher throughout the nervous system in the FAAH-/- mice (Cravatt et al., 2001; Clement et al.,

2003; Cravatt et al., 2004). This drastic change in brain chemistry was interrelated with a CB_1 -dependent analgesic phenotype in FAAH-/- mice that was observed in many different pain sensation assays (Cravatt et al., 2001; Lichtman et al., 2004b). Thus, FAAH appears to set an endocannabinoid tone that regulates pain perception in the nervous system. In the periphery, studies have shown that this enzyme may control other undetermined FAA-signaling pathways that modulate inflammation (Richardson et al., 1998; Cravatt et al., 2004; Lichtman et al., 2004a; Lichtman et al., 2004b). The FAAH-/- mice display no defects in motility, weight, or body temperature, therefore "indicating that the inactivation of FAAH produces a provocative subset of the behavioral effects caused by direct CB_1 agonists" (McKinney and Cravatt, 2005). This pattern of results suggests that FAAH may represent a therapeutic target for the treatment of pain and inflammatory disorders and has stimulated interest in the development of specific inhibitors of this enzyme.

The objective of this thesis is to elucidate whether endogenous cannabinoid levels play a role in THC dependence. Previous work has shown that FAAH-/- mice have normal CB_1 receptor numbers and functions (Lichtman et al., 2002; Cravatt et al., 2004). However, with much higher endogenous cannabinoid levels, we might conclude that FAAH-/- mice would show less withdrawal signs as they are able to produce elevated cannabinoid levels. Therefore, it is hypothesized that because FAAH-/- mice are able to maintain increased levels of anadamide, they will show less THC withdrawal signs. This effect may be seen because the endocannabinoid system may generate anandamide to

occupy CB_1 receptors and to compete with rimonabant while going through rimonabant-precipitated withdrawal.

In order to test this hypothesis, I have examined the expression of THC dependence in FAAH +/+ mice and FAAH-/- mice. I compared the degree of withdrawal effects in both genotypes using a low and high THC dosing regimen and examined the rimonabant dose-response curve on precipitated withdrawal to assess whether its potency is affected by genotype.

Methods

Subjects

Male and female mice at least 8 weeks of age were used in all studies. No significant sex differences were observed in any of the following studies. FAAH (-/-) mice were derived from congenic FAAH (-/-) breeding pairs (12^{th} generation back-cross on a C57BL/6J (Jackson Laboratory, Bar Harbor, ME) background). The FAAH (+/+) mice were derived from FAAH (+/+) breeding pairs, which were the offspring of the 11^{th} generation FAAH (+/-) breeding pairs on the C57BL/6J background. Mice were housed 2 - 4 per cage and maintained on a 12 hr light/dark cycle with food and water available *ad libitum*. All studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Drugs

THC and SR141716 (rimonabant) were provided by the National Institute on Drug Abuse (Bethesda, MD). All drugs were dissolved in a 1:1 mixture of absolute ethanol and alkamuls-620 (Rhone-Poulenc, Princeton, NJ) and diluted with saline to a final ratio of 1:1:18 (ethanol:alkamuls:saline). The ethanol:alkamuls:saline combination was used as the vehicle treatment. THC injections were administered s.c. at an injection volume of 10 ml/kg. Rimonabant was administered i.p. at an injection volume of 10ml/kg.

Experimental Apparatus

The observation apparatus for monitoring and data collection was an 8" x 8" closed cage, with the floor, ceiling, and side walls composed of a white acrylic plastic cube. The cube was lowered into a larger clear acrylic enclosure through which a webcam (Fire-i firewire color webcam, Unibrain, San Ramon, CA) recorded the contents of the entire box. The rear of the box contained a mirror insert that allowed the reflection to be seen by the recording webcam that was connected to a computer running the AnyMaze software (Stoelting, Wood Dale, IL). Accordingly, the observer was able to watch and record behavior without disturbing the test subject. This observation chamber was kept inside a sound attenuating chamber. Confounds related to odor were addressed by cleaning both the maze and escape box with an ammonia based cleaner Whistle (Johnson Diversey, Inc., Sturtevant, WI) after each trial. Subjects were recorded for a 30 min acclimation period, followed by a one hr withdrawal period. The mouse was given its last subchronic injection and immediately placed in the observation box to acclimate to the environment. Thirty min later, the mouse was given an i.p. injection of vehicle or rimonabant and placed back into the observation box to record withdrawal for one hr. These video recordings were then scored using a key pad for the following measures: paw flutter, head twitch and hind leg scratching. These three behaviors were used after preliminary studies showed these to be the most significant.

Dependence protocol

Mice were treated with a low THC dosing regimen protocol or a high THC dosing regimen protocol. In the high dose regimen, mice were subcutaneously injected with THC (50 mg/kg) or vehicle twice each day for 5.5 days. Low dose regimen followed the same protocol except mice were given THC (10 mg/kg) once each day. On the sixth day, mice were brought up to the lab and received their final treatment in the morning and placed in the test recording boxes to acclimate. Thirty minutes after the final treatment the mice were administered vehicle or 10 mg/kg rimonabant and were placed back into the recording boxes. They were recorded on the AnyMaze system during acclimation and after administration of vehicle or rimonabant. Time sampling was used in counting the number of observed paw flutters, head twitches and hind leg scratching for a total of an hour. Past experiments showed that the high dosing regimen did not show any differences between FAAH+/+ mice and FAAH-/- mice, therefore only FAAH+/+ mice were used in the inter-rater-reliability experiment. A group that was subchronically administered THC and given a challenge dose of vehicle was not used because several studies have shown there is no abrupt withdrawal these mice (Cook et al., 1998; Lichtman et al., 2001a; Lichtman et al., 2001b).

Evaluation of the dose-response relationship of rimonabant in precipitating withdrawal

The high dosing regimen was followed for the analysis of the effective dose of rimonabant. Both FAAH +/+ and FAAH-/- mice were tested in groups of 5-6 split into

different challenge dose groups. The challenge doses included vehicle or rimonabant (1, 3, or 10 mg/kg). The maximal dose of 10 mg/kg was used because of the limit of solubility. The testing procedures followed that of the high and low dosing regimen as explained in the preceding paragraph.

Scoring Protocol

The three behaviors of interest included paw flutters, head twitches and hind leg scratching. These three behaviors were focused on because past studies showed these to be the most significant and readily able to score. Paw Tremors: the visible shaking of one or both paws simultaneously in a manner inconsistent with normal motions and movements of the paw, and manifests itself during the lifting of one or both paws. Head Twitching: the quick, successive movement of the head (at least twice in opposite directions) in a counterclockwise/clockwise fashion rather than a normal ordinal direction. This is usually followed by an immediate righting to the original position. Hind Leg Scratching: the rapid movement of a hind paw over a region on the topside of the animal (usually near whiskers, behind the ear, or rear end) in a repeated scratching/grooming motion. Generally paired in rapid succession with hind paw licking.

The mice were videotaped for over a 60 min period in 5-minute bins separated by 5-min break periods. A time sampling was used to score the number of observed paw flutters and headshakes for a total of an hour. The time sample intervals were 5-10 minutes, 15-20, 25-30, 35-40, 45-50 and 55-60 minutes following rimonabant challenge.

Thus, observations were recorded during the following intervals following rimonabant administration 5-10, 15-20, 25-30, 35-40, 45-50, and 55-60 minutes.

Statistical analysis

A multifactorial ANOVA was conducted as appropriate to evaluate the effects using Statview for Windows version 5 (SAS Institute, Minneapolis, MN, USA). The two common factors of these experiments were genotype and treatment. Significant ANOVA analyses were followed by a unifactorial or multifactorial Tukey/Kramer post-hoc and/or a Dunnett's post-hoc analyses. All differences were considered significantly different at $p<0.05$.

To determine potency of rimonabant in eliciting paw fluttering, the data were transformed to maximum percent effect (MPE) by taking the total response for each specific animal and dividing it by the Emax (i.e., average of the 10 mg/kg rimonabant dosing group) and multiplying this value by 100. ED50 values for rimonabant were then determined by least-squares linear regression followed by calculation of 95% confidence limits (Bliss, 1967). All differences were considered significantly different at $p<0.05$.

Results

Experiment 1: Replication of Somatic Rimonabant-Precipitated Withdrawal in THC-Dependent Mice

It was important to assess the ability of the experimenter to recognize, properly track and reliably reproduce past effects using the same methods as previous work. To ensure that there was adequate inter-rater-reliability, a preliminary study was conducted in which another experienced investigator and the author independently scored a set of data. In this pilot study, C57Bl/6j mice were treated using the high dosing regimen described in the Methods section to establish THC dependence. The mice were then challenged with rimonabant (10 mg/kg) to elicit strong somatic signs of THC-precipitated withdrawal. The mice were videotaped during the withdrawal session using the AnyMaze System described in the Methods section. Both experimenters independently viewed and scored the tapes. A Pearson correlation coefficient was then calculated to determine the consistency between the experimenters.

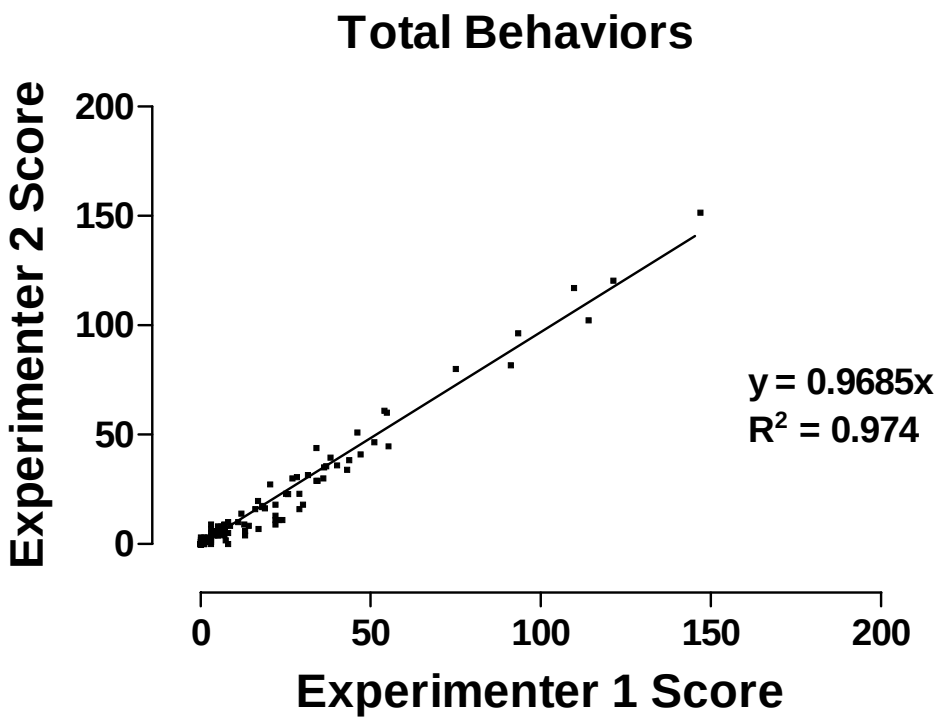

Figure 4: Correlation of all somatic behaviors measured between experimenter 1 and present experimenter (2). The r value was found to be a strong correlation of 0.99 with a slope of 0.97.

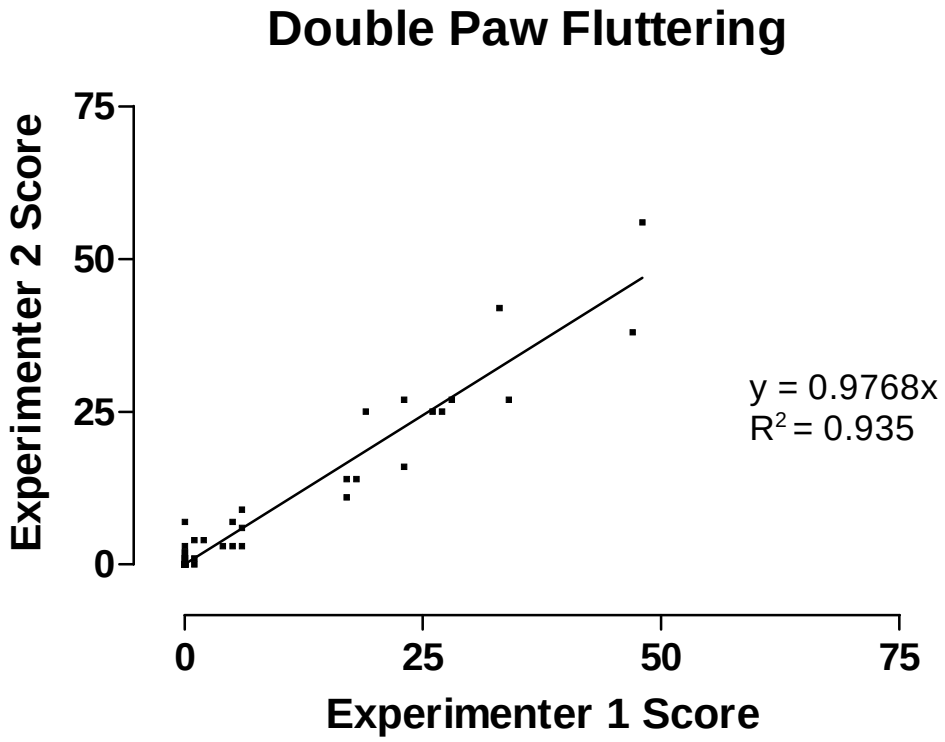

Figure 5: The number of incidents in which mice simultaneously fluttered both arms was scored as described in the Methods section. A strong correlation of double paw flutters between experimenter 1 and present experimenter (2) was found with r =0.97 with a slope of 0.98.

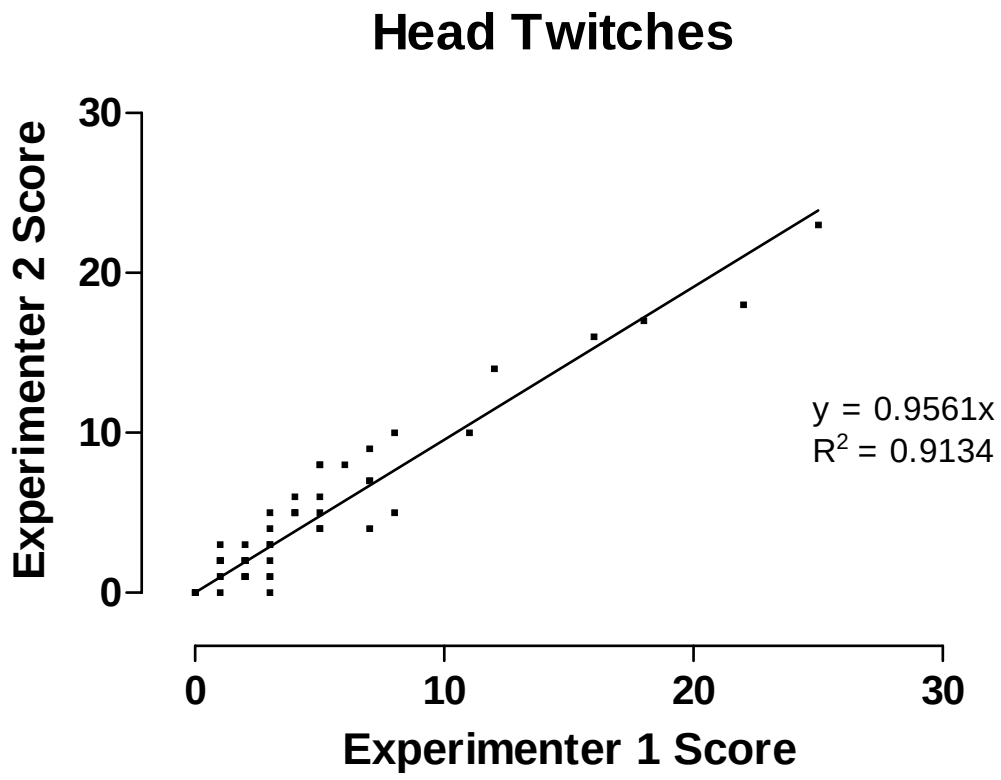

Figure 6: The number of incidents in which head twitches occurred was scored as described in the Methods section. Correlation value of head twitch behavior between experimenter 1 and experimenter 2 was found to be r =0.95 with a slope of 0.96.

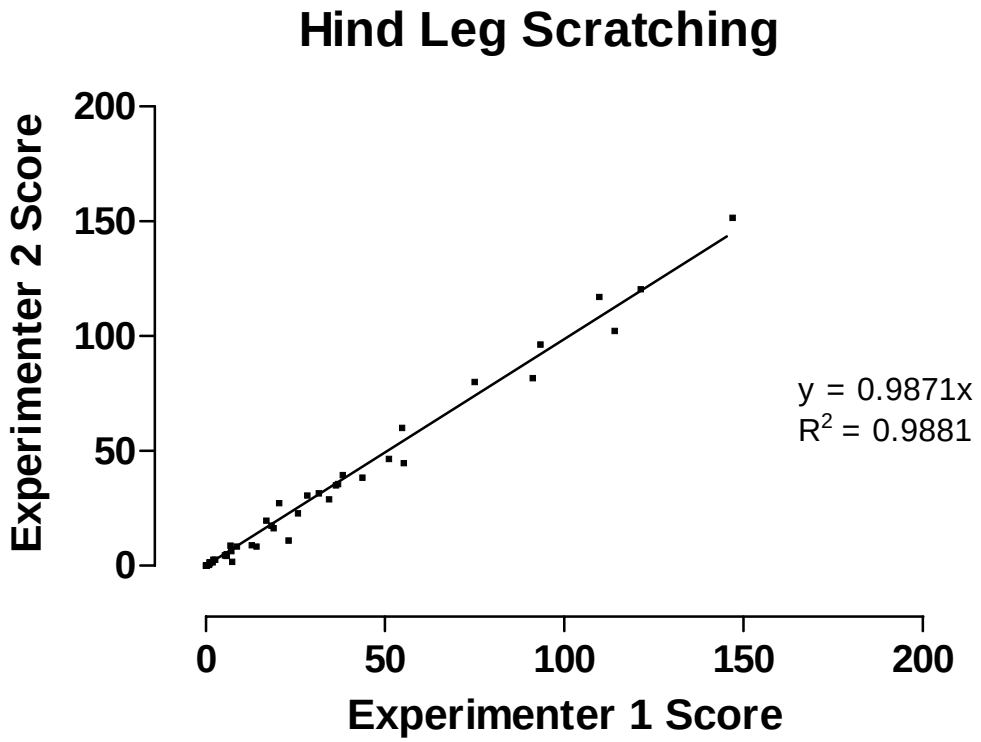

Figure 7: The amount of time that the mice spent scratching with their hind leg scratching was recorded in seconds (s). Correlation of hind leg scratching behavior between experimenter 1 and present experimenter (2) was found to be r = 0.99 with a slope of 0.99.

For all behaviors combined, the correlation between experimenter 1 and experimenter 2 is shown in figure 4 and was found to be 0.99 with a slope of 0.97. For the behavioral measure of double paw flutters during withdrawal, the correlation was found to be 0.97 with a slope of 0.98 shown in figure 5. Figure 6 shows the correlation for head twitches and was found to be 0.95 with a slope of 0.96. The hind leg scratching correlation is shown in figure 7 and was found to be 0.99 with a slope of 0.99. For the inter-rater-reliability experiment, a sample size of 6 mice was used for all measures. The results from this high dosing regimen show strong somatic signs of THC precipitated withdrawal in C57Bl/6j mice and consistently the correlation between experimenters was close to 1 and the slope was approaching 1.

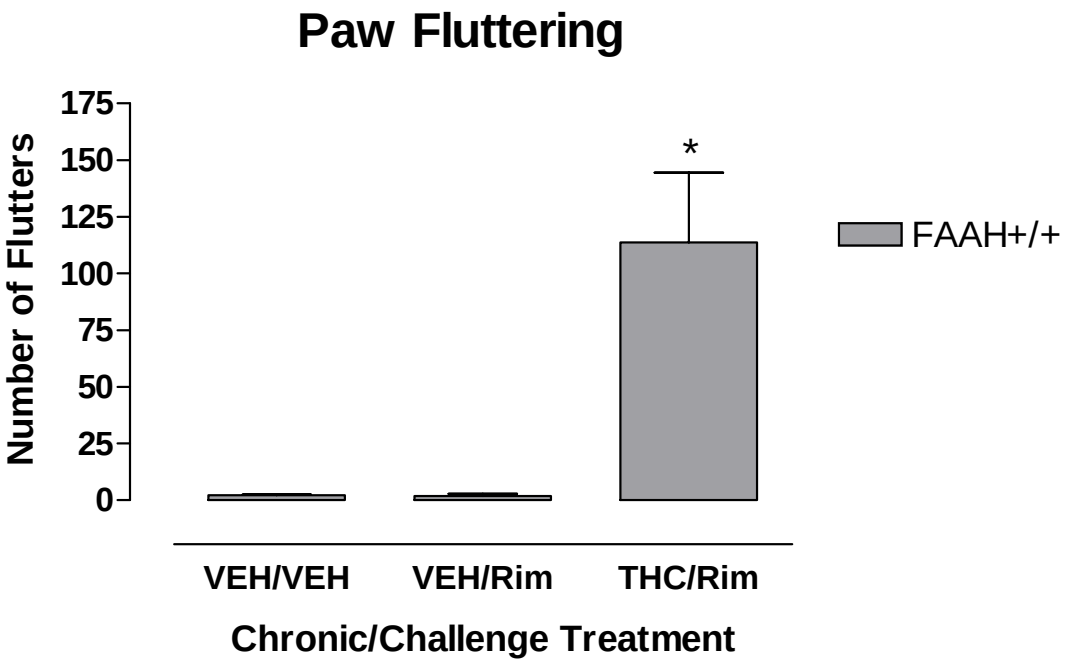

Figure 8: Rimonabant (Rim) precipitates an increase of paw flutters in THC-dependent mice. Mice were given vehicle or 50 mg/kg THC twice a day for 5.5 days and administered vehicle (VEH) or 10 mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (VEH or THC) were given s.c., acute injections (rimonabant) were given i.p. *= $p<.05$ versus vehicle, N=6 mice/group.

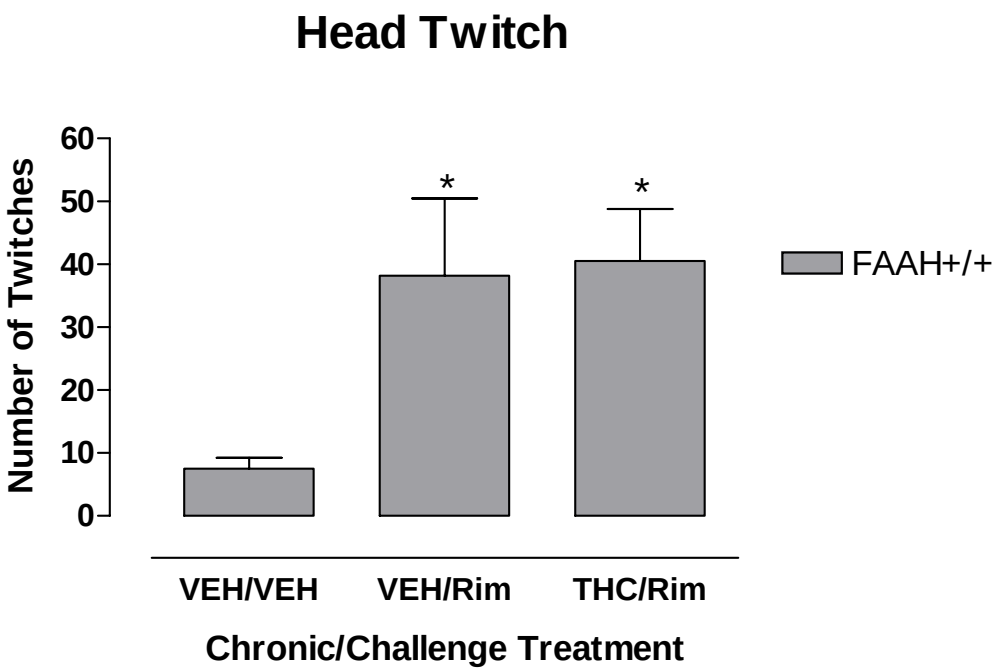

Figure 9: Rimonabant (Rim) precipitates an increase in head twitching behavior. Mice were given vehicle or 50 mg/kg THC twice a day for 5.5 days and administered vehicle or 10mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (veh or THC) were given s.c., acute injections (rimonabant) were given i.p. *= $p<.05$ versus vehicle, N=6 mice/group.

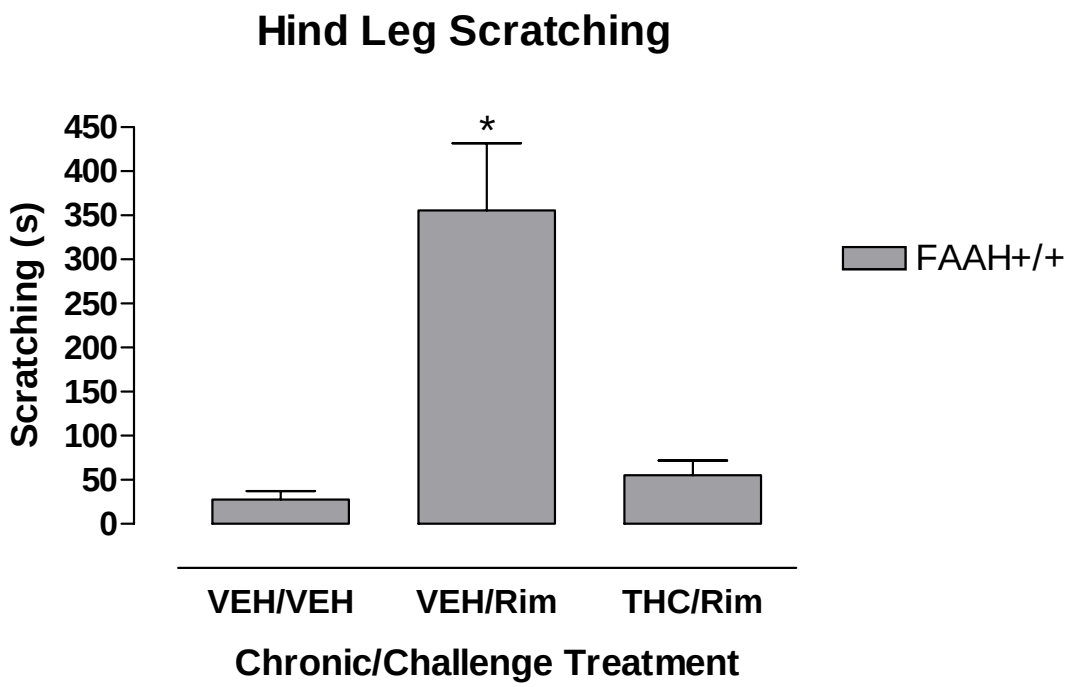

Figure 10: Rimonabant (Rim) precipitated an increase in hind leg scratching in mice treated chronically with vehicle. Mice were given vehicle or 50 mg/kg THC twice a day for 5.5 days and administered vehicle or 10 mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (veh or THC) were given s.c., acute injections (rimonabant) were given i.p. *= $p<0.05$ versus vehicle, N=6 mice/group.

From the data presented, it can be seen that rimonabant elicited a complex pattern of somatic signs across the three dependent measures. Rimonabant precipitated a significant increase in paw flutter in mice assigned to the high THC dosing regimen [$F(2,15)=13$, $p<.001$]. However, rimonabant elicited significant increases in head twitching regardless of subchronic treatment [$F(2,15)=5$, $p<.05$]. Lastly, figure 10 shows that rimonabant elicited a significant increase in hind leg scratching in mice that had been given repeated injections of vehicle, but not in mice that had received THC [$F(2,15)=16$, $p<.001$]. Rimonabant precipitated an increase of paw flutters in THC-dependent mice but not mice subchronically treated with vehicle. Rimonabant precipitated an increase in head twitching behavior irrespective of subchronic THC treatment. Rimonabant elicited an increase in hind leg scratching in mice treated subchronically with vehicle but not THC treated mice.

Experiment 2: Somatic THC Withdrawal in FAAH+/+ Mice vs. FAAH-/- Mice Using a Low Dose Regimen

Based on pilot work in our laboratory, we employed a low to moderate THC dosing regimen described in the Methods section. A sample size of 8-12 mice/group was used. It was found that rimonabant challenge in mice assigned to this moderate THC dosing regimen, precipitated significant increases in paw fluttering.

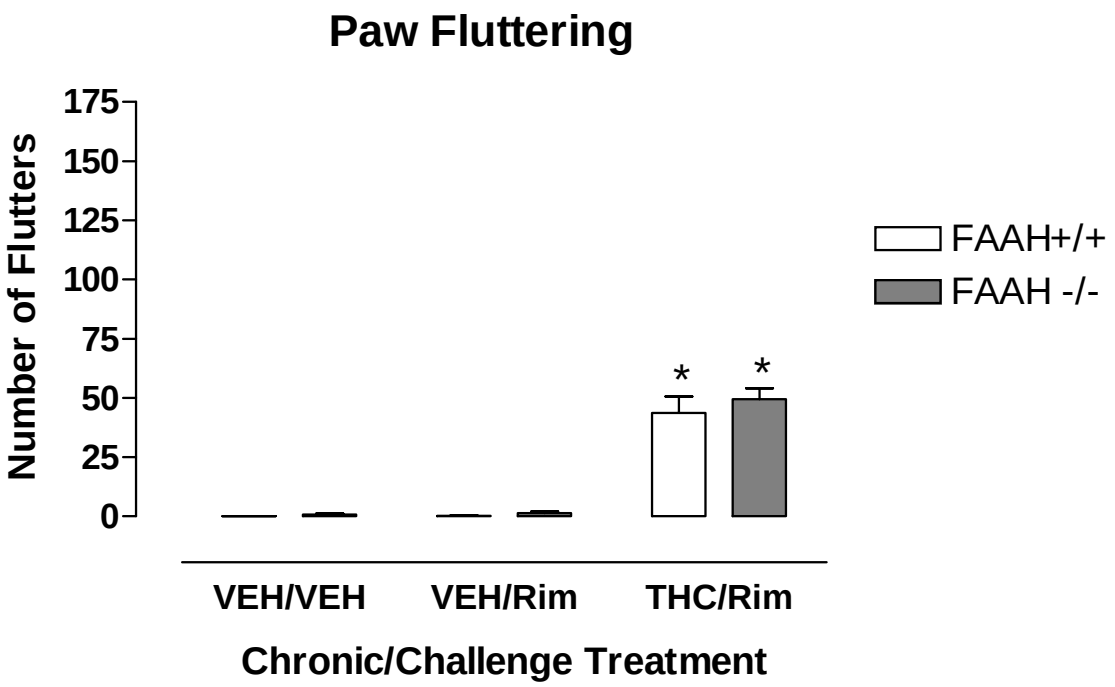

Figure 11: Rimonabant (Rim) precipitated an increase in paw fluttering behavior in mice given a moderate subchronic THC dosing regimen. Mice were given vehicle or 10mg/kg THC once a day for 5.5 days and administered vehicle or 10mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (veh or THC) were given s.c., acute injections (rimonabant) were given i.p. *= $p<.05$ versus VEH/VEH and VEH/Rim. Mice chronically treated with THC, followed by acute rimonabant, showed a significant increase in paw fluttering but no significant difference was found between genotypes. N=6 mice/group. Open bars represent FAAH+/+ mice and closed represent FAAH-/- mice.

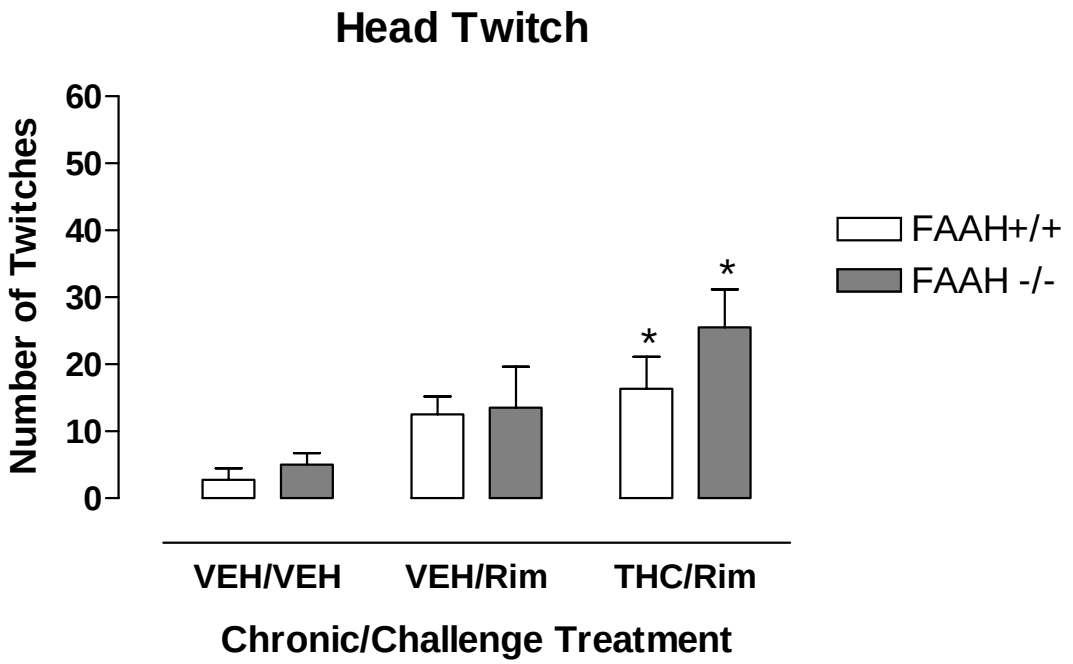

Figure 12: Rimonabant (Rim) precipitated an increase in number of head twitches in THC-dependent mice. Mice were given vehicle or 10mg/kg THC once a day for 5.5 days and administered vehicle or 10mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (veh or THC) were given s.c., acute injections (rimonabant) were given i.p. *= $p<.05$ versus VEH/VEH. Mice chronically treated with THC, followed by acute rimonabant, showed a significant increase in head twitches but no significant difference was found between genotypes. N=6 mice/group. Open bars represent FAAH+/+ mice and closed represent FAAH-/- mice.

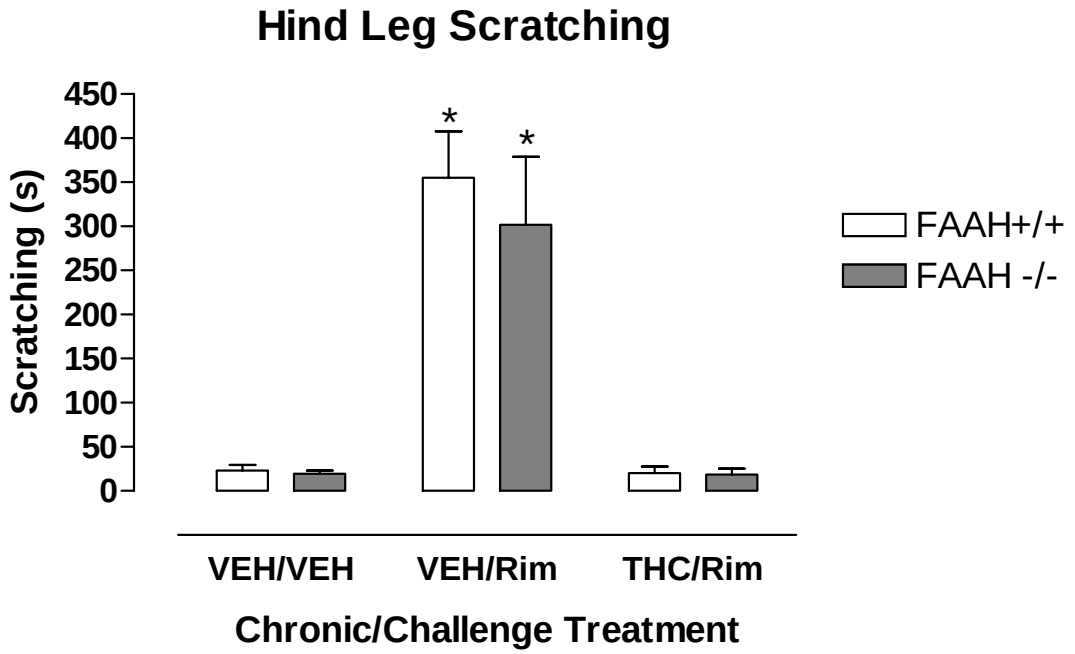

Figure 13: Rimonabant (Rim) precipitated an increase in hind leg scratching in chronically vehicle treated mice. Mice were given vehicle or 10mg/kg THC once a day for 5.5 days and administered vehicle or 10mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (veh or THC) were given s.c., acute injections (rimonabant) were given i.p. *= $p<.05$ versus VEH/VEH and THC/Rim. Mice chronically treated with vehicle followed by acute rimonabant showed a significant increase in hind leg scratching but no significant difference was found between genotypes. N=6 mice/group. Open bars represent FAAH+/+ mice and closed represent FAAH-/- mice.

Analysis of data collected in experiment 2 shows a significant treatment effect [$F(2,26)=98$, $p<.0001$] but no significant difference was found between genotypes [$F(1,26)=1$, $p=.4$] in paw fluttering (figure 11). No interaction was found [$F(2,26)=.3$, $p=.8$]. A Tukey post-hoc test was run and found that a significant increase in paw flutters occurs in the THC/Rim group as compared to the Veh/Rim and Veh/Veh groups. Figure 12 shows the data collected for head twitches and a significant treatment effect was found [$F(2,26)=6$, $p=.01$] but no significant difference was found between genotypes [$F(1,26)=1$, $p=.3$]. A Tukey post-hoc test was run and found that a significant increase in head twitches occurs in the THC/Rim group as compared to the Veh/Veh group. There was no significant difference between the Veh/Rim and THC/Rim groups. A significant treatment effect was found with hind leg scratching behavior [$F(2,26)=35$, $p<.0001$] but no significant difference was found between genotypes [$F(1,26)=.3$, $p=.6$] as shown in figure 13. A Tukey post-hoc test was run and found that a significant increase in hind leg scratching occurs in the THC/Rim group as compared to both the Veh/Rim and Veh/Veh groups.

Experiment 3: Evaluation of the Dose-Response Relationship of Rimonabant in Precipitating Withdrawal in FAAH+/+ Mice vs. FAAH-/- Mice

The purpose of this study was to determine whether rimonabant shows a difference in potency in eliciting withdrawal responses between FAAH+/+ mice and FAAH -/- mice. In order to address this issue, both genotypes were placed through the high dosing regimen

described in the methods section. Thirty min after their final THC injection, each mouse was given an injection of vehicle or rimonabant (1, 3, or 10 mg/kg) with 5 to 6 mice/group

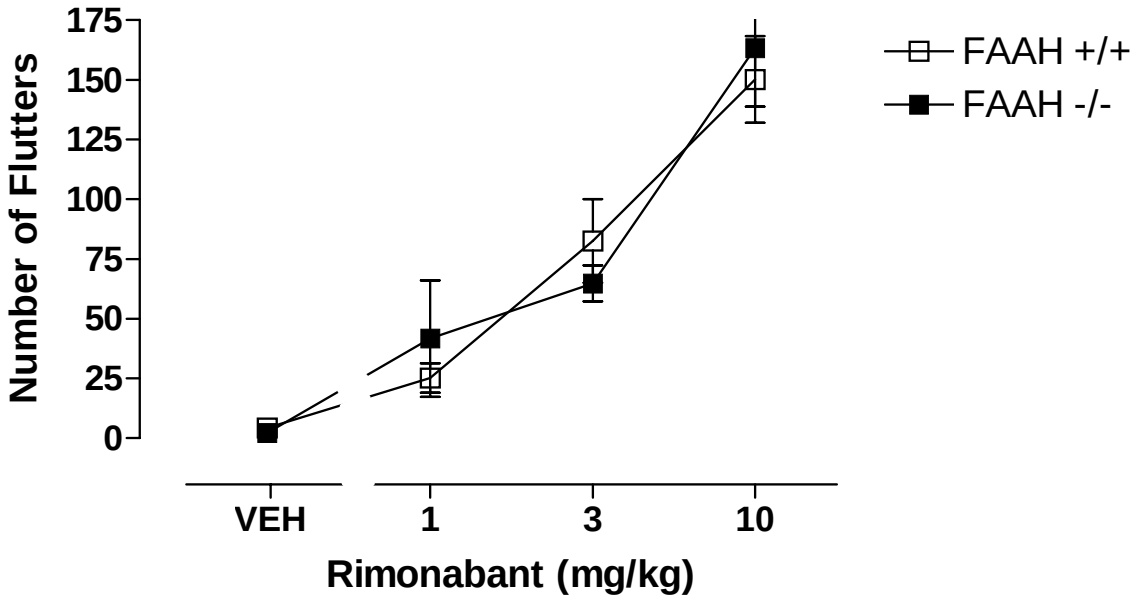

Figure 14: Rimonabant precipitates a dose-related increase in paw fluttering in FAAH-/- mice and FAAH+/+ mice. Mice were given 50 mg/kg THC twice a day for 5.5 days and administered vehicle, 1mg/kg, 3mg/kg or 10mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (THC) were given s.c., acute injections (rimonabant) were given i.p. N=5-6 mice/group. Open symbols represent FAAH+/+ mice and closed represent FAAH-/- mice.

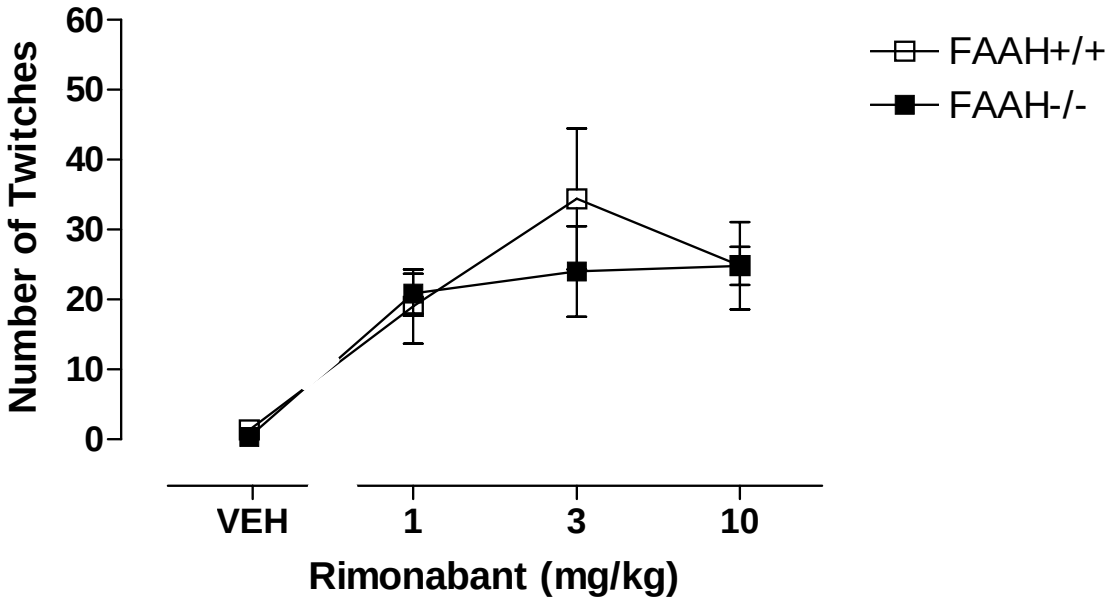

Figure 15: Rimonabant precipitates a significant increase in head twitches in FAAH-/- mice at all doses and in FAAH+/+ mice at 3mg/kg and 10mg/kg. Mice were given 50mg/kg THC twice a day for 5.5 days and administered vehicle, 1mg/kg, 3mg/kg or 10mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (THC) were given s.c., acute injections (rimonabant) were given i.p. N=5-6 mice/group. Open symbols represent FAAH+/+ mice and closed represent FAAH-/- mice.

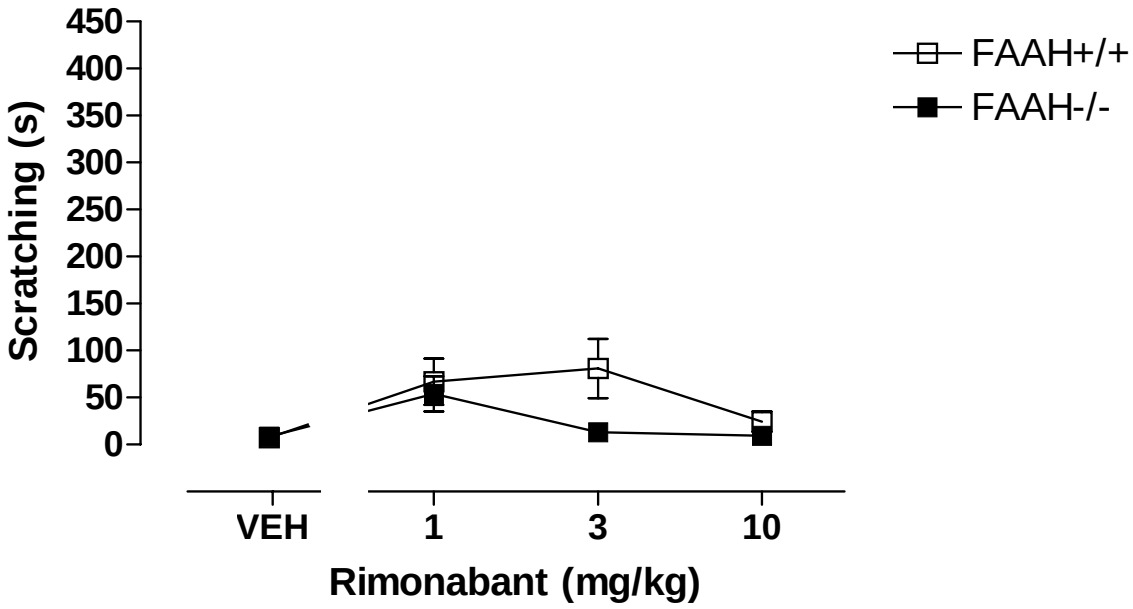

Figure 16: Rimonabant precipitates a significant increase in hind leg scratching at 1mg/kg. Mice were given 50mg/kg THC twice a day for 5.5 days and administered vehicle, 1mg/kg, 3mg/kg or 10mg/kg rimonabant to precipitate withdrawal on day 6. Repeated injections (THC) were given s.c., acute injections (rimonabant) were given i.p. N=5-6 mice/group. Open symbols represent FAAH+/+ mice and closed represent FAAH-/- mice.

Analysis of the range of rimonabant doses shows that for THC withdrawal symptom of paw fluttering, a treatment effect was found [$F(3,37)=38$, $p<.0001$]. There was no significant difference found between genotypes [$F(1,37)=.1$, $p=.8$] and no interaction was found [$F(3,37)=.5$, $p=.7$]. A Dunnett's post-hoc test revealed a significant treatment effect at 3mg/kg and 10mg/kg compared to vehicle. Head twitching data were analyzed and a significant treatment effect was found [$F(3,37)=12$, $p<.0001$]. No significant differences between genotypes [$F(1,37)=.5$, $p=.5$] and no interaction was found [$F(3,37)=.6$, $p=.7$]. A Dunnett's post-hoc test was run showing a significant increase in head twitching at all rimonabant doses when compared to vehicle. A significant treatment effect was also found for hind leg scratching [$F(3,37)=5$, $p<0.01$]. No interaction was found [$F(3,37)=2$, $p=.2$]. Although the statistical analysis shows a significant genotype effect in the hind leg scratching behavior [$F(1,37)=4$, $p<.05$], upon closer inspection and comparison to the other studies, it appears that the magnitude indicates that it is not relevant. The data shows that this genotype difference occurs at a single intermediate dose. A Dunnett's post-hoc test was run showing a significant increase in hind leg scratching only at 1mg/kg compared to vehicle.

From the most significant THC withdrawal behavior, paw fluttering, FAAH+/+ mice have an ED_{50} of 2.9 mg/kg (95% CI 2 to 4 mg/kg) and FAAH-/- mice have an ED_{50} of 2.9 mg/kg (95% CI 2 to 5 mg/kg). The potency ratio was found to be 1 for both genotypes. These findings suggest that there are no differences in the expression on THC withdrawal symptoms between FAAH+/+ mice and FAAH-/- mice and that rimonabant is equipotent.

Discussion

The main objective of this thesis was to investigate if mice lacking fatty acid amide hydrolase, the main enzyme to break down anandamide, had a different pattern of THC dependence. It was hypothesized that because FAAH-/- mice possessed high levels of anandamide, that they would show less THC withdrawal symptoms than wild type mice. Work provided in this thesis could possibly provide proof of principle as to whether elevating endogenous anandamide levels would make effective treatment targets for cannabinoid dependence.

The experiment investigating the ED_{50} of rimonabant in the FAAH +/+ mice and FAAH-/- mice was carried out to investigate if the elevated anadamide levels in FAAH-/- mice could possibly attenuate or prevent the appearance of withdrawal effects under a more mild cannabinoid blockade and withdrawal by creating a rightward shift in the rimonabant dose-response curve. While not necessarily physiologically relevant, the high dosing regimen was chosen for the ED_{50} study because the previous experiments have shown that the behavioral signal decreases with decreased chronic dosing, therefore the high dosing regimen would allow for greater sensitivity to detect differences in effects. From the paw fluttering THC withdrawal behavior studied here, FAAH+/+ mice have an ED50 of 2.9 mg/kg (95% CI 2 to 4 mg/kg) and FAAH-/- mice have an ED50 of 2.9 mg/kg

(95% CI 2 to 5 mg/kg). Even with the deletion of the enzyme FAAH, the potency ratio was found to be 1 for both genotypes.

From the high dosing regimen, the results show a significant increase in paw fluttering compared to the acutely treated vehicle or rimonabant groups. The same is seen in the low dosing regimen with no differences in genotype. Previous work using the same high dosing regimen produced a similar pattern of results with no differences in genotype (Thorpe et al. 2006). From the results showing no significant difference in paw fluttering between FAAH +/+ mice and FAAH-/- mice, it can be concluded that the hypothesis that FAAH-/- mice will express less THC withdrawal symptoms than FAAH +/+ mice cannot be supported.

It is unclear if head twitching represents a precipitated withdrawal sign in FAAH+/+ and FAAH-/- mice from the data collected for this thesis. In the high dose regimen it was seen that along with THC-dependent animals, vehicle-treated mice also displayed a significant increase in the head twitching behavior, but the low dosing regimen does not show a significant increase in head twitching in the vehicle treated animals. From these data, it appears that this measure is dependent on the dose in the chronic treatment. Others have also reported that rimonabant given alone elicits head twitching. Cook et al. (1998) reported that THC-dependent mice exhibited a significant increase in head twitching upon challenge with rimonabant, but these twitches were also elevated in control mice receiving an injection of rimonabant similar to our data. Likewise, Hutcheson et al. (1998) found that rimonabant elicited increases in twitching in both vehicle treated mice and cannabinoid treated mice. Finally, in another study that used a shortened THC dosing

regimen, rimonabant elicited a dose-dependent increase in head twitching regardless of THC treatment (Lichtman et al., 2001a). In general, studies in which head twitching was indicative of withdrawal used higher dosing regimens (Cook et al., 1998; Hutcheson et al., 1998). Thus, high doses of THC may result in the recruitment of other withdrawal behaviors in humans and laboratory animals.

Our data show that the lowest dose to elicit a significant magnitude of hind leg scratching behavior is at a rimonabant dose of 1mg/kg only. At a rimonabant dose of 10mg/kg, the somatic THC withdrawal signs are predominantly characterized by paw fluttering. From these studies, mice placed through precipitated THC-withdrawal show high level of fluttering and do not show the same level of scratching behavior as those mice receiving rimonabant alone. In the present study, low doses of rimonabant produced a slight (but significant) elevation in hind leg scratching behavior in both FAAH+/+ and FAAH-/- THC-dependent mice. Alone, rimonabant has been reported to elicit scratching in rats (Aceto et al., 1996) and mice (Cook et al., 1998) and has also been reported to have other behavioral actions such as anxiety-like responses in the defensive withdrawal test and elevated plus-maze (Navarro et al., 1997), increased locomotor activity (Compton et al., 1996), hyperalgesia (Richardson et al., 1998), anorexic effects (Di Marzo et al., 2001), and memory enhancing effects (Terranova et al., 1996). From the results reported in this thesis along with these studies, it can be concluded that hind leg scratching cannot be designated as a THC-withdrawal effect and is a rimonabant effect.

As a consequence of precipitating a severe and stable withdrawal effect at high THC doses, there were several notable incidences of lethal convulsant events.

Approximately 10% of the entire group of laboratory animals run at a high dosing regimen died before sacrificing was done at the end of the last group of mice run on that particular testing day. All animals died within two hours of high dose rimonabant precipitated withdrawal. These animals displayed severe convulsions immediately before death. Research on the convulsant activity of cannabinoids and in FAAH-/- mice has been mixed (Karler et al., 1974; Clement et al., 2003) and within this study we found similar chances of convulsing across the genotypes.

Other work has shown that the degree of physical dependence, as measured by precipitated increases in paw flutters, was affected by the dose of THC and dosing schedule as this thesis data shows. In one study, rimonabant precipitated approximately 40–55 paw tremors during a 45-min observation period in mice given 10 to 20 mg/kg THC twice a day for 6 days (Hutcheson et al., 1998). In another study, rimonabant given to mice injected with 10 mg/kg THC twice a day for 6.5 day led to approximately 100 paw tremors during a 30-min period (Cook et al., 1998). In yet another study, mice dosed with THC 10 mg/kg twice a day for only 2.5 days and challenged with rimonabant exhibited between 30 and 45 paw tremors (Lichtman et al., 2001a). Compared to the data presented in this thesis, these data indicate that with a higher frequency of low to moderate THC dosing there is an elevated level of paw fluttering withdrawal behaviors similar to those found for the high dosing regimen in the experiments carried out.

The severe regimen used here may have been so severe that the changes in endocannabinoid levels may be masked by the high levels of THC. Therefore, the low dosing regimen was utilized to simulate a physiologically relevant dose of THC as

compared to humans. Inhalation is the usual route of administration in humans and a cannabis cigarette may contain from 10mg of THC but if laced with hashish oil can reach 300mg (approximately between .14 to 4.3 mg/kg for a 70kg individual). About 50% of this amount of THC is inhaled in mainstream smoke and almost all of this THC is absorbed through the lungs (Ashton 2001). While the low dosing regimen employed in the present study may be considered high on the human scale, it has been estimated that on a body weight basis, humans are generally more vulnerable to chemicals than are mice by a factor of 10 (Eaton and Klaassen, 1996).

When looking at antinociception and oral administration of THC, (Noyes et al., 1975) and colleagues found that in humans the ED50=0.24 mg/kg and (Cichewicz and McCarthy, 2003) found that in mice the ED50= 89.4 mg/kg. Our low to moderate dosing (10mg/kg) regimen represents a dose that is consistently lower than the behavioral ED_{50}s of most studies of acute THC effects in mice (Thorpe et al. 2006; (Cravatt et al., 2001). Although mice usually require substantially higher doses of cannabinoids than humans to achieve analogous effects, the fact that cannabinoids elicit similar pharmacological effects, such as motor suppression, increased feeding, antinociception, memory deficits, and tachycardia in both species, supports the utility of the mouse model (Howlett et al., 2002). Along with the fact that humans are more vulnerable to chemicals, Wilson et al. (2006) found that the induction of THC physical dependence, as characterized by rimonabant-precipitated increases in paw fluttering, was related to the amount of marijuana smoke or intravenous THC to which the mice were exposed. One of the most important, and fascinating, observations in the studies presented here was that the same behavioral effects

were seen in the animal groups in the low-dose regimen as were seen in the high dosing regimen, just to a lower degree. Degree of dependence relates to the chronic dosing regimen and is dose dependent.

As mentioned in the Methods section, the maximum dose of rimonabant was 10 mg/kg and this was chosen because of the drug's limits of solubility. Also, (Compton et al., 1996) and colleagues found rimonabant had a direct effect on locomotor activity at doses higher than 3 mg/kg and found that rimonabant had an ED_{50} value of 4.7 (+/- 1.5) mg/kg. This study showed that rimonabant has significant effects at lower doses and supports 10 mg/kg rimonabant as the maximal dose. The rimonabant effects in relation to precipitated withdrawal were shown to be blocked by THC and worked regardless of the THC treatment in our studies.

Although there are two specific procedures to induce states of withdrawal in a drug-dependent animal, abstinence withdrawal and precipitated withdrawal, only precipitated withdrawal using the selective CB_1 antagonist rimonabant was studied here because abstinence withdrawal presents many difficulties with THC having a long half life and a low incident of behavior when trying to quantify the withdrawal symptoms. The selective CB_1 antagonist, rimonabant, was developed and was found in rodents to block the centrally mediated effects of cannabinoids and also block tachycardia and the subjective effects of smoked marijuana in humans (Huestis et al., 2001). Unlike humans where subjective effects can be obtained verbally, laboratory animals present more of a challenge. The long half life of THC and delay of effects contribute to the difficulty in studying withdrawal in non-human animals (Lichtman and Martin 2006). Accordingly, studies on

abstinence withdrawal in laboratory non-human animals following chronic cannabinoid administration has led to varied results. (Kaymakcalan and Deneau, 1972) observed a variety of behavioral effects including tremors, anorexia and hyperirritability. Others have been unsuccessful in observing any withdrawal effects following chronic THC administration in dogs or rats (Leite and Carlini, 1974).

Many human studies have used reports of cannabis abstinence withdrawal syndrome came from outpatient studies and the lack of experimental control made it difficult to associate the results with cannabis use alone and criticism falls on the methodological weaknesses, procedural differences, degree of consistency and degree of control in abstinence THC-withdrawal studies (Smith, 2002). On the other hand, studies on precipitated cannabis withdrawal have shown that many laboratory animals including mice, rats and dogs chronically administered cannabinoid agonists have quantifiable somatic withdrawal symptoms immediately following administration of rimonabant (Lichtman and Martin, 2006). These precipitated withdrawal studies are relevant to human withdrawal because they show that there are withdrawal syndromes across many species. Studies have shown that the CB_1 receptor is highly evolutionarily conserved with 97-99% amino acid sequence identity across species in rat, mouse, and in humans (Howlett et al., 2002). The conservation of this receptor suggests that there is a high likelihood that the cellular adaptations and pharmacological mechanisms are similar (Howlett et al., 2002).

Repeated stimulation of the CB_1 receptors is required for the development of cannabinoid dependence. Studies over the past decade have determined that CB_1 receptors undergo downregulation (i.e., decrease in number of receptors) and desensitization (i.e., G-

protein uncoupling) following chronic administration of THC or synthetic cannabinoid agonists. The adaptations are regionally widespread and of substantial magnitude. These adaptations are thought to influence tolerance to cannabinoid-mediated behavioral effects. It appears that alterations in cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activity may be particularly important in cannabinoid dependence but has been difficult to characterize (Sim-Selley, 2003). This thesis was done in part to determine the ultimate cannabis dependence pathway. Both pharmacokinetic and pharmacodynamic characteristics contribute to the specific withdrawal symptoms, intensity and onset. It is important to investigate the mechanisms of THC dependence and withdrawal syndrome because human participants in THC-withdrawal studies have described the feeling as an unpleasant experience. Uncovering the mechanisms of THC withdrawal in humans may facilitate the development of treatment for those individuals who may fear these unpleasant experiences, which are defined as anxiety, irritability, depression, physical tension and physical discomfort, when trying to discontinue using the drug (Jones and Benowitz, 1976; Jones et al., 1976; Haney et al., 1999b).

Although these pharmacological effects might reflect the blockade of endogenous cannabinoid tone, the responses may also be due to intrinsic effects of rimonabant. Studies have confirmed that cannabinoid agonists stimulate Gi/o-protein activity (Sim et al., 1996; Burkey et al., 1997) but conversely, *in vitro* experiments have suggested that rimonabant actually works as an inverse agonist at the receptor, producing the opposite biochemical effects of the agonist causing a decrease in G-protein activity in many cell types (Landsman et al., 1997; Pan et al., 1998). The relevancy of this inverse agonism in

animals has not yet been determined, but it has been confirmed that rimonabant is 7000-times more potent as an CB_1 receptor antagonist than as an inverse agonist (Sim-Selley et al., 2001). With this in mind, appropriate controls for the effect of rimonabant alone were used throughout these experiments. Taking these results into consideration, it remains to be determined if rimonabant elicits scratching through inverse agonism, action at some other site than the CB_1 receptor, or by inhibition of endogenous cannabinoid activity

Even though *Cannabis sativa* derivatives, such as marijuana and hashish, are the most widely used illicit substances in the world, some believe that it has medicinal value. Although oral THC (Marinol®) is available, inhalation has advantages with its rapid onset, allows the patient to titrate the dose and circumvents the liver's first-pass effect (Ohlsson et al., 1980; Barnett et al., 1982; Chiang and Barnett, 1984; Johansson et al., 1987; Huestis et al., 1992; Cone and Huestis, 1993). In 2006, Johnston and colleagues reported 42.3% of 12^{th} graders have a lifetime prevalence of marijuana use. With this large percentage of young users, more and more are seeking treatment for cannabis related issues and there is a growing amount of literature showing a clinically relevant marijuana withdrawal syndrome (Budney et al., 1999).

The DSM-IV does not include a cannabis withdrawal syndrome and states that "symptoms of cannabis withdrawal…have been described…but their clinical significance is uncertain" (p.235) and this issue of cannabis having a withdrawal syndrome is under constant controversy. There is however a growing body of evidence with past and recent studies that indicate that cannabis-dependent individuals experience a significant physical withdrawal syndrome following the cessation of marijuana smoking (Lichtman and Martin,

2006). Human studies done by several groups have shown that following abrupt cessation from chronic THC, users show symptoms of anxiety, irritability, decrease in appetite, depression, physical tension and physical discomfort (Jones and Benowitz, 1976; Jones et al., 1976).

Information presented in this thesis should contribute to the body of knowledge on the pharmacology of THC and anandamide and the investigation of FAAH. We show that even with the dramatic increase in anandamide in FAAH-/- mice, there are no differences in expression of THC dependence from FAAH+/+ mice. We also show that the FAAH KO mice that were administered vehicle subchronically, then treated with a challenge dose of rimonabant, did not go through precipitated withdrawal showing that the elevated levels of anandamide was not enough to produce withdrawal in FAAH-/- mice. We show that the precipitated withdrawal effects are influenced by dose and regimen. The observation that rimonabant can precipitate withdrawal effects following a low dosing regimen of THC raises concern that even a few consecutive days of recreational or therapeutic cannabinoid use could lead to the development of physical dependence in humans. Finally, the potency of rimonabant to precipitate paw fluttering is not influenced by the FAAH genotype.

The lack of genotype differences between FAAH (-/-) and wild type mice suggests that the elevated levels of anandamide in FAAH -/- have insufficient influence to prevent THC-withdrawal. The results from these studies suggest for additional investigation into studies comparing the cannabinoid activity of anandamide to THC. One question that arises from these results is the efficacy and potency of anandamide to attenuate withdrawal symptoms. THC has been shown to dose-dependently reverse rimonabant-precipitated

symptoms in animal models of precipitated THC-withdrawal (Wilson et al. 2006). While the question of the underlying mechanisms of precipitated withdrawal remains unanswered, the present study indicates that FAAH does not play a substantial role. From the studies showing that THC interferes with the cAMP signaling pathway, cAMP levels could be investigated. Hutcheson and colleagues found that after removing brains from THC-dependent mice administered rimonabant to precipitate withdrawal, there was an increase in basal, forskolin and calcium/calmodulin stimulated adenylyl cyclase activities observed in the cerebellum (1998). Perhaps a cAMP rebound effect drives the severe somatic behavioral symptoms by causing a dramatic increase in exciting the signaling path.

The work completed for this thesis contributed to the scientific field by showing that despite the elevated anandamide levels, FAAH-/- mice show normal responses to exogenous cannabinoids. The results of the present study suggest that FAAH inhibitors may not be sufficient to provide therapeutic efficacy in the treatment of cannabinoid withdrawal, but FAAH inhibitor studies should be conducted because FAAH -/- mice, which have had none of the enzyme throughout ontogeny, may have developed compensatory systems. Therefore, FAAH inhibitor studies may show the prevention of the expression of rimonabant-precipitated withdrawal.

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