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The role of the CB1 receptor in learning, memory and anxiety-like behaviors

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THE ROLE OF THE CB1 RECEPTOR IN LEARNING, MEMORY AND ANXIETY-LIKE BEHAVIORS

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The role of the CB1 receptor in learning, memory, and anxiety-like behaviors

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Several lines of evidence support a role of the endocannabinoid (eCB) system in cognition and anxiety. This study explores cognitive processes and anxiety-like behaviors in wild type ($CB_1^{+/+}$) and CB_1-receptor-deficient ($CB_1^{-/-}$) mice of differing ages. Differences were observed between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice in a Morris Water Maze acquisition task. Furthermore, $CB_1^{-/-}$ mice did not display deficits in extinction during reversal learning. In the Light-Dark Box and Suok tasks, the $CB_1^{-/-}$ mice demonstrated greater anxiety-like behaviors relative the $CB_1^{+/+}$ mice. No differences were observed in the Open-Field task, suggesting that the observed behavioral differences may be related to anxiety rather than cognitive impairments. This study has important implications for neuropsychiatric disorders, including depression and post-traumatic stress disorder.

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The role of the CB1 receptor in learning, memory, and anxiety-like behaviors.

The medicinal and recreational properties of *Cannabis sativa* have been described for thousands of years, dating back to the oldest known pharmacopeia, the Pên-ts'ao Ching (2727 B.C., reviewed in Murray, Morrison, Henquet, & Di Forti, 2007). However, the body's "endocannabinoid" system, which is responsible for mediating the effects of both the endogenous and exogenous cannabinoids, has only been recently discovered and documented. The identification and characterization of Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of cannabis (Gaoni & Mechoulam, 1964), led to the discovery of the endogenous brain (and peripheral) cannabinoid receptors primarily through the advancements in understanding G-protein-coupled receptor signaling and the use of high-affinity radio-labeled ligands and quantitative autoradiography (reviewed in Howlett, 2005). Following the characterization of the pharmacology and localization of the receptor, the cannabinoid receptor was cloned (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990). The cloning of this first cannabinoid receptor (CB_1) led to the successive identification of the mechanism of action for the cannabinoids, the intracellular cannabinoid-mediated signaling pathways, the isolation (and biochemical characterization) of several endogenous ligands binding to the receptor, as well as the identification of a second cannabinoid receptor (CB2).

The endocannabinoid (eCB) system consists of the two cannabinoid receptors (CB_1 and CB_2; Devane, Dysarz, Johnson, Melvin, & Howlett, 1988; Gong et al., 2006; Matsuda et al., 1990; Munro, Thomas, & Abu-Shaar, 1993; Onaivi et al., 2006), their endogenous ligands (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995), and several enzymes that are involved in the synthesis and degradation of the endogenous

cannabinoids (Cravatt et al., 1996; Stella & Piomelli, 2001). The CB_1 receptor, which is activated by the exogenous cannabinoid THC, is likely to be one of the most abundant G protein-coupled receptors in the mammalian brain (Di Marzo, Bifulco, & De Petrocellis, 2004). Advances in the understanding of the molecular functioning of this receptor subtype have initiated a great deal of scientific interest focusing on the elucidation of both the physiological and pathophysiological roles of the endocannabinoid system.

Specifically, the CB_1 receptor has been shown to play a modulatory role in certain types of pain conditions (Calignano, La Rana, Giuffrida, & Piomelli, 1998; Richardson, Aanonsen, & Hargreaves, 1998; Walker, Huang, Strangman, Tsou, & Sanudo-Pena, 1999), appetite control (Di Marzo et al., 2001), motoric functioning (reviewed in Ameri, 1999), and several disorders, both "psychological" and "medical," characterized by cognitive and/or emotional dysregulation, such as schizophrenia, post-traumatic stress disorder (PTSD), stroke, and traumatic brain injury (TBI; see review Pertwee, 2006). The eCB system has also been shown to facilitate several forms of mnemonic processes, such as spatial and working memory (Lichtman, Dimen, & Martin, 1995; Nava, Carta, Colombo, & Gessa, 2001; Varvel, Hamm, Martin, & Lichtman, 2001), as well as memory consolidation (Castellano, Cabib, Palmisano, Di Marzo, & Puglisi-Allegra, 1997) and the extinction of mainly aversive memories (Chhatwal, Davis, Maguschak, & Ressler, 2005; de Oliveira Alvares, Pasqualini Genro, Diehl, Molina, & Quillfeldt, 2008; Marsicano et al., 2002; Varvel & Lichtman, 2002; Varvel, Wise, Niyuhire, Cravatt, & Lichtman, 2007).

Several converging lines of anatomical, electrophysiological, neurochemical, and behavioral evidence suggest a physiological role of the eCB system in the processing of

cognition and of anxiety. For example, the CB_1 receptor and its two distinct endogenous ligands (i.e., anadamide and 2-arachidonoylglycerol) are widely distributed in brain regions related to learning, memory, and anxiety (e.g., hippocampus, amygdala; Di Marzo et al., 2000; Egertova & Elphick, 2000; Eggan & Lewis, 2007; Herkenham et al., 1991; Herkenham et al., 1990; Matyas et al., 2006). Additionally, recent pharmacological and CB_1-receptor-deficient mouse models (e.g., Bilkei-Gorzo et al., 2005; Varvel, Anum, & Lichtman, 2005) have demonstrated that the CB_1 receptor may be responsible for mediating both cognitive and stress-related processes.

Several exogenous CB_1 receptor agonists have been shown to disrupt learning and memory processes in a variety of behavioral paradigms. Specifically, THC (Lichtman et al., 1995; Lichtman & Martin, 1996; Mishima et al., 2001), CP55,940 (Braida & Sala, 2000; Lichtman et al., 1995) and WIN55,212-2 (Lichtman et al., 1995) have been shown to impair working memory in the eight-arm radial maze task, while THC has also been shown to impair performance in the T-Maze alternation task (Braida & Sala, 2000). Additionally, the administration of THC has resulted in acquisition, retrieval, and working memory deficits in the Morris Water Maze task (MWM; Da Silva & Takahashi, 2002; Varvel et al., 2001), one of the primary apparatus used in the current research. Similarly, anandamide, WIN-55,212-2, and THC were all shown to induce deficits in the delayed non-matching-to-sample (DNMTS) task (Hampson & Deadwyler, 1999, 2000; Mallet & Beninger, 1998). THC and methanandamide were also shown to induce impairments in an object recognition task and discrimination task (Brodkin & Moerschbaecher, 1997; Ciccocioppo et al., 2002). The CB_1 receptor antagonist (SR141716A) was shown to reverse the agonist-induced impairments in the above-

mentioned studies, which provides compelling evidence that the observed cognitive deficits are mediated via the CB_1 receptor.

Studies involving the administration of selective CB_1 receptor antagonists, as well as work done with the genetic CB_1-receptor-knockout mouse model, provide additional support for the endogenous involvement of the CB_1 receptor in cognitive and stress-related processes. When administered alone, the antagonist SR141716A (SR; 0.1 – 3 mg/kg) compound has been shown to facilitate memory in a social recognition memory task (Terranova et al., 1996) and the radial maze spatial memory task (Wolff & Leander, 2003). Improvements in acquisition and consolidation have also been reported in the elevated T-maze (Takahashi, Pamplona, & Fernandes, 2005), which provides an animal model for both cognitive processes and anxiety (Carvalho-Netto & Nunes-de-Souza, 2004). However, no differences were observed using 0.5 mg/kg of SR on the elevated T-maze (Nava et al., 2001) or the DNMTS tasks (0.05-2 mg/kg; Hampson & Deadwyler, 1999, 2000; Mallet & Beninger, 1998). Recently, the highly selective full CB_1 receptor antagonist CE (1-[7-(2-Chlorophenyl)-8-(4-chlorophenyl)-2-methyl pyrazolo[1,5-a]-[1,3,5] triazin-4-yl]-3-ethylaminoazetidine-3-carboxylic acid amide benzenesulfonate) has also been shown to enhance memory via consolidation in the radial arm maze paradigm (Wise, Iredale, & Lichtman, 2008).

In these studies the CB_1-receptor-deficient mice were also able to retain memory for longer periods of time relative to their wild type controls in an object recognition task (Maccarrone et al., 2002; Reibaud et al., 1999). In the Morris Water Maze (MWM) task both SR (3mg/kg) treated mice and CB_1-receptor-deficient mice were able to learn the spatial location of a hidden platform without significant difficulty (Varvel, Anum, &

Lichtman, 2005; Varvel & Lichtman, 2002); however, both groups of mice had impairment in the ability to locate the hidden platform when the location of the hidden platform was moved to the opposite quadrant following spatial acquisition. These latter results suggest that activation of the CB_1 receptor may exert an endogenous influence on behavioral flexibility and extinction processes.

A variety of behavioral paradigms, including the elevated plus maze, the light-dark box, and the open-field test (the latter representing two tests employed in the research reported here), have been used to measure endocannabinoid-mediated anxiety-like behaviors. In the elevated plus maze, low doses of SR (0.25 – 1 mg/kg) have generally been shown to have no effect on anxiety-like behaviors in both rat and mouse models (Griebel, Stemmelin, & Scatton, 2005; Haller, Bakos, Szirmay, Ledent, & Freund, 2002; Patel & Hillard, 2006); however, when the SR compound was administered prior to a second elevated-maze trial, open arm exploration was increased, suggesting an anxiolytic effect (Rodgers, Haller, Halasz, & Mikics, 2003). Higher doses of the SR compound (up to 3 mg/kg) have been reported to be either anxiogenic (Arevalo, de Miguel, & Hernandez-Tristan, 2001; Navarro et al., 1997; Patel & Hillard, 2006), anxiolytic (Haller et al., 2002), or ineffective in modifying anxiety (Griebel et al., 2005; Rodgers et al., 2003). Mixed results utilizing the SR compound (0.3 – 3 mg/kg) have also been reported using the Light-Dark box test (Akinshola, Chakrabarti, & Onaivi, 1999). Additionally, previous research has demonstrated that young (6-7 weeks) knockout mice lacking the CB_1 receptor perform similarly to age-matched controls using the open-field, rotarod, and social recognition tasks, whereas older (3-5 month) mice lacking the CB_1 receptor demonstrate significant impairments (Bilkei-Gorzo et al., 2005).

It is noteworthy that the SR compound has been shown partially or even totally to exert its actions via a non-CB_1 receptor mediated mechanism (Haller et al., 2002), which may be contributing to some of the observed heterogeneity in findings between studies. Additionally, the CB_1 receptor antagonist AM251 has been shown to increase anxiety in mice (but not rats) using the elevated plus maze test (Haller et al., 2007; Haller, Varga, Ledent, & Freund, 2004; Patel & Hillard, 2006; Rodgers, Evans, & Murphy, 2005). It has been hypothesized that the AM251 compound may be selective to the CB_1 receptor, which would suggest that the CB_1 receptors are tonically activated to promote anxiolysis during aversive events (i.e., events designed to assess/provoke unconditioned anxiety).

Few studies to date have evaluated the functional consequences of the characteristic age-related changes that occur within the eCB system. Biochemical and pharmacological studies have shown that mRNA levels and CB_1 receptor binding decrease in several brain regions of aged rats, including the basal ganglia and cerebellum (Berrendero et al., 1998; Romero et al., 1998). Others have shown significant increases in mRNA expression within the brain stems of aged mice, while the cortex appears to have region specific changes in receptor density (Liu, Bilkey, Darlington, & Smith, 2003; Wang, Liu, Harvey-White, Zimmer, & Kunos, 2003). Additionally, the levels of endogenous cannabinoids were modestly decreased in aged animals (Maccarrone et al., 2002; Wang et al., 2003).

The primary purpose of this study is to elucidate further the role of the eCB system in age-dependent cognitive processes and anxiety-like behaviors by comparing the behaviors of young (age 4-6 weeks), mature (age 4-5 month), and old (age 12-14 month) CB_1 wild type ($CB_1^{+/+}$) and CB_1 knockout ($CB_1^{-/-}$) mice in a variety of behavioral

paradigms, including the Morris Water Maze, Light-Dark Box, Suok test, and the Open-Field test.

Hypotheses: Based on previous research (Varvel, et al., 2005; Varvel & Lichtman, 2002), we initially hypothesized that (1a) differences would not be evident between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice on the dependent variables associated with pre-acclimation, spatial acquisition, or the 24-hour probe trial in the MWM, and (1b) that the $CB_1^{-/-}$ mice would perseverate to the previously learned platform location during the reversal learning trials. However, our preliminary data suggested differences between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice on the MWM task. In order to understand these differences better, we explored whether our observed differences in the MWM task were dependent on age. We hypothesized (2a) that the young (age 6 – 8 week old) $CB_1^{+/+}$ and $CB_1^{-/-}$ mice would perform similarly on the observed variables, whereas (2b) a significant age-dependent difference would be evident between the mature (4 - 5 month old) $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. These hypotheses were again refined on the basis of the data described in more detail in the Results section.

In order to determine whether these differences were age dependent or genotype dependent, we utilized several behavioral paradigms designed to assess anxiety-like and motor behaviors. We hypothesized that (3) the observed differences in the MWM were due to an increased anxiety response in the $CB_1^{-/-}$ mice, and, thus, we also hypothesized that the $CB_1^{-/-}$ mice would perform poorly relative to their age-matched controls in the (3a) Light-Dark Box and in the (3b) the Suok test, and (3c) that any observed differences between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice on the Open-Field task would represent differences in locomotor activity. It was expected that the null would prevail in the latter hypotheses.

Method

Subjects

Experiments were conducted on young (6 - 8 week old), mature (3 - 5 month old), and old (12 - 14 month old) male $CB_1^{+/+}$ and $CB_1^{-/-}$ mice that had been backcrossed onto a C57BL/6 background. All mice were born in the vivarium at The University of Montana and were derived from a previously described line of breeding pairs (Zimmer, Zimmer, Hohmann, Herkenham, & Bonner, 1999). All mice weighed between 22 and 60 grams at the time of testing, and the mice were housed in groups of three to five mice per cage in a temperature-controlled (20 - 22°C) facility with a 12-hour light/dark cycle. Animals were allowed food and water *ad libitum* while housed in their home cages. Behavioral testing was conducted between 8:00 h and 15:00 h in order to minimize the influence of circadian rhythms and the hypothalamic-pituitary-adrenal (HPA) axis (Valentinuzzi, Menna-Barreto, & Xavier, 2004).

Animals used in the MWM task were not utilized for other behavioral tests. Animals undergoing testing in the Light-Dark Box were also utilized for the Suok test, and these testings occurred two weeks apart, with all animals first undergoing the Light-Dark Box paradigm. Naïve animals were used for the Open-Field test. Following testing, all animals were euthanized and brain tissue was collected for either electrophysiological or microscopy purposes.

Materials and Procedure

Morris Water Maze Apparatus. Young and mature mice were trained and tested in a large, circular, galvanized steel pool (160 cm in diameter, 62 cm high), filled with 22°C +/-1°C water to a height of 24 cm, to find a hidden platform (10 cm in diameter, located

1.5 cm below the surface of the water). In order to render the platform "hidden" or virtually invisible and to facilitate video tracking of the animal, white paint (ProArt ArtWorks Tempera Paint) was added to the pool until the water was opaque. Four different black and white geometric shapes were attached to the inner walls of the N, S, E, and W quadrants of the pool. All visual cues within the testing room remained constant throughout testing.

MWM Testing Procedure. The MWM training procedure described above has been adapted from those previously reported (Varvel et al., 2005; Varvel & Lichtman, 2002). In order to ensure that all mice were able to perform the swim task adequately, the mice were given a pre-acclimation training session (Day zero), which required each animal to swim for five consecutive minutes with no platform present. For the spatial acquisition task, each mouse was subjected to four trials per day for eight consecutive days (Days one though nine; see Figure 1 for schematic of testing protocol). For each trial the mouse was released from a different start point (N, S, E, W) facing the wall of the pool, and the mouse remained in the pool until the platform was located or until 120 s had elapsed. If the mouse failed to reach the platform in the allotted time, the mouse was manually guided to the platform. Each mouse was required to remain on the platform for 10 seconds prior to removal. Upon removal from the pool, the mouse was placed under an infrared light for two minutes in order to warm it before placing the animal back into its home cage. The platform remained in the SE quadrant for all spatial acquisition trials. Animals were defined as meeting "criterion' (or adequately performing the spatial acquisition task) for the next phase of the experiment if they located (and remained on)

the hidden platform within 30 seconds on three out of four trials on *either* day seven or eight of the spatial acquisition task.

A 60-second probe trial (no platform available) was administered one day (Day nine) following completion of the spatial acquisition task. Following the 60-second probe trial, all mice were then subjected to a reversal acquisition task (Days 10 – 18). For the reversal acquisition task, the hidden platform was moved to the NW quadrant, and each mouse was given four trials per day for eight consecutive days. All other reversal-training procedures were identical to the spatial acquisition task.

During the testing phase, one $CB_1^{+/+}$ animal (age 6 - 8 week old) was removed from the MWM task on spatial acquisition day five due to an eye infection. Eight $CB_1^{-/-}$ animals (two 6 - 8 week old and six 4 - 5 month old) were also removed from the study within the first two days of the spatial acquisition task because these animals were near drowning (Table 1). Data from these animals were not included in the statistical analyses.

The AnyMaze automated tracking system (Stoelting Co., Wood Dale, IL) was used to analyze several dependent variables in addition to completion of the MWM task. For the pre-acclimation phase, the dependent variables included total path length traveled, average speed, and percent thigmotaxia (i.e., percent time spent in the peripheral zone of the pool; see Figure 1 for schematic of MWM paradigm). The dependent variables analyzed for the spatial acquisition phase included: latency to (last) escape, latency to first escape, average speed, total path length, percent time spent in specified quadrants(s), number of platform exits, and path efficiency (defined as the straight line distance between the start and end positions divided by the total distance traveled). The dependent variable “latency to first escape” was utilized in addition to “latency to (last)

escape" in order to capture better the amount of time it took the animal to (first) locate and escape to the hidden platform, as the variable "latency to (last) escape" does not account for the behavior of the animals that locate, escape to, and then exit from the hidden platform prior to the 15 second duration required for the behavior to have been recorded as successfully locating the platform (i.e., latency to (last) escape). For example, the mature $CB_1^{-/-}$ mice had a tendency to locate and escape to the hidden platform, remain on the platform for several seconds, and then exit the platform prior to meeting the "15-second criterion for escape". The variable "first latency to escape" appears to capture better the true time required for the mice to locate the hidden platform.

The dependent variables of interest for the 60-second probe trial were total path length, number of platform crossings, and the percent time spent in the target quadrant. For the reversal trials, data on the number of platform crossings, the percent time spent in the initial training quadrant, and the latency to first enter the target platform in the new location (i.e., the quadrant opposite to the initial training quadrant) were analyzed.

Light-Dark Box Task. All testing was completed in a quiet darkened room, illuminated with a red light. Animals were allowed to acclimate to the testing environment for one hour prior to testing. The light-dark box (45 x 27 x 27 cm) consisted of a lit compartment (~200 lux) and a dark compartment (5 lux). The box was open topped and lined with white plastic in one compartment and dark plastic in the other (Costall, Jones, Kelly, Naylor, & Tomkins, 1989; Rutkowska, Jamontt, & Gliniak, 2006). The dark compartment comprised two-fifths of the total area of the box. The lit and dark compartments were connected via a small 7.5 x 7.5 cm passageway/door. The animals were placed in the center of the lit compartment facing the dark compartment and allowed

to explore the entirety of the box for five minutes, beginning with the first entry into the dark compartment. The apparatus was cleaned thoroughly with 30% vol/vol ethanol between animals in order to remove olfactory cues (e.g., urine and/or fecal matter) from the surface of the apparatus. Dependent variables included the time spent in each compartment and the number of transitions between the compartments.

Suok Test. The Suok apparatus consisted of a long (2 m) hollow aluminum tube (2 cm in diameter) that was securely attached to a Plexiglas base (50 x 50 cm^2) on each end. The aluminum rod was mounted 20 cm above the floor of the apparatus, and the rod was separated into 10 cm segments using lines drawn on the rod. During testing, each animal was placed onto the center of the rod and allowed to explore for five minutes. Following a "fall," the animal was picked up and placed back onto the rod in the location (and correct orientation) where it fell. A cushion consisting of Styrofoam was placed underneath the bar throughout testing in order to avoid harm to the animals during falls. Dependent variables included horizontal exploration activity (average distance from the center [start] position, total number of line crossings, total distance traveled, average speed, and time spent immobile) and a vestibular/motor index (number of falls from the rod; Kalueff et al., 2008). All dependent variables, except total number of falls, were automatically calculated using the AnyMaze program. The apparatus was cleaned thoroughly between animals.

Open-Field Test. The dimensions of the open-field apparatus were 80 cm long x 60 cm wide x 60 cm high. The apparatus was divided into 48 square segments (10 cm x 10 cm). All animals were acclimated to the testing room for one hour prior to testing. Upon acclimation the animal was placed into the center of the dimly lit (20-30 lux)

apparatus and allowed to explore for 10 minutes. Dependent variables were automatically recorded and calculated using the AnyMaze program and included total distance traveled, average speed, time spent immobile, and the number of lines crossed. The apparatus was thoroughly cleaned between animals.

Statistical Analyses

For the pre-Acclimation and the 60-second probe trials of the MWM task, as well as the Light-Dark Box, Suok, and Open-Field tasks, groups were compared using a Two-Way (3 x 2) ANOVA (age x genotype). For the MWM spatial acquisition and reversal acquisition tasks, a Three-Way ANOVA (age x genotype x day) with repeated measures was used for statistical analysis; the variable "day" was the within-subjects factor. All graphical data represent the mean +/- the s.e.m. for each dependent variable averaged across trial(s) on a given day. Trial was not included as a factor.

The moderately conservative Huyn-Feldt corrections were used when the sphericity assumption was violated (i.e., when the variance of the difference between conditions was not equal). This inequality of variances causes the *F*-ratio to be positively biased, and the Huyn-Feldt correction alters the significance value of the *F*-ratio by adjusting the degrees of freedom. Tukey post hoc analyses were used for all follow-up comparisons. Additionally, a *chi square* analysis was conducted to determine if group differences were evident in the ability of each genotype and age to complete the MWM task successfully (i.e., not having to be removed from the study).

In order to provide additional information regarding the practical significance of the statistical findings, the partial *Eta* squared effect size (η^2) and the total proportion of variance accounted for by the model (R^2) are reported (when appropriate) for all main

effects and interactions. The partial *Eta* squared index assesses the relationship between the specified independent and dependent variables for the factors of interest (e.g., age, genotype, or the interaction) and provides a direct measure of the proportion of variance accounted for by each factor or by the interaction between these factors. Notably, the partial *Eta* squared value provides the amount of variability that each factor and/or interaction has contributed to the model as though the factor or interaction were the only contributing variable; thus, collectively, the partial *Eta* squared values are not additive (i.e., these values do not sum to equal the total variance, R^2, that is attributed to the overall model; Kirk, 1996).

Additionally, unlike traditional significance tests (e.g., ANOVAs, student *t*-test), effect size measurements are independent of sample size. According to Kirk (1996), partial *Eta* squared effect sizes can be interpreted as small ($\eta^2 > .010$), medium ($\eta^2 > .059$) or large ($\eta^2 > .138$). For comparison and discussion purposes, partial *Eta* squared values were also computed from previous publications that had utilized CB_1-receptor-deficient mice in the Morris Water Maze. These effect size measures were conducted using the following equation: $\eta^2 = F$ value / (F value + degrees of freedom for the error term). All other statistical analyses were conducted using SPSS 16.0 Graduate Student Version for Mac.

Results

Morris Water Maze

The percentage of animals that successfully completed the swim task is shown in Table 1. A *chi-square* test revealed that the inability of an animal to complete the task is associated with the genotype and age of the animal, $\chi^2 (3, N = 92) = 10.59$, $p = .014$, with

the mature $CB_1^{-/-}$ mice having the highest attrition/failure rate. Follow-up post hoc comparisons demonstrated significant differences between the young $CB_1^{+/+}$ and mature $CB_1^{-/-}$ mice, $p = .012$. Specifically, the mature $CB_1^{-/-}$ mice (27%) showed a higher attrition rate relative to the young $CB_1^{+/+}$ mice (zero percent).

Pre-acclimation. During the pre-acclimation stage of the testing (see Hypothesis 1a), a two-way ANOVA indicated a marginally significant interaction between age and genotype, with small effect sizes, for both distance traveled ($F[1, 78] = 3.707$, $p = .058$, $R^2 = .212$, $\eta^2 = .045$) and speed ($F[1, 78] = 3.720$, $p = .057$, $R^2 = .213$, $\eta^2 = .046$). This interaction suggests that the differences observed between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice are dependent on the age of the animal. Post hoc analyses indicated that the mature $CB_1^{+/+}$ mice swam farther and faster than the mature $CB_1^{-/-}$ mice, $p < .01$. A significant main effect for age, $F(1, 78) = 4.514$, $p = .037$ ($R^2 = .037$, $\eta^2 = .055$), was also observed for percent thigmotaxia (defined as time spent in the periphery of the pool), with the mature mice spending a greater amount of time in the periphery of the pool relative to the young mice, regardless of the genotype of the animal. Between-subject differences were not observed among groups for the dependent variable "percent thigmotaxia" (Figure 3).

Spatial Acquisition. Differences were observed between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice on the dependent variables associated with spatial acquisition in the MWM task (see Hypothesis 1a). A highly significant learning response occurred for all groups across training days for latency to (last) escape, $F(5.05, 393.48) = 55.02$, $p < .001$, $\eta^2 = .123$, a medium-sized effect. A significant between-subjects interaction was observed between age and genotype, $F(1, 78) = 5.22$, $p = .025$, $\eta^2 = .063$. This interaction indicates that the $CB_1^{+/+}$ mice decreased the amount of time necessary to locate and escape to the hidden

platform at a faster rate relative to the $CB_1^{-/-}$ mice, whereas the young animals (regardless of genotype) also decreased the time necessary to locate and escape to the hidden platform at a faster rate compared to the mature mice (regardless of genotype).

A significant linear trend with a medium-sized effect was also observed among groups across training days for latency to first escape into the platform zone, $F(4.99, 389.60) = 32.60$, $p < .001$, $\eta^2 = .077$ which also suggests that the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice decreased the amount of time to locate and escape to the hidden platform across days. Distinct from the above-mentioned findings for the dependent variable, latency to (last) escape, no interaction was observed between the variables age and genotype for the variable latency to first escape. However, between-subjects differences were observed for genotype ($F[1, 78] = 7.67$, $p = .007$, $\eta^2 = .089$), which indicates that although the trend for decreasing the amount of time to first escape to the hidden platform zone was similar between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, the $CB_1^{+/+}$ mice first escaped to the platform more quickly, regardless of age. Specifically, differences were evident between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice on days three and eight, $p = .047$ and .028, respectively (Figure 4A).

A significant interaction between the day of testing and genotype emerged for the dependent variable "speed." In general the $CB_1^{+/+}$ mice decreased in speed over time, whereas the $CB_1^{-/-}$ mice increased speed over time, $F(7, 546) = 19.04$, $p < .001$, $\eta^2 = .196$, a large effect. Between-subjects differences were not observed between groups for specific training days.

With respect to distance traveled, a significant linear trend was observed among all groups, $F(6.12, 477.32) = 31.68$, $p < .001$, $\eta^2 = .289$ (a very large effect), indicating that all animals, regardless of age and genotype, decreased the distance traveled across

training days. Consistent with the speed of the groups, a significant interaction between the day of testing and genotype was observed ($F[7, 546] = 12.57$, $p < .001$, $\eta^2 = .139$), indicating that the $CB_1^{+/+}$ mice decreased the overall path length at a faster rate over time relative to the $CB_1^{-/-}$ mice (regardless of age). No between-subjects differences were observed on any particular day (Figure 4B and 4C, respectively). A significant and large linear trend was also observed for all groups across training days for percent thigmotaxia, $F(4.03, 314.49) = 14.98$, $p < .001$, $\eta^2 = .161$, indicating that all groups of animals similarly decreased the amount of time spent in the periphery of the pool across days. Between-subjects differences were not observed for specific training days, suggesting all groups spent a similar amount of time in the periphery zone of the pool across days (Figure 4D).

A highly significant learning response was also noted among all groups across days for the percent time spent in the training/platform quadrant, $F(4.86, 378.67) = 31.79$, $p < .001$, $\eta^2 = .290$ (this is a substantial effect size for this type of work), indicating that over time all groups of animals increased the amount of time in the training quadrant. Between-subjects differences were observed for the genotype variable, $F(1, 78) = 12.03$, $p = .001$, $\eta^2 = .134$, with the $CB_1^{+/+}$ mice spending more time in the training quadrant compared to the $CB_1^{-/-}$ mice, regardless of age. Specifically, the $CB_1^{+/+}$ mice spent more time in the initial training quadrant on days one, three, six, seven, and eight, all p values less than .05 (Figure 5A).

Additionally, the number of exits from the platform zone was measured. A significant interaction occurred between genotype and testing day, suggesting that the $CB_1^{+/+}$ mice decreased the number of platform exits at a faster rate across days relative to

the $CB_1^{-/-}$ mice (regardless of age; F[5.42, 422.59] = 2.26, p = .043, η^2 = .028). Additionally, between-subjects differences were observed between young and mature mice, F(1,78) = 9.81, p = .002, η^2 = .112. Specifically, the mature mice exited the platform more frequently on all observed days, and this difference reached significance on days five and seven, p = .001 and .045, respectively (Figure 5B; Hypothesis 2B). With respect to path efficiency, a significant interaction of day and genotype indicated that the $CB_1^{+/+}$ mice had a greater increase in path efficiency over time relative to the $CB_1^{-/-}$ mice, F(6.49, 466.97) = 3.93, p = .001, η^2 = .052 (Figure 5C). Between-subject differences were observed $CB_1^{+/+}$ and $CB_1^{-/-}$ animals (F[1, 72] = 10.26, p = < .01, η^2 = .125), with the CB1+/+ animals having higher path efficiency on days five, six, seven, and eight (all p values < .05).

24-Hour Probe Trial. Significant group differences were observed between $CB_1^{+/+}$ and $CB_1^{-/-}$ animals during the 24-hour probe trial (see Hypothesis 1a). Specifically, a two-way ANOVA revealed a significant interaction between age and genotype for the average distance from the target platform zone, F[1, 78] = 4.571, p = 0.05, R^2 = .124, η^2 = .055. This interaction indicates that the differences noted between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice are dependent on the age of the animal. Post hoc comparisons revealed significant differences between the mature $CB_1^{+/+}$ and mature $CB_1^{-/-}$ mice; the $CB_1^{-/-}$ mice were on average farther from the platform zone (Figure 6; see Hypothesis 2b).

A significant main effect of genotype (regardless of age) was also observed for the number of crossings into the target platform zone (F[1, 78] = 9.76, p < .01, R^2 = .093, η^2 = .111) during the 24-Hour Probe Trial, with the $CB_1^{-/-}$ mice having fewer number of

platform crossings. A significant main effect for the percent time spent in the target zone was also observed (F[1, 78] = 6.72, p = .01, R^2 = .069, η^2 = .079). The $CB_1^{-/-}$ mice spent less time in the initial training quadrant relative to the $CB_1^{+/+}$ mice (see also Figure 6; see Hypothesis 2b).

Reversal Probe Trial. Differences were noted between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice on the dependent variables associated with the reversal-learning task in the MWM task (see Hypothesis 1b). All groups of mice significantly (and linearly) decreased the number of entries into the initial target platform zone over time, F(3.86, 212.54) = 17.88, p < .01, η^2 = .078. Significant between-subjects differences were observed for genotype (F [1, 55] = 4.13, p = 0.047, η^2 = .070), regardless of age, with the $CB_1^{+/+}$ mice crossing the platform zone a greater number of times. Specifically, the $CB_1^{+/+}$ mice crossed the initial training platform significantly more times on days one and two, whereas the $CB_1^{-/-}$ mice crossed the platform more times on day seven (p = .005, .003, and .029, respectively; Figure 7A). Additionally, a significant linear trend was observed among all groups across training days for the percent time spent in the initial training quadrant, F(6.36, 349.56) = 23.32, p < .01, η^2 = .063, indicating that all groups of mice decreased the amount of time in this quadrant over time. An interaction between genotype and the day of testing was also observed (F[6.36, 349.56] = 7.69, p < .01, η^2 = .020), indicating that the $CB_1^{+/+}$ mice decreased the amount of time spent in the initial training quadrant at a faster rate when compared to the $CB_1^{-/-}$ mice. No significant between-subjects main effects were observed for age or genotype (Figure 7B).

A significant interaction between genotype and the day of testing was observed for the latency to first escape onto the reversal platform, F(6.08, 334.55) = 2.30, p = .033,

η^2 = .007, with the $CB_1^{+/+}$ mice locating and escaping to the hidden platform at a faster rate when compared to the $CB_1^{-/-}$ mice. Additionally, a significant interaction between age and the day of testing also emerged, $F(6.08, 334.55) = 2.34$, $p = .031$, $\eta^2 = .007$. These findings suggest that overall the $CB_1^{+/+}$ mice decrease the latency to first escape more quickly when compared to the $CB_1^{-/-}$ mice, and that the young mice (regardless of genotype) escape to the platform significantly faster over time relative to the mature mice. Between-subjects differences also emerged for the genotype variable, $F(1, 55) = 4.11$, $p = .047$, $\eta^2 = .070$, with the $CB_1^{+/+}$ mice finding the platform location more quickly. Specifically, the $CB_1^{+/+}$ mice had a significantly faster rate of latency to first escape on days five, six, seven, and eight compared to the $CB_1^{-/-}$ mice (all p values less than .05; Figure 7C). Notably, the effect sizes for the set of findings regarding the variable "latency to escape onto the reversal platform" were all very small.

Light-Dark Box

A two-way ANOVA for the time spent in the lit compartment indicated a significant effect of age ($F[2, 43] = 19.62$, $p < .001$, $R^2 = .642$, $\eta^2 = .477$, a very large effect size) and genotype ($F[1, 43] = 51.98$, $p < .001$, $R^2 = .642$, $\eta^2 = .547$, also a very large effect), but no significant interaction was observed between these variables ($F[2, 43] = 0.19$, $p = .831$, $\eta^2 = .009$). This main effect indicates that the time spent in the lit compartment was decreased across the lifespan of the animal, regardless of genotype, and the $CB_1^{-/-}$ mice spent less time in the lit compartment compared to the $CB_1^{+/+}$ mice, regardless of age. Post-hoc analyses using a one-way ANOVA indicated that the older mice (regardless of genotype) spent significantly less time in the lit compartment relative to the amount of time spent in the dark compartment. Specifically, Tukey post analyses

indicated that the mature and old $CB_1^{+/+}$ mice spent less time in the lit compartment relative to the young $CB_1^{+/+}$ mice, and the old $CB_1^{-/-}$ mice spent less time in this compartment relative the mature and young $CB1^{-/-}$ mice. Tukey post hoc analyses for the significant main effect for genotype indicated that the time spent in the lit compartment was significantly higher in young, mature, and old $CB_1^{+/+}$ mice relative to the age-matched $CB_1^{-/-}$ mice (all *p* values less than .001; Figure 8A; see Hypothesis 3a).

A significant main effect for genotype, $F(1, 43) = 46.38$, $p < .001$, $R^2 = .513$, $\eta^2 = .519$, was also noted for the number of crossings into the lit compartment, with the $CB_1^{-/-}$ mice making fewer crossings into the lit compartment. The effect size for this dependent variable was also very large. No significant effect was observed for either age ($F[2, 43] = 2.34$, $p = .123$, $\eta^2 = .093$) or the interaction between age and genotype, $F(2, 43 = 2.21$, $p = .122$, $\eta^2 = .093$), indicating that the number of crossings into the lit compartment was not dependent on the age of the animal or the interaction between the age and genotype of the animal. Post hoc analyses using a one-way ANOVA and follow-up Tukey comparisons indicated that the mature and old $CB_1^{-/-}$ mice had significantly fewer crossings into the lit compartment relative to the age-matched $CB_1^{+/+}$ mice (all *p* values less than .001; Figure 8B; see Hypothesis 3a).

Suok Test.

A two-way ANOVA revealed a significant interaction between age and genotype for the average distance from the center zone, $F(2, 42) = 4.89$, $p = .012$, $R^2 = .437$, $\eta^2 = .189$, a large effect. This interaction demonstrates that the differences observed between the specific age groups (i.e., young, mature, and old mice) are dependent on the genotype of the animal, with the mature and old $CB_1^{-/-}$ mice having a shorter average distance from

the center zone, an age effect not observed for the $CB_1^{+/+}$ mice (see Figure 9A). A significant main effect was also obtained for both age (F[2, 42] = 7.22, $p < .01$, η^2 = .256) and genotype (F[1, 42] = 17.23, $p < .01$, $\eta^2 = .291$), with the mature and old mice (regardless of genotype) and the $CB_1^{-/-}$ mice (regardless of age) having a higher average distance from the center zone compared to the young and $CB_1^{+/+}$ mice, respectively. The magnitudes of the effect sizes for these findings were very large. Post hoc analyses for the main effect of age indicated that the mature and old $CB_1^{-/-}$ mice had a significantly smaller average distance from the center of the Suok apparatus relative to the young $CB_1^{-/-}$ mice, p is less than .01 for both comparisons. Age-dependent differences were not evident for the $CB_1^{+/+}$ mice. With respect to the main effect of genotype, the mature and old $CB_1^{-/-}$ mice had a significantly smaller average distance from the center of the apparatus relative to their age-matched controls ($p < .01$ for both variables; Figure 9A; see Hypothesis 3b).

Significant main effects for genotype, with large effect sizes, were uncovered for both distance traveled (F[1, 42] = 14.19, $p < .01$; $R^2 = .203$, $\eta^2 = .253$, Figure 9B) and average speed in the Suok apparatus (F[1, 42] = 12.38, $p < .01$, $R^2 = .169$, $\eta^2 = .228$; data are not shown in Figure 9), with the $CB_1^{+/+}$ mice traveling both farther and faster than the $CB_1^{-/-}$ mice. However, follow-up post-hoc analyses did not reveal group differences between the age-matched $CB_1^{+/+}$ and $CB_1^{-/-}$ mice for the genotype variable (see Hypothesis 3b).

Additionally, a large main effect for genotype was obtained for the amount of time the animal was immobile during the task (F[1, 42] = 11.51, $p < .01$, $R^2 = .164$, η^2 = .215, Figure 9C); on average the $CB_1^{-/-}$ mice spent more time immobile relative to the

$CB_1^{+/+}$ mice. Between-subjects differences were not observed between age-matched controls, indicating that, on average, the $CB_1^{-/-}$ mice spend more time immobile, regardless of age. A significant main effect for genotype was also obtained for the number of falls from the Suok apparatus, $F(1, 42) = 4.18$, $p = .047$, $R^2 = .126$, $\eta^2 = .119$, with the $CB_1^{-/-}$ animals having a greater number of falls. Significant between-subjects differences were not observed between the age-matched $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. This suggests that the differences observed between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice are not dependent on age (Figure 9D; see Hypothesis 3b).

Open-Field Test. A two-way ANOVA revealed significant and large main effects for both age ($F[2, 48] = 11.059$, $p < 0.01$, $R^2 = .339$, $\eta^2 = .315$) and genotype ($F[1, 48] = 8.359$, $p < 0.01$, $R^2 = .339$, $\eta^2 = .148$) on the total distance traveled in the open-field test, indicating an age-dependent decrease in the total path length traveled (regardless of genotype). The $CB_1^{-/-}$ mice also have a lower total path length compared to the $CB_1^{+/+}$ mice (regardless of age). Similar results were obtained for both age ($F[2, 48] = 11.00$, $p < 0.01$, $R^2 = .337$, $\eta^2 = .314$) and genotype ($F[1, 48] = 8.36$, $p < 0.01$, $R^2 = .337$, $\eta^2 = .148$) for the average speed of the animals, with an apparent age-dependent lower magnitude of average speed, regardless of genotype, and the $CB_1^{-/-}$ mice having a lower average speed compared to the $CB_1^{+/+}$ mice. A significant and large main effect for age and genotype was also observed for the total number of line crossings ($F[2, 48] = 10.09$, $p < 0.01$, $R^2 = .333$, $\eta^2 = .296$ and $F[1, 48] = 8.629$, $p < 0.01$, $R^2 = .333$, $\eta^2 = .152$ respectively), as well as the total time spent immobile ($F[2, 48] = 14.40$, $p < 0.01$, $R^2 = .40$, $\eta^2 = .375$ and $F[1, 48] = 9.59$, $p < 0.01$, $R^2 = .40$, $\eta^2 = .167$ respectively). These results further indicate age-dependent lower numbers of lines crossed and an age-dependent rise in the total time

spent immobile among the young, mature, and old mice (regardless of genotype); the $CB_1^{-/-}$ mice also had fewer line crossings and spent more time immobile compared to the $CB_1^{+/+}$ mice.

A one-way ANOVA for the two factors of interest (age and genotype) revealed no differences between the age-matched $CB_1^{+/+}$ and $CB_1^{-/-}$ mice on any of the Open-Field test dependent variables (i.e., total path length, average speed, number of line crossings, time spent immobile; see Hypothesis 3c). Within each genotype, the only significant difference observed between groups occurred within the $CB_1^{+/+}$ mice. Specifically, the young $CB_1^{+/+}$ mice had a significantly longer path length, higher average speed, and greater number of line crossings, as well as less time immobile compared to the old $CB_1^{+/+}$ mice (all p values < 0.01, Figure 10).

Discussion

The present study uses complimentary approaches to elucidate the role of the CB_1 receptor in learning, memory, and anxiety-like behaviors. In the experiments described a number of phenotype differences emerged between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. To our knowledge, this is the first study to evaluate the role of the CB_1 receptor in mice of differing ages using the MWM. However, Varvel and colleagues (2002, 2005) have studied the behavior of three to five month old $CB_1^{+/+}$ and $CB_1^{-/-}$ mice in the MWM, and others (e.g., Bilkei-Gorzo et al, 2005) have utilized behavioral paradigms similar to those reported in the current study to evaluate the age-dependent role of the CB_1 receptor in cognitive decline. When appropriate, our results will be compared to these findings.

Using the Morris Water Maze task, differences emerged during the pre-acclimation phase between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice that were dependent on the age of the

animal. These findings indicate that the mature $CB_1^{+/+}$ mice swam faster and farther than the age-matched controls. The effect sizes were modest to moderate for these factors. The partial *Eta* squared values were .045 and .046, which means that the interaction between age and genotype accounted for 4.5% and 4.6% of the overall variability in the measured variables, respectively. Previous studies have not published findings regarding total distance traveled and average speed during the pre-acclimation phase, so it is not possible to compare the size of the effects found here with those in previous work.

During the pre-acclimation phase the overall measure of thigmotaxia demonstrated that, on average, the mature mice spent more time in the periphery of the pool relative to the young mice. The factor age alone accounted for 5.5% of the overall variability in thigmotaxia (a medium effect). No detectable differences were found between genotypes. Similar to these results, Varvel and Lichtman (2002) did not report significant differences in percent thigmotaxia between three to five month old $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. However, the authors did report that 20% of the $CB_1^{-/-}$ mice had to be rescued in order to prevent drowning, and about one-half of the $CB_1^{-/-}$ mice stopped swimming and floated for the last several minutes. These latter results are comparable to our findings, where 24% of the mature (age three to five month old) $CB_1^{-/-}$ mice had to be removed from the current study to prevent drowning. Taken together, these results may indicate overall differences that emerge in global functioning in the pool between the mature $CB_1^{+/+}$ and $CB_1^{-/-}$ mice at the onset of the MWM task.

During the spatial acquisition phase, latency to (last) escape appeared to be dependent on the age and genotype of the animal, as the young mice (regardless of genotype) acquired the task at a similar rate, whereas the mature $CB_1^{-/-}$ mice had

difficulty acquiring the task relative to the mature $CB_1^{+/+}$ mice. A moderate effect size was noted, with the interaction between age and genotype accounting for 6.3% of the total variance in acquisition. Notably, an interaction between genotype and day was also observed for the number of exits from the platform zone, with the $CB_1^{+/+}$ mice decreasing the number of exits at a faster rate over time relative to the $CB_1^{-/-}$ mice. The effect of this interaction was small ($\eta^2 = .028$); however, these latter findings may also be involved with the increased time required for the $CB_1^{-/-}$ mice to complete the "latency to (last) escape" task, which could exacerbate the difference between groups. For these reasons we chose to analyze latency to first escape in addition to the latency to last escape.

The results regarding latency to (last) escape contrast with findings published by Varvel and colleagues (2002, 2005), who report near identical escape latencies between three to five month old $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, with the effect size being extremely small ($\eta^2 < .001$; Varvel and Lichtman, 2002). Effect size computations could not be conducted for the number of exits from the platform zone for the above-mentioned studies because the authors did not report findings for this variable. A main effect of age or an interaction involving the factor age was not detected for other dependent variables of interest for the spatial acquisition phase; but, rather, several main effects of genotype were observed.

The $CB_1^{+/+}$ mice in the present study (regardless of age) decreased the latency to first escape and path length to target at a faster rate compared to the $CB_1^{-/-}$ mice. The effect size for latency to first escape was moderate to large, whereas the effect size for total path length was large, with these variables accounting for 8.9% and 13.9% of the total variance, respectively. Additionally, in the current study, the $CB_1^{+/+}$ mice decreased their overall average speed during the acquisition trials, whereas the $CB_1^{-/-}$ mice increased

their speed across days. The effect size for the variable "speed" was large, accounting for 19.6% of the variance. The $CB_1^{+/+}$ mice also demonstrated a greater increase in path efficiency over time relative to the $CB_1^{-/-}$ mice, with the $CB_1^{+/+}$ mice having a greater path efficiency on days five, six, seven, and eight. The interaction between genotype and day accounted for 5.2% of the total variance in path efficiency, while the between-subject differences accounted for 12.5% of the variance.

Previous studies have not published findings regarding the variables "latency to first escape" or "path efficiency," making it difficult to compare the size of the effects found in our study to those found in other reports. Unlike the findings for the current study, Varvel and Lichtman (2002) reported no differences between three to five month old $CB_1^{+/+}$ and $CB_1^{-/-}$ mice for either total path length or average swim speed, with extremely small effect sizes for both dependent variables ($\eta^2 < .001$).

The findings in the current work regarding spatial acquisition in the MWM differ from those in the past literature in terms of statistical significance and the magnitude of the observed effects for a variety of dependent variables (Varvel & Lichtman, 2002; Varvel et al., 2005). These results also contradicted our own initial hypotheses that predicted no detectable differences between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice (the null) during either the pre-acclimation or spatial acquisition phase. Partial *Eta* squared magnitudes in the current study indicate that the differences noted between the current and past literature are not merely dependent on the larger sample size in the present work, but rather that the observed differences between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice may suggest that the endocannabinoid system is tonically active in spatial memory acquisition under specific circumstances.

To our knowledge this study is also the first to assess the role of the CB1 receptor during a 60-second probe trial following the acquisition phase of testing. An age-dependent difference emerged during the 24-hour probe trial, demonstrating that the mature $CB_1^{-/-}$ mice were on average farther from the platform zone, with the interaction between age and genotype accounting for 5.5% of the total variance. A significant main effect for genotype (regardless of age) was noted for the number of platform crossings and the percent time spent in the training quadrant, with the $CB_1^{-/-}$ mice having fewer platform crossings and spending less time in the initial training quadrant. Genotype accounted for 11.1% and 7.9% of the total variance for the number of platform crossings and percent time spent in the training quadrant, respectively. These results support the initial hypotheses of an age-dependent difference in the ability of the $CB_1^{-/-}$ mice to learn a spatial acquisition task; however, given the differences for genotype (regardless of age) during the spatial acquisition task in the current study and previous reports (Varvel & Lichtman, 2002; Varvel et al., 2005) demonstrating no effect of genotype during spatial acquisition utilizing a similar aged mouse (three to five months old), the role of the endocannabinoid system in facilitating spatial learning seem less clear.

In order to evaluate behavioral flexibility and extinction processes, the current study also analyzed the behavior of a subset of animals that met criterion for having successfully learned the location of the platform during the spatial acquisition trials (i.e., animals that had reached the platform zone in less than 30 seconds on either days seven or eight) on the reversal-learning task. The results for the reversal-learning paradigm suggest that the $CB_1^{+/+}$ mice (regardless of age) crossed the platform zone significantly more times on days one and two, spent less time in the initial training quadrant over time,

and learned the location of the "new" platform location at a greater rate compared to the $CB_1^{-/-}$ mice: these difference were significant on days five, six, seven, and eight. The effect sizes for these variables indicate that the factor "genotype" has a moderate effect for the number of entries into the initial platform zone, with only a small effect on both the time spent in the initial training quadrant and the latency to escape to the new platform location. Genotype accounts for 7%, 2% and .7% of the total variance in these models, respectively.

Although this study is the first, to our knowledge, to assess reversal learning for eight consecutive days following a spatial acquisition and 60-second probe trial, our findings appear to differ from previous reports evaluating extinction learning in the MWM. Varvel and Lichtman (2002) presented $CB_1^{+/+}$ and $CB_1^{-/-}$ mice with seven acquisition training sessions consisting of fours trials per session. Following the acquisition phase, mice were subjected to a reversal test of four trials (one session). The $CB_1^{-/-}$ mice demonstrated greater escape latencies, higher path lengths, and a greater number of entries into the previous learned platform location relative to the $CB_1^{+/+}$ mice, which the authors interpreted as a deficit in extinction learning. The previous effect size indices for these variables indicate that the factor "genotype" has a moderate effect on both escape latency ($\eta^2 = .05$) and path length ($\eta^2 = .049$), with a small effect for the number of entries into the previous platform location ($\eta^2 = .033$). Although the design of the current study differs in terms of a number of variables (e.g., the number of acquisition sessions, the number of reversal sessions), it is clear that the findings in the current study do not support the earlier premise that the $CB_1^{-/-}$ mice perseverate to the previously learned platform location.

In a follow-up study, Varvel and colleagues (2005) evaluated $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, as well as $CB_1^{+/+}$ mice treated with the CB_1 antagonist SR141716A using either a massed or spaced extinction protocol. Specifically, the mice were trained to locate a hidden platform, the platform was then removed, and the mice were subjected to either a massed (i.e., five consecutive sessions consisting of four 120-second trials) or a spaced (a single one-minute trial every two to four weeks) extinction protocol. No differences were found between SR141716A-treated and vehicle-treated mice or between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice when the massed extinction protocol was utilized; however, differences were observed for the spaced extinction protocol. Specifically, the SR141716A-treated and $CB_1^{-/-}$ mice had impaired extinction learning, with both groups of mice having greater escape latencies ($\eta^2 = .069$ and $\eta^2 = .057$, respectively), and the $CB_1^{-/-}$ mice having a greater path length to target (($\eta^2 = .023$) when compared to vehicle-treated and $CB_1^{+/+}$ mice, respectively.

Varvel and colleague's (2005) results are consistent with previous findings that suggest the endocannabinoid system is involved in extinction processes within the MWM; however, this study illustrates that the endocannabinoid system may only facilitate extinction processes under specific circumstances. The authors initially hypothesize that the observed discrepancies between the extinction paradigms may have been related to an increase in the amount of stress experienced by the animals. Specifically, the massed extinction protocol is likely to be more stressful than the spaced extinction paradigm. The increase in stress associated with the massed extinction paradigm may have interfered with endocannabinoid-mediated extinction learning.

Notably, the design of the reversal-learning paradigm used in the current study resembles the massed extinction protocol discussed above. It is possible that the animals undergoing the reversal-learning paradigm in the current study experienced increased levels of stress as a result of the 24-hour probe trial and the reversal-learning session that began immediately following the probe test. This protocol could have led to an increase in the level of stress, which may have contributed to the $CB_1^{-/-}$ mice having fewer perseverative behaviors during the reversal-learning trials relative to other studies. However, this hypothesis is unlikely given the findings of the 24-hour probe trial, where the $CB_1^{-/-}$ mice were farther from the platform zone, with fewer crossings into the platform zone, and spending less time in the initial training quadrant.

The observed discrepancies between our results and those previously published were unexpected. Given the above-mentioned findings on spatial acquisition and extinction learning, as well as the extensive body of literature that illustrates enhanced memory performance in $CB_1^{-/-}$ mice on a variety of behavioral paradigms, including object recognition (Reibaud et al., 1999; Maccarone et al., 2002), active avoidance (Martin, Ledent, Parmentier, Maldonado, & Valverde, 2002), and partner recognition (Bilkei-Gorzo et al., 2005), it is likely that the genotype-specific deficits observed in the current study are related to an additional CB_1-receptor-dependent mechanism, such as an age-dependent or anxiety-like response, rather than deficits in pure learning and/or memory processes.

Consistent with this hypothesis, Bilkei-Gorzo et al (2005) demonstrated that memory enhancement in $CB_1^{-/-}$ mice is indeed dependent on age, something that provided the rationale for operationalizing the levels of the independent variable “age” in the

current study. Specifically, young $CB_1^{-/-}$ mice (age 6 – 8 week) demonstrate enhanced or similar performance on an operant-learning paradigm, the partner-recognition and rotorod tasks, as well as the open-field task, while mature (age 3 - 5 month) and old (age 14 – 17 month) $CB_1^{-/-}$ mice perform poorly relative to their age-matched controls. Regarding an anxiety-like response, the MWM is designed to assess spatial memory in rodents; however, due to the aversive nature of being placed into water, it can be assumed that the water maze also evokes a certain degree of stress.

Upon exposure to an aversive (or novel) stimulus, the hypothalamic-pituitary-adrenal axis (HPA) is activated, a response that is consistent across species; this region contains a dense population of corticosterone-releasing hormone (CRH) neurons within the paraventricular nucleus (PVN) of the hypothalamus. Upon activation the CRH neurosecretory cells release CRH, which subsequently releases adrenocorticotropic hormone (ACTH) into the portal blood stream from the pituitary gland. The release of ACTH leads to an increase in secreted glucocorticoids (and other steroids) from the adrenal glands, which allow the organism to mobilize resources in order to contend with the perceived threat (Herman, Tasker, Ziegler, & Cullinan, 2002; Herman et al, 2003). Both *in vitro* and *in vivo* studies suggest that the eCB system regulates the activation of this stress circuit.

Patel and colleagues (2004) have postulated a "gatekeeper theory" for how the eCB system modulates this stress circuitry. According to this hypothesis high levels of endogenous cannabinoids are present within the PVN during non-aversive events, resulting in an inhibition of glutamatergic excitatory inputs into the HPA axis. During an aversive event, the levels of endogenous cannabinoids rapidly decline via an

undetermined mechanism, and the HPA axis is activated by disinhibition of the glutamatergic inputs (Patel, Roelke, Rademacher, Cullinan, & Hillard, 2004). Similarly, $CB_1^{+/+}$ mice receiving a cannabinoid-receptor antagonist (e.g., SR141716A) and CB_1-receptor-deficient mice would be expected to present with an exaggerated activation of the HPA axis and an increased stress response. Consistent with this hypothesis, a CB_1-receptor antagonist has been shown to induce a dose-dependent HPA axis response in non-stressed animals (Manzaneres, Corchero, & Fuentes, 1999; Patel et al., 2004; Wade, Degroot, & Nomikos 2006).

In order to assess the age-dependent effects of the CB_1 receptor on anxiety-like behaviors as a possible mechanism mediating the observed spatial deficits in the MWM, we utilized the Light-Dark Box and Suok tasks. The Light-Dark Box results demonstrated that the time spent in the lit compartment was lower across the developmental lifespan (regardless of genotype) for both the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, with age accounting for 47.7% of the observed variance. Differences between age-matched $CB_1^{+/+}$ and $CB_1^{-/-}$ mice were striking in all age groups for this variable, as were the differences between the mature and old age-matched mice for the number of crossings into the lit compartment. The effect size indices for "genotype" were remarkably large for both the time spent in the lit compartment and number of crossings into the lit region, accounting for 54.7% and 51.9% of the variance in the dependent variable. These findings are in stark contrast to findings published by Maccarone and colleagues (2002), who report a mild decrement in anxiety-like behaviors in four-month-old $CB_1^{-/-}$ relative to age-matched $CB_1^{+/+}$ controls ($\eta^2 = .119$). However, the authors of this study utilized

mice that were backcrossed onto an albino CD1 mouse strain, whereas our mice are backcrossed onto the C57BL6 strain, which could account for the observed differences.

In the Suok task, the $CB_1^{-/-}$ mice spent more time near the center (start position) of the apparatus ($\eta^2 = .291$), traveled less distance ($\eta^2 = .253$), spent more time immobile ($\eta^2 = .215$) and had significantly more falls from the apparatus ($\eta^2 = .119$) compared to the $CB_1^{+/+}$ mice, something that might either be related to either an increased anxiety-like response or impaired motor/balance difficulties. The partial *Eta* squared values for these dependent variables were all moderate to large; the factor genotype accounts for a considerable amount of the variance within the model. To our knowledge, previous studies have not utilized the Suok task to evaluate the role of the CB1 receptor in anxiety-like behaviors; thus it is not possible to compare the sizes of the effects found here with those in previous work.

In contrast to the Light-Dark Box results, the findings from the Suok task did not appear to be dependent on age. However, for all of the observed dependent variables (i.e., average distance from the center [start] position, distance traveled, time immobile, number of falls), there was a noticeable trend (albeit non-significant) with a large effect for age-dependent differences, with the mature and old mice traveling less distance from the center (start) position, displaying an overall shorter average distance traveled, and spending more time immobile, with the mature mice also demonstrating a greater number of falls relative to the young and old mice.

Our results suggest that the $CB_1^{-/-}$ mice exhibit more anxiety-like behaviors relative to the $CB_1^{+/+}$ mice. In order to validate these findings further and to assess horizontal motor differences between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, we utilized the Open-Field

test, as this behavioral paradigm has commonly been used to assess exploratory motor behavior in mice (Sousa, Almeida, & Wotjak, 2006). No differences were observed between the age-matched $CB_1^{+/+}$ and $CB_1^{-/-}$ mice on any of the dependent variables of interest (e.g., total distance traveled, average speed, number of line crossings), which further supports the hypothesis that the observed behavioral effects in the MWM, Light-Dark Box, and Suok tasks may be related to anxiety-like behaviors, rather than more pure learning and memory processes or motor/coordination difficulties in the $CB_1^{-/-}$ mice. These findings are similar to other reports that demonstrate no difference in horizontal behaviors (i.e., distance traveled) between age-matched $CB_1^{+/+}$ and $CB_1^{-/-}$ mice under similar conditions (Bilkei-Gorzo et al., 2005). However, the results of the Open-Field test do not necessarily rule out possible deficits in skilled movement, or "clumsiness," that may be underlying the observed genotype-specific differences noted in the Suok task. Additionally, the current study did not covary out the activity of the animal (e.g., horizontal behaviors) within the Light-Dark Box, due to the limitations of the current data acquisition equipment; however, future research should consider this as an additional analysis.

Additional evidence to support the above-mentioned anxiety-based hypothesis comes from data evaluating the role of the eCB system in long-term potentiation (LTP). In brief, LTP of synaptic transmission is the leading contemporary experimental paradigm for understanding the molecular mechanisms underlying learning and memory (Malenka and Nicoll, 1999). Consistent with the behavioral studies that employ either the CB_1 receptor antagonists (e.g., SR141716A) or the CB_1-receptor-deficient mouse model, hippocampal slice preparations from $CB_1^{-/-}$ mice (11-month old) have shown an enhanced

LTP response (Bohme, Laville, Ledent, Parmentier, & Imperato, 2000). Consistently, hippocampal slices that have been treated with an exogenous CB_1-receptor agonist have been shown to have a decreased LTP response (Davies, Pertwee, & Riedel, 2002), something that is consistent with the observed behavioral decrements in agonist-treated animals. To date, very few studies have evaluated the age-dependent and/or stress-related differences in LTP in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice (Bohme, et al., 2000; Maccarone et al., 2002).

Preliminary age-dependent studies focusing on the LTP response from our laboratory are currently underway. The initial results of these studies suggest that LTP is enhanced in $CB_1^{-/-}$ mice, and that this phenomenon may be occurring in an age-dependent manner. Specifically, these initial data suggest no difference in LTP between young (age 3 -5 week old) $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, but the mature (age 4 – 5 month old) and old (age 12 – 14 month old) $CB_1^{-/-}$ animals appear to have an enhanced LTP response relative to their age-matched controls. If these results prove to be accurate, our LTP experiments would suggest that learning and memory processes would be likely to be enhanced by the loss of CB_1-receptor signaling in mature animals. As stated above, our MWM behavioral data suggest deficits in learning and memory processes in the $CB_1^{-/-}$ mice relative to the $CB_1^{+/+}$ mice, which is contradictory to other reports that demonstrate either no difference or enhanced learning and memory in the $CB_1^{-/-}$ mice in a variety of behavioral paradigms.

The results of the present study suggest that the observed spatial learning deficits observed in the $CB_1^{-/-}$ mice are related to the anxiogenic effects of the loss of eCB signaling. In order to validate the anxiety-like behaviors observed in this study, we plan to utilize a chronic-restraint-stress paradigm. This restraint-stress paradigm will be used

to determine how age interacts with anxiety-like behaviors using a variety of behavioral paradigms and electrophysiological techniques. Additionally, further research needs to be conducted in order to evaluate the changes in neuronal type and density, as well as alterations in the cytoskeletal and synaptic architecture, that may be underlying the observed differences.

The findings of the current study have several clinical implications. An inability to adapt to chronic stress has been associated with the development of several neuropsychiatric disorders, including major depression and post-traumatic stress disorder (PTSD; Korte, Koolhaas, Wingfield, & McEwan 2005). Gorzalka and colleagues (2008) have recently hypothesized that depression may result from a compromised endocannabinoid signaling system which does not allow the individual to adapt effectively to his/her chronic life stressors. Recently, it has been demonstrated that individuals who have been diagnosed with major depressive disorder have significantly lower levels of plasma 2-AG, which is an endogenous cannabinoid, compared to matched control subjects (Hill et al, 2006; Hill et al, 2008), indicating that the facilitation of the eCB system may have antidepressant-like effects.

Additionally, if the lack of endocannabinoid signaling is indeed associated with impairments in the extinction of aversive memories, and if the findings that demonstrate mutant CB_1-receptor-deficient mice have difficulties "forgetting" traumatic/aversive memories are accurate, then $CB_1^{-/-}$ mice may prove to be a useful model of PTSD (Fride, Suris, Weidenfeld, & Mechoulam, 2005). Consistent with this observation, a recent study reported that the synthetic CB_1 receptor agonist, nabilone, significantly reduced the number and intensity of nightmares, night sweats, and daytime flashbacks in patients

diagnosed with PTSD (Fraser, 2009). Moreover, the anti-obesity drug rimonabant (i.e., SR141716A), a CB_1-receptor antagonist, has been removed recently from FDA-approved drug trials due to the high number of patients reporting increased symptoms of anxiety and depression relative to individuals receiving the placebo drug (Pi-Sunyer, et al., 2006; van Gaal et al., 2005). The endocannabinoid system may ultimately provide a therapeutic target for facilitating adaptive stress responses, which may have implications in the treatment of a variety of clinical disorders.

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Table 1

Sample size (n) for behavioral paradigms and percentage of subjects completing the MWM task.

	Behavioral Paradigm(s)				
Subjects	MWM (*n*)	Completed MWM task (%)	Light-Dark Box (*n*)	Suok Test (*n*)	Open-Field (*n*)
CB1+/+					
Young	23	100	8	8	9
Mature	20	95	9	8	9
Old	0	N/A	8	8	9
CB1-/-					
Young	22	90.9	8	8	9
Mature	25	76	8	8	9
Old	0	N/A	8	8	9
Total	90	90.5	49	48	54

Figure Captions

Figure 1. Summary of the MWM protocol.

Figure 2. Schematic or the MWM and testing room.

Figure 3. Pre-acclimation phase of the MWM task in young (4 -6 week) and mature (4 – 5 month) $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. Data represent the mean +/- s.e.m. for (A) total distance traveled, (B) average speed, and (C) percent thigmotaxia. Significant age-matched differences are denoted as (**; $p < .01$).

Figure 4. Spatial acquisition phase in young (4 – 6 week) and mature (4 – 5 month) $CB_1^{+/+}$ and $CB_1^{-/-}$ mice for days one through eight. Data points represent the mean +/- s.e.m. averaged across trials per day for (A) latency to first entry into the training platform, (B) total distance traveled, (C) average speed, and (D) percent thigmotaxia. (*) represents a significant main effect of genotype ($p < .05$).

Figure 5. Spatial acquisition phase in young (4 -6 week) and mature (4 – 5 month) $CB_1^{+/+}$ and $CB_1^{-/-}$ mice for days one through eight. Data points represent the mean +/- s.e.m. averaged across trials per day for (A) percent time spent in the training quadrant, (B) the number of exits from the platform zone, and (C) path efficiency. (*) represents a significant main effect of genotype, and (#) represents a significant main effect of age (both at the $p < .05$ level of significance).

Figure 6. 24-Hour Probe in young and mature $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. Data points represent the mean +/- s.e.m. per day for (A) the average distance from the platform zone, (B) the number of platform zone crossings, and (C) the percent time spent in the training quadrant. Significant differences between age-matched $CB_1^{+/+}$ and $CB_1^{-/-}$ mice are denoted with an (*) and represent a *p* level of less than .05.

Figure 7. Reversal acquisition in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice for days one through eight. Data points represent the mean +/- s.e.m. averaged across trials per day for (A) number of entries in the initial training platform zone, (B) percent time in the initial training quadrant, and (C) the latency to first escape to the reversal platform. (*) represents a significance level of $p < .05$, whereas (**) represents significance at the $p < .01$ level.

Figure 8. Effects of age and genotype on anxiety-like behaviors in the Light-Dark Box. Data points represent the mean +/- s.e.m. for (A) time spent in the lit compartment and (B) the number of crossings into the lit compartment. Significant difference between age-matched $CB_1^{+/+}$ and $CB_1^{-/-}$ mice are denoted with an (**, $p < .01$), whereas age-dependent differences (within each genotype) are denoted with a (#, $p < .01$).

Figure 9. Effects of age and genotype on exploratory and anxiety-like behaviors in the Suok task. Data points represent the mean +/- s.e.m. for (A) the average distance from the center (start point) of that apparatus, (B) total distance traveled, (C) time spent immobile, and (D) the number of falls. Significant age-matched differences between genotypes are denoted with an (**, $p < .01$).

Figure 10. Exploratory and locomotor activity of $CB_1^{+/+}$ and $CB1^{-/-}$ mice in the open-field apparatus. Data points represent the mean +/- s.e.m. for (A) total path length, (B) average speed, (C) number of line crossings, and (D) the total time spent immobile. Significant age-dependent differences (within each genotype) are denoted with a (#, $p < .01$).

Figure 1. Summary of the MWM protocol

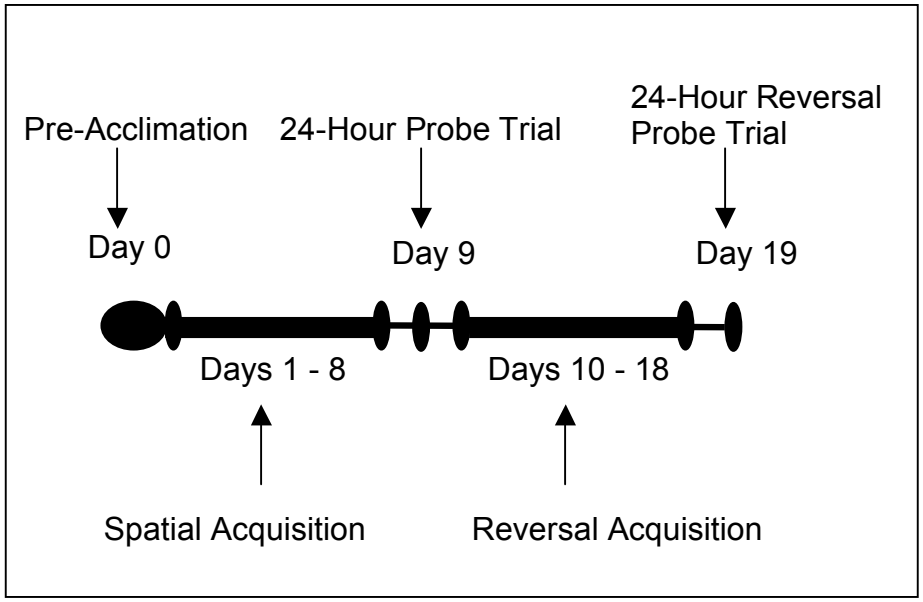

Figure 2. Schematic of the MWM and testing room

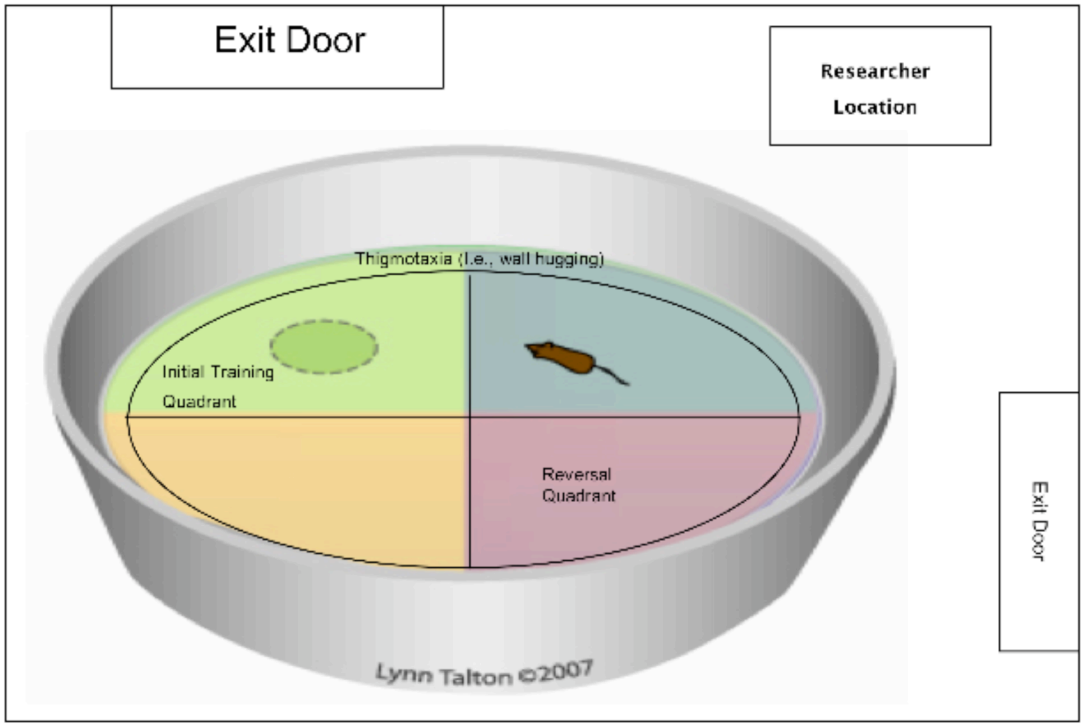

Figure 3. Pre-acclimation phase of the MWM task

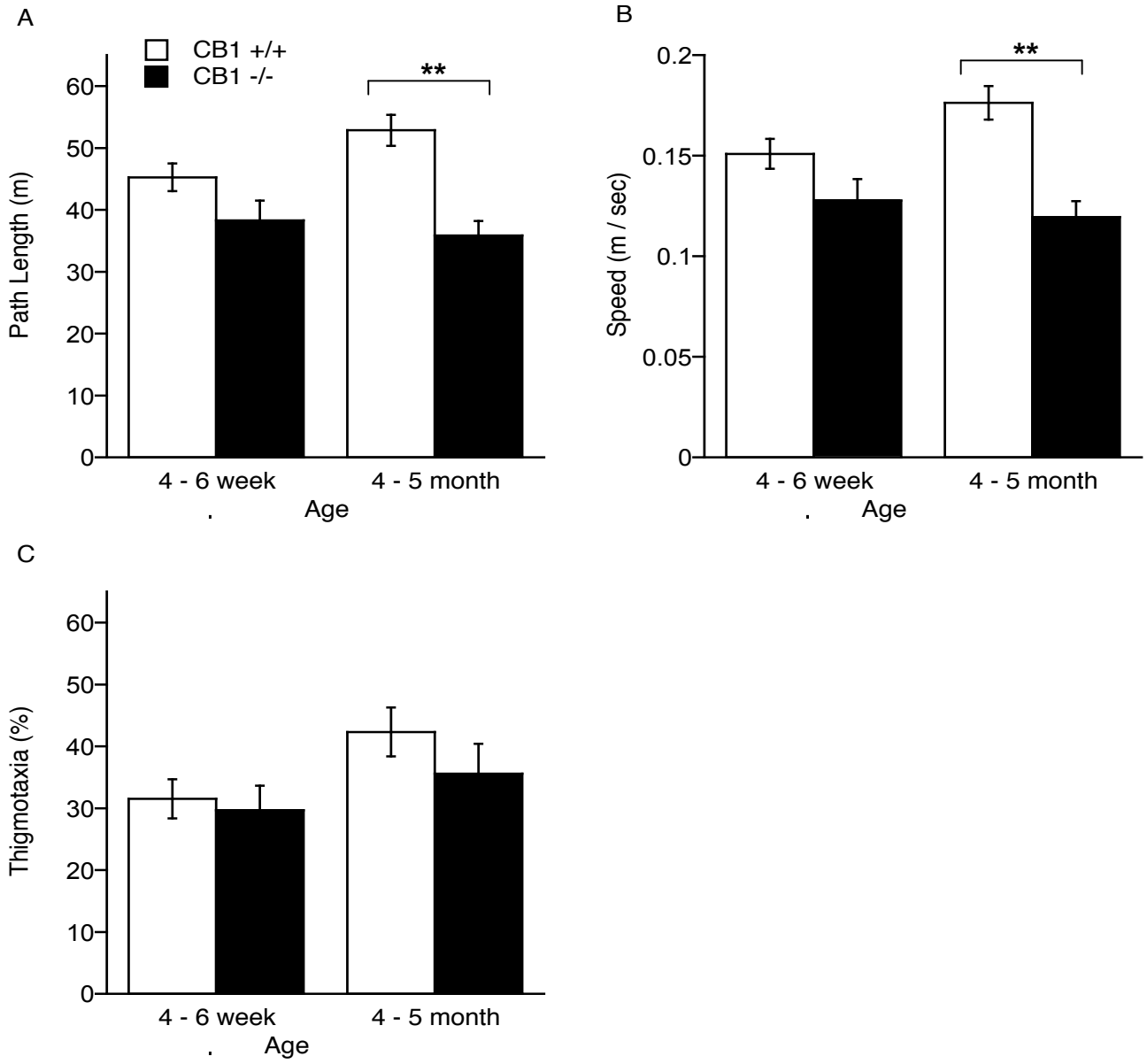

Figure 4. Spatial acquisition phase of the MWM task

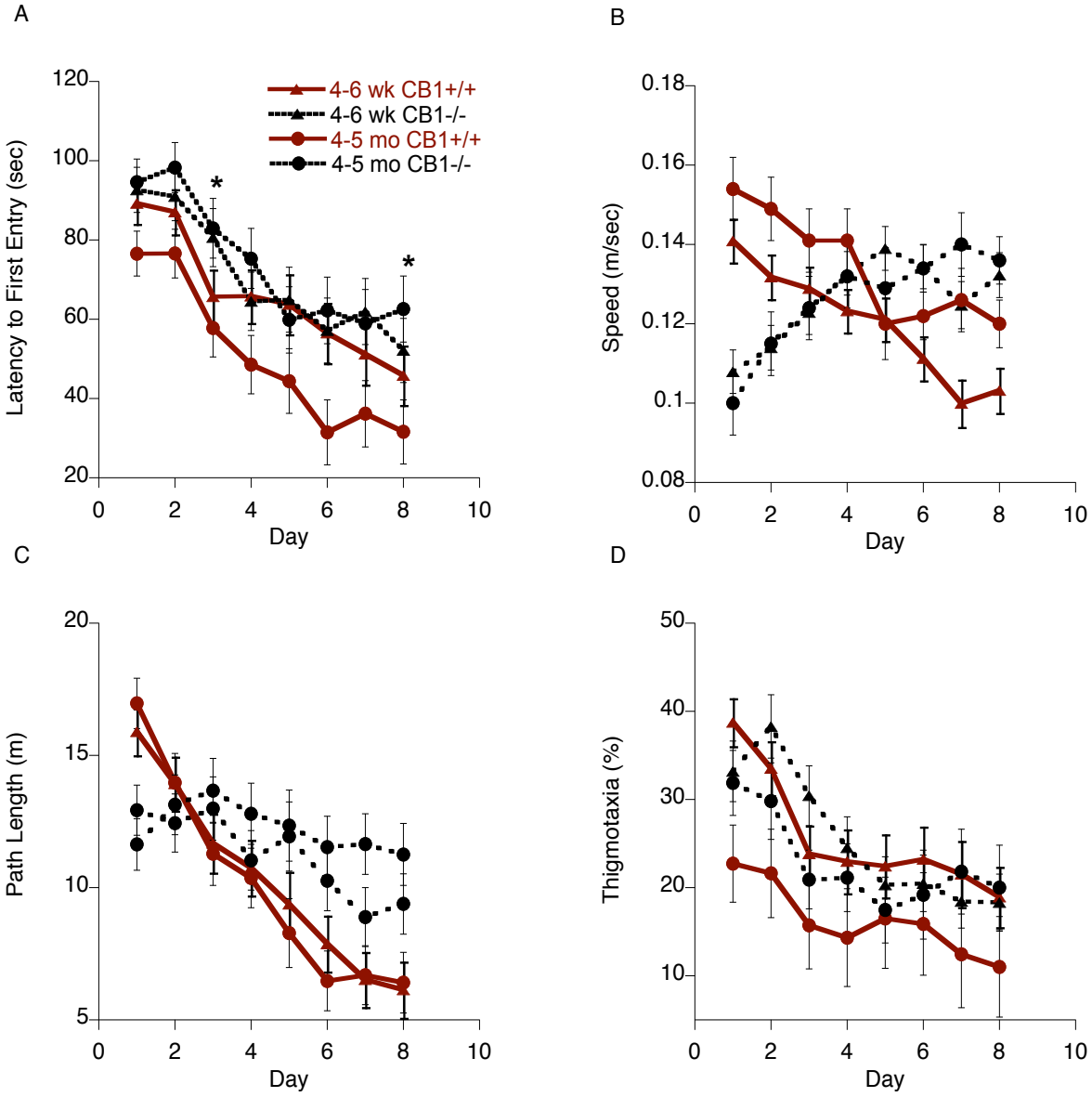

Figure 5. Spatial acquisition phase of the MWM task, continued

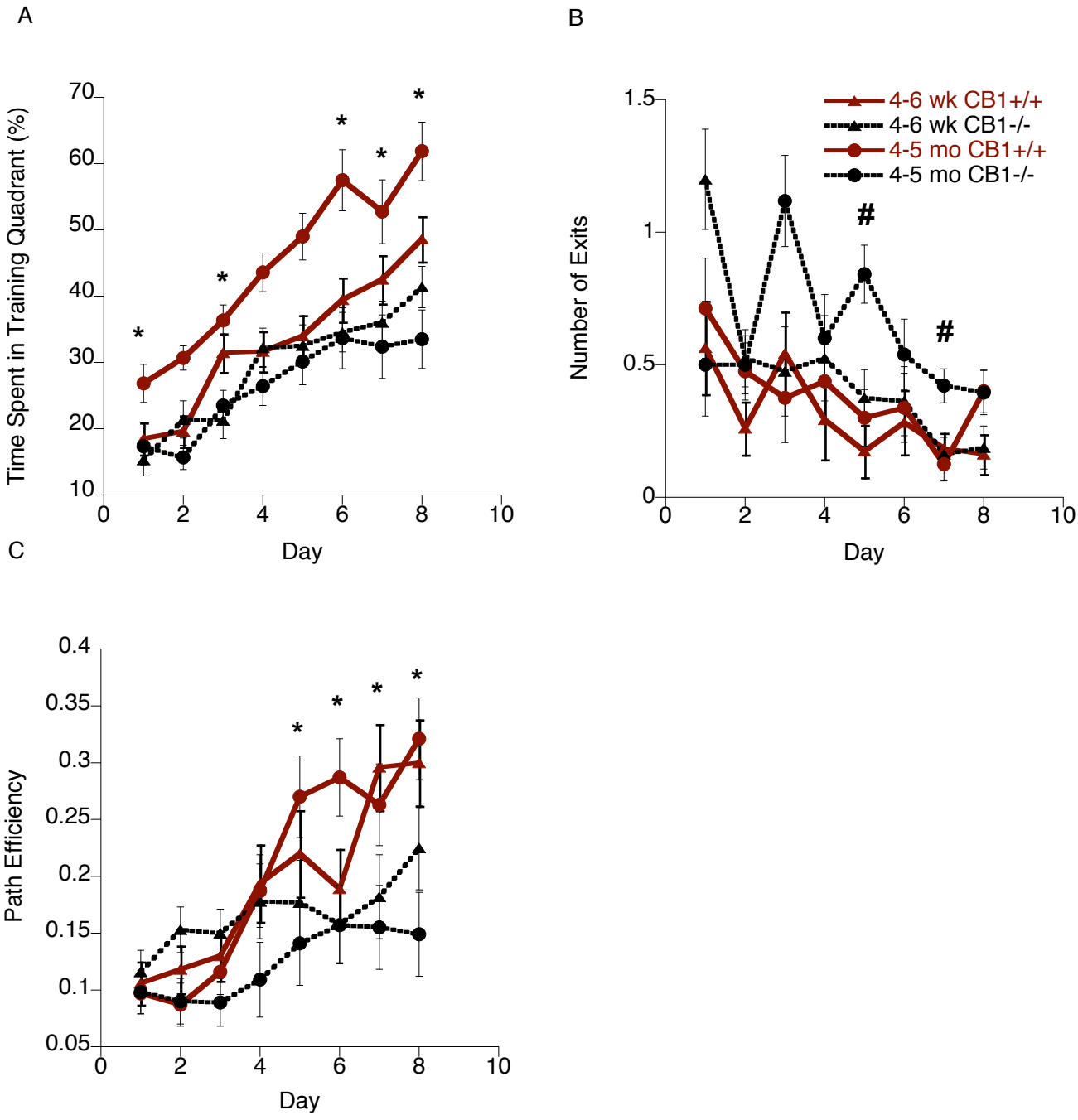

Figure 6. 24-hour probe trial of the MWM task

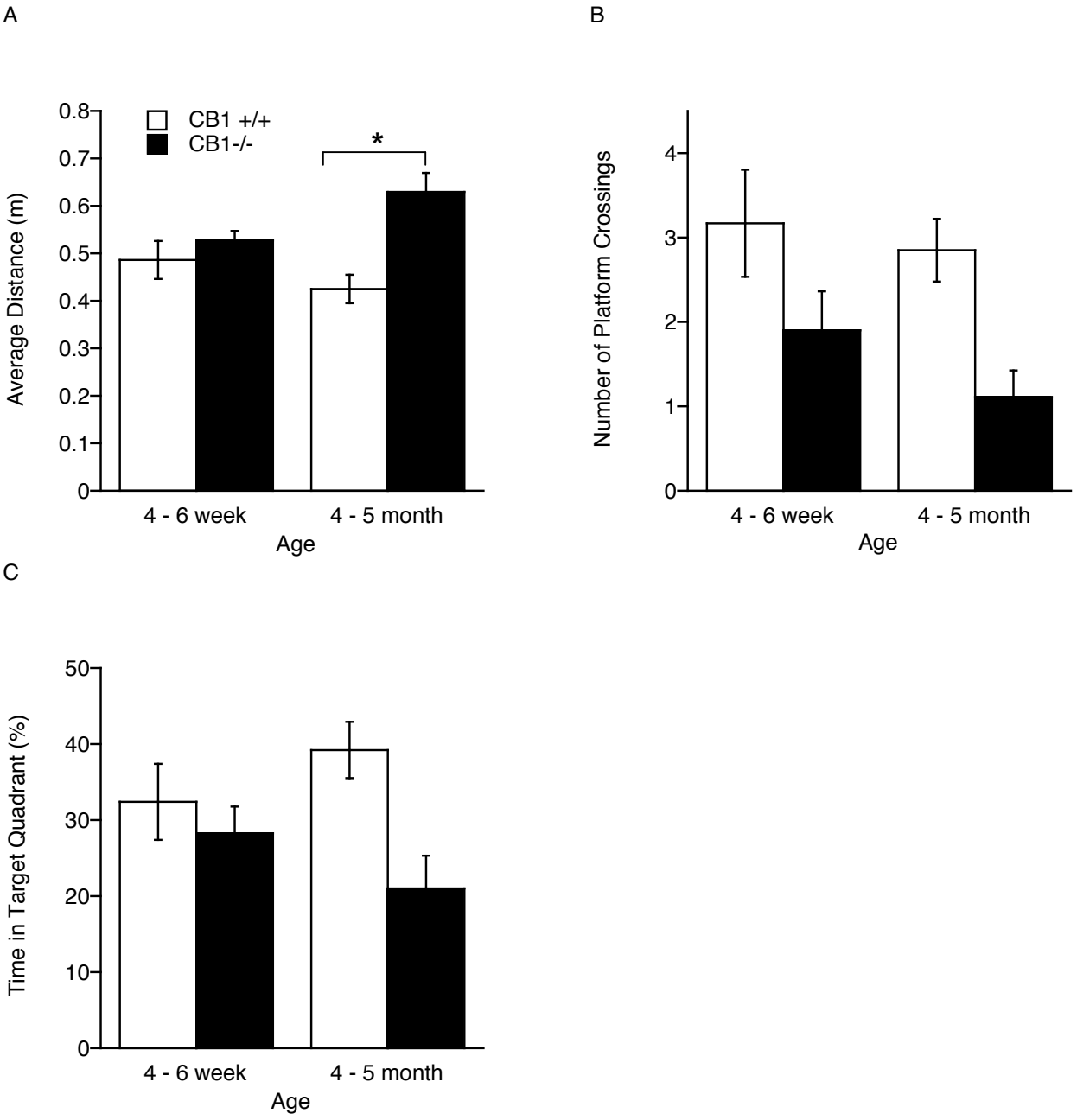

Figure 7. Reversal-learning phase of the MWM task

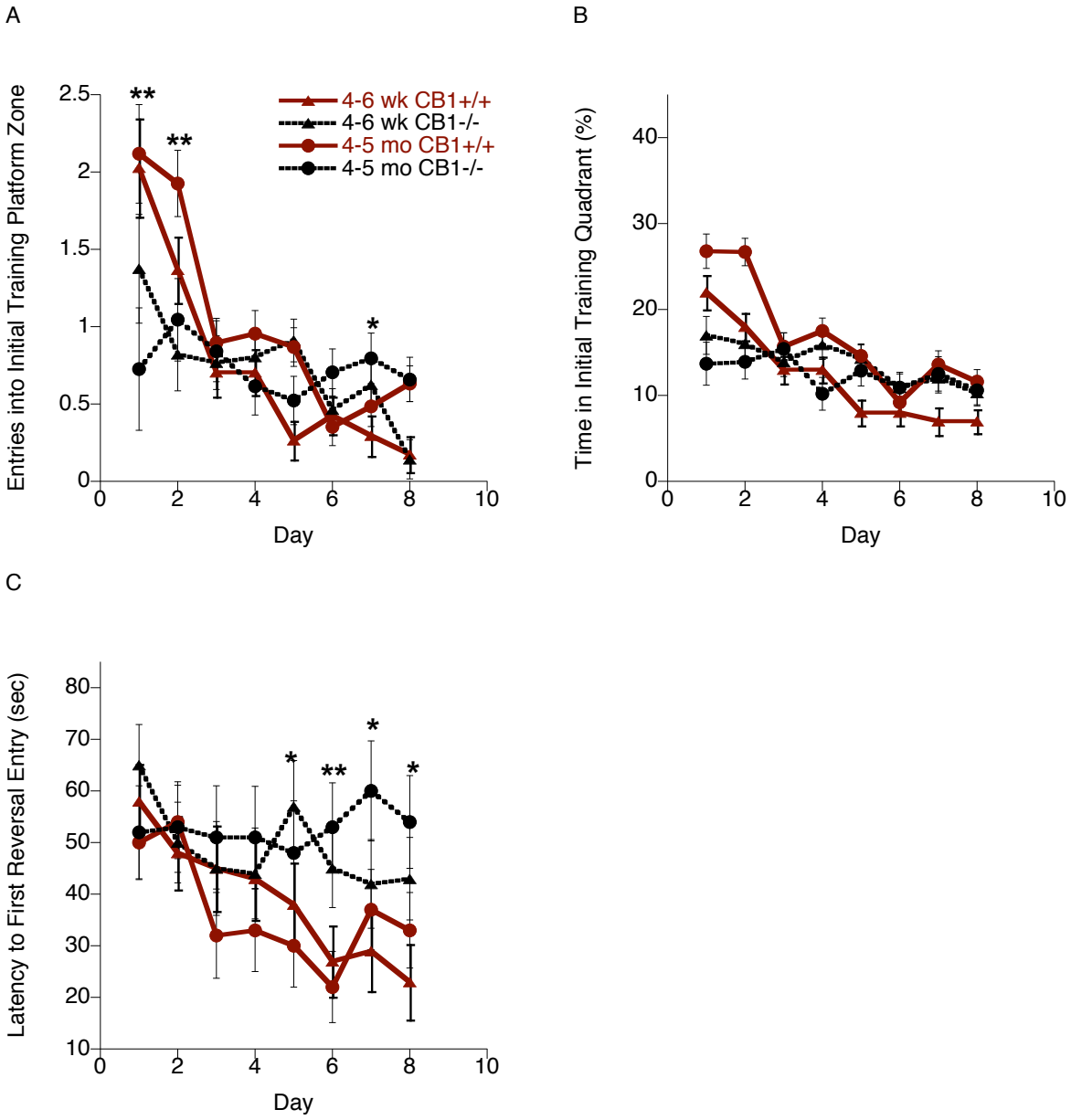

Figure 8. Light-Dark Box

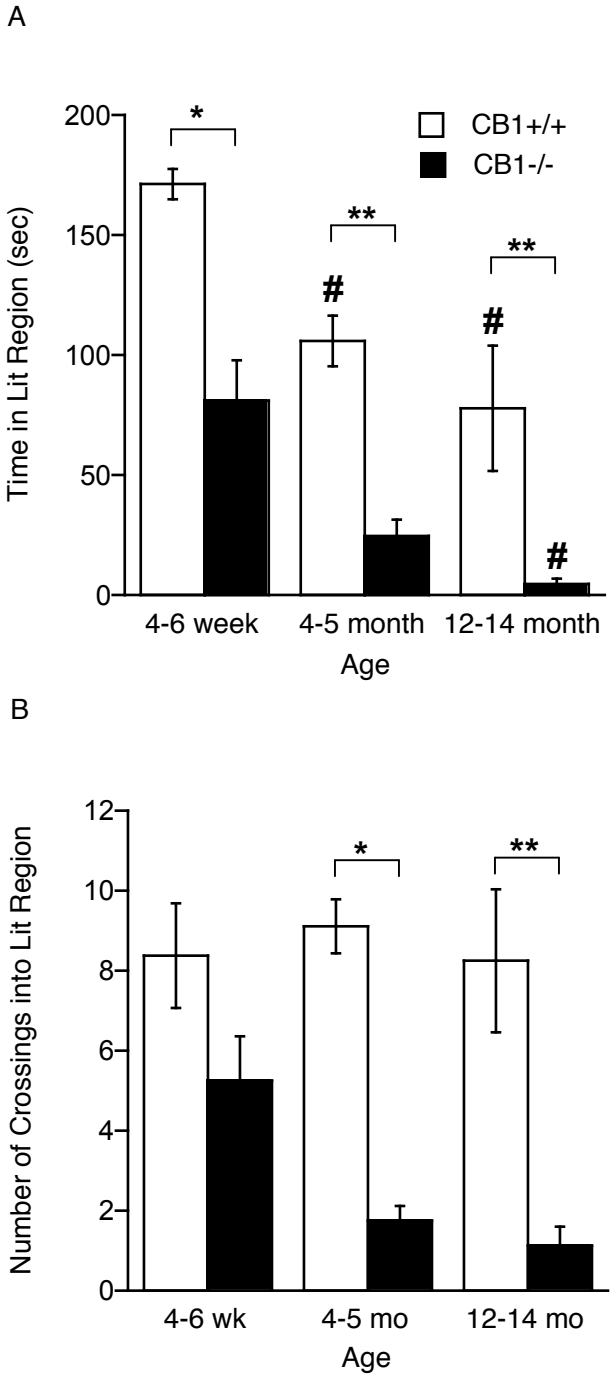

Figure 9. Suok Task

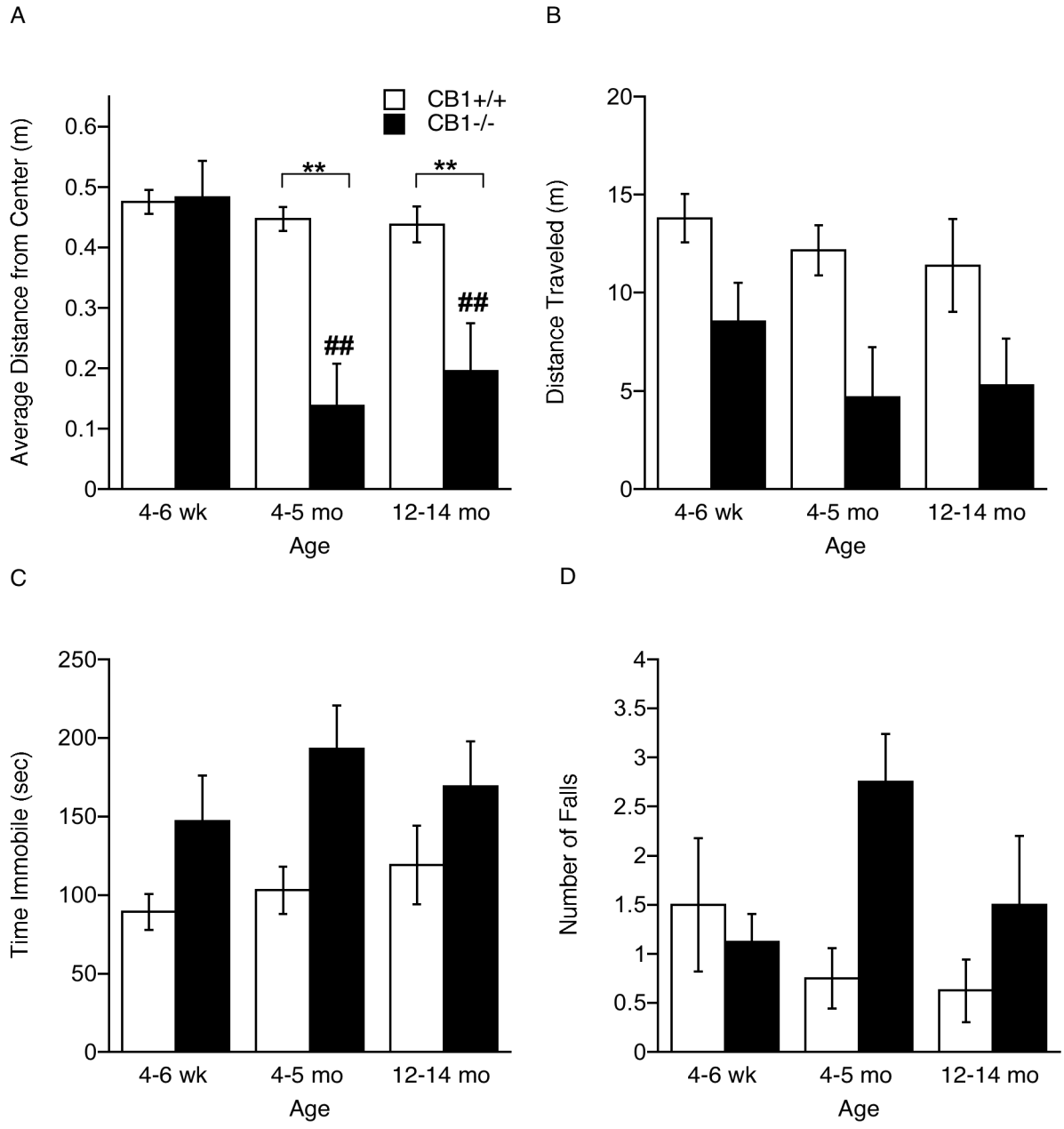

Figure 10. Open-Field task