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Endocannabinoid Modulation of Spatial Memory in Aversively and Appetitively Motivated Barnes Maze Tasks

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ENDOCANNABINOID MODULATION OF SPATIAL MEMORY IN AVERSIVELY AND APPETITIVELY MOTIVATED BARNES MAZE TASKS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University Medical Center.

by

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Then there's the one who struggled with me since day one, when she was 3 months old:

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LIST OF ABBREVIATIONS

2-AG: 2-arachidonoylglycerol
AEA: Arachadonylethanolamine or anandamide
ANOVA: Analysis of variance
cAMP: Cyclic adenosine monophosphate
CB_1: Cannabinoid receptor subtype 1
CB_1 -/-: Transgenic animal devoid of CB_1 receptors
CB_1+/+: Wild-type control animal with no genetic alterations
CB_2: Cannabinoid receptor subtype 2
CNS: Central nervous system
CS: Conditioned stimulus
DAG: Diacylglycerol
DMTS: Delayed match to sample
DNMS: Delayed non-match to sample
eCB: Endocannabinoid
FAAH: Fatty acid amide hydrolase
FAAH -/-: Transgenic animal devoid of the FAAH gene
FAAH +/+: Wild-type control animal with no genetic alterations
GAD: Generalized anxiety disorder
GPCR: G-protein coupled receptor
ITI: Intertrial interval
MAGL: Monoacylglycerol lipase
NAE: *N*-acyl ethanolamines
NAPE: *N*-arachidonoyl-phosphatidylethanolamine
NAT: *N*-acyl transferase
PA: Phosphatidic acid
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PI: Phosphoinositide
PLC: Phospholipase C
PLD: Phospholipase D
PMSF: Phenylmethylsulfonyl fluoride
PTSD: Posttraumatic stress disorder
RIM: Rimonabant
SEM: Standard error of the mean
THC: Δ^9 Tetrahydrocannabinol
US: Unconditioned stimulus

ABSTRACT

ENDOCANNABINOID MODULATION OF SPATIAL MEMORY IN AVERSIVELY AND APPETITIVELY MOTIVATED BARNES MAZE TASKS

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A Dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy, at Virginia Commonwealth University

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Genetic deletion or pharmacological blockade of the CB_1 receptor has been reported to impair extinction learning in aversive conditioning (i.e., conditioned fear and Morris water maze) paradigms, but not in operant procedures in which food reinforcement is earned. It is difficult to discern whether the differential effects caused by CB_1 receptor disruption on extinction result from the hedonics (i.e., aversive vs. appetitive) or is related to the required responses associated with these disparate tasks. In order to evaluate whether the hedonics is the determining factor, we used either aversive

(i.e., escape from bright lights and air turbulence) or appetitive (i.e., to gain access to water) motivators in the Barnes maze task, a model in which mice are required to enter a hidden goal box. Administration of the CB_1 receptor antagonist, rimonabant, disrupted extinction learning under aversive conditions, but not under appetitive conditions. This is the first study to show a differential effect of rimonabant on extinction in a task that required identical motor behaviors, but only differed in hedonic nature of the reinforcer. In addition, genetic ablation of CB_1 receptor signaling impaired acquisition of the task under both aversive and appetitive conditioning procedures. Conversely, enhancing endocannabinoid signaling, via genetic deletion of the FAAH enzyme, accelerated acquisition of the task under aversive, but not appetitive, conditioning procedures. Accordingly, these data strongly support the hypothesis that the endogenous cannabinoid system plays a necessary role in the extinction of aversively motivated behaviors, but is expendable in appetitively motivated behaviors. While these findings underscore concerns over potential side effects associated with CB_1 receptor antagonists, they also suggest that stimulating the endogenous cannabinoid system may be a pharmacological approach to treat maladaptive behaviors that arise from stress or trauma.

INTRODUCTION

THE ENDOCANNABINOID SYSTEM

> "Numerous observers have described the Indian hemp as producing in the natives of the East, who familiarly use it instead of intoxicating spirits, sometimes a heavy, lazy state of agreeable reverie, from which the individual may be easily roused to discharge of any simple duty – sometimes a cheerful, active state of inebriation causing him to dance, sing and laugh, provoking the venereal appetitive, and increasing the desire for food – and sometimes a quarrelsome drunkenness, leading to acts of violence. During this condition pain is assuaged and spasm arrested….On the whole, it is a remedy which deserves a more extensive inquiry than any hitherto instituted." (Christison, 1848)

Documented use of marijuana as a therapeutic spans thousands of years, treating an unparalleled range of general [e.g. pain, edema, migraines] and specific [e.g. infantile convulsions, hemorrhoids, malaria, sexual dysfunction] afflictions. In spite of its intriguing history of medical use, European physicians such as O'Shaughnessy, Christison, and Aubert-Roche began documenting side effects, such as cognitive disruption, subjective effects, and anxiety (Christison, 1848). Eventually, these reports would shift public opinion of marijuana to that of an intoxicant following the 20th century. This shift in public attitude regarding marijuana, from therapeutic to intoxicant, would eventually spread around the world, and in the United States, resulted in the federal anti-marihuana act of 1937 and its classification as a controlled substance

(National Commission on Marihuana and Drug Abuse, 1972). Despite its legal status being restricted in most western countries, the prolonged debate over potential medical utilization has continued. Inexplicably, the therapeutic potential of manipulating the system marijuana activates, as an alternative to cannabis use, rarely enters the public debate.

The discovery of delta-9-tetrahydrocannabinol (THC) as the primary active constituent of marijuana (Gaoni and Mechoulam, 1964) opened the door for scientific investigation into what is currently referred to as the endocannabinoid system. The system is comprised of THC's primary sites of action, the CB_1 (Matsuda *et al.*, 1990) and CB_2 receptors (Munro *et al.*, 1993), as well as THC's endogenous counterparts, the endocannabinoids. This class of THC-like compounds, distinguished by their activity at the CB_1 and CB_2 receptors, include anandamide [AEA] (Devane *et al.*, 1992), 2-arachidonoylglycerol [2-AG] (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995), noladin ether (Hanus *et al.*, 2001), virodhamine (Porter *et al.*, 2002), and *N*-arachidonoyldopamine (Huang *et al.*, 2002). Enzymes responsible for the inactivation of the two primary endocannabinoids, anandamide and 2-AG, are respectively fatty acid amide hydrolase [FAAH] (Cravatt *et al.*, 1996) and monoacylglycerol lipase [MAGL] (Dinh *et al.*, 2002).

Unlike the vast majority of traditional neurotransmitters (e.g., dopamine, acetylcholine), endocannabinoids such as anandamide and 2-AG are not stored in vesicles, but are synthesized and released 'on demand' from membrane bound precursors (Di Marzo *et al.*, 1994). The biosynthesis of anandamide occurs through multiple

pathways, common to each is the formation of the precursor *N*-arachidonoyl-phosphatidylethanolamine (NAPE). The enzyme *N*-acyl transferase (NAT), catalyzes the transfer of arachidonic acid from phosphatidylcholine (PC) to phosphatidylethanolamine (PE), resulting in the formation of NAPE. Previous research in rat cortical neurons has implicated the involvement of two intracellular second messengers controlling NAT activity: Ca^{2+} and cyclic AMP (cAMP). While cAMP is thought to enhance the activity of NAT via protein kinase A-dependent phosphorylation, evidence for calcium-dependence is supported by the observation that NAT is inactive in the absence of Ca^{2+} (Cadas *et al.*, 1996). Three different pathways have been identified through which NAPE is converted into AEA. First, PLC catalyzes the formation of the intermediary p-AEA, which is subsequently converted into AEA by phosphatases. Alternatively, anandamide can be produced by NAPE-PLD. However, this pathway appears to be a 'salvage pathway,' utilized when the PLC/phosphatase pathway is compromised (Schmid *et al.*, 1990; Cadas *et al.*, 1997; Sugiura *et al.*, 2002; Sugiura and Waku, 2002; Leung *et al.*, 2006; Liu *et al.*, 2006; Gomez-Ruiz *et al.*, 2007; Liu *et al.*, 2008). Finally, conversion of NAPE, by $sPLA_2$/Abhd4, to lysoNAPE, which can be subsequently converted into AEA by Lyso-PLD. Inactivation of anandamide is primary mediated by the enzyme FAAH, producing arachidonic acid and ethanolamine. However, evidence also exists for cyclo-oxygenase-2, and lipo-oxygenase as enzymatic regulators of anandamide degredation (Kozak and Marnett, 2002).

The biosynthesis of 2-AG is thought to occur through two pathways, distinguished by the formation of diacylglycerol (DAG). In the first, phospholipase C (PLC) hydrolyzes phosphoinositides (PI) to produce DAG (Stella *et al.*, 1997). Alternatively, phosphatidic acid (PA) phosphohydrolase can hydrolyze PA to synthesize DAG (Bisogno *et al.*, 1999). Common to both pathways, DAG lipase then catalyzes DAG hydrolysis to form the endocannabinoid 2-AG (Farooqui *et al.*, 1989; Bisogno *et al.*, 2003). Mediating the metabolism of 2-AG are the enzymes monoacylglycerol lipase (MGL) (Konrad *et al.*, 1994), ABHD12, ABHD6 (Blankman *et al.*, 2007), and FAAH (Di Marzo *et al.*, 1998). Collectively, MGL, ABHD12, and ABHD6 account for ~98% of 2-AG hydrolysis [respectively: ~85%, 9% and 4%], indicating a negligible role for FAAH (Blankman *et al.*, 2007). Irrespective of the degrading enzyme, arachidonic acid and glycerol are the products of 2-AG degradation (Di Marzo *et al.*, 1999). Together, these receptors, endogenous agonists, and the enzymes regulating them, comprise the endocannabinoid system.

The CB_1 receptor is one of the most abundant receptors in the CNS, exhibiting its highest densities in the cerebellum, hippocampus, striatum, globus palladum, and substantia nigra (Herkenham *et al.*, 1991; Matsuda *et al.*, 1993). The CB_1 receptor, and the predominantly peripheral CB_2, belong to the seven trans-membrane receptor family of G-protein-coupled receptors (GPCR). Interestingly, the CB_1 receptor is the most abundant GPCR found in the brain (Howlett *et al.*, 1990; Herkenham *et al.*, 1991). Further classification of cannabinoid receptors puts them in the family of inhibitory

GPCRs known as $G_{i/o}$. Activation of the cannabinoid receptors results in a cascade of effects, beginning with the inhibition of adenylyl cyclase, subsequent decrease in cAMP, and ensuing decrease in protein kinase A dependent phosphorylation (Devane *et al.*, 1988). Additionally, activation can produce inhibition of calcium through N (Mackie and Hille, 1992), L (Gebremedhin *et al.*, 1999), and P/Q type ion channels (Twitchell *et al.*, 1997) as well as activation of inwardly rectifying K channels. Collectively, the effects serve to decrease neurotransmitter release, primarily through presynaptic inhibitory processes (Mackie *et al.*, 1995).

CANNABINOIDS AND COGNITION

The past 30 years have seen great strides in our understanding of how CB_1 agonists affect learning and memory. However, these advances have not been easy, often requiring novel approaches to address various inconsistencies or uncertainties. Moreover, factors such as cognitive terminology hamper the interpretation and extrapolation of data. For example, the terms learning, memory, performance, and cognition are all related and at times employed interchangeably. Across academic fields, however, these terms can have different definitions and connotations and can be devided into numerous different cognitive processes. Further classification of memory includes working (short-term) and reference (long-term) memory. A third category, recognition memory, is based on an animal's inherent desire to explore unfamiliar objects, scents, or conspecifics (Wotjak, 2005). The inherent difficulty then, is that the investigator must separate these different facets of cognition when designing an experiment to determine, and accurately report, which aspect is affected.

To clarify the terminology used in this dissertation, memory is defined as changes in an animal's behavior some time after learning, and involves processes of acquisition, consolidation and retrieval. Memory acquisition is defined as a learned response. For

example, acquisition in contextual conditioned fear involves the association between a context, and a shock. During consolidation, learned information is encoded into memory by alterations in interneuronal communication, moving the memory from a labile, to a more fixed state (Abel and Lattal, 2001). The persistence in these changes can then be categorized by short-term and long-term memory based on the duration of memory retention, and biosynthetic changes in memory consolidation (McGaugh, 2000). Upon reintroduction into the conditioning context, the previously learned association (memory) is retrieved, and results in behavioral alterations. In the example of contextual fear conditioning, reintroduction into the conditioning context results in the retrieval of memory associating prior experience in the context with shock, and is behaviorally manifested as freezing behavior. The persistence of a memory is limited by either its decay, or the inability to retrieve it. Conversely, the original memory may be actively suppressed by an inhibitory learning process known as extinction learning (Wotjak, 2005).

While this is somewhat of a superficial overview of learning, it serves to introduce a greater problem facing behavioral research. Varvel & Lichtman (2005) provide a precise summary:

> "…learning and memory is not directly measured, but is inferred based on changes in performance. In particular alterations in attentional, sensorimotor, and motivational processes can affect performance, independently of cognition."

Perhaps the greatest issue for the researcher is the inescapable reliance on interpreting performance as a manifestation of learning. Bouton & Moody (2004)

expertly discuss these issues in an enlightening review. Almost immediately, the authors place emphasis on the central idea that 'what an animal does in an…experiment is not the same as what it knows.' Learning, the authors continue, represents a hypothetical psychological and physiological change within the brain and is a separate entity from performance, the expression of learning through behavior (Bouton and Moody, 2004).

In the early 20th century, Edward Tolman was the first to illustrate the separation of learning and performance. In their innovative experiment of latent learning, the authors trained two groups of rats to navigate a maze; the first group was rewarded for successful completion of the maze task, while the second was not. As might be expected, rats rewarded for completing the task performed to a superior degree, committing fewer errors than their non-rewarded counterparts. However, once the non-rewarded group received reward for completing the task, their efficiency at completing the task increased on the following trial. The authors concluded that even though the behavior of the non-reinforced group did not reflect learning, it was nonetheless taking place. Thus, the function of reward was to motivate the animal to perform (Tolman and Honzik, 1930). Summarized by Bouton & Moody (2004), 'learning is not the same as performance. Motivation is required for translation.'

Measuring learning independent of confounds including, emotional state, ambulatory function, attentional state, and motivation are a primary concern for an investigator. These variables represent a challenge to investigators in the field of cannabinoids, as genetic and pharmacological manipulation of the endocannabinoid system can affect all

of the above-mentioned processes, at times making it difficult to infer the occurrence of learning. As will be discussed, controlling for non-mnemonic factors was a primary consideration during the design of the studies presented in this dissertation.

BACKGROUND & SIGNIFICANCE

> "For the Egyptians, hemp is the plant par excellence, not for the uses they make of it in Europe and many other countries, but for its peculiar effects. The hemp cultivated in Egypt is indeed intoxicating and narcotic."
> (Rouyer, 1810)

Marijuana use has long been associated with perturbations of working memory that reflects adapting one's behavior to new information (Varvel and Lichtman, 2005). One of the predominant models for studying this task is the delayed non-match to sample (DNMS) or the delayed match to sample (DMTS) operant task. These experiments rely on the subject's ability to remember a stimulus (e.g. previous choice) over a variable delay, an increase in which requires a greater demand on working memory. Typically, the paradigm involves the presentation of a sample stimulus, followed by a delay in absence of the stimulus. Following the delay, subjects are exposed to both the sample stimulus and a novel stimulus. The task requires that the subject makes an active response, (e.g., a lever press), indicating the novel-stimulus (DNMS) or the previously exhibited sample stimulus (DMTS) for reward. Based on the correct percentage of responses, THC (2mg/kg) administration delay-dependently impaired performance, resulting in performance indistinguishable from hippocampally-lesioned rats pre-trained in the task (Hampson and Deadwyler, 1998; Deadwyler *et al.*, 2007).

Results from a two-component operant task in rats provides further evidence for THC disrupting working memory (Mallet and Beninger, 1998). In this experiment, subjects pressed one of two levers, depending on the presence of two different stimuli (cued conditional discrimination component). Training prior to drug administration allowed subjects to learn to make the correct choice on all subsequent trials. By definition, in trained animals, this component requires reference memory as the subjects had already learned the task (Honig, 1978). In the second component of the session (delayed non-match to position), no cues were presented and rats were required to press the lever opposite the one pressed during the first component. Even after learning this new rule, greater-than-chance responding could only be achieved by remembering which of the two levers was pressed during the first component of that particular trial. By definition, this component requires working memory (Honig, 1978). THC, as well as anandamide co-administered with the non-specific amidase inhibitor phenylmethylsulfonyl fluoride (PMSF), disrupted the working memory aspect, but left reference memory intact. Consistent with a CB_1 receptor mechanism of action, administration of the CB_1 antagonist, rimonabant, reversed the performance deficits caused by exogenously administered CB_1 agonists.

In addition to impairing memory in operant tasks, THC impairs learning in spatial memory paradigms, which generally rely on the use of spatial cues in the environment to navigate a maze. Performance in these paradigms requires hippocampal-dependent processes, an area characterized by dense CB_1 receptor localization, as well as taking

advantage of an animal's natural tendencies such as foraging or predator avoidance (Olton, 1987; Lichtman *et al.*, 2002). For example, THC dose-dependently disrupts performance in the 8-arm radial arm maze. During acquisition of the task, rats use spatial cues to find food in all but one of the arms. Subjects then must remember the unexplored arm on a subsequent exposure following a temporal delay. The dependent measure, errors prior to entering the correct arm, assesses performance. Lichtman *et al*., (1995) demonstrated that systemic administration of THC disrupted performance as assessed by the increased number of errors committed. In a follow-up study THC's disruptive effect was exhibited as delay-dependent, and dose-dependently reversed by rimonabant administration (Lichtman and Martin, 1996).

Another commonly used method of assessing spatial memory is the Morris water maze, a task requiring subjects to use spatial cues to locate and swim to a hidden platform. THC administration disrupted performance in a working memory version of this task, where the location of the hidden platform is variable, exhibited by longer path lengths and latencies to locate the hidden platform. THC also disrupted a reference memory version of the task, in which the hidden platform location was consistent across trials. However, the disruptive effects occurred at much higher doses (100 mg/kg) when compared to the working memory model (3 mg/kg), suggesting an increased susceptibility to THC's disruptive effects in working memory compared to reference memory (Varvel *et al.*, 2001).

Studies utilizing exogenous cannabinoid administration provide rationale for the involvement of the endogenous cannabinoid (eCB) system in cognitive processes, but cannot closely mimic the actions of an endogenous system firmly integrated in neural circuits and eliciting precise biochemical responses to specific contexts (Varvel and Lichtman, 2005). Moreover, confounding the interpretation of the data are side effects associated with exogenous administration of CB_1 agonists, such as alterations in motor behavior and motivation. The development of genetic and pharmacological tools specific to endogenous CB_1 receptor signaling have provided alternative means to study the eCB system. The following section is designed to review the literature surrounding the use of these tools, as well as their effects on acquisition and extinction learning. Moreover, the focus is specific to the work presented in this dissertation, exploring acquisition and extinction following pharmacological CB_1 receptor antagonism, as well as acquisition effects associated with the deletion of the CB_1 receptor and FAAH enzyme.

THE eCB SYSTEM AND ACQUISITION

RIMONABANT

The CB_1 receptor antagonist/inverse agonist, rimonabant, has been a highly useful tool to investigate the endocannabinoid system (Rinaldi-Carmona *et al.*, 1994). As a pharmacotherapeutic, rimonabant has shown potential for treating multiple disorders. Clinical trials revealed its utility in treating type 2 diabetes, increasing HDL-LDL ratios, and reducing risk factors associated with cardiovascular disease (Despres *et al.*, 2005; Van Gaal *et al.*, 2005; Pi-Sunyer *et al.*, 2006; Scheen *et al.*, 2006). Likewise, published research has shown its value as a possible pharmacotherapeutic for treating dependence associated with ethanol (Arnone *et al.*, 1997), tobacco (Cahill and Ussher, 2007) opiates (Navarro *et al.*, 2001), cocaine (De Vries *et al.*, 2001), and cannabis (Tanda *et al.*, 2000; Justinova *et al.*, 2005).

Currently under the trade name Acomplia® (Zimulti® in the United States), rimonabant has won approval for the treatment of obesity in the European Union, and is marketed in countries including Argentina, Austria, Denmark, Finland, Germany, Ireland, Norway, Sweden, Greece, Brazil, Mexico, and the United Kingdom. Conversely, in the United States an FDA advisory panel (2007) composed of outside experts voted unanimously in favor of rejecting approval citing inadequate safety data. More recently,

the possibility of FDA approval for rimonabant was dealt a severe setback following the release of data from a meta-analysis of four clinical trials. In their study, the authors reported a 40% increase in adverse events ranging from depression and anxiety to suicidal thoughts, despite the exclusion of patients with a history of depression or psychiatric illnesses during clinical trials (Christensen *et al.*, 2007). Currently, it would appear that sales within the United States might never materialize. Nonetheless, rimonabant remains a useful pharmacological tool to investigate cannabinoid receptor agonists as well as the eCB system.

In rodent models of learning and memory, rimonabant exhibits a spectrum of cognitive effects; improving, disrupting, and at times exhibiting no effect on learning. Terranova *et al*., (1996) published the first report showing that rimonabant administration enhanced memory in a social recognition paradigm. In this paradigm, the amount of time a mature mouse spent investigating a juvenile conspecific during their first meeting vs. follow-up meetings, measures 'remembering.' Thus, decreased time spent investigating on the second exposure to the juvenile would indicate that they remembered the first exposure. In this paradigm, rimonabant administration 5 min after the first exposure, but not 15 or 90 min after, decreased the time spent investigating during the second exposure. Given the narrow window in which rimonabant administration improved memory functions, the data would suggest an enhancement of consolidation (Terranova *et al.*, 1996).

Reports from the radial arm maze task further support rimonabant enhancing memory processes. In the acquisition of this procedure, rats have access to seven of the eight arms. In the test phase six hours later, errors prior to entering the remaining arm provide a measure of how well the subject remembered. Rimonabant administration prior to the first acquisition phase, but not after the first phase or 20-min before the test phase, decreased the number of re-entry errors (Lichtman, 2000; Wise *et al.*, 2007). In a similar radial arm maze task, rimonabant was observed to dose-dependently decrease re-entry errors when administered immediately after the acquisition phase, but not before the test phase (Wolff and Leander, 2003). Possible explanations for differential results when administration of rimonabant occurred after the test phase include differences in procedure (blocking one vs. four arms), or the choice of vehicle. Alternatively, when administered after the test phase, the absence of enhanced acquisition may be a result of the drug failing to absorb quickly enough to affect consolidation, or a critical period in which consolidation processes are active.

Similarly, rimonabant administration before or immediately after the acquisition phase, but not the test phase, enhances avoidance behavior in the elevated T-Maze (Takahashi *et al.*, 2005), and intrahippocampal administration prior to training enhances memory in food-storing birds for the location of hidden food rewards (Shiflett *et al.*, 2004). Collectively, the results from procedures associating enhanced acquisition with rimonabant suggest the drug is acting on, and improving, the acquisition and/or consolidation of memory rather than retrieval.

Procedural differences appear to provide a possible explanation for reports in which rimonabant administration fails to affect acquisition of other tasks. For example, rimonabant administration does not affect acquisition rates in the Morris water maze spatial memory task (Varvel *et al.*, 2005), passive avoidance tasks (Mazzola *et al.*, 2003; Niyuhire *et al.*, 2007), operant conditioning (Mallet and Beninger, 1998; Niyuhire *et al.*, 2007), or conditioned fear (Marsicano *et al.*, 2002; Suzuki *et al.*, 2004; Kamprath *et al.*, 2006; Niyuhire *et al.*, 2007) paradigms. The collective literature regarding cognitive alterations following rimonabant administration supports two conclusions. First, rimonabant enhances acquisition and/or consolidation of memory, but does not affect retrieval. Second, procedural components appear critical in determining the absence or expression of rimonabant's effects. Specifically the temporal components, but not the hedonic nature of the reinforcer, may be primary. Thus, tasks demanding memory processes lasting for minutes to hours show enhancement, while those on the order of seconds, do not (Lichtman *et al.*, 2002). However, this explanation appears insufficient as rimonabant has been shown to enhance performance in a delayed non-match to sample paradigm. At delays longer than 10s, rimonabant administration resulted in a greater percentage of correct responses, as well as increasing the frequency of 'strong' SmR code strength, a measure of hippocampal information encoding at the time of sample responding during DNMS trials. Furthermore, rimonabant impaired acquisition of delay eyeblink conditioning (Kishimoto and Kano, 2006). In this study, the conditioned stimulus (tone) co-terminates with the unconditioned stimulus (shock), and the learned association between US and CS results in a conditioned startle response (eyeblink) during

subsequent CS presentations. During acquisition of the task, rimonabant treated subjects exhibited a significant reduction in the percentage of conditioned responses compared to vehicle treatment. However, indistinguishable performance occurred between treatment groups during the first acquisition day, and acquisition differences between groups were not observed until subsequent acquisition trials had been performed. Thus, the relevance of these data, with respect to the hypothesis of Lichtman *et al*., (2002), is undetermined.

CB_1 -/- MICE

Converging evidence provided by genetic correlates of pharmacological antagonism provide an alternative means of verifying results from pharmacological studies by inactivating specific biological processes genetically. Furthermore, utilizing both genetic and pharmacological approaches provides evidence that results are independent of confounds associated with drug administration (e.g., inverse agonism, non-specific effects, interactions, alternative sites of action) and/or genetic knockout animals (i.e., compensatory mechanisms, genetic drift, downstream developmental changes). To this end, CB_1 receptor knockout mice provide a complementary approach along with the use of CB_1 receptor antagonists to block eCB signaling. Reibaud *et al.,* (1999) were the first to show enhanced acquisition in CB_1 -/- mice during a two-trial object recognition task. In this experiment, mice received a ten-min open field trial in the presence of a novel object. Following a 3, 24, or 48 h delay, subjects were re-exposed in the presence of both the familiar, as well as a new, unfamiliar object. Comparing the amount of time spent investigating each object provides a measure for how well the subject 'remembers' the first encounter, and chance occurrence is set at 50%. Following a 3 h delay, both genotypes exhibited an indistinguishable increase in time spent analyzing the new object compared to the familiar object. However, 24 and

48 h delays resulted in chance performance in wild-type mice, while CB_1 -/- mice continued to show preference for investigating the unfamiliar object. These results were later replicated using CB_1 -/- mice on the CD-1 background (Maccarrone *et al.*, 2002), in both old and young mice. While the effect was repeated in both young and old knockout mice, the authors reported age-related adaptive changes in knockout mice. Specifically, deletion of the CB_1 receptor resulted in an age-related increase in FAAH activity, and subsequent decrease in AEA content. Furthermore, in both cases these changes exhibited the most profound alterations in the hippocampus.

Evidence for improved acquisition in CB_1 -/- mice has also been exhibited in an active avoidance test (Martin *et al.*, 2002). In this paradigm, the subject is placed in an apparatus composed of two chambers connected by a door. The subject must learn that the onset of a light is predictive of an impending shock, and avoiding the shock requires charging into the unlit compartment. While both genotypes exhibited similar baseline performance on day one, CB_1 -/- mice showed a significant enhancement of avoidance learning, demarcated by increased conditioned charges compared to wild-type mice.

Converging electrophysiological evidence provides further support for enhanced acquisition in CB_1 -/- mice. Long-term potentiation (LTP) is thought to underlie the formation of memory by strengthening synaptic connections following repeated stimulation of a synaptic pathway. *In vitro* analysis of hippocampal CB_1 -/- brain slices reveals an enhanced capacity to strengthen synaptic connections, resulting in an almost 50% greater response compared to wild-type controls (Bohme *et al.*, 2000).

Under certain conditions, CB_1 -/- mice exhibit impaired acquisition. In models of cued conditioned fear, subjects learn to associate the onset of a cue with impending shock. Following repeated pairings, analysis of freezing behavior in probe trials where the cue is presented in a new context denotes acquisition of the task. In this paradigm, genetic ablation of the CB_1 receptor leaves acquisition unaffected (Marsicano *et al.*, 2002; Cannich *et al.*, 2004; Kamprath and Wotjak, 2004). Similarly, contextual conditioned fear involves testing the subject in the same context as training. Unlike results from the cued procedure, CB_1 -/-, but not CB_1 +/+, mice on the CD-1 background exhibited very little freezing upon re-exposure to the context, resembling results from unshocked CB_1 -/- and +/+ control mice (Mikics *et al.*, 2006). While the strain differences may have influenced the results, differences in brain areas associated with the two procedures may also account for the disparity in results. Lesions of the amygdala impair cue-induced fear, while lesions of the hippocampus impair context-induced fear (Phillips and LeDoux, 1994; McNish *et al.*, 1997; Bast *et al.*, 2003; Ahi *et al.*, 2004). Cued conditioned fear is largely reliant on the amygdala, whereas contextual conditioned fear is dependent on the dorsal hippocampus.

Conditioned eyeblink paradigms are categorized primarily by temporal differences in the presentation of the unconditioned stimulus (US) and conditioned stimulus (CS). Delay paradigms require US (shock) and CS (tone) co-termination, whereas trace paradigms utilize a stimulus-free interval between US and CS presentation. Whereas delay paradigms are dependent on the cerebellum (McCormick and Thompson, 1984; Thompson *et al.*, 1997), trace conditioning requires both the cerebellum and

hippocampus (Moyer *et al.*, 1990; Weiss *et al.*, 1999; Kishimoto *et al.*, 2006). Comparison of CB_1 -/- and +/+ mice in the delay procedure reveals acquisition deficits in -/-, but not +/+ mice (Kishimoto and Kano, 2006). Furthermore, electromyogram (EMG) recordings on the final acquisition day revealed significantly lower average amplitudes in response to the CS, presumably due to behavioral differences in conditioned responding. Investigation into potential confounds revealed no significant genotype differences with regards to spontaneous eyeblink frequency, motor coordination, startle responses, or the intensity of the US to elicit an eyeblink response. In opposition to the acquisition of the delay conditioning paradigm, CB_1 -/- and +/+ mice showed equal acquisition performance in the trace conditioning procedure.

Recent reports underscore the importance of age as a determining factor when studying the cognitive performance of CB_1 -/- mice. Bilkei-Gorzo *et al.,* (2005) demonstrated that deletion of the CB_1 receptor results in age-specific cognitive effects. In the social recognition paradigm, subjects are exposed to a novel, juvenile conspecific on two occasions separated by a variable interval. A decrease in time investigating the partner on the second exposure is indicative of remembering. Young (6-7 weeks of age) CB_1 -/- mice exhibited enhanced acquisition compared to wild-type age-matched controls. However, a striking decline in performance was observed in mature (3-5 months) CB_1 -/- mice, exhibiting impairment similar to old (14-17 months) wild-type mice. To determine if the observed effects were strain-dependent, the experiment was replicated using juvenile and mature CB_1 -/- and +/+ mice on the CD-1 background. Again, young CB_1 -/- exhibited enhanced retention compared to controls, as well as a

rapid age-related decline in performance of CB_1 -/- mice. Performance of the same groups in an operant condition paradigm revealed a similar age-related decline in the cognitive performance of CB_1 -/- mice. Conversely, deficits in the wild-type group were only observed in the old group, exhibiting impaired performance similar to mature CB_1 -/- mice. Analysis of neuronal density within the different age groups provides an intriguing correlate to the behavioral deficits. Compared to wild-type mice, CB_1 -/- mice show a rapid decline in the density of hippocampal neurons as they age, in the most extreme case exhibiting a 70% reduction in old knockout mice compared to their age-matched controls.

The expression of cognitive differences in CB_1 -/- mice appears to depend on the hedonic nature of the reinforcer (e.g. aversive vs. appetitive). In an operant conditioning procedure in which subjects were given food-reward for a correct nose-poke response of an illuminated hole, CB_1 -/- mice exhibited normal acquisition of the task (Holter *et al.*, 2005). Importantly, CB_1 -/- mice showed reduced motivation to work for reward following moderate food restriction. Increasing the level of deprivation proved necessary to achieve equivalent performance between genotypes. Furthermore, the age of the mice was the same as mature subjects from the experiment by Bilkei-Gorzo *et al.,* (2005). As this group reported an age-dependent decline in performance in a similar operant task, effects observed by Holter *et al.*, may be influenced by differences in cognitive ability.

Similar to the experiment by Holter *et al.*, baseline differences in the acquisition of an operant conditioning procedure were observed in CB_1 -/- mice trained to nose-poke for either corn oil or the sweetened reinforcer Ensure (Ward and Dykstra, 2005; Ward *et*

al., 2007). Under either condition of reinforcement, both genotypes exhibited the ability to acquire the task. However, regardless of the reinforcer used, fewer CB_1 -/- mice achieved maximal responding than their wild-type counterparts. Higher levels of responding (e.g. active nose-poke hole responding) were observed during maintenance sessions in wild-type mice compared to CB_1 -/- mice trained to respond for Ensure, and substitution of sweetened reward for the fat-reinforcer corn oil produced a similar decrease in responding. As CB_1 -/- mice primarily exhibited differences in responding, rather than the acquisition of the task, the hedonic value of the different appetitive rewards may account for observed genotype differences.

Results from the aversively conditioned Morris water maze spatial memory task suggest acquisition differences between genotypes result from differences in procedure, as genetic deletion of the CB_1 receptor leaves spatial memory intact (Varvel and Lichtman, 2002). Following repeated acquisition trials, the subject learns to use spatial cues surrounding the maze to locate and swim to a hidden platform. In a fixed platform protocol, where the location of the hidden platform is constant, both genotypes exhibited similar latencies, as well as path lengths, to discover the target location. While both genotypes displayed a similar overall reduction in thigmotaxia by the end of acquisition training, the decrease occurred more gradually in knockout mice than in wild-type mice. The possibility of an anxiogenic phenotype was further supported by the observation that one-half of the CB_1 -/- mice stopped swimming in favor of floating, in some cases requiring rescue to prevent sinking. Working memory was also assessed by shifting the platform to a new location each acquisition day. Again, both genotypes showed similar

ability to acquire the task, however notable phenotypic differences were observed. CB_1 -/- mice performed with a pronounced inconsistency, resulting in the removal of 50% of the subjects for failing to reach criteria. In extreme cases, seizures, and eventual death, occurred in five of the mice. Furthermore, the authors reported reduced body weights, labored swimming, and dysfunctional search strategies (i.e., swimming in circles). Importantly, the observed differences were absent in wild-type mice.

In a follow-up experiment by the same group, CB_1 -/- showed no differences in acquiring a fixed-platform task compared to wild-types (Varvel *et al.*, 2005). While the authors did not report genotype differences in thigmotaxia, alternative evidence for an anxiogenic phenotype was reported when non-contingent swimming was assessed. In this experiment, naive subjects were exposed to the maze in the absence of an escape platform during massed probe trials. Across trials, a gradual reduction in swim speed was observed in wild-type, but not CB_1 -/- mice. Thus, it would appear that while spatial memory is intact, differences in experimental design may produce cognitively unrelated effects arising from a possible anxiogenic phenotype.

FAAH -/- MICE

The recent genesis of mice lacking the gene for the FAAH enzyme provides a useful model for studying enhanced eCB signaling by inhibiting the metabolism of anandamide (Cravatt *et al.*, 2001). Varvel *et al.,* (2006) were the first to report acquisition differences in FAAH -/- mice, compared to their wild-type controls in the Morris water maze spatial memory task. During acquisition of a fixed platform procedure, both genotypes exhibited similar acquisition profiles, measured by escape latency, and the corresponding path length. Conversely, in a working memory paradigm where the location of the hidden platform was placed in a new location each day, FAAH -/- mice acquired the task significantly faster than their wild-type littermates. Of interest, the authors note a non-significant trend (p=.06) of enhanced acquisition within the first acquisition day.

In a follow-up experiment by the same group, FAAH -/- mice exhibited enhanced acquisition of a fixed-platform task (Varvel *et al.*, 2007). As these data are in contrast to results from the previous experiment, procedural differences may have accounted for the disparate nature of the results. In the previous experiment, the hidden platform was placed arbitrarily towards the front of the tank (i.e. closest to the entrance to the enclosure). Conversely, the current experiment placed the hidden platform in the back of

the tank (i.e. furthest from the entrance to the enclosure). Apparently, wild type mice display a steeper acquisition curve when the platform is placed in the front aspect of the tank than in the back of the tank. Thus, placing the hidden platform in the back of the tank unmasked phenotypic differences.

Extinction is the suppression of a previously learned behavior, following non-reinforced trials. Disruption of CB_1 receptor signaling has been shown to impair extinction learning in aversively reinforced models of conditioned freezing (Marsicano *et al.*, 2002; Suzuki *et al.*, 2004; Niyuhire *et al.*, 2007), passive avoidance (Niyuhire *et al.*, 2007), and spatial memory (Varvel *et al.*, 2005). In the cued conditioned fear paradigm, a subject learns that a tone is predictive of an impending footshock. With each successive tone presentation in the absence of a footshock, the percentage of time spent immobile decreases, demarcating the occurrence of extinction learning. Marsicano *et al.*, (2002) were the first to show that administration of rimonabant prior to extinction trials resulted in a perseverance of freezing behavior, while vehicle treated subjects exhibited a gradual reduction in freezing behavior following repeated extinction trials. Similarly, in the Morris water maze spatial memory task, mice learn to swim to a hidden platform during acquisition trials. Following removal of the platform during extinction trials, subjects gradually decrease the amount of time spent in the quadrant that previously contained the hidden platform. Thus, while vehicle-treated mice increasingly search other areas of the maze across extinction trials, rimonabant treated animals continue to perseverate in the irrelevant quadrant (Varvel *et al.*, 2005). Interestingly, in the water

maze, the disruption of extinction was only seen when weekly, but not massed (multiple probes separated by a short temporal delay), extinction trials were administered, suggesting temporal components of the task may be critical. Finally, in the passive avoidance paradigm subjects learn to associate shock with one of two chambers. Following conditioning, daily extinction trials are administered and the latency to enter the chamber associated with shock is recorded. In vehicle-treated animals, subjects exhibited a gradual reduction in the latency to enter the chamber associated with shock across extinction sessions. Conversely, subjects administered rimonabant exhibited consistently elevated latencies across trials (Niyuhire *et al.*, 2007).

There is evidence that rimonabant does not affect extinction learning in appetitively reinforced tasks. For example, rimonabant administration did not affect extinction of operant conditioning, either in daily or weekly trials (Niyuhire *et al.*, 2007). Importantly, the results by Niyuhire *et al*., (2007) are in agreement with a previous operant conditioning study using CB_1 -/- mice trained to nose poke for food reward (Holter *et al.*, 2005). Collectively, these results support the hypothesis that the nature of the reinforcer (e.g. aversive vs. appetitive) is primarily responsible for the expression of disrupted extinction in models of attenuated CB_1 receptor signaling (Holter *et al.*, 2005).

SUMMARY

An underlying theme in the review presented above is the inherent difficulty in comparing results from qualitatively different behavioral tasks. As the models discussed in the review fluctuate primarily on either the source of reinforcement (i.e. appetitive vs. aversive), or behavioral demands (i.e. learning to lever-press or find a hidden goal), the necessity for a paradigm in which either is controlled is apparent. The recent adaptation of the Barnes maze for use in mice presents the unique possibility of using dissimilar sources of reinforcement to motivate learning the same goal. If validated, utilization of a modified Barnes maze would facilitate addressing three outstanding questions in the literature.

First, the specific conditions in which rimonabant affects extinction learning have yet to be determined, but appear to depend on whether the behavior is learned in an appetitive or aversive task (Holter *et al.*, 2005; Niyuhire *et al.*, 2007). However, there are two outstanding issues with the latter hypothesis. First, rimonabant may produce a decrease in hedonic value of the reward used in both studies (food pellet or sweetened milk). Previous research has shown that rimonabant, and CB_1 receptor deletion, decreases food consumption (Kirkham and Williams, 2001), salience of food reward (Ward and Dykstra, 2005), and preference for sweetened foods (Arnone *et al.*, 1997;

Higgs *et al.*, 2003). Predictably, CB_1 -/- mice required a greater level of food restriction to reach asymptotic acquisition performance as wild-type littermates (Holter *et al.*, 2005). Again, the difficult nature of resolving differences in hedonics (i.e. aversive vs. appetitive) and the disparate behavioral demands of the tasks preclude interpretation.

Second, many reports suggest genetic deletion of the CB_1 receptor affects acquisition learning. However, conflicting reports exist regarding how acquisition is affected (i.e. facilitated, impaired, or unaffected), and under what reinforcement conditions. For example, CB_1 -/- mice have exhibited a broad spectrum of acquisition effects in aversively reinforced paradigms. Genetic disruption of CB_1 receptor signaling enhances acquisition of an active avoidance paradigm (Martin *et al.*, 2002); impairs learning of contextual conditioned fear sometimes (Mikics *et al.*, 2006), but not always (Suzuki *et al.*, 2004)**,** and delay eyeblink conditioning (Kishimoto and Kano, 2006); but does not affect spatial memory (Varvel and Lichtman, 2002; Varvel *et al.*, 2005), cued conditioned fear (Marsicano *et al.*, 2002; Cannich *et al.*, 2004; Kamprath *et al.*, 2006), or trace eyeblink conditioning (Kishimoto and Kano, 2006). Appetitively reinforced models suggest acquisition is intact following CB_1 receptor deletion. Bilkei-Gorzo *et al.,* (2005), were the first to report intact acquisition of an operant conditioning procedure in young (6-7 weeks of age) CB_1 -/- mice compared to age-matched controls. In agreement with these results, deficits in acquisition learning were not reported in operant conditioning experiments utilizing mature CB_1 -/- and +/+ mice trained to nose-poke for food pellets (Holter *et al.*, 2005), Ensure (a sweetened protein drink), or corn-oil (Ward *et*

al 2007; Ward, personal communication, 2007). An important caveat of these studies is the increased level of food deprivation in the knockout mice to increase their motivation to work for food reward. Thus, similar acquisition profiles in a task where behavioral demands are constant, and only the source of reinforcement is variable, would suggest disparate acquisition performance in the literature result from procedural differences. Importantly, controlling for confounds associated with CB_1 -/- mice, such as age and motivation for food reward, must be considered in future studies.

Finally, there is a need for further research evaluating the cognitive impact of enhancing eCB signaling through FAAH inhibition. The increased levels of brain anandamide in FAAH -/- mice is correlated with accelerated acquisition rates in both working (Varvel *et al.*, 2006) and fixed-platform (Varvel *et al.*, 2007) Morris water maze tasks. As these reports represent the extent of the acquisition literature in FAAH -/- mice, and utilize inherently aversive procedures, it is unknown whether limitations exist in the expression of enhanced acquisition. Thus, performance in the aforementioned modified Barnes maze provides an opportunity to determine if reinforcement conditions dictate the expression of enhanced acquisition in FAAH -/- mice.

THE BARNES MAZE

Carol Barnes developed the first working model of the Barnes maze (fig. 1) in 1979 to study senescence in rats (Barnes, 1979). She hypothesized that the advantage of this task is its superior control for different levels of stress between groups. For example, many earlier tasks required a greater degree of food deprivation in older mice in an attempt to generate 'equal' levels of motivation (Goodrick, 1968). Furthermore, motor confounds related to age might be avoided by using an easily traversed land maze that did not require more demanding motor performance such as with swimming. In her words (personal communication):

> "I developed this task in the mid-1970s for old rats - the idea was to come up with a spatial memory task that didn't require shock, or food restriction - in those days, you had to "grow your own" old rats, and I wanted them to "want to participate" without risking health issues (if they died, there were no replacements). This apparatus was the outcome - in its original incarnation we put females (the rats in the study were males) in a cage below the platform as extra incentive - but this turned out not to be necessary for good performance. We also moved the platform to a huge room, because I noticed that the rats became more 'comfortable' staying out on the platform surface (even under bright lights) if the walls were close to the edge."

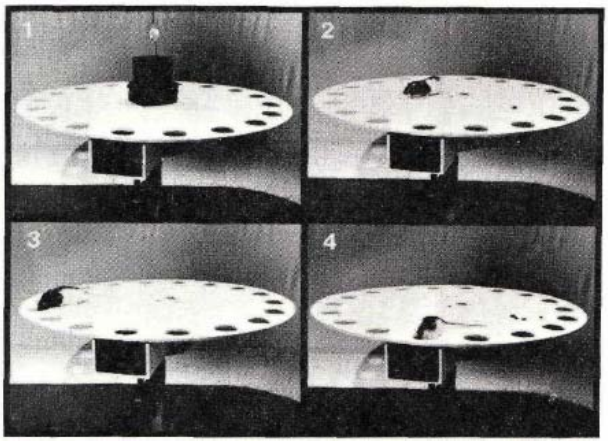

Figure 1: The Barnes Maze (Barnes, 1979)

The Barnes maze utilizes natural sources of motivation such as the tendency to avoid bright lights and air turbulence in favor of an enclosed dark area, thereby avoiding confounds relating to food deprivation, footshock, stress, and stamina during studies of senescence (Barnes, 1979). Years after its initial development for rats, the maze was adapted for use in mice to evaluate transgenic CaMKII -/- (Bach *et al.*, 1995). In their short description, the authors reported an increase in the number, and reduction in the size, of the holes along the perimeter of the maze, as well as the inclusion of aversive stimuli (i.e. bright lights and a buzzer). Fox *et al*., (1998) reported the first detailed description of the adapted maze to evaluate traumatic brain injury (fig. 2). However, little credit has been given to both Fox *et al.,* and Bach *et al.,* for being the first to adapt the Barnes maze for use in mice.

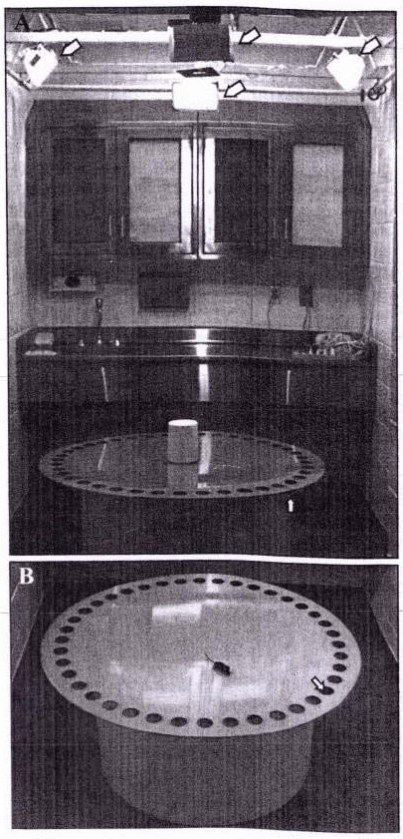

Figure 2: **T**he Barnes maze for mice (Fox *et al.*, 1998)

The current absence of an established Barnes maze apparatus/procedure appears related to the development of 'pseudo-Barnes mazes,' and begins with a report by Pompl *et al*., (1999). In this study, the investigators utilized a 'downsized circular platform' (fig. 3) to evaluate the cognitive performance of APP_{sw}-/- mice. Departing from the traditional, unenclosed open field, the perimeter of the maze contained a second set of cues. In their report, the authors extend on the advantages (vs. the Morris water maze) listed by Barnes (1979), adding that extensive pre-training is unnecessary, there is a minimal reliance on sensorimotor skills, fewer required testing days than other spatial memory tasks, and an inability for mice to use odor cues or exact motor sequences to solve the task. Furthermore, they were the first to report the Barnes maze as less stressful than the Morris water maze, an unsubstantiated claim that spread through the literature for years thereafter. In their defense, there remains evidence that the stress induced by other spatial memory models may affect results. For example, water temperature in the Morris water maze can affect performance in a glucocorticoid dependent manner (Sandi *et al.*, 1997; Sandi, 1998). However, no work exists comparing these two spatial memory paradigms directly (Harrison *et al.*, 2006).

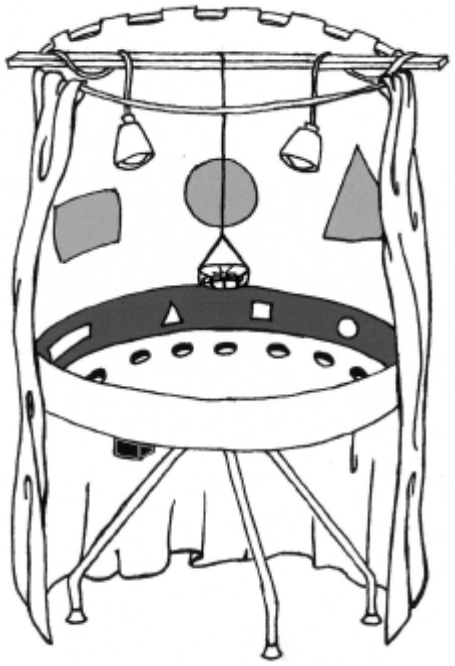

Figure 1:The Barnes maze (Pompl *et al.*, 1999)

While only a handful of reports have been published using the adapted Barnes maze for mice, most have relied on similar sources of reinforcement. For example, many reports utilize escape from aversive stimuli, such as bright lights (Bach *et al.*, 1995; Fox *et al.*, 1998; Pompl *et al.*, 1999; Inman-Wood *et al.*, 2000; Williams *et al.*, 2003; Bredy *et al.*, 2004), air turbulence (Pompl *et al.*, 1999; Inman-Wood *et al.*, 2000) rock and techno music (Fabricius *et al.*, 2008), and tones (Bach *et al.*, 1995; Pompl *et al.*, 1999; Inman-Wood *et al.*, 2000; Bredy *et al.*, 2004). Alternatively, some authors report the use of appetitive (i.e. food reward) reinforcement (Grootendorst *et al.*, 2001; Williams *et al.*, 2003). Moreover, some reports have suggested the return to the home cage, as well as gentle handling (Harrison *et al.*, 2006), are the primary source of reinforcement (Blizard *et al.*, 2003; Williams *et al.*, 2003). Unlike other spatial memory tasks, the Barnes maze is unique as it allows different sources of motivation (i.e. aversive vs. appetitive) to drive the same behavior (e.g. entering the goal box) [for a summary of the literature, see appendix A].

In the following dissertation, we capitalized on the unique nature of the Barnes maze by comparing acquisition and extinction learning utilizing disparate sources of reinforcement. Specifically, bright lights and air turbulence motivated learning under aversive conditions (fig. 4 and 5), and access to water motivated learning under appetitive conditions (fig. 6). Unlike previous publications, we chose to depart from sweetened food reward under appetitive conditions in favor of water reward. As the present study focuses on manipulations to the endocannabinoid system, water reward circumvented

possible confounds related to appetite and hedonic value. Importantly, rimonabant has been shown to leave water consumption unaffected (Arnone *et al.*, 1997) at moderate doses (Colombo *et al.*, 1998). To our knowledge, this is the first report to use access to water to motivate learning in the Barnes maze task.

Figure 4: The Barnes maze (Harloe, 2007)

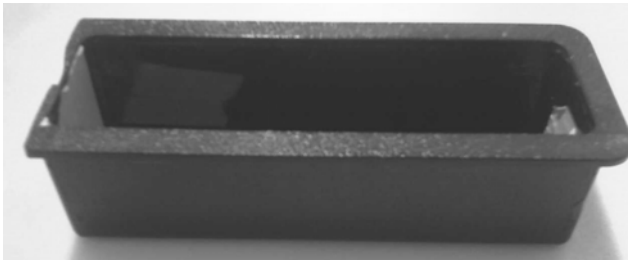

Figure 5: Unmodified goal box for aversive reinforcement conditions**.**

Figure 6: Goal box following modification for appetitive reinforcement conditions.

EXPERIMENT 1

INTRODUCTION

The environmental conditions necessary to observe disrupted extinction learning following rimonabant administration are uncertain, but appear dependent on the source of reinforcement. For example, rimonabant disrupts extinction in aversive conditioning paradigms such as the Morris water maze, conditioned fear, and passive avoidance, but not in appetitively reinforced operant conditioning procedures (Marsicano *et al.*, 2002; Holter *et al.*, 2005; Varvel *et al.*, 2005; Niyuhire *et al.*, 2007). It is difficult to reconcile between the qualitative hedonic value of the reinforcer (i.e., eliciting appetitively-motivated versus aversively motivated behavior) and the disparate behavioral demands of differing tasks. Thus, in order to discern whether the hedonic value of the reinforcer plays a determining role in the outcome of dissimilar behavioral models, it is critical to utilize a paradigm in which the nature of the reinforcement is varied but the behavioral demands of the task remain constant. A unique aspect of the Barnes maze is that different sources of reinforcement can be utilized to drive the same behavior (i.e., finding and entering the goal box) to escape aversive stimuli or obtain appetitive reinforcement. Here, we utilized bright lights and air turbulence to motivate learning under aversive

conditions and access to drinking water under appetitive conditions. As future studies will focus on the endocannabinoid system, water reward circumvented confounds related to the observations that genetic disruption or pharmacological inhibition of the CB_1 receptor often reduces operant responding for and the intake of palatable food (De Vry and Jentzsch, 2004; Holter *et al.*, 2005; Ward and Dykstra, 2005), though not always (Jarrett *et al.*, 2005). Importantly, compromising CB_1 receptor signaling has been shown to leave water consumption unaffected at moderate doses (Arnone *et al.*, 1997; Colombo *et al.*, 1998). To the best of our knowledge, the studies presented in this dissertation are the first to use access to drinking water to motivate learning in the Barnes maze task.

The initial objective of the present study was to characterize aversively- and appetitively conditioned acquisition and extinction learning in the Barnes maze. Moreover, these experiments allowed us to test the hypothesis that mice will exhibit acquisition and extinction learning during both and appetitively and aversively motivated Barnes maze task. In addition, a third group of subjects was assessed under conditions common to both the aversive and appetitive procedures. In this control group, no bright lights, air turbulence, water access, or water deprivation were used. Finally, a control experiment was performed to differentiate between extinction learning and the gradual decay of memory, or forgetting. In subsequent experiments, manipulations of the endogenous cannabinoid system were evaluated in these appetitive and aversive tasks.

METHODS

SUBJECTS

A total of 79 C57BL/6J (Jackson Labs, Bar Harbor, ME) mice, weighing between 20-30 g, and housed individually, were used as subjects. All subjects were housed in a temperature-controlled (20-22º C) environment, with a 12-h light/dark cycle and ad libitum access to food. Mice in the ambient (*n*=8) or aversive condition (*n*=35) were allowed ad libitum access to water in their home-cage for the entirety of the study, while mice in the appetitive condition (*n*=36) were only given access to water for 2 h per day (see procedure below). All experiments have been approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

APPARATUS

The Barnes maze (Hamilton-Kinder, Poway, CA) consisted of a round board (122 cm diameter) fabricated from PVC with 40 holes (2.54 cm diameter) surrounding the perimeter of the maze. The maze was divided into six zones, each containing a possible location for the goal box (19.5 cm X 5.5 cm). A square 152 cm X 152 cm aluminum frame enclosure surrounded the apparatus and was used to hang contextual cues (i.e. various dark shapes) on white curtains that encircled the maze. A circular starting tube (7.62 cm. internal diameter) was placed in the center of the maze to ensure that all subjects began each trial from the same location. The tube was attached to a cord and pulley system, which the investigator could raise from outside the enclosure. The trial began 3 s after the subject was placed in the starting tube. A digital camera (Panasonic BP-330), connected to a nearby computer running AnyMaze software (Stoelting, Wood Dale, IL), allowed the observer to watch and record without disturbing the subject. Both the maze and goal box were wiped with an ammonia based cleaner (Whistle [JohnsonDiversey Inc., Sturtevant, WI]) after each trial.

PROCEDURE

Three types of testing conditions were used: aversive, ambient, and appetitive. In the aversive procedure, bright lights (two, 500 watt halogen bulbs) and two, 60 cm wide fans (Holmes, Milford, MA) for air turbulence were located 120 cm above the maze and remained on during all stages and trials. In the appetitive procedure, a modified goal box containing access to water was used as a reinforcer for water-deprived mice. Subjects were given 22 h of daily water deprivation before each session and were weighed for comparison to a pre-deprivation baseline. Immediately after each session, the mice were returned to their home cages, given access to water for 2 h, and weighed. Finally, in the ambient condition, stimuli were limited to those inherent to the laboratory environment (e.g. fluorescent lighting) and the Barnes maze apparatus (e.g. entering the goal box) during appetitive and aversive procedures. No additional stimuli or water restriction were introduced.

Shaping

All subjects were acclimated to the apparatus and basic procedure before formal acquisition training began. The subject was placed in the start cylinder and was released

once the experimenter had closed the curtain and initiated the software. Three min later, the subject was placed in the goal box, which was then slid into one of the six corresponding target locations. A metal lid was placed over the escape hole to prevent the mouse from exiting. Following two min of acclimation to the goal box, the mouse was placed into its home cage for a 30 s intertrial interval (ITI). After the ITI, the goal box was placed back into its corresponding location and the subject was guided from the center of the maze to the entrance of the goal box. Shaping was concluded after at least two consecutive entries into the goal box without provocation from the investigator.

Acquisition

Each mouse was given four acquisition trials per day for ten days. Each trial ended when either three min had elapsed or the subject entered the goal box, whichever occurred first. In the event that the mouse failed to enter the goal box within the three min trial, it was placed in the center of the maze and the experimenter led it to the goal box where it remained for 30 s before being returned to its home cage for the 30 s ITI. If the mouse repeatedly found the goal box, but failed to enter, it was given additional shaping in which it was again placed in the center of the maze and led to the escape hole, a process that was repeated until the mouse entered the goal box without provocation from the experimenter. Acquisition measures included test duration (latency to enter the hidden goal box), total time spent immobile, distance traveled, and adjusted speed.

Extinction

In order to assess extinction, the goal box was removed and subjects were given a single, three-min probe trial per day for a total of 10 days. Extinction was inferred to have occurred when the percentage of time spent in the target zone was significantly reduced compared to the first probe trial or to chance levels (18%). Additionally, as with acquisition, total time immobile, adjusted speed and distance traveled were analyzed to assess locomotor effects.

To differentiate between forgetting and extinction, the mice were given 10 days of acquisition training in either aversive (n=15) or appetitive (n=17) conditions, as described above, and were then divided into two separate groups. The first group received 10 days of extinction training. The second group remained in the vivarium during the first nine days of extinction. On the tenth day after acquisition, this group was given a 3 min probe trial to assess whether they still recalled the location of the hidden escape hole.

STATISTICAL ANALYSES

AnyMaze (Stoelting, Wood Dale, IL) software was used to accumulate most of the dependent measures of interest. The maze was divided into six zones to determine the duration of time spent in the target zone (the zone that contained the goal box). Other dependent measures of interest included adjusted speed (distance traveled/(latency to enter – time immobile)), distance traveled, time spent immobile, and latency to enter the goal box (test duration).

Results from comparison studies were analyzed using two-way mixed design ANOVAs (treatment by session). A significant effect of motivating condition was further analyzed for each condition by a one-way repeated measures ANOVA. Dunnett's post-hoc analysis with comparison to day one values was used when appropriate. Significant interactions were analyzed in the same manner, but also included comparison of reinforcing condition within each acquisition or extinction session using the Tukey post-hoc test. Finally, a student's t-test was used to distinguish between forgetting and extinction. The accepted level of significance for the tests was $p<0.05$.

RESULTS

Acquisition of aversive, appetitive, and ambient conditioning groups

Figure 7A-D illustrates each of the dependent measures for acquisition in the aversive, appetitive, and ambient conditions. A significant effect of acquisition day was found for all dependent measures including distance [fig. 7A; $F(9,396)=25.0$, $p<0.0001$], latency to enter [fig. 7B; $F(9,396)=52.0$, $p<0.0001$], adjusted speed [fig. 7C, $F(9,396)=26.0$, $p<0.0001$], and time immobile [fig. 7D; $F(9,396)=7.7$, $p<0.0001$].

While subjects learned the task in each condition, differences in acquisition rates, as well as locomotor effects, were detected. Specifically, a significant effect of conditioning procedure was found for latency to enter [$F(2, 396)=15.0$, $p<0.0001$], distance traveled [$F(2,396)=6.4$, $p<0.01$], time immobile [$F(2,396)=9.9$, $p<0.001$], and adjusted speed [$F(2,396)=8.2$, $p<0.001$]. In each situation, no differences were found between the aversive and appetitive conditioning group, but both were significantly different from the ambient condition.

Significant interactions between conditioning procedure and acquisition day were found for adjusted speed [$F(18,396)=2.1$, $p<0.01$] and time immobile [$F(18,396)=3.5$, $p<0.0001$]. While subjects in the aversive and appetitive condition reached asymptotic

performance based on latency to enter the hidden goal box, subjects under the ambient condition showed significantly longer latencies to enter the goal box than the other two groups. Additionally, subjects in the ambient condition showed no change in the amount of time spent immobile [$F(9,63)=1.5$, $p=0.1$] over the 10 acquisition days. When speed was adjusted to account for time spent immobile, all groups showed a gradual increase in running speed over acquisition days (appetitive [$F(9,162)=14.0$, $p<0.0001$]; aversive [$F(9,171)=22.7$, $p<0.0001$]; ambient [$F(9,63)=3.8$, $p<0.001$]).

Finally, water restricted subjects were weighed twice daily to investigate whether they would be able to maintain a proper range of body weight following water-deprivation. While subjects showed an initial drop in body weight, they quickly adjusted to the schedule, never falling below 75% of their baseline weight (fig. 8). No apparent differences in water consumption were observed, however considerable leakage from the water bottle prevented accurate measurement.

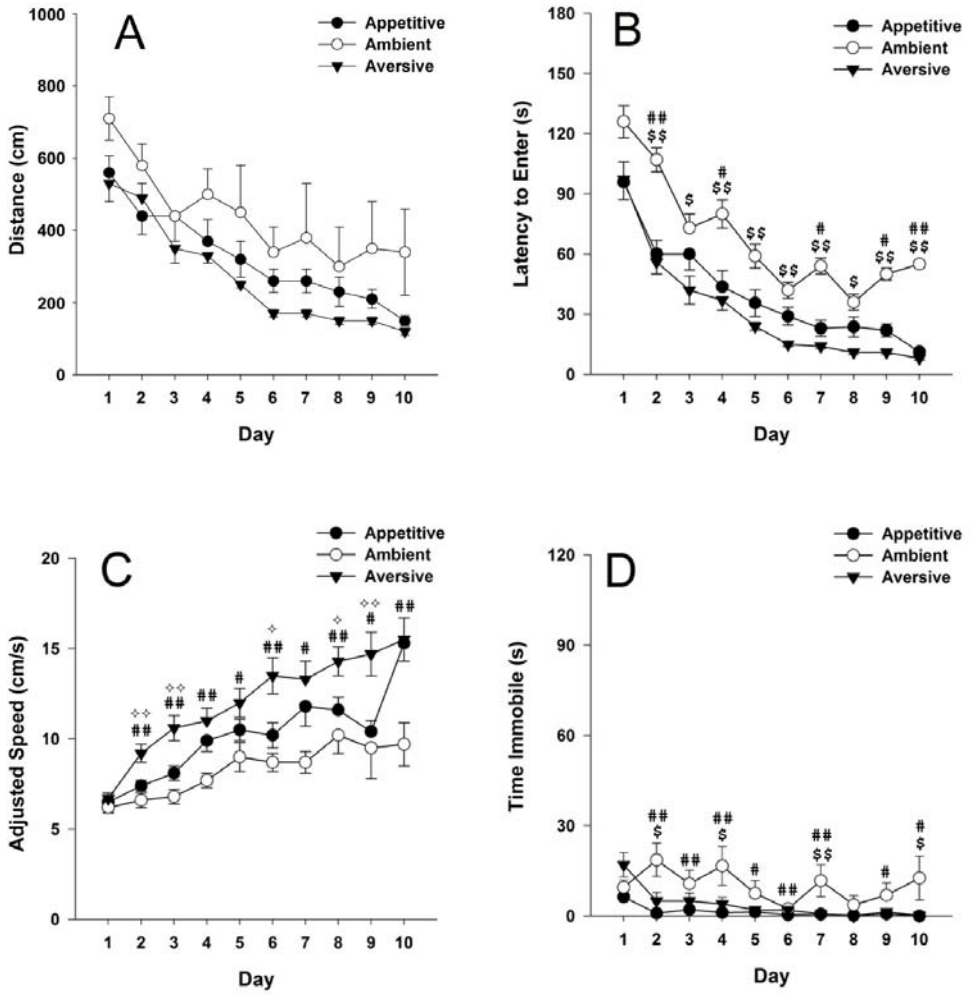

Figure 7: Mice learn to enter a hidden goal box in the Barnes maze under aversive, appetitive, and ambient conditioning procedures. Panel A. Distance traveled to enter the box (cm). Panel B. latency to enter the goal box. Panel C. Adjusted running speed (cm/s) was determined by the following formula: [distance/(latency to enter – time immobile)]. Panel D. average time spent immobile. The data for each session represent the average of four daily trials ± SEM. # $p<0.05$ ## $p<0.01$ # vs. the appetitive condition. $ $p<0.05$ $$ $p<0.01$ vs. aversive condition. ✧ $p<0.05$ ✧✧ $p<0.01$ ✧ vs. ambient reinforcement. N=8-20 mice/group. The data for each session represent the average of four daily trials ± SEM. N=8-20 mice/group.

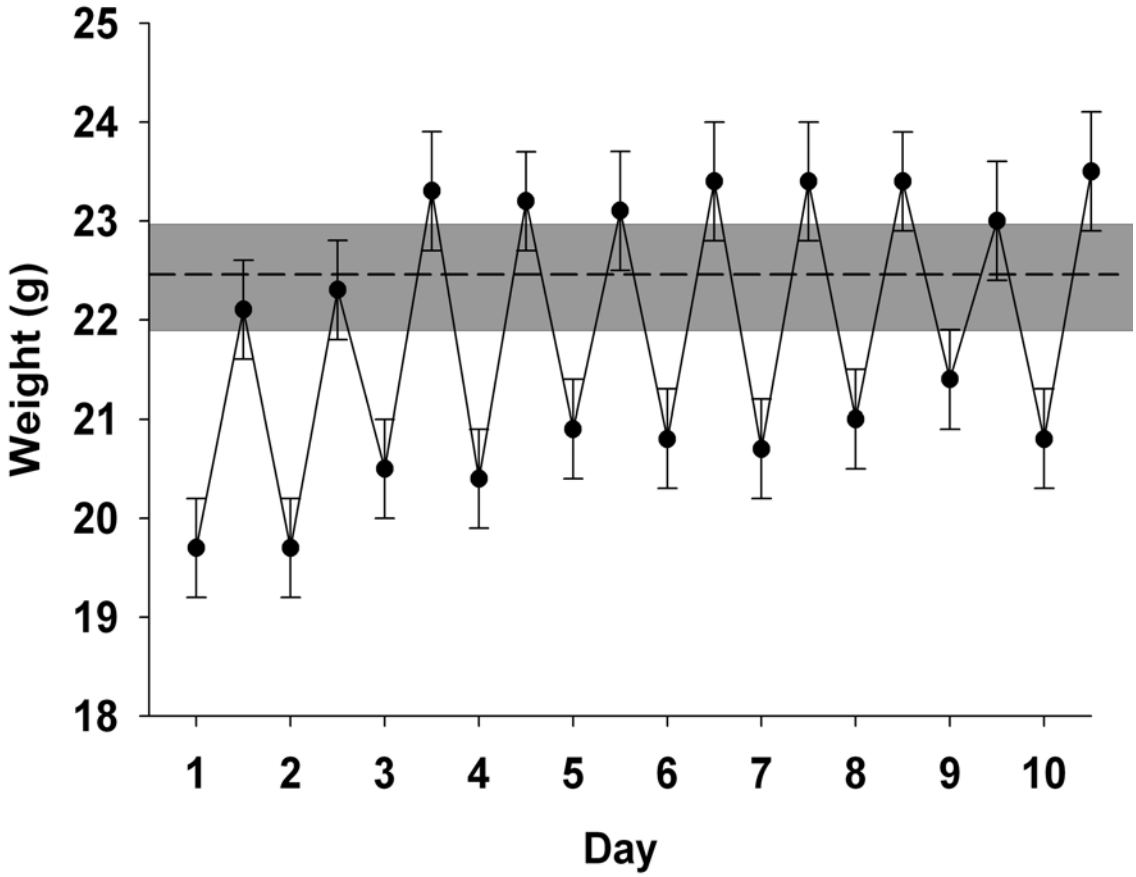

Figure 8: Mice rapidly adapt to water deprivation. Average daily weight (g) recorded before and after 2-h access to water following 22-h of deprivation. --------- indicates average baseline weight prior to testing and the shaded error represents the standard error of baseline weight. All data are represented as mean ± SEM. *N*=10.

Extinction of aversive, appetitive, and ambient conditioning groups

Significant reductions in the percentage of time spent in the target zone across the ten probe trials in the aversive conditioning procedure [fig. 9A; $F(9,63)=6.4$, $p<0.05$], the appetitive conditioning procedure [fig. 9C; $F(9,36)=3.5$, $p<0.01$], and under ambient conditions [fig. 9E; $F(9,63)=4.4$, $p<0.001$]. Additionally, the mice displayed a significant decrease in adjusted speed across extinction trials in the aversive [fig. 9B; $F(9,63)=9.3$, $p<0.0001$] and ambient [fig. 9D; $F(9,63)=2.1$, $p<0.05$], but not appetitive (fig. 9F; $p=0.51$) condition. These results indicate that a 3 min daily extinction trial is sufficient to produce extinction learning.

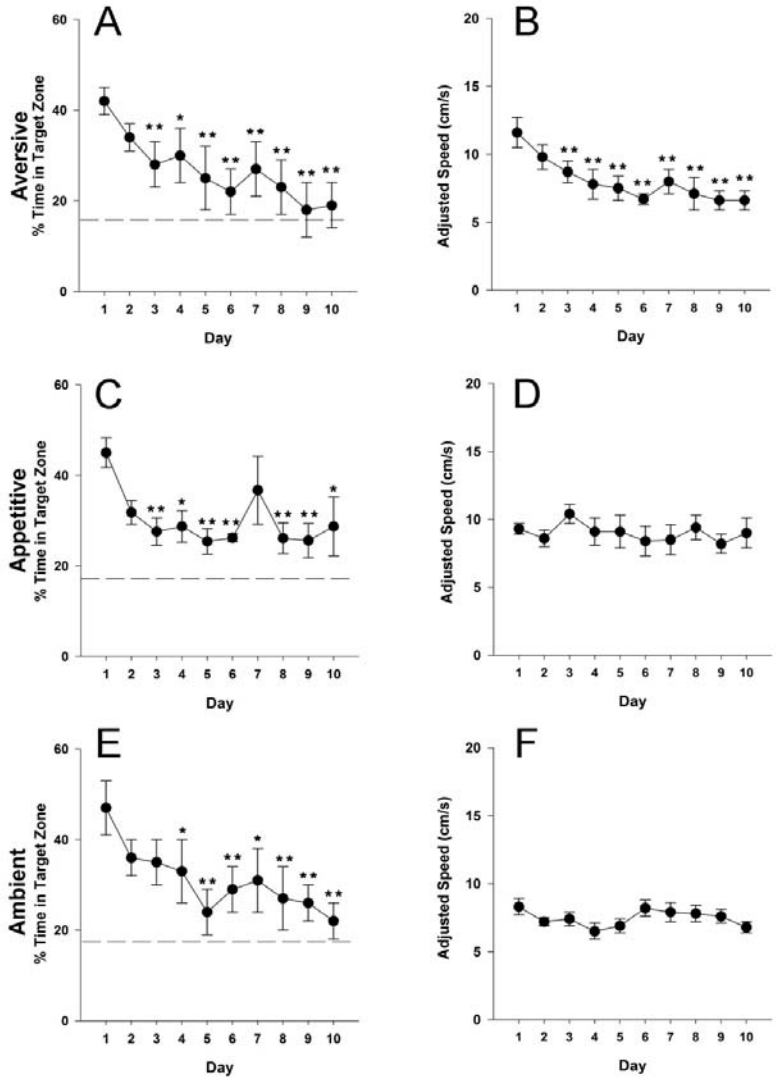

Figure 9: Exposure to the Barnes maze after removal of the goal box leads to extinction. Mice were given ten day of acquisition training in aversive (top panels), appetitive (middle panels), or ambient (bottom panels) conditioning procedures. The percentages of time spent in the target zone (i.e., the area formerly associated with the goal box) are shown for each condition (panels A, C, and E). The dotted line from the 18% point of the ordinate spanning to the width of the abscissa indicates chance performance. Adjusted speeds [distance/(latency to enter – total time immobile)] are represented for aversive (panel B), appetitive (panel D), and ambient (panel F) conditions. * $p<0.05$ ** $p<0.01$ vs. extinction day 1 values. ------- Represents chance performance. All data are represented as mean ± SEM. N=5-8 mice/group.

Forgetting vs. extinction

In order to distinguish between extinction learning and forgetting, naive mice were given 10 days of acquisition training in either the aversive conditioning procedure or the appetitive conditioning procedure. The mice in each conditioning procedure were divided into two groups, with the first group receiving a daily extinction trial for 10 days (Group Extinction) and the second group (Group No Extinction) receiving only a single probe trial that coincided with extinction day 10 for Group Extinction. As can be seen in fig. 10A, Group Extinction in the aversive conditioning procedure displayed a significant decrease in the percentage of time spent in the target zone across the ten probe trials, [$F(9,54)=2.8$, $p<0.01$]. In contrast, Group No Extinction, which was given a single probe trial ten days after acquisition, spent significantly more time in the target zone than Group Extinction [$t(13)=3.0$, $p<0.01$]. Moreover, no differences were observed when comparing the percentage of time spent in the target zone between the single probe trial of Group No Extinction and the first extinction trial of Group Extinction [$t(13)=.1$, $p=0.93$]).

A similar pattern of results was found when appetitive conditions were employed. Again, Group Extinction showed a significant decrease in the percentage of time spent in the target zone across the ten probe trials [fig. 10B, $F(9,81)=4.1$, $p<0.001$]. Group No Extinction appeared to remember the location of the target box ten days after acquisition, as they spent a similar amount in the target zone as Group Extinction on their first

extinction trial [t(15)=1.1, p=0.26]. Additionally, Group No extinction spent a significantly greater percentage of time in the target area compared to Group Extinction's tenth trial [t(15)=4.9, p<0.001]. Thus, under both aversive and appetitive conditions, subjects still recall the location of the escape box ten days after training. However, daily three min exposures to the Barnes maze with no goal box present were sufficient to elicit extinction under both conditioning regimens.

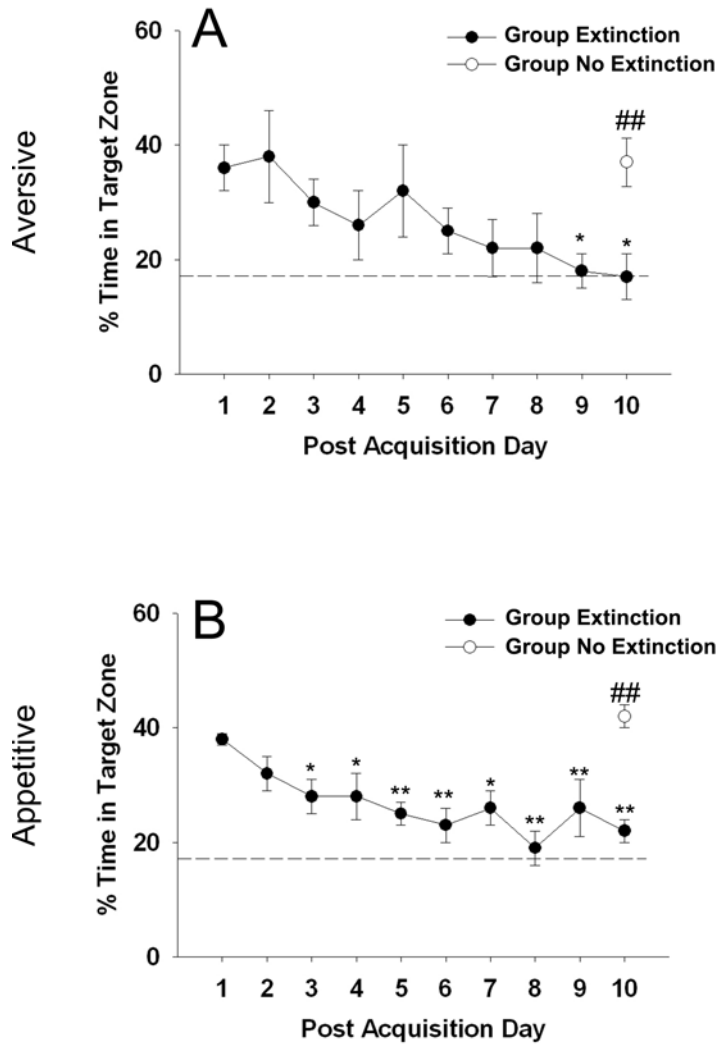

Figure 10: Extinction learning is independent of forgetting. Percentage of time spent in the target zone (i.e., the zone previously containing the escape box) during extinction of an aversively (panel A) and appetitively (panel B) conditioned Barnes maze tasks. All subjects were given ten days of acquisition training, as described in the methods. Following acquisition, subjects in Group Extinction were given ten daily three min exposures to Barnes maze, but the goal box was removed. Group No Extinction received a single three min trial in the Barnes maze without the goal box that coinciding with extinction day 10 for Group Extinction. The dotted line from the 18% point of the ordinate spanning to the width of the abscissa indicates chance performance. *$p< 0.05$; **$p<0.01$ vs. Group Extinction. All data are represented as mean ± SEM. N=7-10 mice/group.

DISCUSSION

While our lab has previously employed the Morris water maze to evaluate the role of the endogenous cannabinoid system on extinction, this task is inherently aversive (Morris, 1984). This notion is supported by the observation that water maze training activates the pituitary adrenal axis, causing an increase in corticosterone (Sandi *et al.*, 1997; Akirav *et al.*, 2001). Rimonabant disrupted extinction learning in the Morris water maze (Varvel *et al.*, 2005), as well as other aversively conditioned paradigms such as passive avoidance and conditioned fear (Marsicano *et al.*, 2002; Niyuhire *et al.*, 2007). Conversely, rimonabant does not affect extinction learning in appetitively conditioned operant tasks (Holter *et al.*, 2005; Niyuhire *et al.*, 2007). In contrast, the Barnes maze spatial memory paradigm presented a unique opportunity of comparing different sources of reinforcement, either aversive or appetitive (i.e. food and entering the goal box), to motivate the same goal. Only a handful of published reports have employed the Barnes maze to evaluate mice and most of this work has relied on similar sources of aversive reinforcement, such as bright lights, air turbulence, or auditory stimuli (Bach *et al.*, 1995; Fox *et al.*, 1998; Pompl *et al.*, 1999; Inman-Wood *et al.*, 2000; Williams *et al.*, 2003; Bredy *et al.*, 2004). Conversely, other studies have utilized positive reinforcement, such as food, gentle handling, or the return to the home cage to motivate Barnes maze learning

(Grootendorst *et al.*, 2001; Blizard *et al.*, 2003; Koopmans *et al.*, 2003; Williams *et al.*, 2003; Harrison *et al.*, 2006).

To focus on the qualitative nature of the reinforcer, we modified the Barnes maze task in the present study to evaluate appetitively and aversively motivated conditioning procedures on acquisition and extinction, but required the same motor responses (i.e., searching and entering the goal box). Subjects acquired the Barnes maze task irrespective of reinforcement condition, illustrated by significant reductions in both path length and test duration across acquisition days. Removal of the goal box following acquisition trials produced a gradual decrease in the percentage of time spent in the target zone for all conditions, indicating the occurrence of extinction learning. Importantly, control experiments demonstrated extinction learning was independent of forgetting, under both appetitive and aversive conditions. Collectively, the results support the utilization of this model as a viable method of assessing spatial memory in a paradigm in which the nature of reinforcement is variable, but the behavioral demands of the task remain constant.

The Barnes maze is traditionally used to assess spatial memory, requiring the subject to use spatial cues to find a hidden location (Bach *et al.*, 1995). It has been reported that animals progress through random, serial, and direct search strategies during acquisition of the Barnes maze task (Barnes, 1979). While we initially attempted to record these three search strategies, we elected not to use this measure. There is difficulty in objectively scoring these strategies because subjects often, in no particular order, utilize all three strategies within a single acquisition day, though by the completion

of acquisition training, nearly every subject adopted the direct strategy. Accordingly, we elected to rely on path length and latency to enter the hidden goal box measures to infer learning and percentage of time spent in the target zone to infer extinction learning.

Results garnered from the ambient condition indicate that mice placed on the Barnes maze, without any extra environmental manipulations (e.g., bright lights, air turbulence, or access to water) will learn to enter the hidden box, though they do not enter the goal box as quickly as mice in the aversive or appetitive conditioning procedures, suggesting environmental manipulations increased acquisition rates. Additionally, when the hidden goal box was removed, the mice in the ambient condition extinguished searching in the target zone at a similar rate as the mice in the other two groups. Thus, mere placement onto the Barnes maze is a sufficient motivator for mice to learn to find and enter the goal box. The finding that the mice prefer the goal box to remaining on the open maze is not surprising given that open fields generally provoke anxiety-like states in rodents (Crawley, 1985).

EXPERIMENT 2

INTRODUCTION

A growing body of literature has implicated the eCB system in extinction learning in which learned behavior becomes suppressed when reinforcement is withheld. Disruption of CB_1 receptor signaling, through either its genetic deletion or administration of a receptor antagonist impairs extinction learning in a variety of aversively motivated tasks, including conditioned freezing (Marsicano *et al.*, 2002; Suzuki *et al.*, 2004; Kamprath *et al.*, 2006; Niyuhire *et al.*, 2007), passive avoidance (Niyuhire *et al.*, 2007), and the Morris water maze spatial memory task (Varvel *et al.*, 2005). A common aspect of these tasks is that each uses an aversive unconditioned stimulus. In contrast, disruption of CB_1 receptor signaling failed to affect extinction learning in operant conditioning tasks that use palatable food as the reinforcer (Holter *et al.*, 2005; Niyuhire *et al.*, 2007; Ward *et al.*, 2007). Specifically, CB_1 -/- mice displayed similar rates of extinction as wild type mice in nose-poking tasks for food pellets (Holter *et al.*, 2005) as well as for Ensure^R (a sweetened protein drink) or corn oil (Ward *et al.*, 2007). To account for the differential consequences of CB_1 deletion on extinction learning in operant and other behavioral paradigms, Holter *et al* (2005) hypothesized that the eCB system plays an important role in extinction of aversively-motivated learned behavior,

but is dispensable for the extinction of appetitively-motivated behavior. Similarly, rimonabant treatment failed to affect extinction rates in a lever pressing operant task for sweetened condensed milk (Niyuhire *et al.*, 2007), providing pharmacological evidence the CB_1 receptor does not play a role in extinction of appetitively-motivated behavior.

It is difficult to reconcile between the qualitative hedonic value of the reinforcer (i.e., eliciting appetitively-motivated versus aversively motivated behavior) and the disparate behavioral demands of differing tasks. Thus, in order to discern whether the hedonic value of the reinforcer plays a determining role in the activation of the endogenous cannabinoid system, it is critical to utilize a behavioral paradigm in which the nature of the reinforcement is varied but the behavioral demands of the task remain constant. In the present study, we used modified versions of the Barnes maze (Barnes, 1979), characterized and evaluated in the previous chapter, to examine the consequences of pharmacologically blocking endocannabinoid signaling on extinction learning in mice. A unique aspect of the Barnes maze is that different sources of reinforcement can be utilized to drive the same behavior (i.e., finding and entering the goal box) to escape aversive stimuli or obtain appetitive reinforcement. Here, we utilized bright lights and air turbulence to motivate learning under aversive conditions and access to drinking water under appetitive conditions. As the present study focuses on manipulations to the endocannabinoid system, water reward circumvented confounds related to the observations that genetic disruption or pharmacological inhibition of the CB_1 receptor often reduces operant responding for and the intake of palatable food (De Vry and Jentzsch, 2004; Holter *et al.*, 2005; Ward and Dykstra, 2005), though not always (Jarrett

et al., 2005). Importantly, compromising CB_1 receptor signaling has been shown to leave water consumption unaffected at moderate doses (Arnone *et al.*, 1997; Colombo *et al.*, 1998).

The primary objective of this study was to test the hypothesis that the endogenous cannabinoid system plays a differential role in modulating extinction in aversive and appetitive conditioning paradigms. While the behavioral demands (i.e., locating and entering the escape box) for conditioning was kept constant, the qualitative value of the reinforcement was experimentally manipulated. In the present study, we evaluated the effects of rimonabant administration in appetitive and aversive Barnes maze tasks. Accordingly, we sought to determine the outstanding question of whether observed differences of rimonabant on extinction learning were due to hedonics or the disparate nature of the tasks utilized.

METHODS

SUBJECTS

A total of 71 C57BL/6J (Jackson Labs, Bar Harbor, ME) mice, weighing between 20-30 g, and housed individually, were used as subjects. All subjects were housed in a temperature-controlled (20-22º C) environment, with a 12-h light/dark cycle and ad libitum access to food. Mice in the aversive condition (*n*=53) were allowed ad libitum access to water in their home-cage for the entirety of the study, while mice in the appetitive condition (*n*=18) were only given access to water for 2 h per day (see procedure below). All experiments have been approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

DRUGS

Rimonabant (SR-141716A) was obtained from the National Institute on Drug Abuse (Rockville, MD). The drug was dissolved into a vehicle consisting of ethanol, alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline at a ratio of 1:1:18, and a 3 mg/kg dose was administered i.p. 30 min prior to testing at a volume of 10 ml/kg.

APPARATUS

The Barnes maze (Hamilton-Kinder, Poway, CA) consisted of a round board (122 cm diameter) fabricated from PVC with 40 holes (2.54 cm diameter) surrounding the perimeter of the maze. The maze was divided into six zones, each containing a possible location for the goal box (19.5 cm X 5.5 cm). A square 152 cm X 152 cm aluminum frame enclosure surrounded the apparatus and was used to hang contextual cues (i.e. various dark shapes) on white curtains that encircled the maze. A circular starting tube (7.62 cm. internal diameter) was placed in the center of the maze to ensure that all subjects began each trial from the same location. The tube was attached to a cord and pulley system, which the investigator could raise from outside the enclosure. The trial began 3 s after the subject was placed in the starting tube. A digital camera (Panasonic BP-330), connected to a nearby computer running AnyMaze software (Stoelting, Wood Dale, IL), allowed the observer to watch and record without disturbing the subject. Both the maze and goal box were wiped with an ammonia based cleaner (Whistle [JohnsonDiversey Inc., Sturtevant, WI]) after each trial.

PROCEDURE

Two types of testing conditions were used: aversive and appetitive. In the aversive procedure, bright lights (two, 500 watt halogen bulbs) and two, 60 cm wide fans (Holmes, Milford, MA) for air turbulence were located 120 cm above the maze and remained on during all stages and trials. In the appetitive procedure, a modified goal box containing access to water was used as a reinforcer for water-deprived mice. Subjects were given 22 h of daily water deprivation before each session and were weighed for comparison to a pre-deprivation baseline. Immediately after each session, the mice were returned to their home cages, given access to water for 2 h, and weighed.

Shaping

All subjects were acclimated to the apparatus and basic procedure before formal acquisition training began. The subject was placed in the start cylinder and was released once the experimenter had closed the curtain and initiated the software. Three min later, the subject was placed in the goal box, which was then slid into one of the six corresponding target locations. A metal lid was placed over the escape hole to prevent the mouse from exiting. Following two min of acclimation to the goal box, the mouse was placed into its home cage for a 30 s intertrial interval (ITI). After the ITI, the goal

box was placed back into its corresponding location and the subject was guided from the center of the maze to the entrance of the goal box. Shaping was concluded after at least two consecutive entries into the goal box without provocation from the investigator.

Acquisition

Each mouse was given four acquisition trials per day for ten days. Each trial ended when either three min had elapsed or the subject entered the goal box, whichever occurred first. In the event that the mouse failed to enter the goal box within the three min trial, it was placed in the center of the maze and the experimenter led it to the goal box where it remained for 30 s before being returned to its home cage for the 30 s ITI. If the mouse repeatedly found the goal box, but failed to enter, it was given additional shaping in which it was again placed in the center of the maze and led to the escape hole, a process that was repeated until the mouse entered the goal box without provocation from the experimenter. Acquisition measures included test duration (latency to enter the hidden goal box), total time spent immobile, distance traveled, and adjusted speed.

Extinction

In order to assess extinction, the goal box was removed and subjects were given a single, three-min probe trial per day for a total of 10 days. Extinction was inferred to have occurred when the percentage of time spent in the target zone was significantly

reduced compared to the first probe trial or to chance levels (18%). The latency to find the target hole, time spent immobile, and the number of nose pokes into the target hole were assessed during extinction. Additionally, as with acquisition, adjusted speed and distance traveled were analyzed to assess locomotor effects.

STATISTICAL ANALYSES

AnyMaze (Stoelting, Wood Dale, IL) software was used to accumulate most of the dependent measures of interest. The maze was divided into six zones to determine the duration of time spent in the target zone (the zone that contained the goal box). Other dependent measures of interest included adjusted speed (distance traveled/(latency to enter – time immobile)), distance traveled, time spent immobile, and latency to enter the goal box (test duration).

Results from comparison studies were analyzed using two-way mixed design ANOVAs (treatment by session). A significant effect of treatment was further analyzed for each drug condition by a one-way repeated measures ANOVA. Dunnett's post-hoc analysis with comparison to day one values was used when appropriate. Significant interactions were analyzed in the same manner, but also included comparison of treatment within each acquisition or extinction session using the Tukey post-hoc test. The accepted level of significance for the tests was $p<0.05$.

RESULTS

Rimonabant fails to affect Barnes maze acquisition

Rimonabant (3 mg/kg) failed to alter path lengths under both aversive (fig. 11A; p=0.14) and appetitive (fig. 12A; p =0.75) conditioning tasks, though the drug-treated mice had significantly longer path lengths than vehicle-treated mice on day 4, only. Similarly, rimonabant failed to affect the latency to enter the goal box during acquisition in aversive (fig. 11B; p=0.09) and appetitive (fig. 12B; p=0.18) conditioning Barnes maze procedures. Rimonabant failed to affect speed during acquisition in the aversive (fig. 11C; p=0.68) and appetitive (fig. 12C; p=0.13) conditioning tasks. During the first few conditioning sessions, the rimonabant-treated mice displayed more immobility than vehicle-treated mice, as indicated by significant interactions between drug treatment and conditioning day in aversive [fig. 11D; $F(9,315)=2.3$, $p<0.05$] and appetitive [fig. 12D; $F(9,144)=2.0$, $p<0.05$] procedures.

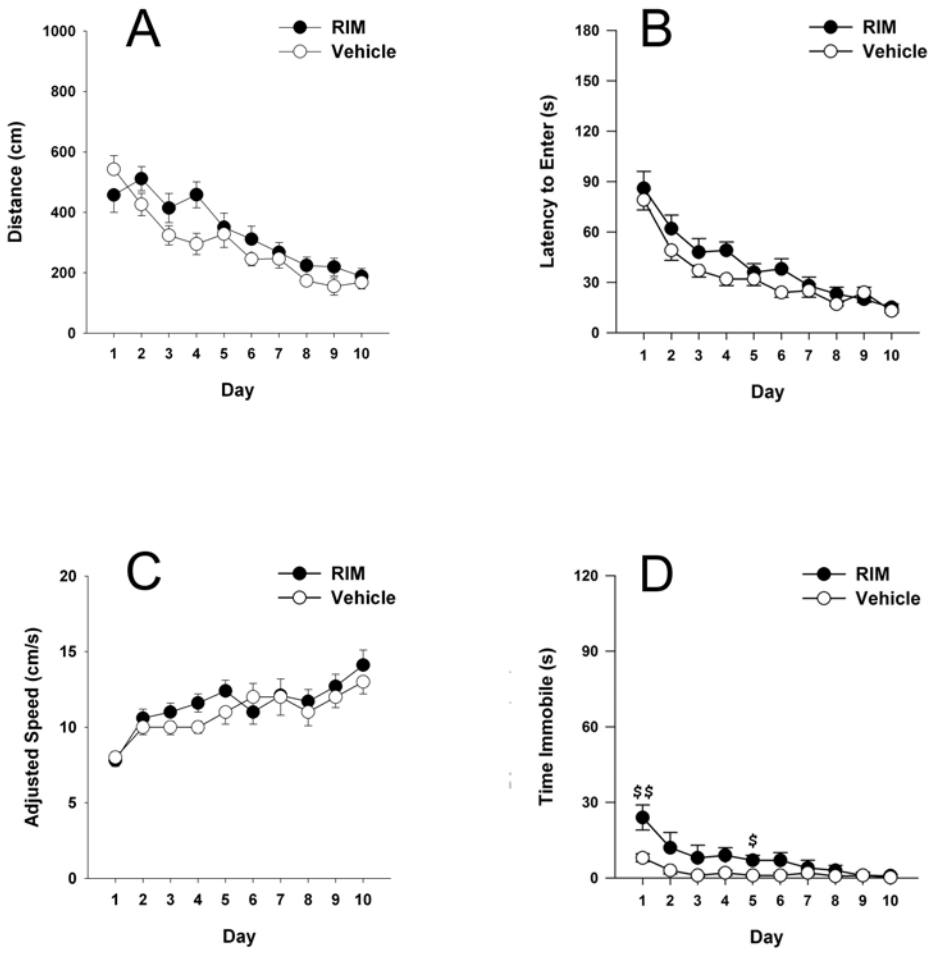

Figure 11: Rimonabant administration (3 mg/kg) increases immobility time, but does not affect acquisition of an aversively conditioned Barnes maze task. The average distance (cm) traveled (panel A), and the corresponding latency (s) to enter the goal box (panel B), did not significantly differ between groups. Panel C. No treatment differences were observed for adjusted speed [distance/(latency to enter – total time immobile)]. Panel D. Rimonabant increased time spent immobile during the first few conditional trials. $ p<0.05 $$ p<0.01 vs. the corresponding vehicle-treated mice. The data for each acquisition session are represented as the average of four daily trials ± SEM. $ denotes a significant difference from vehicle treatment. N=17-20 mice/group.

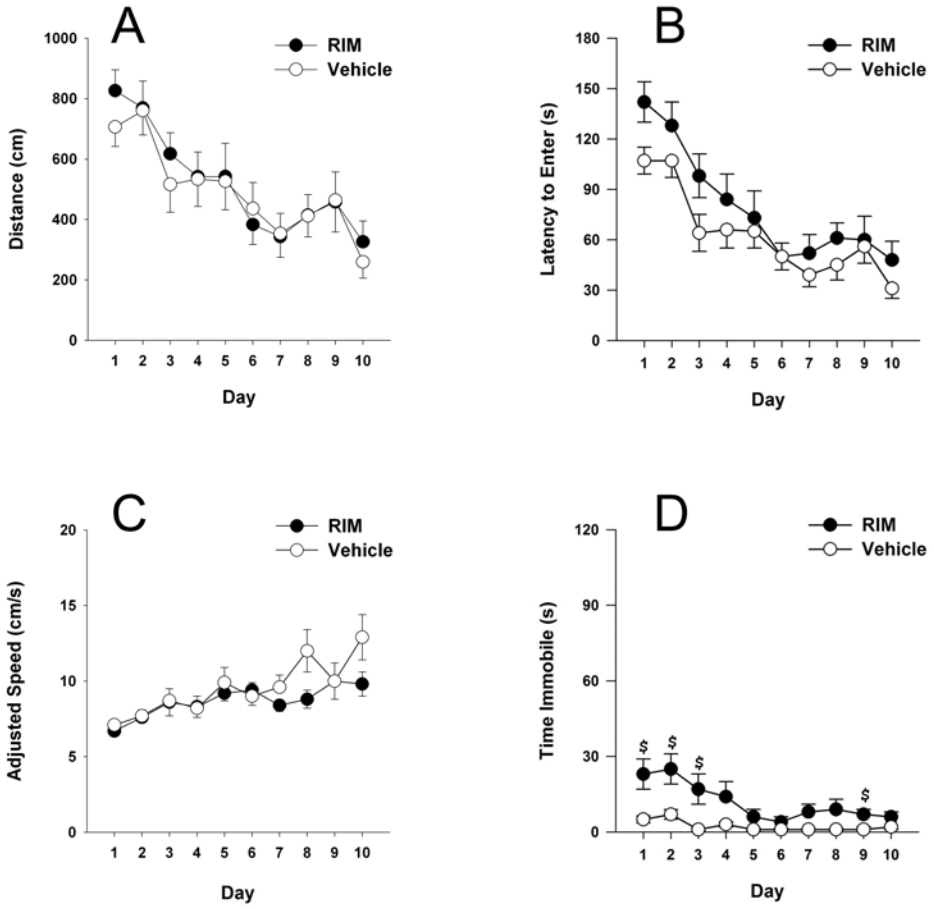

Figure 12: Rimonabant administration (3 mg/kg) increases immobility time, but does not affect acquisition, in an appetitively conditioned Barnes maze task. The average distance (cm) traveled (panel A), and the corresponding latency (s) to enter the goal box (panel B), did not significantly differ between groups. Panel C. No differences were observed for adjusted speed [distance/(latency to enter – total time immobile)]. Panel D. A significant increase in time spent immobile was observed following rimonabant treatment. $ $p<0.05$ vs. vehicle group. The data for each acquisition session are represented as the average of four daily trials ± SEM. N=9 mice/group.

Rimonbant disrupts extinction learning in an aversive, but not in an appetitive, Barnes maze task

In an initial experiment, subjects were administered rimonabant or vehicle during both acquisition and extinction. The percentage of time spent in the target zone in both groups across extinction trials is shown in fig. 13A, and track plots of a representative mouse for each treatment group on Day 1 and Day 10 of extinction are shown in fig. 15 (top traces). A significant drug by day interaction [$F(9,306)=2.0$, $p<0.05$] was found, indicating that the rimonabant-treated mice displayed a significant delay in extinction rate. Whereas the vehicle control group underwent extinction following repeated trials without the goal box present [$F(9,171)=9.1$, $p<0.0001$], the rimonabant-treated group failed to display any evidence of extinction ($p=0.76$). Specifically, the vehicle-treated mice spent significantly less time in the target zone by extinction day 2, while rimonabant-treated animals continued to perseverate in the target zone throughout all 10 extinction trials. Rimonabant treatment did not affect speed (fig. 13B; $p=0.79$), or distance traveled ($p=0.60$; data not shown), but did significantly increase the amount of time spent immobile [fig. 13C; $F(1,306)=4.4$, $p<0.05$].

In the next experiment, rimonabant was administered before each extinction session, but not during acquisition. Again, administration of rimonabant led to extinction deficits, as indicated by the percentage of time spent in the target zone [fig. 13D;

F(9,126)=2.2, $p<0.05$]. The vehicle-treated mice showed a reduction in the percentage of time spent in the target area [F(9,63)=8.2, $p<0.0001$], with a significant decrease in the target zone by day 3. On the other hand, rimonabant-treated mice continued to perseverate in the target zone across all ten extinction sessions, with no reductions ($p=0.45$). As in the previous experiment, rimonabant failed to affect adjusted speed (fig. 13E; $p=0.58$); however, rimonabant treatment failed to produce a significant effect on immobility time [fig. 13F; F(1,126)=3.6, $p=0.07$].

In the appetitive Barnes maze conditioning paradigm, rimonabant failed to alter extinction learning. Both rimonabant- and vehicle-treated subjects exhibited a gradual reduction in the percentage of time spent in the target zone [fig. 14A; F(9,126)=6.1, $p<0.0001$]. However, neither a main effect of drug ($p=0.68$) nor an interaction between drug and extinction day ($p=0.99$) was observed. Finally, there were no significant effects of treatment condition on adjusted speed (fig. 14B; $p=0.57$), distance traveled ($p=0.59$; data not shown), or time spent immobile (fig. 14C; $p=0.79$). Representative traces of vehicle-treated and rimonabant-treated mice on Days 1 and 10 of extinction are shown in Figure 15 (bottom panel).

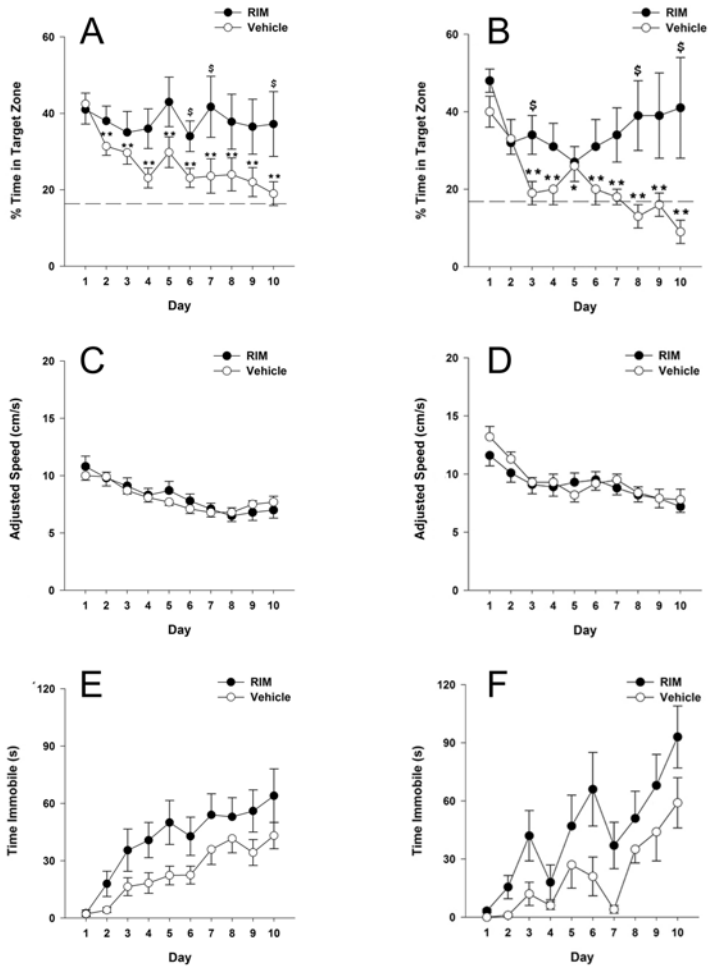

Figure 13: Rimonabant impairs extinction learning in the aversively motivated Barnes maze task. In the first experiment (left panels), subjects were administered vehicle or rimonabant(3 mg/kg) before each acquisition and extinction trial, whereas rimonabant was only administered before each extinction session in the second experiment (right panels). In both experiments, rimonabant, but not vehicle, treatment produced a perseverant effect in the target zone across ten daily, 3-min extinction trials, based on the percentages of time spent in the zone that previously contained the escape box (panels A and D). The dotted line from the 16.7% point of the ordinate spanning to the width of the abscissa indicates chance performance. Panels B and E. No treatment effect was observed for either experiment on adjusted speed [distance/(180 – total time immobile)]. Rimonabant treatment significantly increased time spent immobile (s) in experiment 1 (panel C), but not in experiment 2 (panel F, p=0.07). * p< 0.05; ** p<0.01 vs. extinction day 1 for each respective group. $ p<0.05 vs. the vehicle group. All data are represented as mean ± SEM. N=8-20 mice/group.

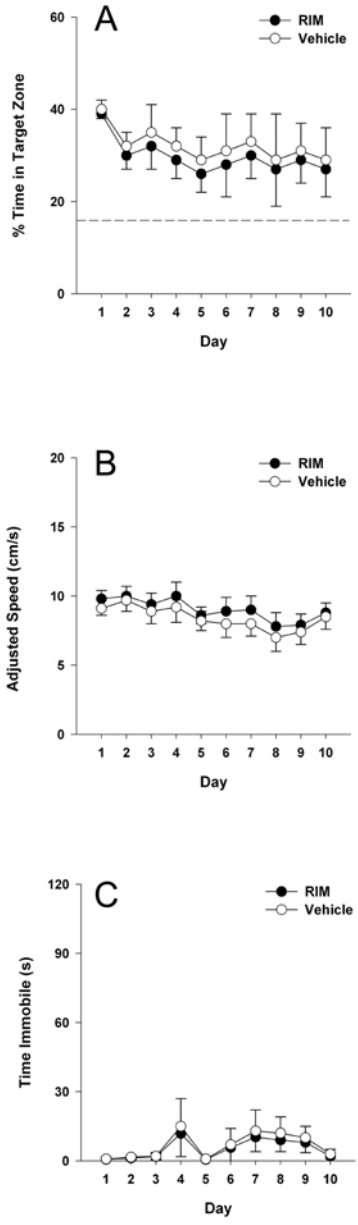

Figure 14: Rimonabant fails to affect extinction learning in an appetitively conditioned Barnes maze task. Panel A. Both rimonabant and vehicle treatment groups exhibited a significant decrease in the percentage of time spent in the target zone, which previously contained the goal. The dotted line from the 16.7% point of the ordinate spanning to the width of the abscissa indicates chance performance. Adjusted running speeds [distance/(180 – total time immobile); panel B], as well as total time spent immobile (panel C) were unaffected by treatment condition. All data are represented as mean ± SEM. *N*=7-9 mice/group.

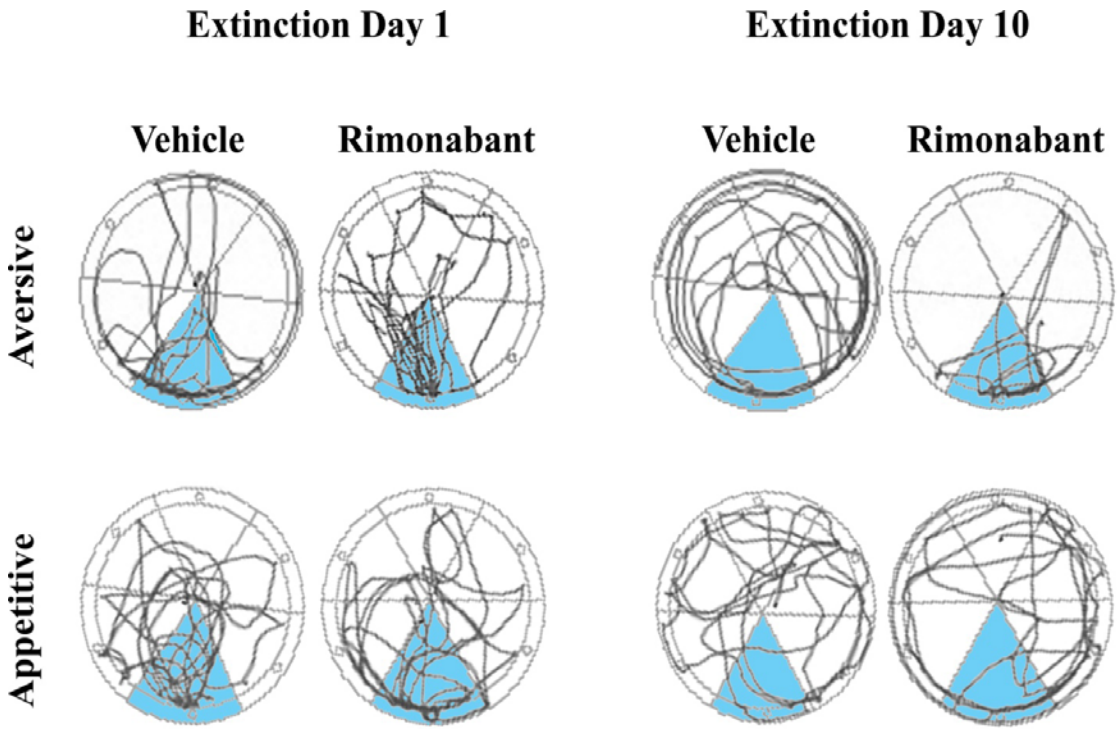

Figure 15: Track plots of representative vehicle- and rimonabant-treated mice in aversive (top panels) and appetitive (bottom panels) conditioning procedures on Days 1 and 10, from the experiments presented in figures 13 and 14. The target zone (i.e., the area that previously contained the goal box) for each trace is highlighted. Additionally, the plots have been rotated to display the target zone at the bottom.

DISCUSSION

Rimonabant treatment disrupted extinction learning under aversive, but not under appetitive conditions. These data strongly support the hypothesis that the endocannabinoid system mediates learning under aversive conditions, but is dispensable for appetitively-motivated learning (Holter *et al.*, 2005). Unlike previous reports, this study represents the first case investigating the neurochemical mechanisms underlying extinction learning in which the same behavioral demands were required (i.e. locating and entering the goal box) and only the reinforcement was varied. Control mice trained in either the aversive or appetitive Barnes maze conditioning paradigm showed a gradual decline in the percentage of time spent in the target zone across the extinction trials. The rimonabant-treated mice trained in the aversive conditioning procedure continued to spend significantly more time in the zone that previously contained the escape box than each of the other zones throughout extinction training. In contrast, the vehicle-treated mice showed a gradual decline in the percentage of time spent in the target zone across the extinction trials. Conversely, in the appetitive task, rimonabant-treated mice showed a virtually identical decrease in the percentage of time spent in target zone as vehicle-treated mice. Importantly, previous control experiments demonstrated extinction learning was independent of forgetting, under both appetitive and aversive conditions.

These data support the initial hypothesis of Holter *et al.,* (2005) that the endocannabinoid system is dispensable for extinction learning in an appetitively-motivated learning tasks. Specifically, they found CB_1 -/- and +/+ mice displayed similar extinction rates in an operant nose-poke for food paradigm. Similarly, Niyuhire *et al,* (2007) reported that rimonabant administration (1 or 3 mg/kg) failed to alter extinction rates in an appetitively-motivated operant conditioning paradigm in which the mice were trained to press a lever for access to sweetened milk, though rimonabant administration produced a reduction in the extinction burst on the first extinction trial. Finally, Ward *et al* (2007) found no differences in extinction learning between CB_1 -/- and +/+ mice.

There are three alternative explanations for the apparent lack of evidence supporting endocannabinoid modulation of appetitively motivated extinction. First, disruption of CB_1 receptor signaling has been demonstrated to decrease salience of food reward (Ward and Dykstra, 2005), decreased salience for palatable sucrose solution (Higgs *et al.*, 2003), and reduce operant responding for food (De Vry and Jentzsch, 2004; Holter *et al.*, 2005; Ward *et al.*, 2007). It is important to note that Holter *et al.,* (2005) employed a more stringent deprivation schedule in the CB_1 -/- mice than the wild type counterparts in order to ensure that both genotypes displayed similar response rates. Similarly, Ward *et al.,* (2007) analyzed extinction as a percentage of baseline responding during maintenance sessions. Nonetheless, in both experiments the CB_1 -/- mice still displayed equivalent extinction rates as the wild type mice. Second, recent evidence has emerged showing that CB_1 -/- mice exhibit an accelerated and age-related deficit in cognitive ability, which was associated with the loss of hippocampal neurons beginning

at three months of age (Bilkei-Gorzo *et al.*, 2005). Importantly, Holter *et al.*,(2005) utilized CB_1 -/- mice that were 11-14 weeks and Ward *et al.*, (2007) used 7-8 month-old mice (Ward, October 2007, Personal Communication), corresponding with significant cognitive deficits found by Bilkei-Gorzo *et al.*(2005) in other learning paradigms. Thus, the age-related neurodegeneration in the CB_1 -/- mice may play a contributing role in the reported extinction deficits, though this explanation would not hold for rimonabant-treated animals.

A third challenge in interpreting the role of the endogenous cannabinoid system on extinction learning is the difficulty in comparing the results of experiments across different behavioral paradigms. Each of the reports examining appetitively motivated conditioning tasks employed an operant procedure, which has qualitatively different behavioral demands than learning paradigms employing either electric shock as the unconditioned stimulus (i.e., conditioned freezing and passive avoidance) or the Morris water maze. Thus, it may be that extinction of operant behavior, rather than the hedonic value of the reinforcer, is refractory to CB_1 receptor disruption. Moreover, the reinforcers in the conditioned freezing, passive avoidance, Morris water maze, and operant tasks are qualitatively different and not interchangeable. To focus on the qualitative nature of the reinforcer, we modified the Barnes maze task in the present study to evaluate appetitively and aversively motivated conditioning procedures on extinction, but required the same motor responses (i.e., searching and entering the goal box). Additionally, we departed from employing highly palatable food rewards in favor of water to limit confounding variables related to motivational factors. Notably, rimonabant administration does not

affect water consumption (Arnone *et al.*, 1997), an observation supported by the present study. The differential effects of rimonabant on extinction in our two Barnes maze tasks support the hypothesis that the endocannabinoid system is dispensable for the extinction of appetitively motivated behaviors.

While we have previously employed the Morris water maze to evaluate the role of the endogenous cannabinoid system on extinction, this task is inherently aversive (Morris, 1984) . This notion is supported by the observation that water maze training activates the pituitary adrenal axis, causing an increase in corticosterone (Sandi *et al.*, 1997; Akirav *et al.*, 2001). In contrast, the Barnes maze spatial memory paradigm presented a unique opportunity of comparing different sources of reinforcement, either aversive or appetitive (i.e. food and entering the goal box), to motivate the same goal. Only a handful of published reports have employed the Barnes maze to evaluate mice and most of this work has relied on similar sources of aversive reinforcement, such as bright lights, air turbulence, or auditory stimuli (Bach *et al,* 1995; Fox *et al,* 1998; Williams *et al,* 2003; Pompl *et al,* 1999; Inman-Wood *et al,* 2000; Bredy *et al,* 2004). Conversely, other studies have utilized positive reinforcement, such as food, gentle handling, or the return to the home cage to motivate Barnes maze learning (Grootendorst *et al,* 2001; Williams *et al,* 2003; Blizard *et al,* 2003; Koopmans *et al,* 2003; Harrison *et al,* 2006). In concert with results from the Morris water maze spatial memory paradigm, rimonabant treatment disrupted extinction learning in the aversive conditioning task. Furthermore, our results indicate that rimonabant administration during acquisition is not necessary to

affect extinction in aversive conditions. In contrast, rimonabant failed to affect extinction learning in the appetitive version of the task.

Rimonabant did not affect acquisition of either an appetitively- or an aversively-conditioned Barnes maze task. These data are in agreement with previous results from the Morris water maze (Varvel *et al.*, 2005), passive avoidance (Mazzola *et al.*, 2003; Niyuhire *et al.*, 2007), conditioned fear (Marsicano *et al.*, 2002; Suzuki *et al.*, 2004; Kamprath *et al.*, 2006; Niyuhire *et al.*, 2007), and operant conditioning paradigms (Mallet and Beninger, 1998; Niyuhire *et al.*, 2007). Of interest, rimonabant treatment significantly increased immobility time under both aversive and appetitive conditions of reinforcement. One possible explanation for the increased immobility time is an anxiogenesis, and is supported by the observation that rimonabant dose-dependently increases plasma corticosterone (Patel *et al.*, 2004; Wade *et al.*, 2006; Steiner *et al.*, 2008). However, indistinguishable performance during the first extinction trial would suggest that both vehicle and rimonabant treated subjects acquired the task to a comparable degree.

Pharmacotherapies directed at the endocannabinoid system hold potential promise for the treatment of a variety of maladies including, pain and inflammation (Lichtman *et al.*, 2004; Hohmann *et al.*, 2005), obesity (Rinaldi-Carmona *et al.*, 1994), drug abuse (Arnone *et al.*, 1997), diabetes (Anthenelli and Despres, 2004), anxiety (Gaetani *et al.*, 2003; Kathuria *et al.*, 2003), depression (Gobbi *et al.*, 2005; Hill and Gorzalka, 2005), and possibly post traumatic stress syndrome (Marsicano *et al.*, 2002; Chhatwal *et al.*, 2005; Varvel *et al.*, 2007). The results presented here provide compelling evidence that

the endocannabinoid system mediates the extinction of behaviors that are associated with aversive memories, leaving extinction of learned behaviors from appetitively reinforced tasks intact. While it is unknown whether the endocannabinoid system is involved in the extinction of other forms of positively reinforced (e.g. mating) behavior, the system's impact on aversively motivated learning is clear. These results underscore the concern over the therapeutic use of rimonabant or other cannabinoid receptor antagonists. Specifically, contraindication might be warranted for patients diagnosed with posttraumatic stress syndrome, as this disorder is believed to contain an element in which patients display deficits in extinguishing certain maladaptive behaviors associated with anxiety or panic attacks (Rothbaum and Davis, 2003). This observation is further supported by a recent meta-analysis of rimonabants clinical trials, reporting a 40% increase in side effects ranging from depression and anxiety to suicidal thoughts (Christensen *et al.*, 2007). Conversely, pharmacotherapies that enhance endocannabinoid signaling, such as FAAH inhibitors or a cannabinoid receptor agonists, may accelerate extinction of aversively motivated behaviors. In conclusion, the results of the present study are the first to show a differential effect of rimonabant on extinguishing a learned behavior that only differed in the hedonic nature of the reinforcer.

EXPERIMENT 3

INTRODUCTION

A growing body of research has employed genetically altered mice to examine the role that the endocannabinoid system plays on learning. However, conflicting reports exist regarding how acquisition is affected, and under what conditions. Genetic disruption of CB_1 receptor signaling enhances acquisition of an active avoidance paradigm (Martin *et al.*, 2002), but impairs contextual conditioned fear (Mikics *et al.*, 2006), and delay eyeblink conditioning (Kishimoto and Kano, 2006). On the other hand, CB_1 -/- mice display similar acquisition as wild-type mice in the Morris water maze (Varvel and Lichtman, 2002; Varvel *et al.*, 2005), cued conditioned fear (Marsicano *et al.*, 2002; Cannich *et al.*, 2004; Kamprath *et al.*, 2006), and trace eyeblink conditioning (Kishimoto and Kano, 2006) tasks. Differences in procedural demands may underlie the disparate nature of these reports.

Under conditions of appetitive reinforcement, a more uniform collection of results have been reported. Bilkei-Gorzo *et al.,* (2005), were the first to report intact acquisition of an operant conditioning procedure in young (6-7 weeks of age) CB_1 -/- mice compared to age-matched controls. However, mature (3-5 months old) and old (14-17 months) CB_1 -/- mice exhibited an accelerated age-dependent decline in acquisition of the task

compared to controls, suggesting differences in age warrant consideration when interpreting results from studies utilizing CB_1 -/- mice . For example, acquisition learning was unaffected in operant conditioning experiments utilizing mature CB_1 -/- and +/+ mice trained to nose-poke for food pellets (Holter *et al.*, 2005), Ensure (a sweetened protein drink), or corn-oil (Ward *et al* 2007; Ward, personal communication, 2007). However, as CB_1 -/- mice exhibit reduced motivation for food-reward, the extent to which phenotypic differences in hedonics may have contributed to these results remains unknown (Holter *et al.*, 2005; Ward and Dykstra, 2005; Ward *et al.*, 2007).

For this dissertation, I developed a novel Barnes maze procedure in which a variable source of reinforcement (i.e., aversive or appetitive) is used to motivate the acquisition of a consistent goal (i.e., finding and entering the goal box). Application of this new procedure presented the opportunity to clarify the relative importance of procedural demands and hedonics, with regards to the expression of genotypic differences in acquisition learning. Age-matched CB_1 -/- and +/+ litter-mate controls were given acquisition training under conditions of aversive or appetitive reinforcement conditions. In the appetitive procedure, the mice were water restricted and access to water served as the appetitive reinforcer. Importantly, water consumption is unaffected by genetic deletion of the CB_1 receptor (Poncelet *et al.*, 2003; Thanos *et al.*, 2005). The initial objective of this study was to provide a complementary approach to previous studies utilizing rimonabant during extinction. However, the data presented here revealed an impaired acquisition phenotype in CB_1 -/- mice, confounding the interpretation of extinction results. Thus, the purpose of this study was to compare the

consequence of CB_1 receptor deletion in acquisition of appetitive and aversive Barnes maze conditioning procedures. To this end, we tested the hypothesis that acquisition deficits associated with CB_1 receptor deletion are dependent hedonics, and CB_1 -/- mice would exhibit impaired acquisition and extinction learning under aversive, but not appetitive, reinforcement conditions.

METHODS

SUBJECTS

Subjects included CB_1 -/- (*n*=17) and CB_1 +/+ (*n*=17) mice between 8-16 weeks of age, on the C57BL/6 background that were born from breeding pairs at Virginia Commonwealth University. The original breeding pairs were obtained from Zimmer *et al*., (1999). All subjects were housed in a temperature-controlled (20-22º C) environment, with a 12-h light/dark cycle and ad libitum access to food. In the appetitive condition, the same methodology as previously described was utilized. In short, subjects were deprived of access to water for 22 h per day. Upon completion of each acquisition session, subjects were allowed access to water for 2 h per day. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

PROCEDURE

Two types of testing conditions were used: aversive and appetitive. In the aversive procedure, bright lights (two, 500 watt halogen bulbs) and two, 60 cm wide fans (Holmes, Milford, MA) for air turbulence were located 120 cm above the maze and remained on during all stages and trials. In the appetitive procedure, a modified goal box containing access to water was used as a reinforcer for water-deprived mice. Subjects were given 22 h of daily water deprivation before each session and were weighed for comparison to a pre-deprivation baseline. Immediately after each session, the mice were returned to their home cages, given access to water for 2 h, and weighed.

Shaping

Shaping occurred as described previously. In summary, all subjects were acclimated to the apparatus and basic procedure before formal acquisition training began. The subject was placed in the start cylinder and released. Three min later, the subject was placed in the goal box, which was then slid into one of the six corresponding target locations. Following two min of acclimation to the goal box, the mouse was placed into its home cage for a 30 s intertrial interval (ITI). After the ITI, the goal box was placed back into its corresponding location and the subject was guided from the center of the

maze to the entrance of the goal box. Shaping was concluded after at least two consecutive entries into the goal box without provocation from the investigator.

Acquisition

Again, the same acquisition procedure as previously described was employed. Each mouse was given four acquisition trials per day for ten days. Each trial ended when either three min had elapsed or the subject entered the goal box, whichever occurred first. In the event that the mouse failed to enter the goal box within the three min trial, it was placed in the center of the maze and the experimenter led it to the goal box where it remained for 30 s before being returned to its home cage for the 30 s ITI. If the mouse repeatedly found the goal box, but failed to enter, it was given additional shaping in which it was again placed in the center of the maze and led to the escape hole, a process that was repeated until the mouse entered the goal box without provocation from the experimenter. Acquisition measures included test duration (latency to enter the hidden goal box), total time spent immobile, distance traveled, and adjusted speed [distance/(latency to enter – time immobile)].

STASTICAL ANALYSES

AnyMaze (Stoelting, Wood Dale, IL) software was used to accumulate the dependent measures of interest. As reported, measures of interest included adjusted speed (distance traveled/(latency to enter – time immobile)), distance traveled, time spent immobile, and latency to enter the goal box (test duration).

Results from comparison studies were analyzed using two-way mixed design ANOVAs (genotype by session). A significant effect of genotype was further analyzed for each genotype condition by a one-way repeated measures ANOVA. Dunnett's post-hoc analysis with comparison to day one values was used when appropriate. Significant interactions were analyzed in the same manner, but also included comparison of genotype within each acquisition session using the Tukey post-hoc test.

RESULTS

CB_1 -/- mice exhibit deficits in acquiring an aversively conditioned Barnes maze task

Under the aversive conditioning procedure, both CB_1 -/- and +/+ mice acquired the task in a manner consistent with previous experiments, exhibiting a gradual reduction in distance traveled [fig. 16A; $F(9,144)=20.6$, $p<0.0001$], latency to enter [fig. 16B; $F(9,144)=30.9$, $p<0.0001$], and time immobile [fig. 16C; $F(9,144)=4.7$, $p<0.0001$].

Genetic ablation of the CB_1 receptor disrupted acquisition learning, exhibited by a significant genotype effect of distance [$F(1,144)=18.5$. $p<0.001$], as well as the corresponding latency to enter [$F(1,144)=26.4$, $p<0.0001$]. While no genotype differences were observed for adjusted speed [fig. 16D; $p=0.76$], CB_1 -/- mice spent significantly more time immobile than CB_1 +/+ mice [$F(1,144)=18.9$, $p<0.001$].

Significant interactions were observed for both latency to enter [$F(9,144)=1.95$, $p<0.05$] and time immobile [$F(9,144)=2.20$, $p<0.05$]. In each case, post-hoc analysis revealed significantly different values starting on acquisition day one ($p<0.01$). Furthermore, significant differences on the final acquisition day suggest acquisition deficits in CB_1 -/- mice perseverate despite continued acquisition training.

Analysis of the first four acquisition trials on day one resulted in a similar pattern of results. A significant effect of trial was observed for the latency to enter [Fig. 17B;

$F(3,48)=4.9$, $p<0.01$], distance traveled [Fig. 17A; $F(3,48)=10.8$, $p<0.0001$], and adjusted speed [fig. 17D; $F(3,48)=3.9$, $p<0.05$]. In contrast, no significant effect of trial on immobility time was found [Fig. 17C; $F(3,48)=2.0$, $p=0.11$]. Again, CB_1 -/- mice required a greater amount of time to enter the goal box, resulting in a significant effect of genotype [$F(1,48)=14.1$, $p<0.01$]. Notably, none of the CB_1 -/- mice entered the goal box on the first trial, likely contributing to the significant difference on day one, as shown in fig. 17B. Despite similar performance on the first trial, the disparity in immobility time between genotypes seen in the final three trials was sufficient to produce a significant effect of genotype [$F(1,48)=11.6$, $p<0.01$]. Furthermore, control animals exhibited no change in the amount of time spent immobile across trials, while CB_1 -/- mice exhibited a consistent increase in immobility, following the first trial. Finally, the genotypes did not differ with regards to adjusted speed [$F(1,48)=.35$, $p=0.55$].

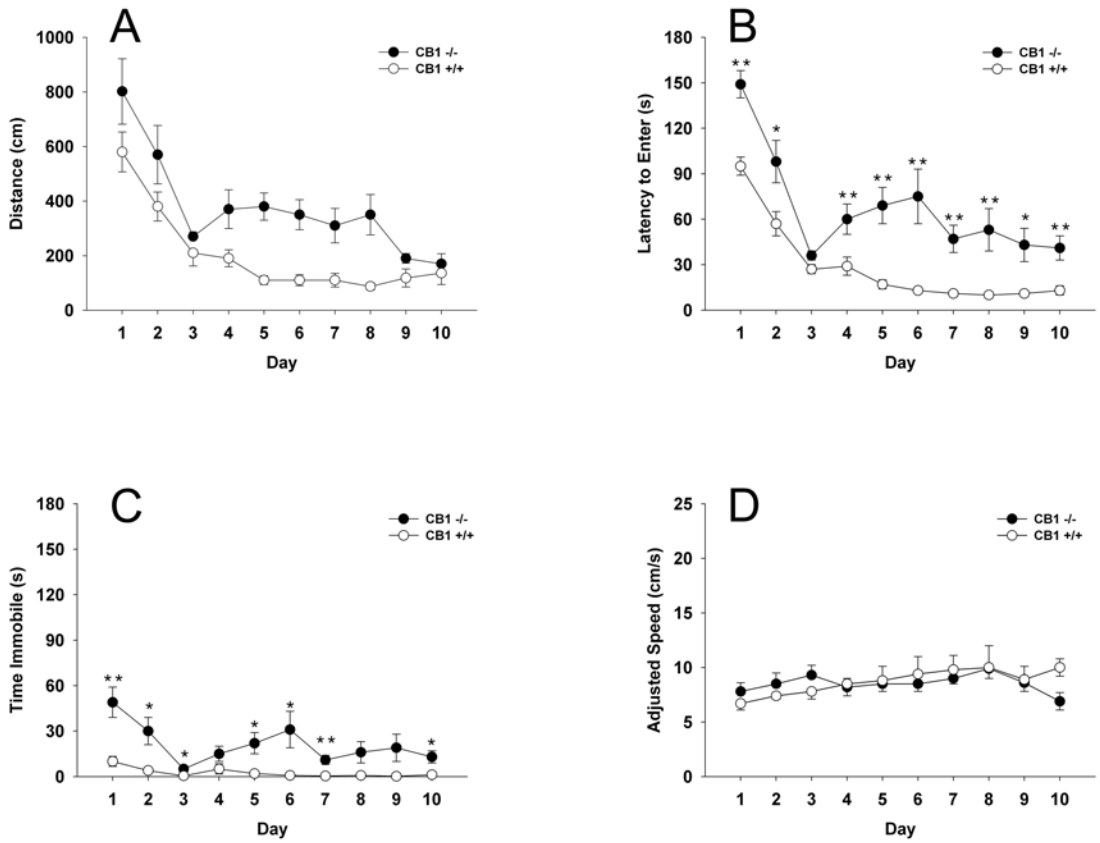

Figure 16: CB_1 -/- mice exhibit deficits in acquiring an aversively motivated Barnes maze task. Distance (cm) traveled (panel A), and corresponding latency (s) to enter (panel B) were significantly elevated in CB_1 -/- mice compared to wild-type controls. Panel C. A significant effect of genotype was observed for time immobile (s). Panel D. No genotype differences were observed for adjusted speed [distance/(latency to enter – total time immobile)]. * $p<0.05$ ** $p<0.01$ vs. the corresponding CB_1 (+/+) mice. The data for each acquisition session are represented as the average of four daily trials ± SEM. N=9 mice/group.

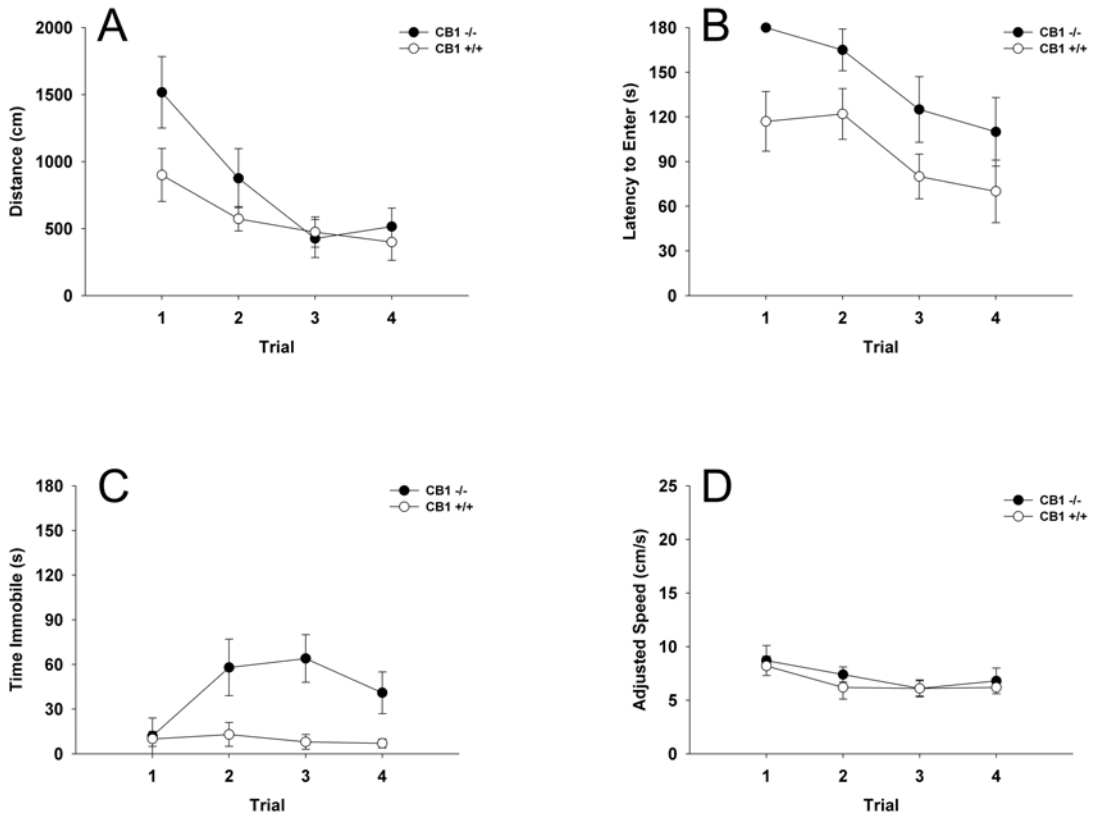

Figure 17: CB_1 -/- mice exhibit impaired acquisition on the first day of an aversively motivated Barnes maze task. No genotype differences were observed for distance (cm) traveled (panel A). Significantly greater latency (s) to enter (panel B) the goal box, as well as time (s) spent immobile (panel C) were observed in CB_1 -/- mice compared to CB_1 +/+ mice. The data are represented as the mean ± SEM. *N*=9 mice/group.

CB_1 -/- mice exhibit deficits in acquiring an appetitively conditioned Barnes maze task

Figure 18 A-D illustrate the primary dependent measures for acquisition of an appetitively conditioned Barnes maze task across ten days of acquisition. A significant effect of day was observed for distance [fig. 18A; $F(9,126)=30.3$, $p<0.0001$], latency to enter [fig. 18B; $F(9,126)=37.6$, $p<0.0001$], time immobile [fig. 18C; $F(9,126)=4.0$, $p<0.0001$], and adjusted speed [fig. 18D; $F(9,126)=4.9$, $p<0.0001$]; indicating both CB_1 -/- and CB_1 +/+ mice acquired the task.

While both genotypes learned the task, significant genotype differences in acquisition were observed for both latency to enter [$F(1,126)=7.2$, $p<0.05$], and distance traveled [$F(1,126)=8.9$, $p<0.01$]. In both cases, CB_1 -/- mice consistently traveled further, and took more time to enter the escape box, than their wild-type littermates, illustrating impaired acquisition of the task. In agreement with the aversive paradigm, genetic deletion of the CB_1 receptor resulted in a significant increased time immobile compared to wild-type controls [$F(1,126)=4.6$, $p<0.05$]. Finally, analysis of adjusted speed revealed a significant genotype by day interaction [$F(9,126)=2.5$, $p<0.01$]. However, post-hoc analyses revealed that genotypes only differed significantly on days seven and nine ($p<0.05$).

Unlike the aversive condition, analysis within the first acquisition day failed to yield any significant genotype differences [distance: $p=0.08$ (Fig. 19A), latency to enter: $p=0.08$ (Fig. 19B), time immobile: $p=0.15$ (Fig. 19C), adjusted speed: $p=0.48$ (Fig. 19D)]. Notably, mean values for all dependent measures on trial one were

indistinguishable between groups, suggesting significant differences across all ten acquisition days were not confounded by differences in initial baseline performance.

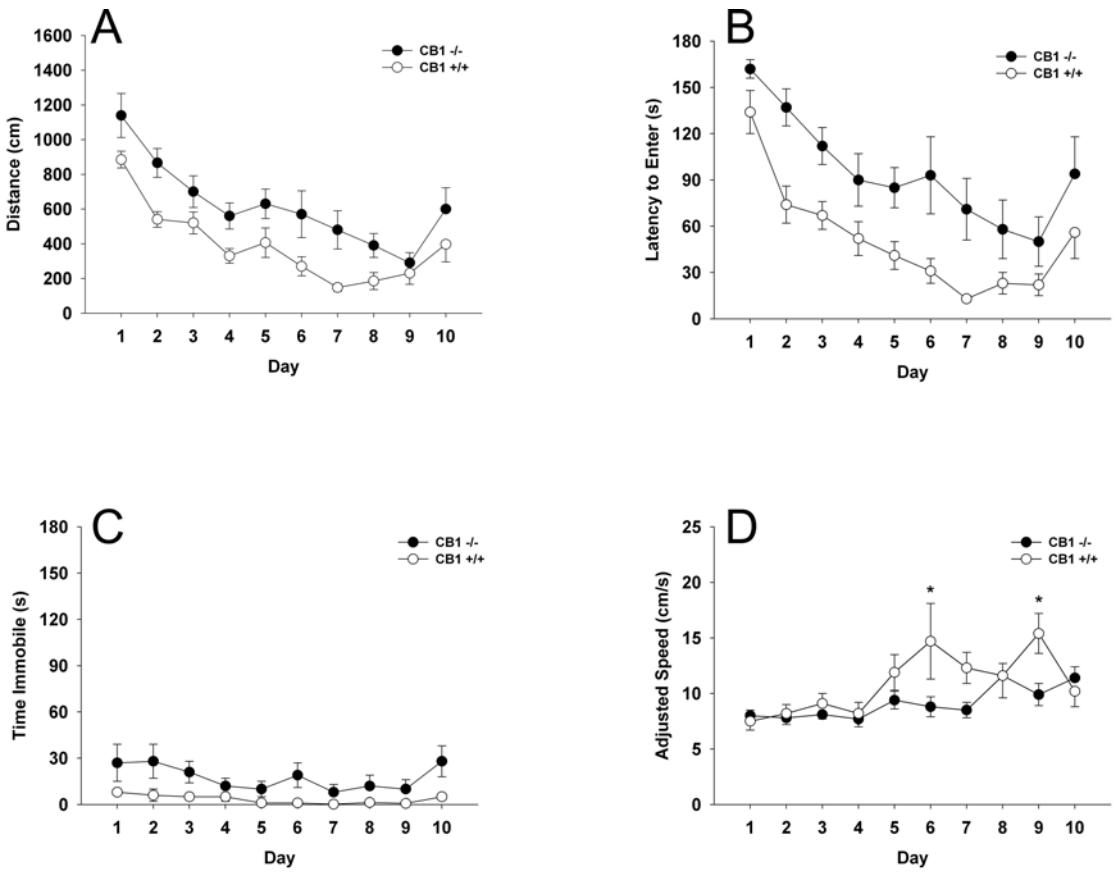

Figure 18: CB_1 -/- mice exhibit impaired acquisition of an appetitively reinforced Barnes maze task. Distance (cm) traveled (panel A), and corresponding latency (s) to enter (panel B) were significantly elevated in CB_1 -/- mice compared to wild-type controls. Panel C. CB_1 -/- spent significantly more time immobile (s) than their CB_1 +/+ controls. Panel D. a significant genotype by day interaction was observed for adjusted speed [distance/(latency to enter – total time immobile)]. * indicates a significant difference from CB_1 -/- mice. * $p<0.05$ The data for each acquisition session are represented as the average of four daily trials ± SEM. N=8 mice/group.

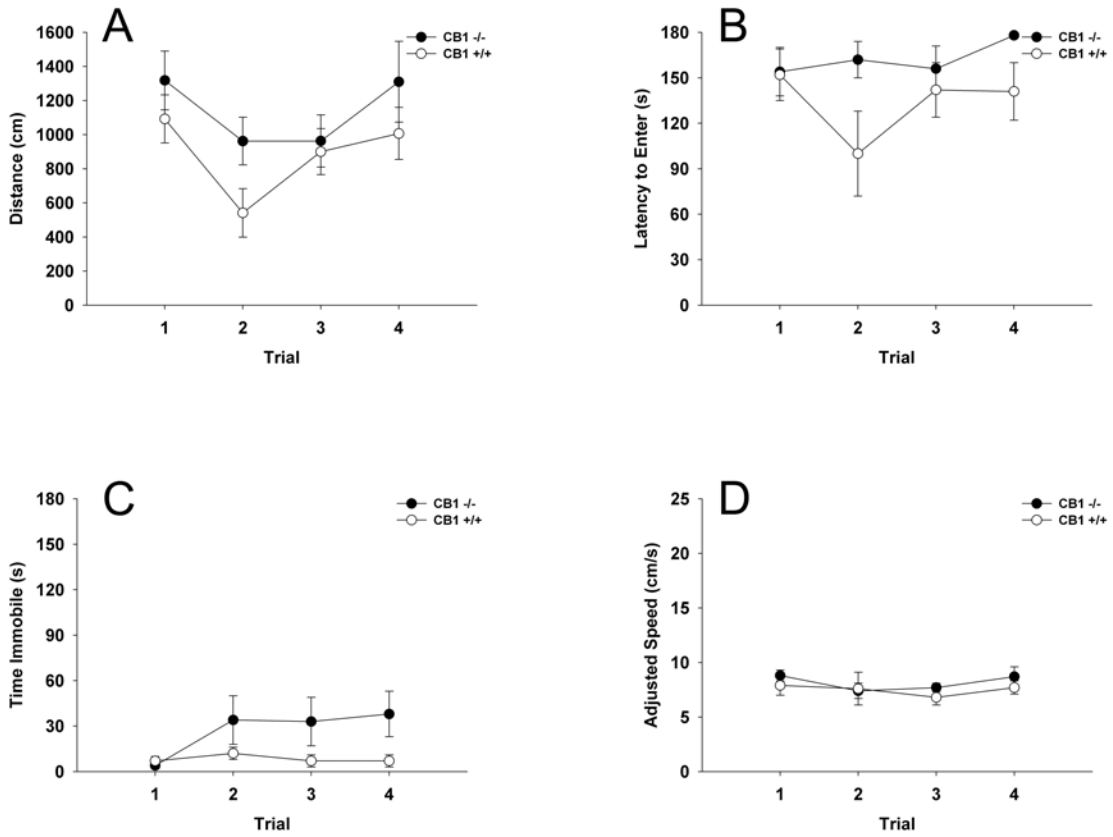

Figure 19: CB_1 -/- mice acquire the Barnes maze task under appetitive conditions on acquisition day one. The average distance (cm) traveled (panel A), and the corresponding latency (s) to enter (panel B), did not significantly differ between genotypes. Panel C. No genotype differences were observed for time immobile (s). Panel D. Adjusted speed [distance/(latency to enter – time immobile)] did not differ between genotypes. The data for each trial are represented as mean ± SEM. *N*=8 mice/group.

DISCUSSION

CB_1 receptor -/- mice displayed impaired acquisition learning under both aversive and appetitive conditions. These data suggest that acquisition deficits in CB_1 -/- mice are independent of the hedonic nature of the reinforcer. While we initially sought to evaluate extinction learning in CB_1 -/- mice as a complementary approach to previous studies utilizing rimonabant, the impaired acquisition phenotype of CB_1 -/- mice confounded interpretation of results. Unlike previous reports investigating acquisition learning following genetic deletion of the CB_1 receptor, the procedure employed here utilized the same behavioral demands (e.g. locating and entering the goal box) under varied conditions of reinforcement (i.e. aversive and appetitive). Under both aversive and appetitive conditions, CB_1 +/+ mice exhibited acquisition learning on par with our previous results, exhibiting a gradual decline in the latency to enter the goal box, as well as a reduction in the concurrent distance traveled. Conversely, CB_1 -/- mice displayed an increased path length, and required more time to enter the goal box relative to their wild-type littermates. CB_1 -/- mice exhibited impaired acquisition under both aversive and appetitive conditions, suggesting that the behavioral demands of the task, and not the source of reinforcement, are primarily responsible for the results presented here.

These data are in agreement with previous reports illustrating acquisition deficits in CB_1 -/- mice in aversively reinforced paradigms including contextual conditioned fear

(Mikics *et al.*, 2006), and delay eyeblink conditioning paradigms (Kishimoto and Kano, 2006). However, alternative paradigms dependent on aversive reinforcement have exhibited enhanced, or intact acquisition performance. For example, CB_1 -/- mice exhibited enhanced acquisition in the active avoidance paradigm(Martin *et al.*, 2002), as well as social and object recognition tasks (Reibaud *et al.*, 1999; Maccarrone *et al.*, 2002; Bilkei-Gorzo *et al.*, 2005); and normal acquisition of spatial memory (Varvel and Lichtman, 2002; Varvel *et al.*, 2005), cued conditioned fear (Marsicano *et al.*, 2002; Cannich *et al.*, 2004; Kamprath *et al.*, 2006), and trace eyeblink conditioning (Kishimoto and Kano, 2006). Conversely, in appetitively reinforced paradigms, CB_1 -/- mice show no impairment in acquiring operant conditioning (Bilkei-Gorzo *et al.*, 2005; Holter *et al.*, 2005; Ward *et al.*, 2007).

There are five possible explanations that may account for the lack of continuity in the literature. First, the primary challenge in interpreting these data is the confounding nature of comparing results across dissimilar behavioral paradigms. Differences in the nature of reinforcement, as well as behavioral demands associated with qualitatively different learning paradigms, preclude direct comparison across studies. For example, previous reports utilizing appetitive reinforcement have relied on operant condition paradigms, which have little in common with the behavioral demands of the Barnes maze task. Ultimately, differences in procedural demands may be fundamental in unmasking differences in acquisition performance between genotypes.

Second, exogenous administration of CB_1 receptor agonists have been demonstrated to increase the salience of food reward (Abel, 1975; Ward and Dykstra,

2005). Concurrently, pharmacological or genetic attenuation of CB_1 receptor signaling has been demonstrated to decrease the salience and motivation for a diverse array of palatable substances including high carbohydrate, high fat, and standard lab chow food-pellets (Verty *et al.*, 2004; McLaughlin *et al.*, 2006); sweet sucrose solutions (Arnone *et al.*, 1997; Higgs *et al.*, 2003; Sanchis-Segura *et al.*, 2004); the high-protein solution Ensure® (Ward and Dykstra, 2005); and high-fat corn-oil (Ward and Dykstra, 2005). Together, these reports illustrate the problematic nature of interpreting acquisition performance following eCB manipulation, independent of changes in the hedonic value of appetitive reward. In the current study, access to water within the goal box reinforced acquisition learning in water-deprived subjects. As water consumption is unaffected by manipulations of the eCB system (Arnone *et al.*, 1997; Colombo *et al.*, 1998; Poncelet *et al.*, 2003; Thanos *et al.*, 2005) the results presented here are suggested as independent of confounds related to changes in the hedonic value of the appetitive reward.

Third, the importance of controlling for age in CB_1 -/- mice has been underestimated until recently, and may also account for the lack of continuity among results. Bilkei-Gorzo *et al.,* (2005) were the first to report an accelerated age-related decline in both cognitive performance and the density of hippocampal neurons, beginning at three months of age. As subjects in the aversive paradigm were approximately twelve to sixteen weeks of age, the aforementioned decline in cognitive performance may have contributed to the impairment of acquisition learning. However, despite controlling for age in the appetitive condition (i.e. subjects were six to eight weeks of age), acquisition

deficits were still evident, suggesting that while age may exacerbate acquisition deficits, it does not fully account for its occurrence.

Fourth, given the age-related decline in hippocampal morphology following CB_1 receptor deletion, it is important to note that hippocampal injury is correlated with impaired acquisition learning in the Barnes maze (Fox *et al.*, 1998; Paylor *et al.*, 2001; Deacon and Rawlins, 2002; Raber *et al.*, 2004) and other spatial memory tasks (Morris, 1984; Logue *et al.*, 1997). Moreover, disruption of hippocampal function impairs contextual fear conditioning (Phillips and LeDoux, 1992; Logue *et al.*, 1997), and spatial mapping (Sutherland *et al.*, 1982). In contrast, damage to the hippocampus appears independent of performance in cued spatial memory tasks (Morris, 1984; Fox *et al.*, 1998), as well as cued conditioned fear (Phillips and LeDoux, 1992; Logue *et al.*, 1997). Together, these reports underscore differences in substrate demands associated with disparate learning tasks, and may contribute to the conflicting results presented here, and those previously established.

Finally, in addition to disrupting acquisition of the Barnes maze task, genetic attenuation of CB_1 receptor signaling produced a significant increase in immobility time, independent of the conditioning procedure. These data would suggest that processes unrelated to learning and memory may be contributing to the observed acquisition deficits. As the aversive paradigm is assumed to be more anxiogenic than the appetitive paradigm, this hypothesis is further supported by the observation that all CB_1 -/- mice failed to enter the goal box on the first trial of day one in the aversive paradigm. Importantly, the caveat that CB_1 -/- mice failed to enter the goal box on the first trial

because they found the Barnes maze less aversive than wild-type mice deserves attention. However, this explanation appears unlikely as repeated Barnes maze exposures would result in habituation to the stressful stimuli, gradually decreasing the anxiety provoked by aversive stimuli. In turn, habituation to the stress of Barnes maze exposure would increase the probability of electing not to enter the goal box in favor of remaining on the maze. In contrast, both CB_1 -/- and +/+ mice exhibited a reduction in the latency to enter across successive trials. Further supporting the hypothesis that processes unrelated to learning may be responsible for the effects reported here is the report of similar phenotypic differences in CB_1 null mutant mice by Varvel & Lichtman (2002) in the Morris water maze. Unlike wild-type controls, CB_1 -/- mice were characterized by labored motor behavior, a propensity for floating, seizures that ultimately resulted in death, and the development of incompatible swim strategies (i.e. repetitive circling behaviors). Ultimately, half of these mice were removed from the study for failing to reach inclusion criteria. Similar methodological considerations designed to normalize performance between genotypes have been reported, including increased food deprivation (Holter *et al.*, 2005), or comparison to baseline performance (Ward *et al.*, 2007). Together, the diversity in methodology, statistical transformations, and inclusion criteria may play a primary role in determining whether anxiety-like behaviors are reported.

There is a growing body of literature supporting eCB modulation of stress and anxiety. CB_1 receptors expression occurs at different levels controlling the HPA axis, such as the hippocampus and amygdala (Marsicano and Lutz, 1999; Mackie, 2005), hypothalamus (Cota *et al.*, 2003), the pituitary (Wenger *et al.*, 1999), and adrenal glands

(Galiegue *et al.*, 1995). Administration of the CB_1 receptor antagonist rimonabant dose-dependently increases plasma corticosterone and ACTH (Manzanares *et al.*, 1999; Wade *et al.*, 2006; Steiner *et al.*, 2008), and is potentiated by restraint stress (Patel *et al.*, 2004; Steiner *et al.*, 2008). In rats, chronic unpredictable stress significantly increases plasma corticosterone, and reduces hippocampal, but not limbic forebrain, CB_1 receptor protein expression by 50% (Hill *et al.*, 2005a). Similarly, CB_1 -/- mice exhibit higher basal levels of plasma corticoserone and ACTH (Barna *et al.*, 2004; Cota *et al.*, 2007; Steiner *et al.*, 2008), and hyper-responsiveness to stress (Barna *et al.*, 2004; Steiner *et al.*, 2008), as well as CRH- and forskolin-induced ACTH secretion (Cota *et al.*, 2007). While HPA axis dysregulation in CB_1 -/- mice has been postulated to arise from developmental deficits (Wade *et al.*, 2006), a recent report by Steiner *et al*., (2007) would suggest this is not the case, as results in CB_1 -/- mice were replicated following rimonabant administration. Together, these reports, as well as the data presented here, support a role of CB_1 receptor signaling as a mechanism for dampening stress-induced corticosterone secretion; independent of genetic background, type of stressor, or method of CB_1 receptor disruption (e.g. pharmacological vs. genetic). In relation to the present study, differences in basal corticosterone secretion may contribute to the observed increase in immobility time. Observationally, CB_1 -/- mice spent an unusual amount of time immobile adjacent to the goal box entrance prior to entering. This observation would explain for consistently greater values for latency to enter and time immobile, and accounts for the eventual asymptotic performance between genotypes with respect to distance traveled.

However, anything beyond speculation warrants further elucidation of stress responses following Barnes maze exposure.

The impact of chronic eCB disruption, either through cannabis use or pharmacological antagonism, on cognition remains unclear. The results presented here provide considerable evidence that long-term inactivation of eCB signaling disrupts both aversively and appetitively reinforced acquisition learning. Furthermore, as the qualitative behavioral demands of the task were unchanged between reinforcement conditions, these data are the first to compare acquisition performance while controlling for procedural differences. A notable caveat that remains undetermined is the possible involvement of confounds related to genetic knockout mice, including genetic drift, compensatory changes, and downstream developmental alterations (Taft *et al.*, 2006). These data are in conflict with previous results indicating that acquisition learning is unaffected by pharmacological CB_1 receptor antagonism. In fact, the only consistent result between studies was increased immobility time in both aversively and appetitively motivated procedures. The lack of continuity between studies would suggest that impaired acquisition learning in CB_1 -/- mice is a result of confounds associated with gene deletion, or chronic attenuation of CB_1 receptor signaling. To address this uncertainty, future studies evaluating Barnes maze acquisition learning following chronic administration of rimonabant are warranted. Nonetheless, these data raise the intriguing possibility that chronic disruption of eCB signaling may ultimately take a substantial toll on certain mnemonic processes.

EXPERIMENT 4

INTRODUCTION

The results of studies utilizing direct acting cannabinoid agonist administration has contributed in a growing acceptance that the eCB system modulates cognitive processes. However, confounds associated with exogenous CB_1 agonist administration, such as disruptive effects on motor behavior and memory, preclude the interpretation of results as simulating endogenous activity (Pamplona and Takahashi, 2006). In contrast, the recent availability of FAAH -/- mice (Cravatt *et al.*, 2001), and FAAH inhibitors provide an alternative approach to evaluate eCB function by inhibiting the metabolism of the endocannabinoid anandamide, in effect magnifying and prolonging eCB signaling. Already, a growing body of research would suggest FAAH inhibitors as a potential pharmacotherapeutic with regards to disorders of depression (Gobbi *et al.*, 2005; Naidu *et al.*, 2007), anxiety (Kathuria *et al.*, 2003; Patel and Hillard, 2006; Naidu *et al.*, 2007), pain (Lichtman *et al.*, 2004), and cognition (Varvel *et al.*, 2006; Varvel *et al.*, 2007).

The disruptive effects of exogenously administered CB_1 agonists on acquisition learning have been exhibited in rodents (Varvel *et al.*, 2001) and humans (Chait and Pierri, 1992). However, the impact of enhancing endogenous eCB signaling on cognitive processes remains enigmatic. Varvel *et al.,* (2006) were the first to report an

enhancement of working memory in FAAH -/- mice during acquisition of the Morris water maze spatial memory task. In this experiment, FAAH -/- mice displayed accelerated acquisition rates compared to wild-type littermates. In a follow-up experiment by the same group, FAAH -/- mice again exhibited enhanced acquisition, this time of a fixed-platform task (Varvel *et al*, 2006). While these data suggest enhancing eCB signaling facilitates acquisition learning, the lack of alternative studies in the literature underscore the current inability to elucidate the conditions and extent to which these effects are unmasked.

Procedural differences associated with disparate tasks, as well as the hedonic nature of the reinforcer can determine the absence or expression of eCB modulated cognitive effects. For example, the disruption of extinction learning following rimonabant administration is primarily dependent on the nature of the reinforcer. As we have shown, rimonabant disrupts extinction learning under aversive, but not appetitive conditions. Conversely, CB_1 -/- mice exhibit impaired acquisition of both aversive and appetitively conditioned Barnes maze spatial learning. However, as these studies evaluated attenuated CB_1 receptor signaling, the extent to which these observations generalize to enhanced eCB signaling remain unknown.

The present study utilized the Barnes maze procedure in which a variable source of reinforcement (i.e. aversive or appetitive) motivates the acquisition of a consistent goal (i.e. entering the goal box). Application of this new procedure presented the opportunity to clarify the relative importance of procedural demands and hedonics, with regards to the expression of genotypic differences in acquisition learning. The primary purpose of this

experiment was to test the hypothesis that FAAH -/- mice would exhibit enhanced acquisition under aversive, but not appetitive, conditioning procedures. Furthermore, we postulated a CB_1 receptor mechanism of action for enhanced acquisition in FAAH -/- mice. To this end, a separate study was conducted in which FAAH -/- were administered the CB_1 receptor antagonist, rimonabant, or vehicle. As the same procedure was used, differences were controlled and only the source of reinforcement was manipulated, we sought to determine if the enhancement of acquisition learning observed in FAAH -/- mice is dependent on the nature of the reinforcer.

METHODS

SUBJECTS

FAAH -/- mice (*n*=44) were derived from FAAH -/- congenic breeding pairs that had been back-crossed onto a C57BL/6J background approximately 14 generations. FAAH +/+ (*n*=32) mice used in the study were produced by FAAH +/+ parents derived from a cross between a FAAH -/- congenic parent mated with a C57BL/6J from Jackson labs. The resulting +/- offspring were then crossed with C57BL/6J mice to derive +/+ breeding pairs. All FAAH -/- and FAAH +/+ mice were bred from breeding pairs in Virginia Commonwealth University (Lichtman *et al.*, 2004). All subjects were housed in a temperature-controlled (20-22º C) environment, with a 12-h light/dark cycle and ad libitum access to food, and in the aversive condition, water. In the appetitive condition, the same methodology as previously described was utilized. In short, subjects were deprived of access to water for 22 h per day. Upon completion of acquisition trials, subjects were allowed access to water for 2 h per day. All experiments have been approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

PROCEDURE

Two types of testing conditions were used: aversive and appetitive. In the aversive procedure, bright lights (two, 500 watt halogen bulbs) and two, 60 cm wide fans (Holmes, Milford, MA) for air turbulence were located 120 cm above the maze and remained on during all stages and trials. In the appetitive procedure, a modified goal box containing access to water was used as a reinforcer for water-deprived mice. Subjects were given 22 h of daily water deprivation before each session and were weighed for comparison to a pre-deprivation baseline. Immediately after each session, the mice were returned to their home cages, given access to water for 2 h, and weighed.

Shaping

Shaping occurred as described previously. In summary, all subjects were acclimated to the apparatus and basic procedure before formal acquisition training began. The subject was placed in the start cylinder and released. Three min later, the subject was placed in the goal box, which was then slid into one of the six corresponding target locations. Following two min of acclimation to the goal box, the mouse was placed into its home cage for a 30 s intertrial interval (ITI). After the ITI, the goal box was placed back into its corresponding location and the subject was guided from the center of the

maze to the entrance of the goal box. Shaping was concluded after at least two consecutive entries into the goal box without provocation from the investigator.

Acquisition

Again, no deviations in acquisition procedure were employed. Each mouse was given four acquisition trials per day for ten days. Each trial ended when either three min had elapsed or the subject entered the goal box, whichever occurred first. In the event that the mouse failed to enter the goal box within the three min trial, it was placed in the center of the maze and the experimenter led it to the goal box where it remained for 30 s before being returned to its home cage for the 30 s ITI. If the mouse repeatedly found the goal box, but failed to enter, it was given additional shaping in which it was again placed in the center of the maze and led to the escape hole, a process that was repeated until the mouse entered the goal box without provocation from the experimenter. Acquisition measures included test duration (latency to enter the hidden goal box), total time spent immobile, distance traveled, and adjusted speed [distance/(latency to enter – time immobile)].

STASTICAL ANALYSES

AnyMaze (Stoelting, Wood Dale, IL) software was used to accumulate the dependent measures of interest. As reported, measures of interest included adjusted speed [distance traveled/(latency to enter – time immobile)], distance traveled, time spent immobile, and latency to enter the goal box (test duration).

Results from comparison studies were analyzed using two-way mixed design ANOVA (genotype by session). A significant effect of genotype was further analyzed for each genotype condition by a one-way repeated measure ANOVA. Dunnett's post-hoc analysis with comparison to day one values was used when appropriate. Significant interactions were analyzed in the same manner, but also included comparison of genotype within each acquisition session using the Tukey post-hoc test.

RESULTS

FAAH -/- mice exhibit enhanced acquisition of an aversively conditioned Barnes maze task

Under conditions of aversive reinforcement both FAAH -/- and +/+ mice acquired the Barnes maze task, resulting in a significant effect of day for latency to enter [fig. 20B; $F(9,126)=35.2$, $p<0.0001$], distance traveled [fig. 20A; $F(9,126)=18.7$, $p<0.0001$], time immobile [fig. 20C; $F(9,126)=3.5$, $p<0.001$], and adjusted speed [fig. 20D; $F(9,126)=13.4$, $p<0.0001$].

Comparison between genotypes resulted in a significant effect of latency to enter, characterized by accelerated improvement in the task by FAAH -/- mice compared to wild-type controls, resulting in both a significant effect of genotype [$F(1,126)=22.2$, $p<0.001$] as well as a genotype by day interaction [$F(9,126)=2.1$, $p<0.05$]. While FAAH -/- mice spent significantly less time immobile than FAAH +/+ mice, resulting in a significant effect of genotype [$F(1,126)=11.7$, $p<0.01$], the FAAH -/- mice also showed significantly higher mean running speed [$F(1,126)=9.9$, $p<0.01$] (data not shown), which persisted when time immobile was accounted for (i.e. adjusted speed) [$F(1,126)=9.7$, $p<0.01$].

Analysis within the first day of acquisition resulted in a significant effect of trial for duration [fig. 21B; $F(3,42)=4.0$, $p<0.05$], but not distance [fig. 21A; $p=0.14$], time immobile [fig. 21C; $p=0.19$], or adjusted speed [fig. 21D; $p=0.07$].

No significant genotype effects were observed on the first acquisition day for all measures [distance: $p=0.13$, time immobile: $p=0.8$, adjusted speed: $p=0.15$] with the exception of latency to enter [$F(1,42)=4.9$, $p<0.05$]. Notably, genotype differences for latency to enter were most prominent on trial three and four whereas on the first trial, both genotypes exhibited similar starting latencies. The absence of any significant genotype differences during the first trial would suggest that differences between genotypes on day one in figure 20 are not confounded by learning that may have occurred during shaping.

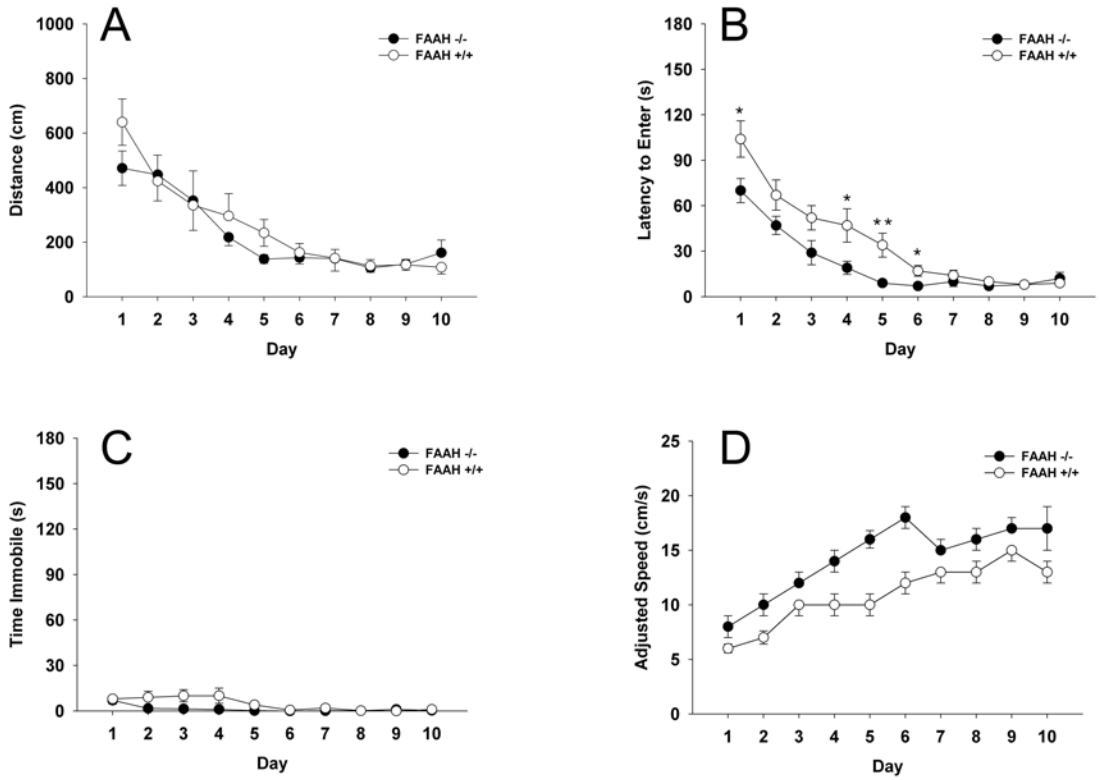

Figure 20: FAAH -/- mice show enhanced acquisition of an aversive Barnes maze procedure. No differences between genotypes were observed for distance (cm) traveled (panel A), however, the corresponding average latency (s) to enter (panel B) significantly differed between genotypes. FAAH -/- mice spent significantly less time (s) immobile (panel C), and displayed a greater adjusted speed [distance/(latency to enter – total time immobile)] compared to FAAH +/+ controls. * $p<0.05$ ** $p<0.01$ vs. FAAH -/- mice. The data for each acquisition session are represented as the average of four daily trials ± SEM. * denotes a significant difference from FAAH -/- mice. N=8 mice/group.

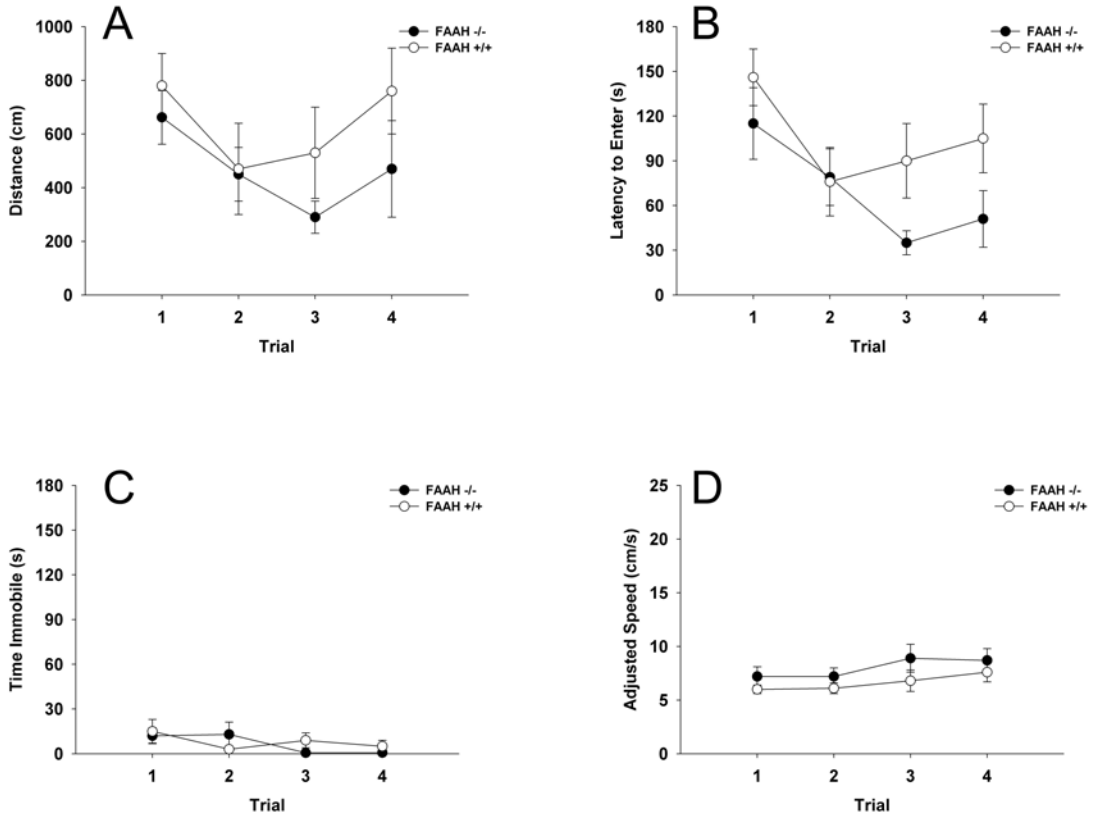

Figure 21: FAAH -/- mice exhibit enhanced acquisition within the first acquisition day of an aversive Barnes maze procedure. No genotype differences were observed for distance (cm) traveled (panel A). Panel B. A significant genotype effect was observed for latency (s) to enter the goal box with FAAH -/- acquiring the task faster than wild-type controls. No genotype differences were observed for the amount of time (s) spent immobile (panel C), or adjusted speed [distance/(latency to enter – time immobile)]. All data are represented as mean ± SEM. *N*=8 mice/group.

FAAH -/- mice display normalized acquisition under appetitive conditions

Under appetitive conditions, analysis of FAAH -/- and +/+ mice across ten days of acquisition resulted in a significant effect of acquisition day for distance traveled [fig. 22A; $F(9,162)=17.9$, $p<0.0001$], latency to enter [fig. 22B; $F(9,162)=41.3$, $p<0.0001$], time immobile [fig. 22C; $F(9,162)=12.5$, $p<0.0001$], and adjusted speed [fig. D; $F(9,162)=10.4$, $p<0.0001$].

While these results illustrate the occurrence of acquisition learning, only time immobile significantly differed between genotypes [$F(1,162)=7.6$, $p<0.05$]. In contrast to the aversive paradigm, exposure to the appetitive condition increased the average amount of time spent immobile in the FAAH -/- mice compared to FAAH +/+ controls. However, no other genotype differences were observed (latency to enter: $p=0.15$, distance traveled: $p=0.40$, adjusted speed: $p=0.70$).

Within the first acquisition day, a significant effect of trial was observed for distance traveled [fig. 23A; $F(3,54)=10.3$, $p<0.0001$], latency to enter [fig. 23B; $F(3,54)=6.9$, $p<0.001$], time immobile [fig. 23C; $F(3,54)=2.9$, $p<0.05$], and adjusted speed [fig. 23D; $F(3,54)=3.5$, $p<0.05$]. However, no significant differences between genotypes (latency to enter: $p=0.10$, distance traveled: $p=0.77$, time immobile: $p=0.81$, adjusted speed: $p=0.06$), or genotype by day interactions, were observed, indicating that performance was not affected by the shaping procedure.

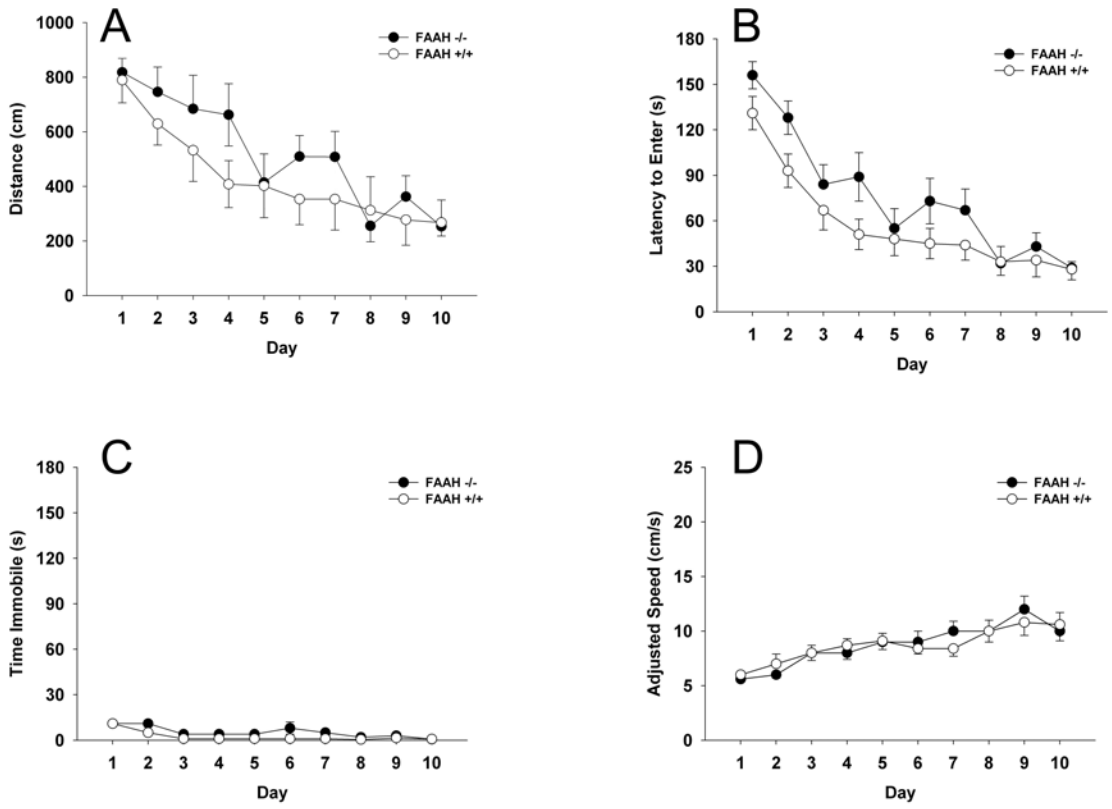

Figure 22: FAAH -/- mice show normal acquisition in an appetitively motivated Barnes maze task. No genotype effects were observed for distance (cm) traveled (panel A), or the corresponding latency (s) to enter the goal box (panel B). A significant effect of genotype on the amount of time (s) spent immobile (panel C) was observed. Panel D. Analysis of adjusted speed [distance/(latency to enter – time immobile)] revealed no differences between genotypes. The data from each acquisition session are represented as the average of four daily trials ± SEM. *N*=10 mice/group.

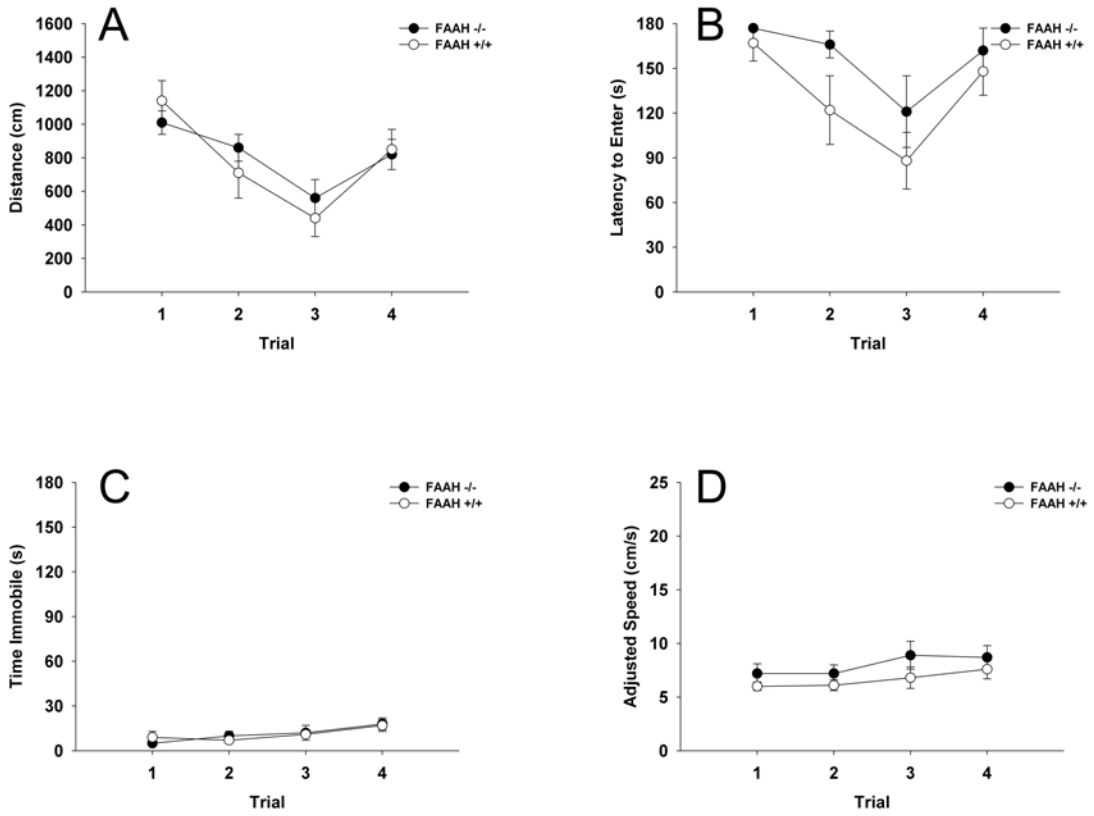

Figure 23: FAAH -/- mice show normal acquisition of an appetitive Barnes maze task, within the first day. No genotype effects were observed for distance (cm) traveled (panel A), or the corresponding latency (s) to enter the goal box (panel B). No differences were observed between genotypes based on time (s) spent immobile (panel C), or adjusted speed [distance/(latency to enter – time immobile)]. All data are represented as the average of each trial within the first acquisition day ± SEM. *N*=10 mice/group.

Rimonabant (1mg/kg) Administration does not affect Barnes Maze Acquisition

Future experiments would utilize rimonabant to test the hypothesis that the enhancement of acquisition, observed in FAAH -/- mice, is dependent on CB_1 receptor signaling. However, as a 3 mg/kg dose of rimonabant has been show to increase immobility during acquisition of the Barnes maze task, characterization of a lower dose was necessary to determine if similar effects would be elicited. A 1 mg/kg dose was chosen as it has been shown to attenuate the behavioral effects of i.v. THC, without producing locomotor disturbances when administered alone (Compton *et al.*, 1996).

Administration of rimonabant or vehicle 30 min before exposure to the aversively conditioned Barnes maze task did not affect acquisition learning, indicated by the absence of significant treatment effects (Fig. 24A; distance: p=0.94; Fig 24B.; duration: p=0.16; Fig. 24C; time immobile: p=0.16; Fig. 24D adjusted speed: p=0.36), or treatment by day interactions (duration: p=0.59; distance: p=0.92; time immobile: p=0.29; adjusted speed: p=0.57). Independent of treatment, the occurrence of acquisition learning was denoted by a significant effect of acquisition day for all dependent measures, including distance [$F(2,20)=8.8$, $p<0.01$], the corresponding latency to enter [$F(2,20)=27.3$, $p<0.0001$], time immobile [$F(2,20)=11.7$, $p<0.001$], and adjusted speed [$F(2,20)=8.2$, $p<0.01$].

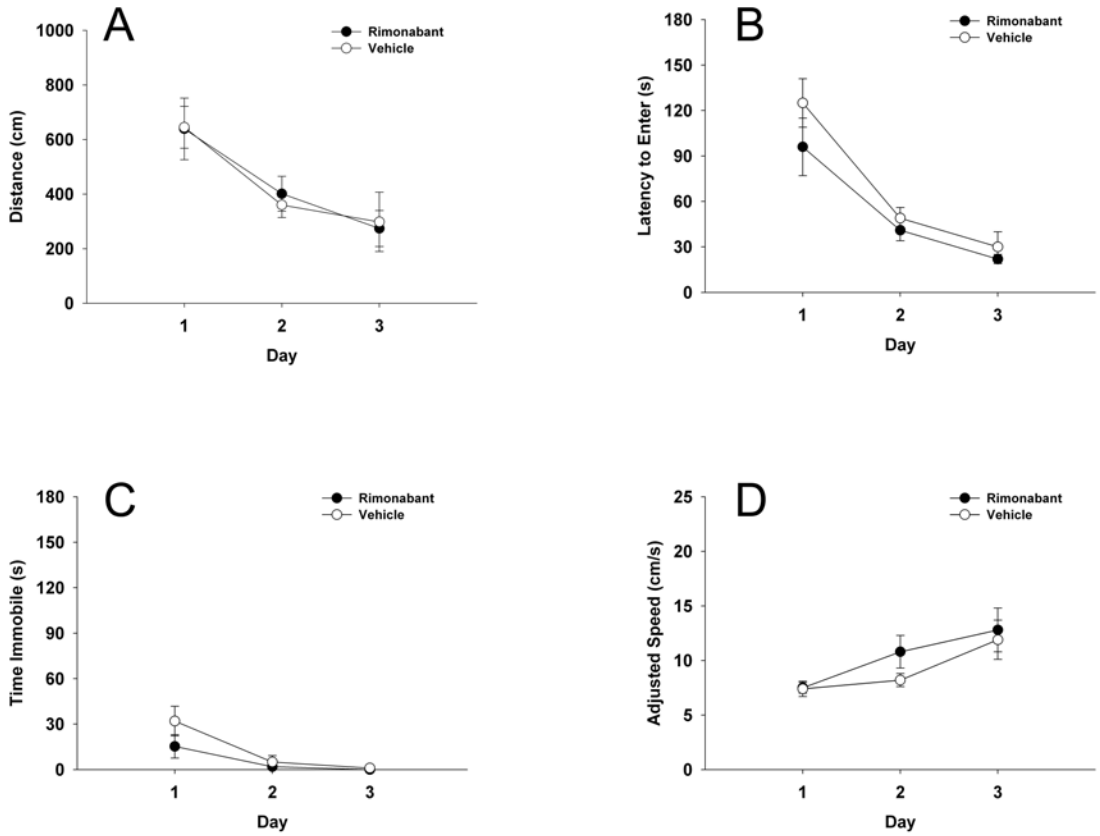

Figure 24: Rimonabant (1mg/kg i.p.) does not affect acquisition learning in an aversively reinforced Barnes maze task. No differences were observed for distance traveled, or the corresponding latency to enter the goal box. Panel C. Rimonabant treatment does not affect the average time spent immobile. Panel D. No differences were observed for adjusted speed [distance/(duration – time immobile)]. All data are represented as the average of four daily trials ± SEM. *N*=6 mice/group.

Rimonabant attenuates the enhancement of acquisition in FAAH -/- mice under aversive conditions

To determine if genotype differences in acquisition were CB_1 mediated, FAAH -/- mice were administered either 1 mg/kg rimonabant or vehicle and compared to vehicle-injected FAAH +/+ controls. A significant effect of trial was observed for distance traveled [fig. 25A; $F(9,333)=61.6$, $p<0.0001$], latency to enter [fig. 25B; $F(9,333)=104.8$, $p<0.0001$], time immobile [fig. 25C; $F(9,333)=10.7$, $p<0.0001$], and adjusted speed [fig. 25D; $F(9,333)=26.0$, $p<0.0001$].

Comparison between groups failed to indicate differences in motor behavior between groups, either for distance traveled [$F(2,333)=1.6$, $p=0.21$] or adjusted speed [$F(2,333)=1.4$, $p=0.25$]. However, a significant group difference for latency to enter [$F(2,333)=3.6$, $p<0.05$], as well as a group by day interaction [$F(18,333)=1.7$, $p<0.05$], was observed. Post-hoc analysis indicated that FAAH -/- mice administered vehicle were significantly different than FAAH -/- mice administered rimonabant ($p<0.05$). However, comparison to respective day one values using Dunnett's test indicated that all groups showed significant improvement ($p<0.01$) starting on day two.

Further differences were observed between groups when time spent immobile was analyzed by repeated measures ANOVA. In this case, a significant effect of group [$F(2,333)=4.2$, $p<0.05$] as well as a group by day interaction [$F(18,333)=2.0$, $p<0.01$] were observed. Similar to duration, Tukey post-hoc analysis revealed a significant difference between FAAH -/- mice administered rimonabant or vehicle. However, only

FAAH -/- mice administered vehicle exhibited a significant ($p<0.01$) reduction in time spent immobile by day 2, following Dunnett's comparison to the first acquisition day.

Analysis within acquisition day one revealed a significant effect of trial for the dependent measures distance [fig. 26A; $F(3,111)=6.0$, $p<0.001$], and latency to enter [fig. 26B; $F(3,111)=3.6$, $p<0.05$], but not time immobile (Fig. 26C, $p=0.15$) or adjusted speed (Fig. 26D, $p=0.71$). No significant effects of group (duration: $p=0.20$; distance: $p=0.44$; time immobile: $p=0.15$; adjusted speed: $p=0.92$), or group by trial interactions (duration: $p=0.86$; distance: $p=0.93$; time immobile: $p=0.39$; adjusted speed: $p=0.55$), were observed.

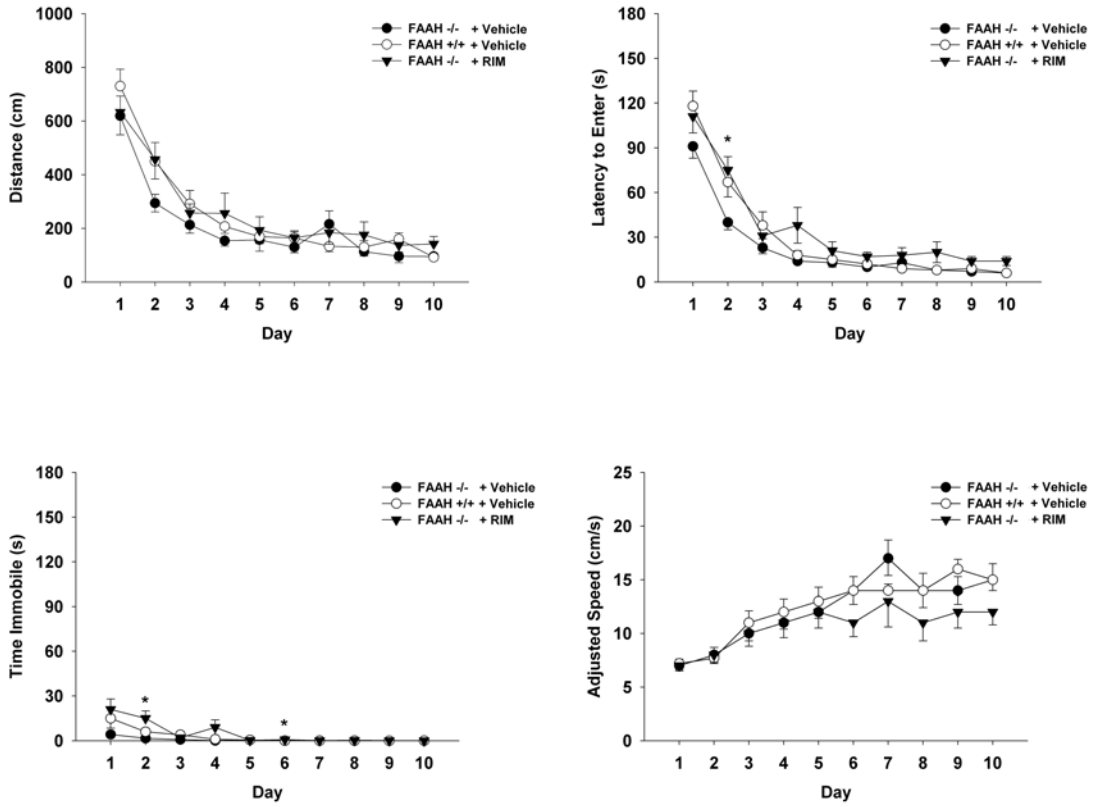

Figure 25: Rimonabant administration (1 mg/kg) attenuates the enhancement of acquisition in FAAH -/- mice under aversive conditions. Rimonabant or vehicle was administered i.p. 30-min prior to testing on each acquisition day. No differences were observed on distance (cm) traveled, however a significant effect of condition was observed for the corresponding latency (s) to enter the goal box (panel B). A significant effect of condition was also observed for time (s) spent immobile (panel C). Panel D. Analysis of adjusted speed [distance/(latency to enter – time immobile)] failed to reveal any significant differences between conditions. * indicates a significant difference between FAAH -/- + RIM and FAAH -/- + Vehicle groups. * $p<0.05$. All data are represented as the average of four daily trials ± SEM. N= 13-14 mice/condition.

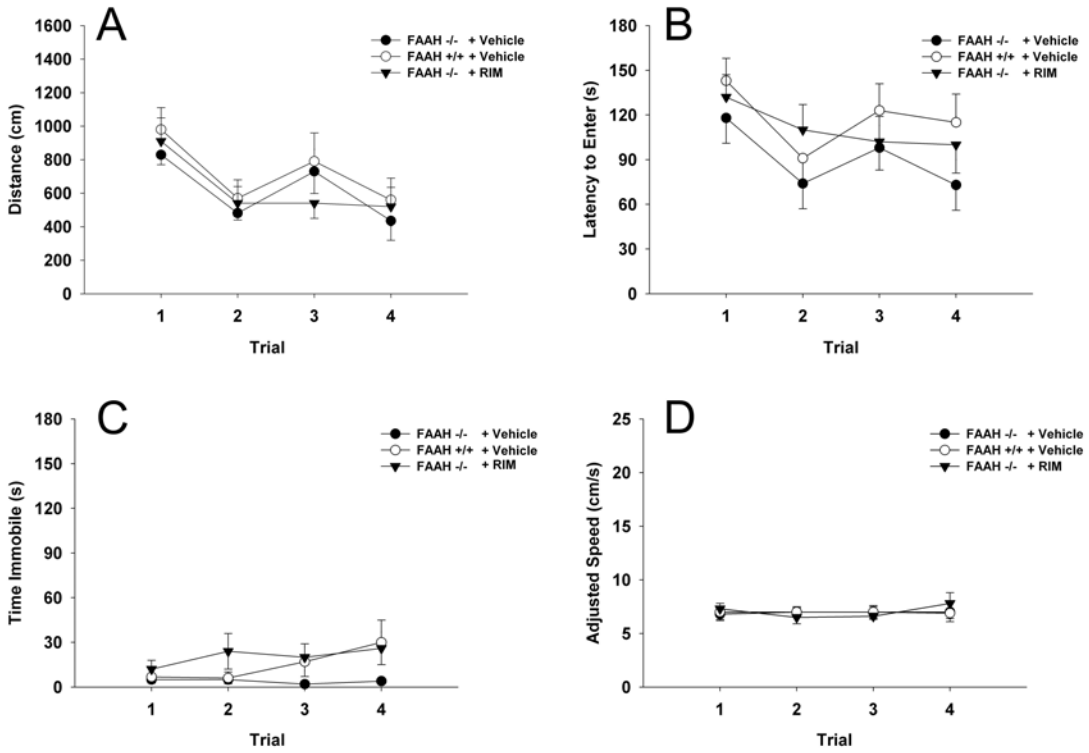

Figure 26: Rimonabant (1 mg/kg) does not affect acquisition in FAAH -/- mice within the first acquisition day of an aversively conditioned Barnes maze task. Rimonabant or vehicle was administered i.p. 30-min prior to the first acquisition trial. No differences between conditions were observed for distance (cm) traveled (panel A), or the corresponding latency (s) to enter the goal box (panel B). Panel C. No differences were observed for time immobile (s). Panel D. Adjusted speed [distance/(latency to enter – time immobile)] failed to yield any significant differences between conditions. All data are represented as the average of each trial ± SEM. *N*=13-14 mice/condition.

DISCUSSION

FAAH -/- mice exhibited enhanced acquisition learning in an aversive, but not an appetitive, Barnes maze task. These data support the hypothesis that stimulating eCB signaling enhances acquisition of an aversively reinforced spatial memory task (Varvel *et al.*, 2007). Furthermore, as the behavioral demands (i.e. locating and entering the goal box) remained unchanged between reinforcement conditions, to our knowledge this is the first report to illustrate the enhancement of acquisition following FAAH deletion as dependent on reinforcement conditions. The data also extend the hypothesis that the eCB system mediates extinction learning under aversive conditions, but is dispensable for appetitively-motivated learning (Holter *et al.*, 2005) to include acquisition learning. While we initially proposed evaluating extinction learning, FAAH +/+ mice failed to exhibit decreased perseverant behavior in the target zone, confounding interpretation of results.

During initial acquisition sessions in the aversive paradigm, FAAH -/- mice required less time than their wild-type counterparts to achieve the goal of the task, entering the hidden goal box. A similar pattern of results was observed within the first acquisition day, as FAAH -/- mice required less time to enter the goal box, resulting in a significant effect of genotype. As both genotypes exhibited similar latencies within the

first trial, the observed effects are independent of learning that may have occurred during shaping. Importantly, the CB_1 receptor antagonist rimonabant attenuated the enhancement, indicating the effects were specific to the FAA anandamide. In the appetitive conditioning procedures, no differences between genotypes were observed with regards to the latency to enter the goal box. Consequently, the aforementioned effects on acquisition learning are specific to aversive conditions.

An unexpected finding in these experiments was that the adjusted speed under aversive conditions of the FAAH -/- mice was significantly quicker than for the FAAH +/+ mice, suggesting that the decreased escape latencies in the FAAH -/- mice can be accounted by differences in motor behavior, and is further supported by the observation that genotypes did not differ in the distance traveled to complete the task. While initial reports utilizing exogenous administration of AEA have shown a hypomotile response, (Crawley *et al.*, 1993; Fride and Mechoulam, 1993; Smith *et al.*, 1994) it is generally accepted that this approach does not mimic endogenous function given AEA's short half-life *in vivo* (i.e. <5 min.) (Willoughby *et al.*, 1997). In contrast, inhibition of FAAH provides an alternative approach by inhibiting the metabolism of endogenously released anandamide, in effect magnifying and prolonging the physiological response. Genetic and pharmacological approaches to FAAH inhibition have suggested motor behavior is unaffected in the open-field test (Cravatt *et al.*, 2001; Cippitelli *et al.*, 2007; Moreira *et al.*, 2008), and elevated plus maze (Naidu *et al.*, 2007). Conversely, in the fixed platform Morris water maze (Varvel *et al.*, 2007) paradigm, the authors report a significant increase in swim speed in FAAH -/- mice, as well as enhanced acquisition. Similar to the

aversive conditioning Barnes maze paradigm, the methodology employed in the Morris water maze utilized aversive reinforcement to motivate learning a fixed goal location in a spatial memory task (Morris, 1982). Thus, aversive reinforcement appears to be necessary to unmask genotype differences in motor behavior and/or acquisition of spatial memory.

Alternatively, the enhancement of acquisition presented here may reflect alterations in emotionality and attentional processes. Specifically, pharmacological inhibitors of FAAH have been reported to produce an anxiolytic response in several murine models of emotionality (Kathuria *et al.*, 2003; Viveros *et al.*, 2005; Bortolato *et al.*, 2006; Patel and Hillard, 2006; Naidu *et al.*, 2007). Likewise, both pharmacological and genetic attenuation of eCB signaling have been shown to produce anxiogenesis (Navarro *et al.*, 1997; Arevalo *et al.*, 2001; Haller *et al.*, 2002; Maccarrone *et al.*, 2002; Martin *et al.*, 2002; Rodgers *et al.*, 2005; Patel and Hillard, 2006). As we have shown previously, CB_1 -/- mice spend more time immobile and exhibit acquisition deficits when exposed to the aversively conditioned Barnes maze paradigm, supporting the initial hypothesis that heightened emotionality contributes to the disruption of spatial memory learning (Ferrari *et al.*, 1999). Furthermore, as FAAH -/- mice spent significantly less time immobile, and acquired the task faster than controls the results, the data would suggest the eCB system facilitates learning under aversive conditions by dampening the expression of anxiety.

In contrast to the results from the aversive paradigm, FAAH -/- mice spent significantly more time immobile than controls in the appetitive conditioning procedure.

As eCB's are presumed to be released on demand in response to aversive stimuli (Di Marzo *et al.*, 1994; Marsicano *et al.*, 2002; Marsicano *et al.*, 2003; Piomelli, 2003; Hohmann *et al.*, 2005), it is possible that the conditions associated with the appetitive paradigm are insufficient to increase eCB signaling. However, this explanation does not account for the increase in immobility time under the appetitive conditioning procedure. However, it deserves mentioning that immobility is a very complex phenotype and may result from multiple factors in addition to freezing, including fear and arousal.

Alternatively, the rewarding value of escaping an aversive situation may be enhanced in FAAH -/- mice. Evidence suggesting the endocannabinoid and dopaminergic systems work in tandem to activate reward processes is further supported by observations that increase FAAH inhibition increases motivation for ethanol and food consumption (Giuffrida *et al.*, 1999; De Vries *et al.*, 2001; Di Marzo *et al.*, 2001; Blednov *et al.*, 2007; Soria-Gomez *et al.*, 2007). However, these observations do not adequately account for the observation that FAAH -/- mice spend significantly more time immobile under appetitive conditions. Instead, compensatory changes associated with genetic FAAH deletion may account for these observations. Given that only the source of reinforcement was altered in the present experiment, differences in hedonics between the aversive and appetitive paradigm would appear to be of primary importance. For example, uncharacterized alterations in hedonics associated water deprivation, or water consumption, might occur following genetic manipulation. However, the latter explanation appears insufficient as FAAH -/- mice show a preference for water containing ethanol, but not sucrose or quinine, in the two-bottle choice paradigm

(Basavarajappa *et al.*, 2006; Blednov *et al.*, 2007). Thus, it appears future experiments are required to elucidate possible genotype differences in motivation following water deprivation.

As FAAH is primarily responsible for the metabolism of many FAAs (i.e. AEA, oleamide, PEA, and OEA) (Cravatt *et al.*, 1996), we used rimonabant to determine whether the phenotypic acceleration in acquisition was CB_1 receptor mediated. Indeed, rimonabant normalized acquisition learning in FAAH -/- mice to the same rate as FAAH +/+ mice, implicating a CB_1 receptor mechanism of action, as well as the involvement of the only FAAH substrate with relevant cannabinoid activity, anandamide. Of interest, rimonabant treatment increased the amount of time spent immobile in FAAH -/- mice to levels indistinguishable from vehicle treated controls, providing evidence that FAAH -/- mice display a CB_1 receptor mediated anxiolytic response to aversive conditions. Importantly, we have shown that the dose of rimonabant used in the current experiment (1 mg/kg) does not affect Barnes maze performance.

In conclusion, the results provided here provide evidence that the eCB system modulates acquisition under aversive conditions, but is dispensable for appetitively-motivated learning. Furthermore, these data suggest FAAH inhibitors be useful as an alternative pharmacotherapy in treating anxiety disorders. Currently, the most predominant pharmacological treatment remains GABA agonists, which are characterized by generalized anxiolysis and side-effects such as learning and memory disruptions, and abuse liability. In contrast, FAAH inhibitors appear to exert their anxiolytic properties under specific conditions such as exposure to aversive conditions

(Naidu *et al.*, 2007), lack side-effects associated with cannabinoid agonists (Lichtman *et al.*, 2004), and facilitate certain mnemonic processes.

GENERAL SUMMARY

The data presented here support the following conclusions: 1) Mice acquire, and extinguish the Barnes maze task under both appetitive and aversive conditions of reinforcement, 2) Rimonabant does not impair the acquisition of either an appetitively or aversively conditioned Barnes maze task, 3) Rimonabant disrupts extinction learning of an aversive, but not an appetitive, Barnes maze task, 4) Extinction learning is independent of forgetting, 5) Compared to CB_1 +/+ mice, CB_1 -/- mice exhibit deficits in acquiring both an aversive and appetitive Barnes maze task, and 6) FAAH -/- mice acquire an aversively, but not appetitively, conditioned Barnes maze task faster than FAAH +/+ controls.

The initial objective of these studies was to develop, and characterize, a paradigm in which the source of reinforcement varied, and the goal of the task remained constant. To this end, we adapted the Barnes maze task (Barnes, 1979) so that bright lights and air turbulence motivated learning under aversive conditions, and access to water reinforced learning under appetitive conditions. Importantly, previous research has demonstrated that alterations in eCB signaling can affect a subject's motivation to work for palatable food (De Vry and Jentzsch, 2004; Holter *et al.*, 2005; Ward and Dykstra, 2005), but does not alter water consumption (Arnone *et al.*, 1997; Colombo *et al.*, 1998; Poncelet *et al.*, 2003; Thanos *et al.*, 2005). As subsequent studies focused on manipulations to the

endogenous cannabinoid system, we departed from the use of palatable food reward in favor of access to water under appetitive conditions.

We evaluated three conditioning procedures in the Barnes maze task: appetitive, aversive, and ambient. In the aversive condition, bright lights and air turbulence motivated learning the task. Conversely, in the appetitive condition, subjects were water deprived and the goal box was modified to provide access to water. Finally, conditions common to both the aversive and appetitive procedure (i.e., no bright lights, air turbulence, or water deprivation) were utilized for the ambient conditioning paradigm. Subjects in each condition displayed normal acquisition learning, exhibiting gradual improvement in their ability to complete the task, based on the latency and distance traveled to enter the hidden goal box. Under conditions common to both the appetitive and aversive conditions, subjects in the ambient conditioning group consistently traveled further, spent more time immobile, and took longer than their aversively or appetitively conditioned counterparts. These findings suggest that the added source of motivation, either aversive or appetitive, accelerate and improve acquisition of the Barnes maze task. In addition, the results also suggest that Barnes maze exposure is sufficient to motivate learning the task, possibly through an innate aversion to open fields, or the intrinsic reward value of the goal box.

During extinction of the task, the goal box was removed and subjects in each condition were given a three min probe trial, and the percentage of time spent in the target zone, the zone which was formerly associated with the goal box, was used as a measure of extinction learning. Subjects in all three reinforcement conditions

extinguished the task, exhibiting a gradual reduction in the percentage of time in the target zone following repeated exposures to the Barnes maze. Importantly, a control experiment was performed to distinguish forgetting from extinction learning. In this experiment, subjects were divided into two groups following acquisition. While the first group (Group Extinction) received their first nine extinction trials, the second group (Group No Extinction) remained in the vivarium. On the tenth and final extinction trial for Group Extinction, both groups were evaluated. While Group Extinction exhibited a significant reduction in time spent in the target zone, Group No Extinction exhibited a preference for the target zone, indistinguishable from Group Extinctions first trial. These results support the conclusion that mice extinguished the behavior, and did not merely forget the escape box location.

Having characterized Barnes maze acquisition and extinction learning under qualitatively different conditioning procedures, we tested the hypothesis that rimonabant would disrupt extinction learning under aversive, but not appetitive, conditions. The hypothesis that rimonabant is dispensable for appetitively, but not aversively conditioned extinction learning, was first proposed by Holter *et al*., (2005) and recently supported by Niyuhire *et al*., (2007). In both reports, attenuated CB_1 receptor signaling, either pharmacological or genetic, did not significantly impair extinction of operant tasks. In contrast, rimonabant and genetic deletion of the CB_1 receptor disrupts extinction learning in a number of aversively reinforced procedures (Marsicano *et al.*, 2002; Suzuki *et al.*, 2004; Varvel *et al.*, 2005; Kamprath *et al.*, 2006; Niyuhire *et al.*, 2007). However, the validity of the hypothesis by Holter *et al.*, (2005) remains undetermined due in part to the

difficulty of reconciling between differences in hedonics and behavioral demands of disparate tasks. Specifically, behavioral demands required by operant tasks (i.e. learning to press a lever for food reward) are qualitatively different from the Morris water maze (i.e. learning to locate and swim to a hidden platform) or conditioned freezing (i.e. learning a tone is predictive of a shock). Furthermore, the experiments by Holter *et al.,* and Niyuhire *et al.,* utilized palatable food reward to reinforce operant learning. As discussed, genetic and pharmacological antagonism of CB_1 receptor signaling decreases the salience of food reward (Ward and Dykstra, 2005), as well as palatable sucrose solution (Higgs *et al.*, 2003), and reduces operant responding for food (De Vry and Jentzsch, 2004; Holter *et al.*, 2005; Ward *et al.*, 2007). To address these outstanding issues, we evaluated extinction learning in a Barnes maze task utilizing consistent behavioral demands, with a varying source of reinforcement.

Rimonabant treatment disrupted extinction learning under aversive, but not appetitive conditions. These data strongly support the hypothesis that the endocannabinoid system mediates learning under aversive conditions, but is dispensable for appetitively-motivated learning (Holter *et al.,* 2005). Unlike previous reports, this study represents the first case in which the same behavioral demands were required (i.e. locating and entering the goal box) and only the source of reinforcement was varied. Control mice trained in either the aversive or appetitive Barnes maze conditioning paradigms showed a gradual decline in the percentage of time spent in the target zone across the extinction trials. Conversely, rimonabant-treated mice trained in the aversive conditioning procedure spent significantly more time in the zone that previously

contained the escape box than each of the other zones. In contrast, in the appetitive task, rimonabant-treated mice showed a virtually identical decrease in the percentage of time spent in the target zone as vehicle-treated mice.

Rimonabant did not impair acquisition of either the aversively, or appetitively, conditioned Barnes maze task, exhibiting similar latencies and path lengths to enter the goal box across acquisition trials compared to vehicle-treated subjects. However, rimonabant administration resulted in significantly more time spent immobile than vehicle treated mice under both conditions of reinforcement. It is possible that the increased immobility time is an anxiogenic effect of rimonabant administration, as supported by the observation that rimonabant dose-dependently increases plasma corticosterone (Patel *et al.*, 2004; Wade *et al.*, 2006; Steiner *et al.*, 2008). However, future experiments are necessary to determine the validity of this hypothesis.

Our second objective was to characterize acquisition performance of CB_1 -/- mice in both the aversively and appetitively conditioned Barnes maze tasks. Genetic deletion of the CB_1 receptor produces a variety of learning effects which appear dependent on procedure. For example, CB_1 -/- mice exhibit enhanced acquisition of recognition memory in the object (Reibaud *et al.*, 1999; Maccarrone *et al.*, 2002) and social recognition paradigms (Bilkei-Gorzo *et al.*, 2005); impaired acquisition of contextual conditioned fear and delay eyeblink conditioning (Kishimoto and Kano, 2006; Mikics *et al.*, 2006); and normal acquisition of spatial memory (Varvel and Lichtman, 2002; Varvel *et al.*, 2005), cued conditioned fear (Marsicano *et al.*, 2002; Cannich *et al.*, 2004; Kamprath *et al.*, 2006), operant conditioning tasks (Bilkei-Gorzo *et al.*, 2005; Holter *et*

al., 2005; Ward *et al.*, 2007), and trace eyeblink conditioning (Kishimoto and Kano, 2006). However, results from appetitively reinforced operant paradigms are difficult to interpret due to confounds related to age, the choice of appetitive reinforcer, and inclusion criteria. Specifically, Bilkei-Gorzo *et al.,* (2005) were the first to report an accelerated age-related decline in both cognitive performance and the density of hippocampal neurons, beginning at three months of age. The age of subjects from the studies by Ward *et al.*, and Holter *et al.,* utilized mice corresponded with the age-related deficits reported by Bilkei-Gorzo *et al.*, possibly affecting results. Furthermore, methodological considerations designed to normalize performance between genotypes have been reported, including increased food deprivation in CB_1 -/- mice (Holter *et al.*, 2005), comparison to baseline performance (Ward *et al.*, 2007), and the exclusion of subjects for exhibiting strange behavior or failing to reach criteria (Varvel *et al.*, 2005; Ward *et al.*, 2007). To address these concerns, we utilized young, age-matched CB_1 -/- and +/+ mice, departed from food reward in favor of access to water, and excluded acquisition criteria to test the hypothesis that CB_1 -/- would exhibit impaired acquisition independent of reinforcement condition.

CB_1 -/- mice exhibited impaired acquisition of both the aversively and appetitively conditioned Barnes maze task compared to CB_1 +/+ controls. Independent of reinforcement condition, CB_1 -/- mice traveled further, and required more time to achieve the goal of the Barnes maze task across acquisition trials. Furthermore, CB_1 -/- mice consistently spent more time immobile than their wild-type counterparts. Of interest, both genotypes achieved asymptotic performance for only one of the aforementioned

dependent variables, distance traveled. The observation that CB_1 -/- mice tended to travel to the goal box entrance, and remained immobile for a variable interval prior to entering, may account for the consistently greater latency to enter and amount of time spent immobile. Analysis of the first four trials of acquisition day one in the aversively conditioned paradigm revealed that the CB_1 -/- mice had significantly longer latencies to enter the goal box, and increased time immobile compared to CB_1 +/+ mice. Conversely, no significant differences were found during analysis of the first four trials of the appetitively conditioned task.

We initially attempted to assess extinction learning in CB_1 -/- mice to provide a complementary approach to studies utilizing the pharmacological CB_1 antagonist rimonabant. However, acquisition deficits in CB_1 -/- mice confounded the possible interpretation of extinction results. Regardless, these reports support the hypothesis that disparate acquisition results previously reported between CB_1 -/- and +/+ mice are due to differences in behavioral demands of qualitatively dissimilar tasks, rather than the hedonic nature of reinforcement. Furthermore, two explanations may account for the observed differences between genotypes in the results garnered from the present experiments. First, CB_1 receptor deletion may impair critical aspects of acquisition learning, though the exact mechanisms (i.e. neuronal or behavioral) remain undetermined. Alternatively, the act of entering the goal box may be inherently, and insurmountably, more aversive in CB_1 -/- mice. This explanation is supported by consistently greater latencies to enter the goal box, as well as the observation that CB_1 -/- tended to remain immobile adjacent to the escape hole prior to entering.

The purpose of our final objective was to characterize acquisition learning in a genetic model of enhanced eCB signaling. To this end, FAAH -/- and +/+ mice were evaluated in both the aversively and appetitively conditioned Barnes maze task. Currently, only two reports have been published evaluating acquisition performance in FAAH -/- mice. In both cases, FAAH -/- mice exhibited enhanced acquisition of the Morris water maze task compared to FAAH +/+ controls (Varvel *et al.*, 2006; Varvel *et al.*, 2007). However, it is unknown whether these results generalize to alternative memory models. The Barnes maze task allowed us to evaluate two outstanding questions in relation to the works by Varvel *et al*., (2006, 2007). First, would FAAH -/- mice exhibit enhanced acquisition in an alternative spatial memory paradigm with qualitatively different behavioral demands? Second, is the enhancement of acquisition dependent on the conditioning procedure (i.e. aversive or appetitive)? Thus, we evaluated the hypothesis that FAAH -/- mice will exhibit enhanced acquisition of the Barnes maze task under both appetitive and aversive conditioning procedures.

FAAH -/- mice acquired the task faster than their +/+ counterparts in an aversively, but not an appetitively, conditioned Barnes maze task. In relation to the work by Varvel *et al*., (2006, 2007) the present data suggest that enhanced acquisition of FAAH -/- mice are not limited by qualitatively different behavioral responses demanded by dissimilar spatial memory paradigms. Under aversive conditions, FAAH -/- mice required less time to complete the task for the initial six days of acquisition, after which time both genotypes exhibited asymptotic performance. However, no genotype differences were observed for the total distance traveled. FAAH -/- mice also spent

significantly less time immobile, and traveled faster than FAAH +/+ mice. As the measure 'adjusted speed' corrects for differences in immobility time, it is possible that FAAH -/- mice are better at performing the Barnes maze task because they run faster. This alternative explanation is supported by results from the dependent measure distance traveled, considered a more objective measure of learning than latency to enter, as no genotype differences were observed.

Conversely, under the appetitive conditioning procedure, FAAH -/- and +/+ mice did not significantly differ in adjusted speed, the total distance traveled, or corresponding latency to enter the goal box. In contrast with results from the aversively conditioned paradigm, FAAH -/- spent more time immobile than +/+ mice. One possible explanation for the conflicting results is the hypothesis that endocannabinoids are released under aversive conditions. If true, this would suggest that any aversiveness inherent to the appetitively conditioned paradigm, such as being exposed to an open area, is not sufficient to increase eCB signaling. However, this explanation appears insufficient as it cannot account for the *increase* in immobility time observed in FAAH -/- mice under appetitive conditions. Alternatively, compensatory changes associated with genetic deletion of FAAH may explain the increase in immobility time. Regardless, immobility is a very complex phenotype and could be due to many different reasons in addition to freezing.

Our final experiment tested the hypothesis that FAAH -/- mice exhibited enhanced acquisition through a CB_1 mediated mechanism of action. Inhibition of the FAAH enzyme results in significantly elevated levels of anandamide, as well as non-

cannabinoid FAAs (Cravatt *et al*., 2001). Among the non-cannabinoid FAAs are the sleep-inducing compound oleamide (OLE; Cravatt *et al*., 1995), the anti-inflammatory *N*-palmitoylethanolamine (PEA; Lambert *et al*., 2002), and the appetite suppressing agent *N*-oleoylethanolamine (OEA; Rodriguez de Fonseca *et al*., 2002). Anandamide is also an endogenous agonist at the TRPV1 receptor (zygmunt *et al,* 1999; Smart *et al*., 2000; Van der Stelt & DiMarzo, 2004). The heat-activated TRPV1 receptor was first identified by its responsiveness to the compound capsaicin, isolated from hot chili peppers, and is purported to mediate responses to thermal and chemical pain (Caterina *et al*., 1997; Szallasi & Blumberg, 1999). The purpose of this experiment was to determine if non-specific cannabinoid mechanisms were responsible for the enhanced acquisition observed in FAAH -/- mice. To this end, we used rimonabant to test the hypothesis that CB_1 antagonism attenuates the enhancement of acquisition observed in FAAH -/- mice.

Rimonabant (1 mg/kg) administration in FAAH -/- mice resulted in performance indistinguishable from FAAH +/+ mice. These results support the hypothesis that enhanced acquisition in FAAH -/- mice is mediated through a CB_1 receptor dependent mechanism of action. Furthermore, the results support the exclusion of non-cannabinoid FAAs as mediating acquisition effects in FAAH -/- mice. In this experiment, we evaluated three groups: FAAH -/- mice receiving rimonabant, FAAH -/- mice administered vehicle, and FAAH +/+ mice administered vehicle. Importantly, we utilized a dose of rimonabant (1 mg/kg) which we determined did not affect Barnes maze performance. In agreement with previous results, the total distance traveled did not significantly differ between groups. Furthermore, FAAH -/- mice administered vehicle

exhibited consistently lower values for the latency to enter the goal box, as well as immobility time. As these effects were absence in FAAH -/- mice administered rimonabant, the results support a cannabinoid mechanism of action. Finally, departing from previous observations, analysis of adjusted speed failed to reveal any significant differences between groups. The conflicting adjusted speed results, between this study and the initial study comparing genotypes, are perplexing as the only difference in experimental procedure was the administration of drug or vehicle; suggesting the effect is either related to nonspecific effects associated with i.p. injection or an artifact resulting from unknown differences in cohorts.

GENERAL DISCUSSION

We have identified three alternative explanations for the failure of rimonabant to disrupt extinction learning under appetitive conditions. First, reduced motivation might result in a concomitant reduction in the performance, resulting in faster extinction. However, this does not appear likely as we observed asymptotic acquisition performance between appetitive and aversive conditioning procedures during methods development. Furthermore, control mice in the aversive condition exhibited a more robust decline in the percentage of time spent in the target zone during extinction trials when compared to control animals in the appetitive condition. Second, cognitive impairments resulting from water deprivation may potentially contribute to weaker memory formation and faster extinction. Again, this possibility is unlikely because both conditions displayed similar acquisition performance. A third possibility is the eCB system is differentially involved in extinction, depending on the nature of reinforcement during extinction training, rather than during acquisition. For example, extinction learning in classical conditioning models is reinforced by the omission of punishment, and can be regarded as rewarding. Operant conditioning models, however, bear aspects of frustration as they rely on the omission of reward. In the present experiment, the source of reinforcement

between conditions involved a similar aspect, omission of the goal box. As rimonabant disrupted extinction learning in one condition, but not the other, it would appear that the results cannot be attributed to the involvement of eCB signaling in reward processes during extinction learning.

The results presented here are grounded in the assumption that learning, either under the aversive or appetitive conditioning procedure, is motivated primarily by representative stimuli for each condition (i.e., bright lights and air turbulence for aversive procedures vs. access to water in appetitive procedures). This assumption is supported by the observation that additional reinforcement, either aversive or appetitive, improved acquisition performance when compared to ambient reinforcement conditions. These data also suggest that exposure to the Barnes maze is sufficiently aversive to motivate mice to escape an elevated, open field. Alternatively, the goal box could be sufficiently rewarding or merely preferable to the maze.

Water deprivation may be inherently aversive, producing a state of prolonged stress that result in untoward compensatory changes. Rodents, like most animals, have evolved distinct physiological mechanisms enabling them to adapt to prolonged periods in which food or water may be unavailable. As a result, it has been hypothesized rodents are equipped to handle dehydration periods for as long as 24 h without any overt signs of physiological stress or behavioral abnormalities (Rowland, 2007). Among these adaptive mechanisms, is a reduction in food intake in the absence of access to water, resulting in 'dehydration anorexia' (Watts, 1999). Thus, there is potential for water-deprivation to produce a concomitant, food-deprived state. However, in a study using rats subjected to

a dehydration paradigm, in which water was replaced with hypertonic saline, no alterations in feeding were observed until the second day of deprivation, suggesting a relatively slow onset of dehydration anorexia. Furthermore, the authors reported a rapid reversal of anorexia within the first 30 min of free access to water (Watts, 1999). As we have shown, mice subjected to our water restriction protocol quickly adapt to the procedure and are able to maintain their body weight appropriately. Together, these results would support the observation by Rowland (2007) that rodents are able to entrain their ingestive responses to adapt to availability. Water deprivation may also cause alterations in stress hormones. However, in rats subjected to 48 h of water-deprivation, a slight but significant elevation in basal corticosterone, but not ACTH was observed. In response to 15 min of restraint stress, no differences between water-deprived and control animals was observed for corticosterone levels, and ACTH levels decreased (Aguilera *et al*., 1993). Furthermore, in a study where rats were restricted to 15 min access to water per day, no changes were observed with regards to open-field behavior, freezing behavior, or corticosterone levels. In contrast, food-restriction to 80% baseline weight resulted in significantly greater activity in the open-field, and higher mean serum corticosterone (Heiderstadt *et al*., 1999). While these results suggest that water restriction produces a negligible stress response, it is unknown whether adaptations occur with regards to the eCB system. Thus, future studies designed to evaluate possible changes, such as CB_1 receptor density or eCB content, deserve consideration.

A growing body of research has implicated the endogenous cannabinoid system as modulating homeostatic mechanisms related to neuroendocrine responses to stress and

energy balance, and manifested as anxiety (Pagotto *et al.*, 2006; Tasker, 2006). The primary response to a stressor is activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, and subsequent increase in plasma corticosterone. Co-localization of CB_1 receptors at each level controlling HPA axis functioning has been shown, suggesting a multi-tiered role for eCB modulation of the stress response (Galiegue *et al.*, 1995; Marsicano and Lutz, 1999; Wenger *et al.*, 1999; Pagotto *et al.*, 2001; Cota *et al.*, 2003; Mackie, 2005). In support, rimonabant dose-dependently increases basal and stressed-induced plasma corticosterone secretion (Patel *et al.*, 2004; Wade *et al.*, 2006; Steiner *et al.*, 2008). Concurrently, CB_1 -/- mice exhibit increased plasma corticosterone secretion in response to stress (Haller *et al.*, 2002; Barna *et al.*, 2004), and at rest (Barna *et al.*, 2004; Cota *et al.*, 2007; Steiner *et al.*, 2008). Behaviorally, CB_1 -/- mice exhibit an aggressive, and anxiety-like phenotype (Navarro *et al.*, 1997; Martin *et al.*, 2002), which may result from compensatory mechanisms associated with a life-long absence of CB_1 receptors (Wade *et al.*, 2006). Indeed, chronic exposure to unpredictable stress results in adaptive changes in the hippocampus, including a 50% reduction in CB_1 receptor density, and a 40% reduction in 2-AG content (Hill *et al.*, 2005b). In contrast to attenuated CB_1 receptor signaling, enhancement of eCB signaling appears to dampen the stress response. Acute presentation of anxiogenic stimuli increases 2-AG and AEA content in the amygdala (Marsicano *et al.*, 2002; Patel *et al.*, 2004). Administration of the FAAH inhibitors AM-404 or URB-597 both decrease plasma corticosterone in response to restraint stress (Patel *et al.*, 2004), and FAAH -/- mice and URB-treated rodents exhibit anxiolytic-like effects (Kathuria *et al.*, 2003; Patel and Hillard, 2006; Naidu *et al.*, 2007).

There is evidence that the intensity of a stressor can impair or facilitate memory acquisition, and exhibits a U-shaped curve (McGaugh, 1985; Korneyev, 1997). Mild, low-intensity stressors, such as handling or exposure to an intruder, appear to stimulate cognitive function and facilitate acquisition (Shors *et al.*, 1992). In contrast, severe stressors such as inescapable shock inhibit memory formation in rodents (Anderson and Paden, 1966; Jackson *et al.*, 1980), and humans (Pitman, 1989). One particular brain-region exhibiting marked sensitivity to stress-induced alterations is the hippocampus. Specifically, dendritic atrophy and decrements in long-term potentiation have been reported following chronic exposure to stress (Galea *et al.*, 1997; Alfarez *et al.*, 2003). In rodent models of cognition, these physiological changes appear to result in impairments of acquisition and reversal/extinction learning (Luine *et al.*, 1993; Luine *et al.*, 1994; Francis *et al.*, 1995; de Quervain *et al.*, 1998; Vasconcellos *et al.*, 2003; Hill *et al.*, 2005b). Collectively, these reports suggest that eCB manipulations can affect cognitive processes by altering responding to stress, or vice versa. In relation to the present work, it is difficult to distinguish the extent to which, if any, alterations in the physiological response to stress influence performance. For example, the deficits in acquisition associated with CB_1 -/- mice may be a result of a hyperresponsiveness to the stress of open-field exposure, deficits in cognitive processes such as consolidation, or both. In addition, motor deficits associated with CB_1 receptor deletion, such as reduced bone density in CB_1 -/- mice on the C57 background , may result in poorer performance compared to CB_1 +/+ mice (Tam *et al.*, 2006). The observation that CB_1 -/- mice, and rimonabant-treated mice, consistently spent more time immobile than controls, supports

the involvement of eCB-mediated alterations in anxiety during acquisition of the task. Furthermore, in the aversive paradigm, FAAH -/- mice spent significantly less time immobile than FAAH +/+ mice. Together, these results suggest that under aversive conditions, the eCB system is actively engaged in dampening the stress response elicited by aversive conditions. However, acquisition was only affected in CB_1 -/- and FAAH -/- mice, and rimonabant did not impair acquisition of the task under either reinforcement condition. Thus, compensatory mechanisms associated with genetic deletion of the FAAH enzyme or CB_1 receptor may be responsible for the alterations in acquisition learning. Finally, it is unclear why FAAH -/- spent significantly less time immobile under aversive conditions, but spent significantly more time immobile under appetitive conditions. However, mobility is a complicated phenotype and is affected by a wide range of factors including but not limited to fear and arousal. As endocannabinoids are released on demand in response to stressors, it is tempting to speculate that appetitive conditions are insufficient to cause eCB release. The resulting increase in eCB signaling under aversive conditions would produce an anxiolytic response, and is supported by the observation that subthreshold doses of THC produce anxiolytic-like effects (Valjent *et al.*, 2002).

Comparing initial day one baseline acquisition data across studies reveals differences in initial acquisition performance between experiments. However, as we treated each experiment as a separate entity, no analyses were performed comparing data between experiments. Furthermore, these differences appear to be specific to individual experiments. For example, comparing acquisition data from the first day of the appetitive

procedure (i.e. methods development and rimonabant during acquisition) reveals slightly different values. However, in each case within group means and standard errors appear consistent, suggesting differences do not arise from distinct populations of 'fast' and/or 'slow' learning subjects. Regardless, we assume that these differences are an artifact produced by different cohorts of subjects, and do not affect the interpretation of the data.

It is unknown whether rimonabant will disrupt extinction learning in the appetitive task if administered during both acquisition and extinction. In the aversive paradigm, rimonabant was administered during acquisition and extinction in one experiment, and only during extinction in a follow-up experiment. However, in the appetitive model, rimonabant administration only occurred during extinction. While we assumed from results in the aversive paradigm that administration of rimonabant during extinction is sufficient to disrupt extinction learning, the application of this conclusion to the appetitive condition deserves consideration.

There are three uncertainties regarding the data presented here that could be clarified in future studies. First, it is unknown whether exposure to the Barnes maze task results in HPA axis activation. Experiments designed to collect and measure plasma corticosterone at different time-points during acquisition, after handling, and at rest, would provide a framework for future experiments, as well as characterize the physiological consequence of acute and repeated Barnes maze exposure in relation to stress as well as associated adaptive changes in the stress response. Alternatively, if the effects observed during acquisition reflect altered anxiety, supporting evidence may be procured via administration of a sub-threshold dose of a benzodiazepine. Of course a

dose-response curve would be necessary as benzodiazepines have well-document amnesic effects.

Second, experiments designed to characterize eCB content in relevant brain areas such as the hippocampus, amygdala, and PFC, would facilitate our understanding of how the eCB system responds to acute and repeated exposure to the aversive and appetitive Barnes maze task, and later, may aid the identification of purported adaptive changes associated with genetic deletion of the FAAH enzyme. Alternatively, complementary approaches to manipulate the eCB system, aside from pharmacological drug administration, would provide convergent data for results from genetic knockouts. For example, siRNA specific for CB_1 or FAAH would create an acute genetic knockout with transient effects. Thus, this approach would aid the distinction between effects associated with genetic inhibition, or adaptive changes resulting from the life-long absence of a gene.

Third, attentional processes can affect performance during cognitive tasks, resulting in altered performance. In the present context, it is unknown whether our observations result from abnormal attention to the context stimuli, rather than the task itself. Future experiments utilizing a cued procedure, in which a salient proximal cue demarcates the location of the hidden goal, will allow us to demonstrate whether a deficit in acquisition occurs with preserved general attention, motoric abilities, and motivation (Whishaw and Tomie, 1987). For example, septal lesions result in impaired acquisition of the homing board, and Morris water maze, resulting in longer latencies and path lengths to find the hidden goal (Kelsey and Landry, 1988; Brandner and Schenk, 1998).

Following the introduction of a salient cue, the deficits in acquisition were reversed, suggesting deficits in reference memory. Introduction of the same methodology into the Barnes maze model would provide information regarding the nature of the acquisition effects reported here. In the case of our CB_1 -/- mice acquisition data, it appears unlikely that the deficits result from shifted attention to aversive stimuli, as the deficits were also observed in the absence of bright lights and air turbulence. However, as mentioned, a cued procedure would also facilitate investigation into altered motor processes, or motivation. As differences in running speed were observed in FAAH -/- mice, the necessity of running a cued procedure is underscored.

Endocannabinoid-based pharmacotherapies to treat a wide range of disorders are under development. As research in the field of endocannabinoids progresses, so does the evidence for pharmacotherapeutic potential. Already, manipulations of this system hold potential for therapies in the fields of pain, inflammation, obesity, drug abuse, diabetes, anxiety, depression, and cognitive disorders. The results presented here further support the hypothesis that the endocannabinoid system mediates the extinction of behaviors that are specific to aversive conditions, leaving extinction of learned behaviors in appetitively reinforced tasks intact. While it remains questionable whether the physiological role of the endocannabinoid system is involved in other forms of positively reinforced (e.g., sexual) learning, the system's impact on aversively motivated learning is unmistakable. These results underscore the importance of understanding a patient's history prior to administering rimonabant or other cannabinoid receptor antagonists should they become therapeutically available. Specifically, contraindication might include patients with a

history that includes abuse or traumatic events (e.g. combat, criminal assault, severe injury) that interfere with normal, daily functioning. For example, post-traumatic stress disorder has been described as a failure of extinction learning (Rothbaum & Davis, 2003). Treatment of a patient suffering from post-traumatic stress disorder, or similar afflictions (e.g. panic attacks, obsessive-compulsive disorder, and adjustment disorder) would be difficult, if not impossible, following rimonabant administration. Furthermore, administering a drug that interferes with normal extinction learning, such as rimonabant, might exacerbate such an illness.

In contrast to the potential harm associated with rimonabant administration, and possibly CB_1 antagonism in general, these results have an encouraging aspect. First, the results suggest that therapies resulting in increased endocannabinoid signaling, as with a FAAH inhibitor or a cannabinoid receptor agonist, may accelerate extinction learning, thereby presenting a potential therapeutic treatment for psychiatric disorders that are hypothesized to include elements of maladaptive cognitive processes and an inability to adapt to a new environment (i.e., post-traumatic stress disorder [PTSD], phobias, and generalized anxiety disorder [GAD]) (Lutz, 2007). In general, these disorders result from unpleasant prior experiences, are manifested as feelings of intense anxiety and panic, and persist due to inappropriate extinction (Milad *et al.*, 2006). Recently, Varvel et al., (2006) reported that FAAH -/- mice, and mice administered the FAAH inhibitor OL-135, displayed accelerated extinction learning in the Morris water maze, further underscoring the therapeutic potential of endocannabinoid based drugs.

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APPENDIX A

Descend into escape box	18	(Barnes, 1979)	Bright lights	Rats	Evaluate senescence	latency to enter, speed, strategy, distance, total errors, angle of deviation
Descend into escape box	40	(Bach *et al.*, 1995)	Bright lights, tone	Mice	Evaluate CaMKII -/- mice	Total errors, strategy, distance from tunnel, perseverative errors,
Descend into escape box	40	(Fox *et al.*, 1998)	Bright lights	Mice	CCI induced cognitive deficits	Latency to enter, strategy - % of trials used, total errors
Descend into escape box	16	(Pompl *et al.*, 1999)	Bright lights/fan	Mice	Assess APPsw mice in a new model	latency to enter, total errors, error scale
Descend into escape box	40	(Inman-Wood *et al.*, 2000)	Bright lights/fan/tone; all turned off after completion of goal	Mice	Prenatal exposure to cocaine	Total errors, latency to enter, strategy, # of holes from target hole
Descend into escape box	12	(Miyakawa *et al.*, 2001)	Unknown	Mice	Evaluation of *Ng* -/- mice	Latency to find, distance to find, total errors, time spent/hole, speed.
Descend into escape box	12	(Grootendorst *et al.*, 2001)	Return to home-cage, sugar in home-cage	Mice	Stress and ApoE -/- mice	Latency to find, velocity, distance, % time per zone (center, mid, tunnel, outer ring), strategy, total pokes

Descend into escape box	36	(Paylor *et al.*, 2001)	Bright lights	Mice	*LHX5* -/- mice	Latency to find, composite search score, composite search score, site errors
Descend into escape box	12	(Holmes *et al.*, 2002)	Bright lights (?)	Mice	Behavioral profiles of inbred strains	Errors to reach target, target hole preference index, speed, distance, Visits/hole, speed, distance
Descend into escape box	30	(Williams *et al.*, 2003)	fruit loop in goal box or bright lights	Rats	Prenatal meth exposure	latency to first hole, latency to find target hole, total errors
Walk into escape tunnel	12	(Koopmans *et al.*, 2003)	Escape tunnel allows access to home cage	Mice	strain differences in new model	Latency to enter, distance to find, total errors, speed, time in target zone
Descend into escape box	40	(Seeger *et al.*, 2004)	Bright lights, tone	Mice	Evaluation of M2 -/- mice	Latency to enter, total errors, latency to enter, # perseverations
Descend into escape box	40	(Dawood *et al.*, 2004)	Unknown	Mice	Compare training paradigms	latency to find, total errors prior to finding,
Descend into escape box (same as Pompl et al.)	16	(Bredy *et al.*, 2004)	Bright lights/fan	Mice	neonatal handling/paternal care in monogamous mice	Latency to find , total errors prior to finding, time in target zone, target hole approaches
Descend into escape box	40	(Raber *et al.*, 2004)	Bright lights, tone	Mice	Radiation induced cognitive deficits	Errors, distance from escape hole, strategy

Descend into escape box	12	(Harrison *et al.*, 2006)	Gentle handling	Mice	Spatial and non-spatial search strategies in the Barnes maze	Errors prior to finding, latency to find, distance to find, total errors, latency to enter, total distance, strategy - % of trials used, % time in target zone
Descend into escape box	20	(Barr *et al.*, 2007)	Buzzer (80dB), bright lights.	Mice	Evaluation of ApoER2 -/- mice.	Latency to find, errors, perseverations, distance from first hole investigated to target hole, total errors, latency to enter.
Descend into escape box	40	(Dai *et al.*, 2007)	Bright lights	Mice	Cognitive dysfunction in H1 and H2 -/- mice.	Distance to enter.
Descend into escape box	40	(Hong *et al.*, 2007)	Fans, bright light.	Mice	Assess *Emx1* -/- mice	Path-length, velocity, latency to find
Descend into escape box	24	(Mueller and Bale, 2007)	Bright lights and/or air turbulence and/or 100 dB noise	Mice	Prenatal stress on learning performance	Latency to first hole, distance of first hole searched to target hole, errors, latency to find, errors after finding, search strategy, success.

Descend into escape box	19	(O'Connor *et al.*, 2007)	Bright lights, aversive auditory stimulus	rats	Effects of progesterone following traumatic brain injury.	Latency to enter
Descend into escape box	20	(O'Tuathaigh *et al.*, 2007)	Litter from home cage and Honey Loops (food reward) in escape box. Bright Lights.	Mice	Assess NRG1 -/- mice	Latency to enter, distance traveled, number of errors
Descend into escape box	20	(Prut *et al.*, 2007)	Buzzer (80dB), bright lights.	Mice	Assess APP23 -/- mice	Latency to enter, number of errors, search strategy
Descend into escape box	12	(Reiserer *et al.*, 2007)	Gentle handling	Mice	Assess APPsw -/- mice	omission errors, errors/distance/latency to find, total errors, strategy - % of trials used, % time in target zone
Descend into escape box	40	(Rueda-Orozco *et al.*, 2007)	White noise (90dB), bright lights	Rats	Endocannabinoid mediation of search strategy	Total errors, % search strategy, mean time of performance, % errors in target zone, median of the distance.
Descend into escape box	18	(Simola *et al.*, 2008)	Unknown	Rats	Assess perinatal asphyxia in non-spatial memory.	Latency to find, total errors.

Descend into escape box	30	(Vorhees *et al.*, 2007)	Bright lights	Rats	Developmental effects of MDMA on spatial learning.	Latency to find, distance to find, total distance traveled.
Descend into escape box	8	(Xu *et al.*, 2007)	Ambient laboratory conditions	Mice	Assess age differences in Tg-SwDI -/- mice	Latency to find the escape hold
Descend into escape box.	12	(Ambree *et al.*, 2007)	Bright lights	Mice	L-DA in murine models of Alzheimer's (TgCRND8 -/- mice).	Path length, latency to find, latency to enter, % time in target zone.
Descend into escape box	16	(Fabricius *et al.*, 2008)	Bright lights, loud rock and techno music.	Mice	Maternal separation on behavior and hippocampal neuronal count.	Latency to find, distance traveled, error frequency, visits to target hole and adjacent two holes, search strategy.
Descend into escape box.	40	(Fedorova *et al.*, 2007)	Bright lights.	Mice	Spatial learning following n-3 fatty acid deficient diet.	Distance, latency to enter, number of errors, total time mobile/immobile.
Descend into escape tunnel	12	(Moreau *et al.*, 2008)	Tunnel leading to homecage	Mice	Behavioral effects of p75-Saporin immunotoxin.	Visits to target/non-target holes, latency to find, order of visited holes, repetitive errors.

Descend into escape box	20	(Oliveira *et al.*, 2008)	Bright lights	Rats	Involvement of polyamine binding sights at NMDAr in creatine-induced spatial learning enhancement.	Latency to find, total errors.
Descend into escape tunnel	12	(Richter *et al.*, 2008)	Tunnel leading to homecage, bright lights	Mice	Assess TgCRND8 -/- mice in enriched environment.	Path length, latency to enter, time spent in target zone.
Descend into escape box	8(?)	(Trofimiuk and Braszko, 2008)	Unknown	Rats	Alleviation of stress induced spatial memory impairments with St. Johns wort.	Latency to find, total errors.

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EDUCATION

Virginia Commonwealth University Medical Center, Richmond, VA
Ph.D. in Pharmacology & Toxicology **April 2008**
Dissertation: "Endocannabinoid Modulation of Spatial Memory."
Advisor: Aron H. Lichtman

Virginia Commonwealth University Medical Center, Richmond, VA
M.S. in Pharmacology & Toxicology **March 2007**
Thesis: "Rimonabant Disrupts Extinction Learning in an Aversive, but not an Appetitive, Barnes Maze Task."
Advisor: Aron H. Lichtman

Furman University, Greenville, SC
B.A. in Psychology **May 2003**
Areas of Concentration: Neuroscience, Political Science
Mentor: Judith E. Grisel

RESEARCH EXPERIENCE

Graduate Research Assistant **2003-2008**
Department of Pharmacology and Toxicology, School of Medicine
Virginia Commonwealth University, Richmond, Virginia
Mentor: Aron H. Lichtman.

Undergraduate Research Assistant **2002-2003**
Department of Psychology, Furman University,
Mentor: Judith E. Grisel

PUBLICATIONS AND PAPERS

Wilson DM, Varvel SA, **Harloe JP**, Martin BR, Lichtman AH. "SR 141716 (Rimonabant) Precipitates Withdrawal in Marijuana-Dependent Mice." Pharmacol. Biochem. Behav. 2006. **85**(1):105-113.

Harloe JP, Thorpe AJ, Lichtman AH. "Differential Endocannabinoid Regulation of Extinction in Appetitive and Aversive Barnes Maze Tasks." Under Review at Neuropsychopharmacology.

Harloe JP, Lichtman AH. "Alterations in Endocannabinoid Signaling Produce Disparate Acquisition Profiles in an Aversive and Appetitive Barnes Maze Task." Manuscript in Preparation.

ABSTRACTS/SCIENTIFIC PRESENTATIONS

Harloe JP, Lichtman AH. "Rimonabant Disrupts Extinction in an Aversive, but not an Appetitive Barnes Maze Task." Watts Day Symposium. Richmond, VA. Poster. 2007.

Harloe JP, Lichtman, AH. "Rimonabant Disrupts Extinction in an Aversive, but not an Appetitive Barnes Maze Task." Carolina Cannabinoid Collaborative. Wilmington, NC. Poster. 2007.

Harloe JP, Lichtman AH. "Rimonabant Disrupts Extinction in an Aversive, but not an Appetitive Barnes Maze Task." International Cannabinoid Research Society. Quebec, Canada. Poster. 2007.

Harloe JP, Thorpe AJ, Lichtman AH. "SR-141716, the CB1 Antagonist, Impairs Extinction In the Barnes Maze Paradigm." Central Virginia Chapter Society for Neuroscience. Richmond, VA. Poster. 2006.

Harloe JP, Thorpe AJ, Lichtman AH. "SR-141716, the CB1 Antagonist, Impairs Extinction In the Barnes Maze Paradigm." Society for Neuroscience. Atlanta, GA. Poster. 2006.

Harloe JP. "Modulation of Learning and Memory by the Endocannabinoid System." Dept. of Pharmacology & Toxicology Student Seminar. Richmond VA. 2006.

Harloe JP. "Cannabinoids and Memory in the Barnes Maze Paradigm." Dept. of Pharmacology & Toxicology Student Seminar. Richmond, VA. 2006.

Harloe JP, Grisel JE. "Sex-Dependent Effects of Melanocyte Stimulating Hormone (MSH) on Social Interaction." Dept. of Psychology Research & Internship Forum. Greenville, SC. Poster. 2003.

Harloe JP, Grisel JE. "Sex-Dependent Effects of Melanocyte Stimulating Hormone (MSH) on Social Interaction" Nat. Conf. Undergrad. Research. Salt Lake City, UT. Poster. 2003.

AWARDS

Travel Award, International Cannabinoid Research Society, Quebec, Canada	**2007**
Furman Advantage Research Fellowship, Furman University, Greenville SC	**2002**
Travel Award, National Conference of Undergraduate Research, Salt Lake City, UT	**January 2002**

TEACHING EXPERIENCE

Virginia Commonwealth University, Richmond, Virginia - Questers

Lab Leader **6/2005, 06, 07**

Organized, prepared, and implemented curriculum for high school science students.

INSTITUTIONAL SERVICE

VCU Student Government Association

Departmental Representative **2005-2007**

Acted as liaison between the school and our department, Honor Council alternate

Pharmacology & Toxicology Student Government Organization

Student Representative **2005-2007**

Organized and planned departmental functions and activities.

MEMBERSHIPS

Society for Neuroscience	**2006-present**
International Cannabinoid Research Society	**2006-present**
Carolina Cannabinoid Collaborative	**2006-present**

SKILLS

Proficient in experimental models of anxiety, depression, memory, pain, and drug abuse.
Extensive knowledge of electronics, as well as computer hardware and software