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Evaluation of CB_1 Receptor Knockout Mice in the Morris Water Maze

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ABSTRACT

The endocannabinoid system has been proposed to modulate a variety of physiological processes, including those that underlie cognition. The present study tested whether this system is tonically active in learning and memory by comparing CB_1 receptor knockout mice ($CB_1^{-/-}$) to wild-type mice ($CB_1^{+/+}$) in several Morris water maze tasks. Also, the effects of three cannabinoid agonists, Δ^9-tetrahydrocannabinol (Δ^9-THC), *R*-(+)-[2,3-dihydro-5-methyl-3[morpholinyl)methyl]-pyrrolo[1,2,3-de]-1, 4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN 55,212-2), and methanandamide, were evaluated in a working memory procedure. Both genotypes exhibited identical acquisition rates in a fixed platform procedure; however, the $CB_1^{-/-}$ mice demonstrated significant deficits in a reversal task in which the location of the hidden platform was moved to the opposite side of the tank. This phenotype difference was most likely due to an increased perseverance of the $CB_1^{-/-}$ mice in that they continued to return to the original platform location, despite being repeatedly shown the new platform location. In addition, Δ^9-THC (ED_{50} = 1.3 mg/kg), WIN 55,212-2 (ED_{50} = 0.35 mg/kg), and methanandamide (ED_{50} = 3.2 mg/kg) disrupted the performance of $CB_1^{+/+}$ mice in the working memory task at doses that did not elicit motivational or sensorimotor impairment as assessed in a cued version of the task. Furthermore, doses of each drug that were maximally disruptive in $CB_1^{+/+}$ mice were ineffective in either *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl (SR 141716A)-treated $CB_1^{+/+}$ or $CB_1^{-/-}$ mice. These results provide strong evidence that cannabinoids disrupt working memory through a CB_1 receptor mechanism of action, and suggest that the endocannabinoid system may have a role in facilitating extinction and/or forgetting processes.

The existence of an endocannabinoid system in the central nervous system that consists of G protein-coupled CB_1 cannabinoid receptors (Herkenham et al., 1991) and endocannabinoids, including arachidonylethanolamide (i.e., anandamide; Devane et al., 1992) and 2-arachidonoylglycerol (i.e., 2-AG; Mechoulam et al., 1995), has gained general acceptance. Recent reports suggest that this system may serve several physiological functions, including the modulation of pain (Calignano et al., 1998; Richardson et al., 1998; Walker et al., 1999), feeding (Di Marzo et al., 2001), emotional behavior (Martin et al., 2002), and cognition (Terranova et al., 1996; Lichtman, 2000).

Converging lines of anatomical, electrophysiological, neurochemical, and behavioral evidence support the proposal that endocannabinoids play a modulatory role on cognition. CB_1 receptors (Herkenham et al., 1991) as well as anandamide and 2-AG (Di Marzo et al., 2000) are present in hippocampus and other forebrain areas associated with memory at high concentrations. In addition, endocannabinoids modulate glutamatergic (Sullivan, 2000), cholinergic (Gifford et al., 2000), and γ-aminobutyric acidergic (Hampson and Deadwyler, 2000; Wilson and Nicoll, 2001) pathways within the hippocampus. Interestingly, hippocampal slices from mice devoid of CB_1 receptors (i.e., $CB_1^{-/-}$ mice) exhibited enhanced long-term potentiation, an electrophysiological model of synaptic plasticity, compared with the wild-type ($CB_1^{+/+}$) mice (Bohme et al., 2000), although the CB_1 receptor antagonist SR 141716A failed to enhance long-term potentiation in rat hippocampal slices (Terranova et al., 1995). Behavioral data also provide compelling support for the involvement of endocannabinoids in learning and memory. Cannabinoid agonists disrupt aspects of working (i.e., short-term) memory, while leaving retrieval of reference (i.e., long-term) memories largely intact, through a CB_1 receptor mechanism of action (Mallet and Beninger, 1996; Jentsch et al., 1997; Varvel et al., 2001). Moreover, intrahippocampal administration of the potent cannabinoid analog CP 55,940

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ABBREVIATIONS: CB, cannabinoid; 2-AG, 2-arachidonoylglycerol; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl; Δ^9-THC, Δ^9-tetrahydrocannabinol; WIN 55,212-2, *R*-(+)-[2,3-dihydro-5-methyl-3[morpholinyl)methyl]-pyrrolo[1,2,3-de]-1, 4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate; ANOVA, analysis of variance; CI, confidence interval; CP 55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; HU-210, (−)-7-OH-Δ-6-tetrahydrocannabinol-dimethylheptyl.

selectively impaired working memory as assessed in the radial-arm maze task (Lichtman et al., 1995). Indeed, stimulation of CB_1 receptors in the hippocampus disrupts memory in a similar manner as hippocampal removal (Hampson and Deadwyler, 1998). Conversely, the inhibition of the endocannabinoid system has been found to enhance performance in several memory tasks. SR 141716A dose dependently improved the social recognition memory of rats, as well as attenuated the deficits displayed by aged mice and rats in the same task (Terranova et al., 1996). Also, rats trained in a modified eight-arm radial maze task displayed fewer errors after treatment with SR 141716A relative to vehicle-treated controls (Lichtman, 2000). Consistent with these findings is that $CB_1^{-/-}$ mice exhibited an enhanced performance in an object-recognition task compared with the wild-type controls (Reibaud et al., 1999). On the other hand, SR 141716A failed to enhance performance in a variety of operant paradigms (Mansbach et al., 1996; Brodkin and Moerschbaecher, 1997; Mallet and Beninger, 1998; Hampson and Deadwyler, 2000).

One learning and memory paradigm that is particularly well suited for investigating specific mnemonic processes is the Morris water maze (Brandeis et al., 1989; Hodges, 1996), which generally involves rodents learning to navigate in a water-filled tank toward a hidden escape platform based on ambient visual cues. Administration of the cannabinoid agonist HU 210 to rats has been shown to retard the acquisition of a reference memory version in a dose-dependent manner at doses that did not disrupt performance when the platform location was made visible (Ferrari et al., 1999). Additionally, our laboratory has recently shown that Δ^9-THC selectively disrupted a working memory version of the water maze in C57BL/6 mice in which the location of the platform was changed from day to day, an effect that was blocked by SR 141716A (Varvel et al., 2001).

The primary goal of the present study was to elucidate further the function of the endocannabinoid system in learning and memory processes by comparing $CB_1^{-/-}$ and $CB_1^{+/+}$ mice in reference and working memory water maze tasks. In addition, we characterized the effects of three structurally dissimilar exogenously applied cannabinoid agonists, Δ^9-THC, WIN 55,212-2 (a high-efficacy aminoalkylindole analog), and methanandamide (a stable anandamide analog) in the working memory task. To rule out nonspecific sensorimotor or motivational influences, wild-type mice were treated with active doses of each agonist and evaluated in a cued version of the task in which the platform was made known by placing a visible object on it. Finally, the involvement of CB_1 receptors was investigated by comparing the effects of each agonist in wild-type mice to either $CB_1^{-/-}$ mice or SR 141716A-treated wild-type mice.

Materials and Methods

Subjects. The subjects included $CB_1^{-/-}$ ($n = 20$) and $CB_1^{+/+}$ ($n = 21$) mice on a C57BL/6 background. All mice were born in the Virginia Commonwealth University vivarium from breeding pairs ($CB_1^{+/-}$ parents) that were derived from a line that is described previously (Zimmer et al., 1999). All subjects were male, weighed 22 to 30 g, and were housed six animals per cage in a temperature-controlled (20–22°C) facility. The Institutional Animal Care and Use Committee at Virginia Commonwealth University approved all experiments. Mice were given unlimited access to food and water and were maintained on a 12-h light/dark cycle.

Drugs. Δ^9-THC and SR 141716A were provided by the National Institute on Drug Abuse (Bethesda, MD), and WIN 55,212-2 and methanandamide were purchased from Tocris Cookson (St. Louis, MO). Each drug was dissolved in a 1:1 mixture of absolute ethanol and alkamuls-620 (Aventis, Princeton, NJ) and diluted with saline to a final ratio of 1:1:18 (ethanol/alkamuls/saline). All drug injections were given subcutaneously in an injection volume of 0.1 ml/kg. Δ^9-THC, WIN 55,212-2, and SR 141716A were administered 30 min before the initiation of the first trial, whereas methanandamide was administered 15 min before.

Apparatus. The water maze consisted of a large, circular, galvanized steel pool (1.8 m in diameter, 0.6 m in height). A white platform (10 cm in diameter) was placed inside, and the tank was filled with water (22°C) until the top of the platform was submerged 1 cm below the water's surface. A sufficient amount of white paint (Proline-Latex Flat; Martin Senour Company, Cleveland, OH) was added to make the water opaque and render the platform virtually invisible. In addition to the visual cues on the walls of the laboratory (shapes), five sheets of paper with black-and-white geometric designs attached to the sides of the tank served as additional cues. An automated tracking system (Columbus Instruments, Columbus, OH) analyzed the swim path of each subject and calculated escape latencies (the time between being placed in the water and finding the hidden platform), total path lengths, average swim speed, and thigmotaxia (percentage of time spent in periphery).

Acquisition and Reversal Procedures. Before beginning acquisition training mice were given a pretraining acclimation session during which they were allowed to swim in the pool for 5 min without the platform present. Beginning on the following day, mice were given seven acquisition sessions that consisted of four trials per day with an intertrial interval of 10 min. Throughout the course of this acquisition period, the hidden platform remained in the same fixed position for all mice. Four points along the perimeter of the maze arbitrarily designated as N, S, E, and W, served as starting points where the mice were released, facing the wall of the tank, at the beginning of each trial (the order of the starting points was determined randomly, except that each starting point was used only once each session). Once a mouse located the platform, it was allowed to remain there for 30 s before being removed from the tank. If a mouse failed to locate the platform within 120 s, it was manually guided to it. After seven sessions of acquisition training, mice were subjected to a reversal test in which the platform was moved to the opposite side of the tank. Other task parameters remained identical to the acquisition procedures (i.e., 10-min intertrial interval, each trial began from a different release point).

Working Memory Procedure. The training for this task was described previously (Varvel et al., 2001). In brief, the platform was located in one of 24 possible positions, with the determination of the exact platform position on any given day being randomly determined (positions along the perimeter of the tank and in the exact middle were excluded). As in the reference memory procedure, if a mouse failed to locate the platform in 120 s, it was manually guided to it. The second trial began after a period of 30 s on the platform, when the mouse was again released into the water from the same position as the first trial (first trial start positions were still randomly determined). To be eligible for testing with drug or vehicle the subjects were required to locate the platform in less than 30 s on two of the three trials subsequent to the first, and were required to meet this criterion on three of their four most recent training sessions. Drug tests were conducted once or twice per week, with at least 72 h and one training session between tests to ensure drug clearance. In addition, drug tests were conducted identically to training sessions except that only two trials were run.

Cued Procedure. Experiments were also conducted using a cued procedure, in which the location of the platform was made known to the mice by placing a black rubber stopper (height, 3 cm; radius, 1.5 cm) on the platform that extended about 2 cm above the surface of the water. The platform, which remained submerged 1 cm below the

surface of the water, was moved to a new location each day in the same manner as in the working memory procedure. Test sessions consisted of four trials, each starting from one of the four release points. Mice were allowed to rest on the platform for 30 s in between trials.

Statistics. For the initial acquisition and reversal experiments, two-factor repeated measures analysis of variance (ANOVA) tests were conducted to assess the effects of genotype and sessions/trials. These were followed by planned comparisons of genotype at each session/trial. For the working memory experiments, one-way repeated measures ANOVAs were performed for trials 1 and 2. In addition, comparisons were made between trials 1 and 2 for each condition using paired t tests. The raw path length scores were converted into a "savings ratio" by dividing the path length of the first trial by the combined path lengths of the first and second trials, providing a normalized measure of the first trial's path length relative to second trial's path length. Thus, a ratio of 0.5 indicates that path lengths of the two trials were identical, whereas ratios greater than 0.5 indicate the degree of improvement between the first and second trial. The ED_{50} value of each agonist in disrupting this savings ratio was calculated by least-squares linear regression. A Student's t test was used to determine whether SR 141716A antagonized the disruptive effects of Δ^9-THC, WIN 55,212-2, and methanandamide using the normalized dependent measure. All differences were considered significant at the $p < 0.05$. ANOVAs and subsequent planned comparisons were conducted using SigmaStat for Windows, version 2.03 (SPSS, Inc., Chicago, IL).

Results

Comparisons Between $CB_1^{-/-}$ and $^{+/+}$ Mice. Mice were carefully observed during the 5-min pretraining session to detect any phenotype differences in their initial reactions to being placed in the water. At the beginning of the session both genotypes immediately approached the sides of the tank, and spent progressively less time there as the session continued. The overall measure of thigmotaxia during the pretraining session did not differ between $CB_1^{+/+}$ (mean = 54%) and $CB_1^{-/-}$ mice (mean = 49%), $t(39.5) = 0.94$, $p = 0.36$. However, about one-half of the $CB_1^{-/-}$ mice stopped swimming and just floated for the last minute or two, and five (20%) of the $CB_1^{-/-}$ mice had to be rescued before the 5-min session ended to prevent them from sinking. Notably, the swimming style of the $CB_1^{-/-}$ mice seemed more labored than that of the $CB_1^{+/+}$ mice, characterized by slightly more rapid, jerky movements. None of the $CB_1^{+/+}$ mice displayed similar problems. In most cases, the swimming performance of the $CB_1^{-/-}$ mice improved quickly over the subsequent training sessions. It is worthy of note that the $CB_1^{-/-}$ mice weighed significantly less than the $CB_1^{+/+}$ mice at the beginning of the study [means, 25.3 versus 30.8 g, $t(27.9) = 6.7$, $p < 0.001$]. At the time of the 5-min pretraining session mice ranged in age from 3 to 5 months, and a similar difference in weight was maintained throughout the course of the study.

The results of the acquisition experiments are shown in Fig. 1. Two-factor ANOVAs revealed significant decreases in escape latencies, $F(6,265) = 32.9$, $p < 0.001$, and path lengths, $F(6,265) = 20.2$, $p < 0.001$, across training sessions, although no differences were detected between genotypes, $F(1,265) = 0.23$, $p = 0.62$, and $F(1,265) = 0.16$, $p = 0.69$, respectively. Swim speeds significantly increased across sessions, $F(6,265) = 9.9$, $p < 0.05$, but also failed to differ significantly between the two genotypes, $F(1,265) = 3.6$, $p = 0.07$. Thigmotaxia significantly decreased across sessions, closely resembling the observed drops in escape latencies and path lengths, $F(6,265) = 37.1$, $p < 0.05$. Interestingly, there was a significant genotype by session interaction, $F(6,265) = 2.4$, $p < 0.05$, where thigmotaxia dropped slightly faster in the wild-type group than in the knockout group (post hoc

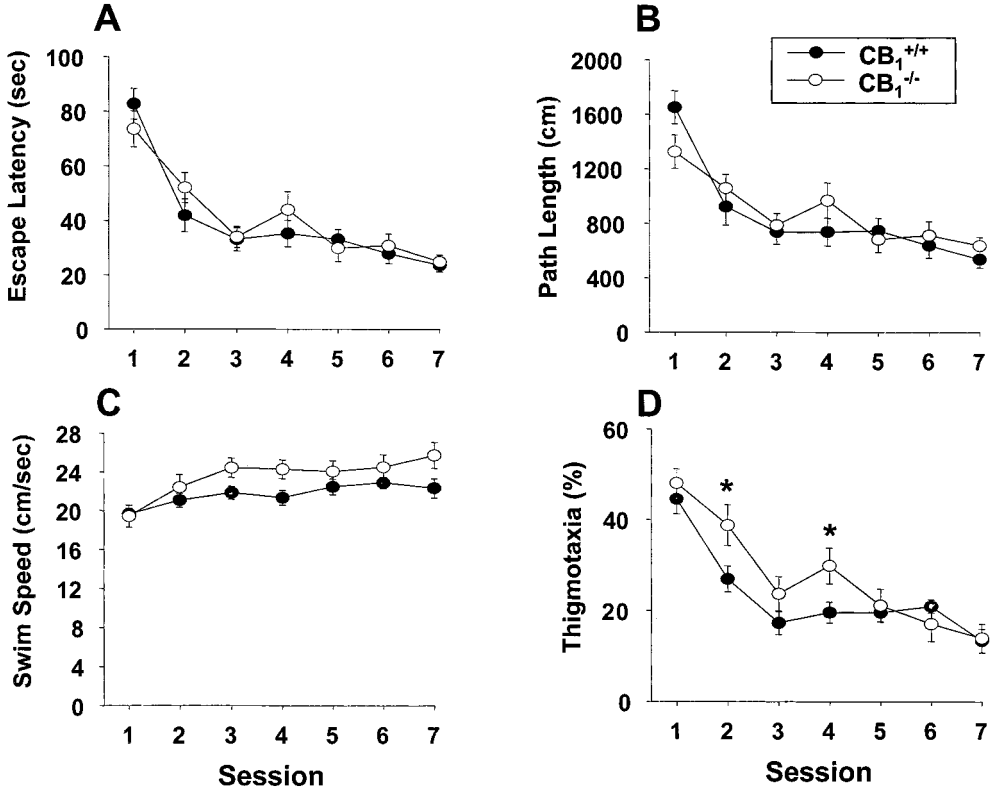

Fig. 1. Escape latency (seconds) (A), path length (centimeters) (B), swim speed (centimeters per second) (C) and thigmotaxia (percentage) (D) for $CB_1^{-/-}$ ($n = 18$) and $CB_1^{+/+}$ ($n = 20$) mice during acquisition of a spatial reference memory task. Values expressed are means (± S.E.) of the four daily trials. Asterisks denote significant effects of genotype for a given session (*, $p < 0.05$).

analysis revealed significant effects of genotype on thigmotaxia during the second and fourth sessions). Further analysis of the results from the first day of the acquisition task by trial also failed to reveal any genotype differences (data not shown).

Figure 2 depicts the results of the reversal test in which the platform was placed on the opposite side of the tank. Escape latencies were significantly affected by both genotype, $F(1,151) = 8.3$, $p < 0.01$, and trial, $F(3, 151) = 4.9$, $p < 0.01$. Planned comparisons between the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice at each trial revealed that escape latencies were significantly elevated in the $CB_1^{-/-}$ mice during trials 3, $t(36.9) = 2.8$, $p = 0.01$, and 4, $t(37.9) = 2.8$, $p < 0.01$. Similarly, significant effects of genotype, $F(1,151) = 7.8$, $p < 0.01$, and of trial, $F(3, 151) = 10.8$, $p < 0.001$, were found for total path length. Subsequent planned comparisons found that $CB_1^{-/-}$ mice also had significantly higher path lengths during trials 3, $t(37.8) = 2.6$, $p < 0.05$, and 4, $t(37.9) = 2.3$, $p < 0.05$. Interestingly, significant effects of genotype, $F(1,151) = 5.2$, $p < 0.05$, and trial, $F(3,151) = 2.8$, $p < 0.05$ were also found on the number of entries to the previous platform location, "returns". Additional planned comparisons revealed that $CB_1^{-/-}$ mice returned to the previous platform position significantly more times than did $CB_1^{+/+}$ mice during trials 3, $t(35.2) = 2.5$, $p < 0.05$, and 4, $t(23.1) = 2.4$, $p < 0.05$.

Despite the observed difficulty of the $CB_1^{-/-}$ mice during the reversal test, most eventually learned to perform the working memory task. The average number of sessions to criteria did not significantly differ between the $CB_1^{-/-}$ mice (mean = 9.0, S.E. = 1.4) and the $CB_1^{+/+}$ mice (mean = 6.3, S.E. = 0.9), $t(28.1) = 1.7$, $p = 0.11$. However, during subsequent training sessions, $CB_1^{-/-}$ mice performed less consistently than did the $CB_1^{+/+}$ mice, and consequently approximately 50% of the $CB_1^{-/-}$ mice were removed from the study due to the development of swim strategies that were incompatible with the task (i.e., repetitive circling behaviors). Furthermore, five of the $CB_1^{-/-}$ mice exhibited seizures while in the pool and died over the course of the study. These problems restricted the number of subsequent experiments that could be conducted with the $CB_1^{-/-}$ mice. Notably, none of the $CB_1^{+/+}$ mice demonstrated similar problems.

Effects of CB_1 Agonists in $CB_1^{+/+}$ Mice. Half of the $CB_1^{+/+}$ mice ($n = 9$) from the acquisition/reversal study were used to evaluate the effects of three agonists in the working memory procedure. The effects of Δ^9-THC are shown in Fig. 3. During trial 1, Δ^9-THC failed to affect both escape latencies, $F(5,53) = 0.38$, $p = 0.8$, and path lengths, $F(5,53) = 0.6$, $p = 0.68$. However, second trial latencies were significantly increased by drug, $F(5,53) = 4.6$, $p < 0.01$, as were path lengths, $F(5,53) = 3.4$, $p < 0.01$. For both measures, the vehicle, 1 mg/kg Δ^9-THC, and SR 141716A plus 10 mg/kg Δ^9-THC conditions exhibited significant enhancement of performance from trials 1 to 2. In contrast, the 3, 10, and 30 mg/kg Δ^9-THC conditions failed to improve performance during trial 2. Analysis of the savings ratio data revealed that Δ^9-THC significantly disrupted working memory performance, $F(5,53) = 3.4$, $p < 0.01$, with an ED_{50} (95% CI) value of 1.3 (0.40–4.1) mg/kg. Treatment with 3, 10, or 30 mg/kg Δ^9-THC significantly disrupted performance compared with the vehicle condition. No significant difference was found between the SR 141716A plus 10 mg/kg Δ^9-THC and 10 mg/kg Δ^9-THC alone conditions ($p = 0.10$) in the savings ratio data. Although Δ^9-THC produced a significant degree of thigmotaxia, $F(5,53) = 4.3$, $p < 0.01$, only the 30-mg/kg dose elicited a significant increase in this measure. No effects on average swim speed were observed at any dose, $F(5,53) = 0.9$, $p = 0.47$.

Figure 4 shows the effects of WIN 55,212-2 in the working memory task. Both escape latencies and path lengths of the first trial were not affected by any dose. However, the second trial escape latencies, $F(4,39) = 9.6$, $p < 0.001$, and path lengths, $F(4,39) = 8.5$, $p < 0.01$, were significantly increased by WIN 55,212-2. For both measures, the vehicle and 0.1 mg/kg WIN 55,212-2 exhibited significant enhancement of

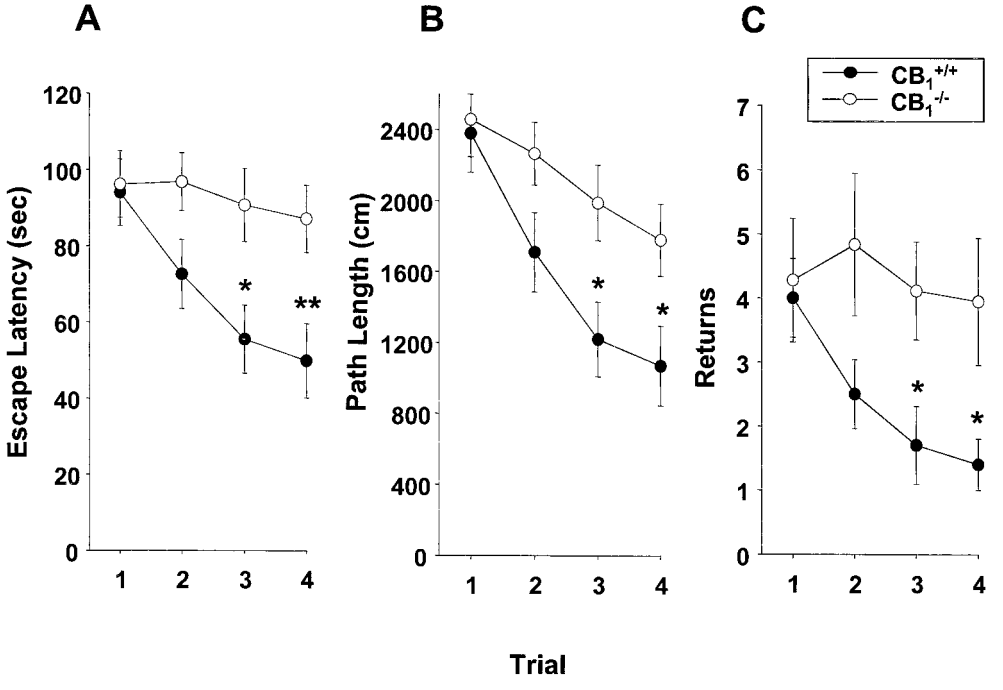

Fig. 2. Escape latency (seconds) (A), path length (centimeters) (B), and number of returns to where the hidden platform had been previously located (C) for $CB_1^{-/-}$ ($n = 18$) and $CB_1^{+/+}$ ($n = 20$) mice performing a reversal learning task, in which the hidden platform was moved to the opposite side of the tank. Values represent mean (± S.E.) of each trial. Asterisks denote significant effects of genotype at a given trial (*, $p < 0.05$; **, $p < 0.01$).

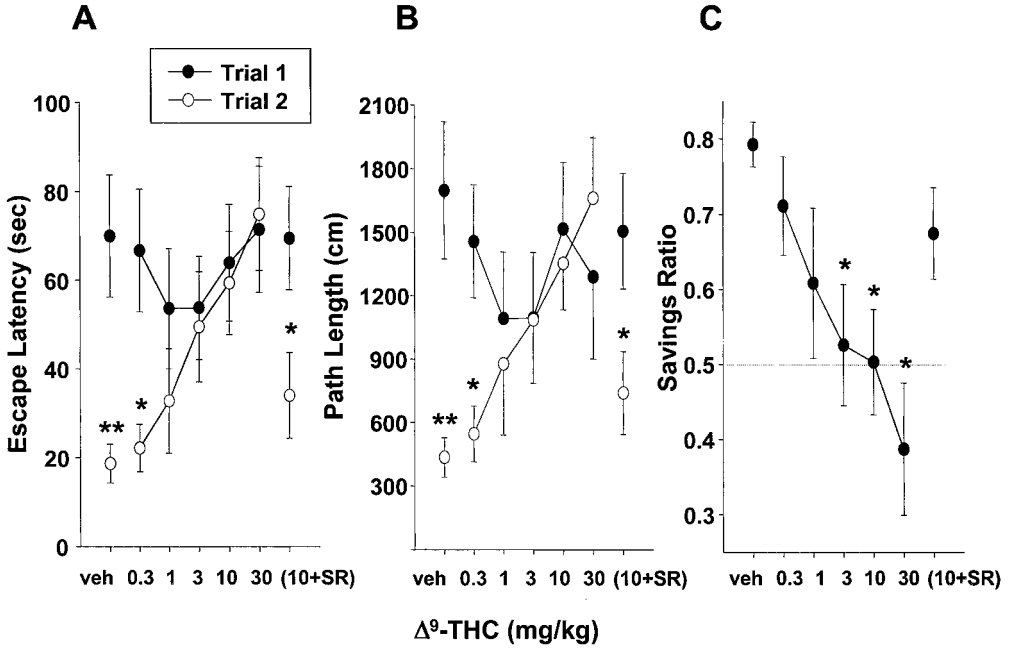

Fig. 3. Effects of Δ^9-THC on escape latency (seconds) (A), path length (centimeters) (B), and savings ratio (C) of $CB_1^{+/+}$ mice ($n = 9$) in a spatial working memory task. Coadministration of 3 mg/kg SR 141716A reversed the effects of 10 mg/kg THC alone. Escape latency and path length values represent means (± S.E.) from each trial; asterisks signify differences between trials 1 and 2 for each condition (*, $p < 0.05$; **, $p < 0.05$). Savings ratios were calculated based on path length data to standardize the improvements seen between the first and second trials (see *Materials and Methods* for details); asterisks significant disruption compared with vehicle (veh) (*, $p < 0.05$).

performance from trial 1 to trial 2. However, the 0.3, 1.0, and 3.0 mg/kg WIN 55,212-2 conditions failed to improve performance during trial 2. The SR 141716A plus WIN 55,212-2 condition failed to exhibit improved escape latencies ($p = 0.10$), but did exhibit improved path lengths ($p < 0.05$). Analysis of the savings ratio data revealed that WIN 55,212-2 significantly disrupted working memory performance, $F(4,39) = 5.8$, $p < 0.01$, with an ED_{50} (95% CI) value of 0.35 (0.20–0.62) mg/kg. Treatment with 1 or 3 mg/kg WIN 55,212-2 significantly disrupted performance compared with the vehicle condition. In addition, the SR 141716A plus 1 mg/kg WIN 55,212-2 condition led to significantly better performance than the 1 mg/kg WIN 55,212-2 alone condition ($p = 0.05$). As shown in Table 1, WIN 55,212-2 produced a significant effect on thigmotaxia, $F(4,39) = 4.5$, $p < 0.01$, with the 3-mg/kg dose differing from vehicle. However, swim speeds were not significantly affected by any dose of WIN 55,212-2, $F(4,39) = 1.4$, $p = 0.24$.

The effects of methanandamide in the working memory task are presented in Fig. 5. Once again, first trial escape latencies and path lengths were not affected by drug, but significant effects were found for second trial escape latencies, $F(3,31) = 3.1$, $p < 0.05$, and path lengths, $F(3,31) = 3.6$, $p < 0.05$. For both of these measures, the vehicle, 1 mg/kg methanandamide, and SR 141716A plus 10 mg/kg methanandamide conditions exhibited improved performance from trials 1 to 2. Savings ratios were significantly disrupted, $F(3,31) = 5.3$, $p < 0.01$, with only the 10 mg/kg anandamide

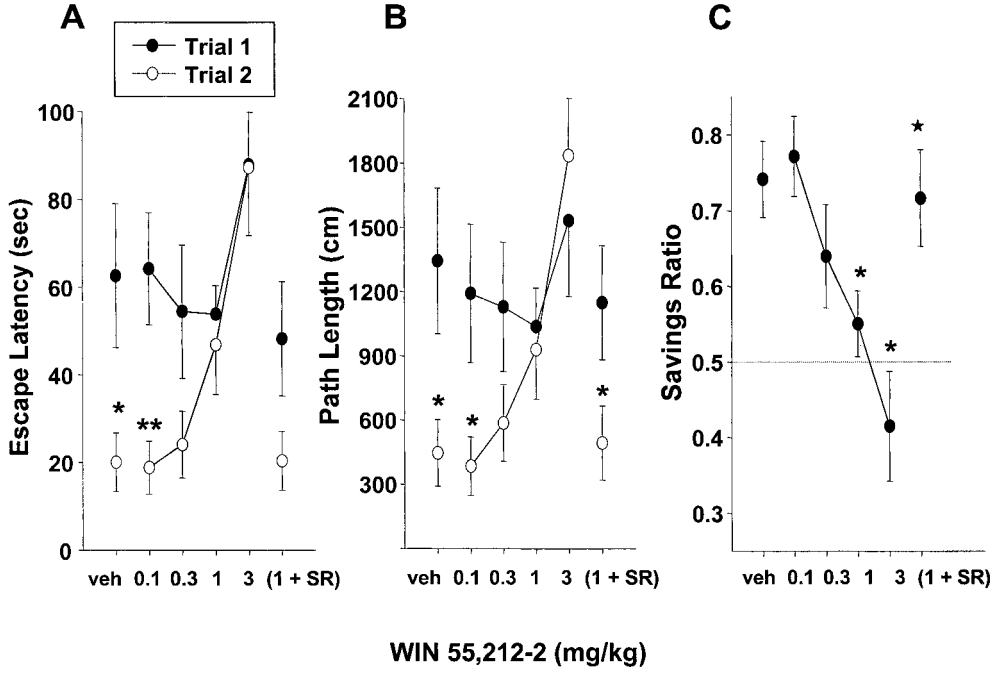

Fig. 4. Effects of WIN 55,212-2 on escape latency (seconds) (A), path length (centimeters) (B), and savings ratio (C) of $CB_1^{+/+}$ mice ($n = 8$) in a spatial working memory task. Coadministration of 3 mg/kg SR 141716A reversed the effects of 3 mg/kg WIN 55,212-2. Escape latency and path length values represent means (± S.E.) from each trial; asterisks signify differences between trials 1 and 2 for each condition (*, $p < 0.05$; **, $p < 0.05$). Savings ratios were calculated based on path length data to standardize the improvements seen between the first and second trials (see *Materials and Methods* for details); asterisks signify significant disruption compared with vehicle (veh) ($p < 0.05$); ⋆, significantly different from the 1 mg/kg WIN 55,212-2 alone condition ($p = 0.05$).

TABLE 1
Effects of Δ^9-THC (n = 9), WIN 55,212-2 (n = 8), and methanandamide (n = 8) on thigmotaxia (percentage of time spent near the perimeter) and average swim speeds in $CB_1^{+/+}$ mice in a working memory water maze task
Data are presented as means (S.E.).

	Thigmotaxia	Swim Speeds
	%	*cm/sec*
Δ^9-THC		
Vehicle	14.0 (2.8)	25.3 (1.3)
0.3	6.7 (1.9)	21.6 (2.9)
1	8.1 (2.0)	23.0 (1.4)
3	17.2 (3.6)	23.5 (1.2)
10	11.5 (3.1)	23.9 (0.9)
30	25.3 (5.9)[a]	20.7 (2.4)
10 + 3 mg/kg SR 141716A	22.5 (7.5)	20.6 (1.1)
WIN 55,212-2		
Vehicle	10.5 (3.0)	21.0 (3.1)
0.1	11.2 (2.6)	22.9 (0.7)
0.3	17.8 (3.2)	22.7 (1.4)
1	17.2 (5.8)	19.5 (1.7)
3	29.7 (3.6)[a]	18.6 (1.0)
1 + 3 mg/kg SR 141716A	10.4 (3.5)	24.8 (0.9)
Methanandamide		
Vehicle	11.5 (4.2)	19.8 (1.3)
1	15.1 (3.7)	21.9 (1.0)
3	15.8 (5.1)	22.6 (0.9)
10	14.4 (5.8)	22.9 (0.9)
10 + 3 mg/kg SR 141716A	24.4 (5.8)	21.1 (0.7)

[a] Asterisks represent significant differences compared to vehicle, $p < 0.05$).

condition differing from vehicle. The ED_{50} (95% CI) was 3.2 (2.0–5.0) mg/kg. SR 141716A significantly blocked the memory disruptive effects of 10 mg/kg methanandamide ($p < 0.01$). No effects of methanandamide were observed on either thigmotaxia, $F(3,31) = 0.2$, $p = 0.88$, or average swim speeds, $F(3,31) = 1.5$, $p = 0.25$.

In contrast, doses of 10 mg/kg Δ^9-THC, 1 mg/kg WIN 55,212-2, and 10 mg/kg methanandamide that significantly disrupted performance in the working memory task had no effects on either escape latency or path length in a cued version of the task in which the location of the platform was visible (data not shown). Escape latencies were less than 20 s and path lengths were less than 350 cm for each drug, both of which did not differ from the vehicle condition.

Effects of CB_1 Agonists in $CB_1^{-/-}$ Mice. Doses of Δ^9-THC, WIN 55,212-2, and methanandamide found to be maximally effective in the $CB_1^{+/+}$ mice described above were subsequently evaluated in $CB_1^{-/-}$ and in a new group of $CB_1^{+/+}$ mice. As can be seen in Fig. 6, $CB_1^{+/+}$ mice displayed significantly lower escape latencies and path lengths in the second trial compared with the first after vehicle administration, and this improvement in both measures was completely prevented by administration of 10 mg/kg Δ^9-THC. In contrast, although $CB_1^{-/-}$ mice displayed similar improvements after vehicle administration, no disruptive effects of 10 mg/kg Δ^9-THC were observed. The same pattern of effects is shown in Figs. 7 and 8 for WIN 55,212-2 and methanandamide, respectively. Both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice exhibited adequate control performance, in that escape latencies and path lengths were significantly decreased in the second trials. Although 1.0 mg/kg WIN 55,212-2 or 10 mg/kg methanandamide prevented this improvement between trials observed under control conditions in the $CB_1^{+/+}$ mice, neither drug impaired the performance of $CB_1^{-/-}$ mice. As shown in Table 2, none of these drugs had any effects on measures of average swim speed or thigmotaxia at the doses tested.

Discussion

Results from the present study suggest three main conclusions. First, these experiments revealed phenotype differences between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, in that the $CB_1^{-/-}$ mice exhibited an increased perseverance in the reversal test. Specifically, they continued to return to the location where the platform had been previously located, which interfered with their finding the new platform position. Second, the lack of cannabinoid-induced memory impairment in either $CB_1^{-/-}$ mice or SR 141716A-treated wild-type mice provides definitive evidence that the disruptive effects of Δ^9-THC, WIN 55,212-2, and methanandamide on working

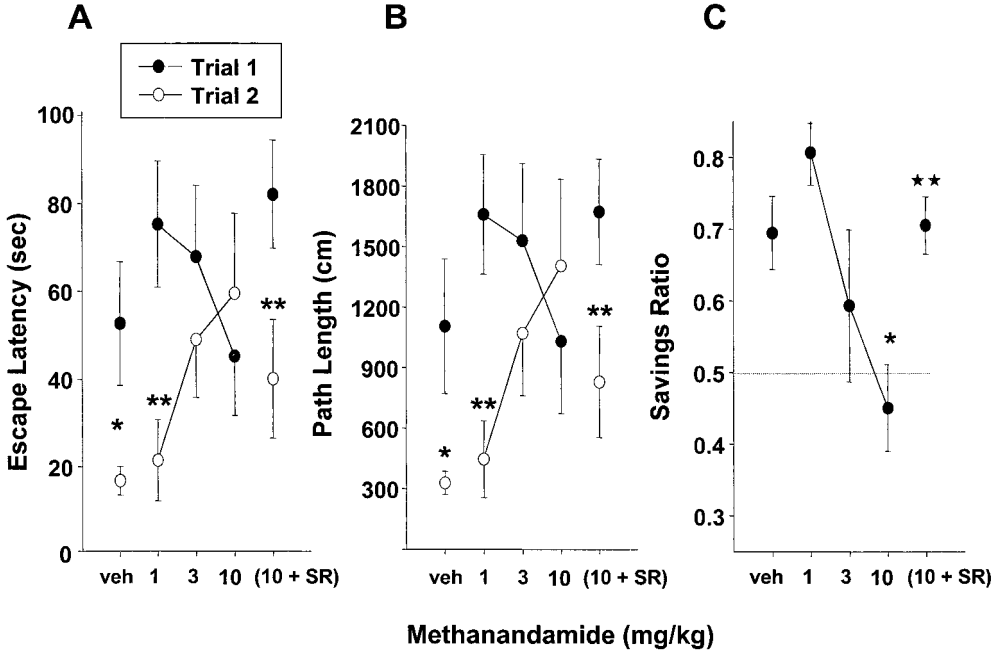

Fig. 5. Effects of methanandamide on escape latency (seconds) (A), path length (centimeters) (B), and savings ratio (C) of $CB_1^{+/+}$ mice (n = 8) in a spatial working memory task. Coadministration of 3 mg/kg SR 141716A reversed the effects of 10 mg/kg methanandamide. Escape latency and path length values represent means (± S.E.) from each trial; asterisks signify effects of genotype at a given trial (*, $p < 0.05$; **, $p < 0.05$). Savings ratios were calculated based on path length data to standardize the improvements seen between the first and second trials (see *Materials and Methods* for details); asterisks denote values significantly below vehicle (veh) (*, $p < 0.05$); ★★, significantly different from the 10 mg/kg methanandamide alone condition ($p < 0.01$).

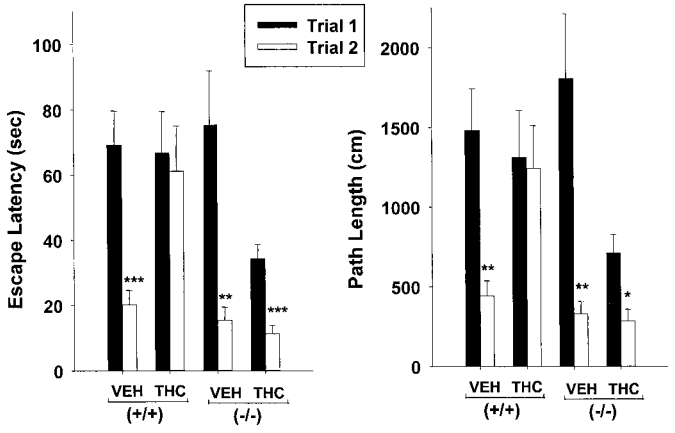

Fig. 6. Effects of administration of vehicle or 10 mg/kg Δ^9-THC on escape latencies (seconds) and path lengths (centimeters) of $CB_1^{+/+}$ ($n = 10$) and $CB_1^{-/-}$ ($n = 8$) mice trained in the working memory task. Both groups of mice demonstrated reliable performance after vehicle (VEH) administration. Δ^9-THC disrupted this performance in $CB_1^{+/+}$, but not $CB_1^{-/-}$ mice. Asterisks represent significant differences between first and second trials (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

memory are mediated via activation of CB_1 receptors. Third, the fact that none of these agonists impaired performance in a cued version of the task argues against motivational or sensorimotor confounds.

An unexpected finding in the present study was the observation that the $CB_1^{-/-}$ mice exhibited a deficit in learning the new platform location during the reversal test. These mice continued to return to the previously learned location, despite being repeatedly shown the new location. The facts that initial learning in the $CB_1^{-/-}$ mice was identical to that of the $CB_1^{+/+}$ mice and that the deficits were only observed when the mice were required to shift away from the behavioral strategy that they had previously learned (always returning to the same spot) to a new one (returning to a new location) argue against an interpretation of a deficit in general mechanisms of acquiring, encoding, or storage of the relevant behaviors, because a deficit in any one of these processes would be expected to result in a disruption of the initial place learning.

One plausible explanation for the impaired performance of the $CB_1^{-/-}$ mice in the reversal task is that the endocannabinoid system may play a role in facilitating a process directed toward memory decay (i.e., forgetting) or extinction of learned behaviors. Extinction is believed to involve active suppression of previously learned associations and seems to involve molecular mechanisms distinct from those associated with normal learning (Lattal and Abel, 2001; Rescorla, 2001). If the endocannabinoid system were involved forgetting and/or extinction processes then disrupting it via pharmacological or genetic deletion of CB_1 receptors may seem in some models as improved memory (Terranova et al., 1996; Reibaud et al., 1999; Lichtman, 2000), because disruption of endocannabinoid signaling prolonged retention compared with control animals. Conversely, in tasks that require the suppression of previously learned responses, endocannabinoid inhibition may actually interfere with learning, as in the reversal test of the present study.

CB_1 receptor antagonism has failed to affect performance in a variety of operant paradigms, particularly those that require rapid relearning of new information such as delayed nonmatch-to-sample (Mallet and Beninger, 1998; Hampson and Deadwyler, 2000), repeated acquisition (Brodkin and Moerschbaecher, 1997), and fixed consecutive number counting (Mansbach et al., 1996) tasks. A critical difference between these studies and those in which disruption of CB_1 receptor signaling altered performance is the temporal components of the task. Although the operant tasks require information to be retained on the order of seconds, the social recognition, object recognition, radial arm maze, and Morris water maze reversal tasks require information to be retained for substantially longer durations (e.g., minutes, hours, or days). Thus, the endocannabinoid system may function in processes related to extinction and/or forgetting of information that is retained for prolonged durations.

Our results also suggest that the endocannabinoid system

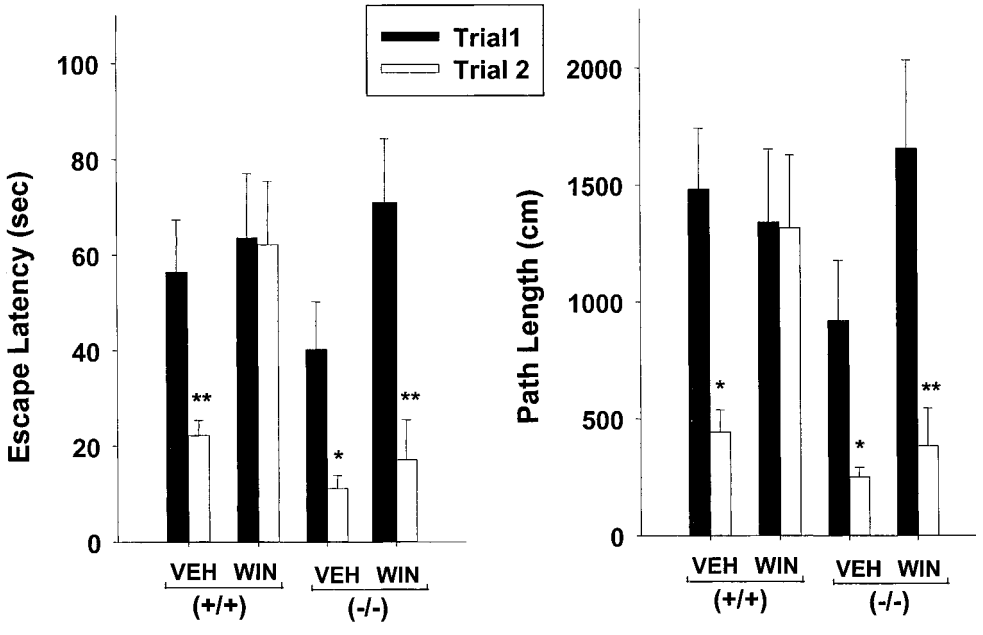

Fig. 7. Effects of administration of vehicle or 1 mg/kg WIN 55,212-2 on escape latencies (seconds) and path lengths (centimeters) of $CB_1^{+/+}$ ($n = 10$) and $CB_1^{-/-}$ ($n = 7$) mice trained in the working memory task. Both groups of mice demonstrated reliable performance after vehicle (VEH) administration. WIN 55,212-2 (WIN) disrupted this performance in $CB_1^{+/+}$, but not $CB_1^{-/-}$ mice. Asterisks represent significant differences between first and second trials (*, $p < 0.05$; **, $p < 0.01$).

TABLE 2

Effects of 10 mg/kg Δ^9-THC, 1 mg/kg WIN 55,212-2, and 10 mg/kg methanandamide on thigmotaxia (percentage of time spent near the perimeter) and average swim speeds in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice in a working memory water maze task

Data are presented as means (S.E.).

	Thigmotaxia	Swim Speeds
	%	*cm/s*
$CB_1^{+/+}$		
Vehicle	26.9 (5.3)	21.1 (0.7)
10 mg/kg THC	35.1 (5.8)	21.1 (1.3)
Vehicle	36.6 (7.1)	21.1 (1.2)
1 mg/kg WIN 55,212-2	34.8 (5.1)	19.7 (0.8)
Vehicle	31.9 (4.7)	21.9 (1.2)
10 mg/kg Meth	26.4 (4.4)	20.6 (0.9)
$CB_1^{-/-}$		
Vehicle	31.6 (5.0)	20.6 (1.4)
10 mg/kg THC	22.5 (5.3)	22.0 (1.4)
Vehicle	13.5 (3.9)	23.3 (1.1)
1 mg/kg WIN 55,212-2	14.9 (5.4)	22.8 (1.1)
Vehicle	16.4 (3.4)	23.0 (1.6)
10 mg/kg Meth	17.2 (4.5)	24.1 (2.1)

Meth, methanandamide.

does not play a critical role in the initial acquisition rate of the spatial memory task. However, it should be noted that the failure to demonstrate phenotype differences between the $CB_1^{-/-}$ and $CB_1^{+/+}$ mice herein might simply be the result of methodological issues. The acquisition task used in the present experiments involves many different processes, including habituating to the stress resulting from the forced swim, learning to swim efficiently, learning that the only way to escape the pool is to find the platform, and learning the location of the platform itself. Thus, an enhancement in one of these processes could have been offset by a deficit in another one.

Although the results of the present study implicate the involvement of endocannabinoids in forgetting and/or extinction, alternative interpretations related to the use of knockout models must be considered. The elimination of the $CB_1^{-/-}$ receptor may impact in unanticipated ways the development of these animals, leading to behavioral changes that are not the direct result of acute disruption of cannabinoid transmission. For example, in the present study the $CB_1^{-/-}$ mice had reduced body weights, swam poorly during their first exposure to the pool, performed inconsistently after being trained in the working memory task, and some exhibited seizures that resulted in death. However, it is likely that these phenotype differences result directly from the absence of the $CB_1^{-/-}$ receptor. In particular, the weight differences between the genotypes are consistent with a recent report in which $CB_1^{-/-}$ mice ate less than wild-type controls and SR 141716A reduced food intake in wild types (Di Marzo et al., 2001). Also, $CB_1^{-/-}$ mice are known to have an increased mortality rate as well as a decreased locomotor activity compared with their wild-type littermates (Zimmer et al., 1999). To address potential confounds related to transgenic models, it will be important in future studies to assess the effects of SR 141716A in analogous Morris water maze tasks. Given that the performances of the $CB_1^{-/-}$ and $CB_1^{+/+}$ mice were essentially identical during acquisition in the fixed platform task, it is unlikely that the increased perseverance exhibited by the $CB_1^{-/-}$ mice during the reversal task was selectively caused by these other phenotype differences.

Our results also show that three structurally dissimilar cannabinoids impaired performance in a spatial working memory task. Given the nature of the task itself, it is possible that such performance disruptions may not reflect memory impairment directly, but rather some combination of sensorimotor deficits, motivational deficits, or increased levels of anxiety. However, the lack of cannabinoid-induced effects on swim speed or in the cued procedure argues against sensorimotor or motivational deficits. Additionally, the fact that the performance deficits occurred at doses lower than those necessary to elicit thigmotaxia tends to argue against the hypothesis that the performance deficits were due to anxiety, because thigmotaxia is considered to reflect anxiety (Simon et al., 1994).

These experiments also present three compelling lines of

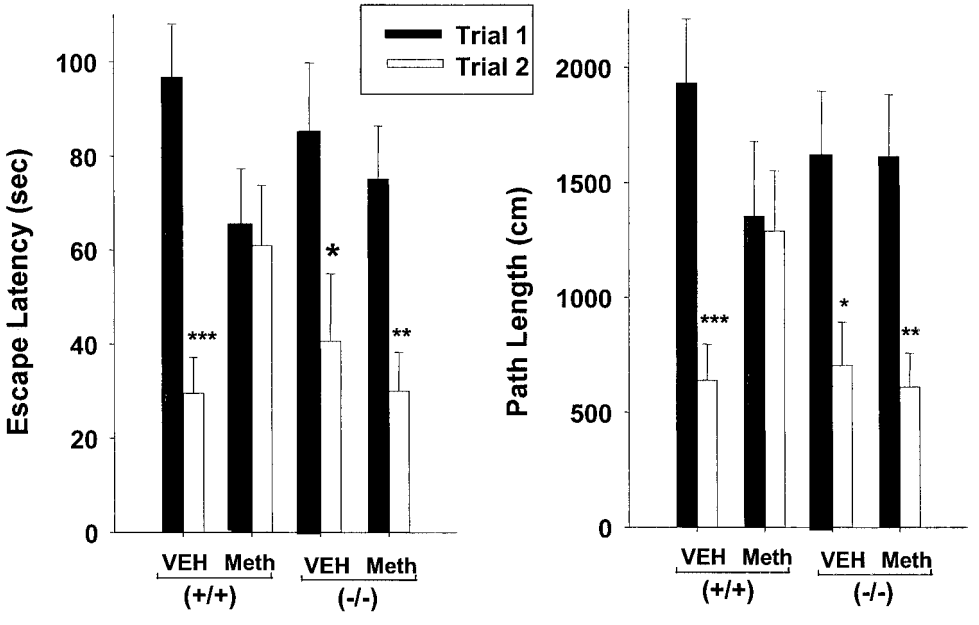

Fig. 8. Effects of administration of vehicle or 10 mg/kg methanandamide on escape latencies (s) and path lengths (cm) of $CB_1^{+/+}$ ($n = 9$) and $CB_1^{-/-}$ ($n = 7$) mice trained in the working memory task. Both groups of mice demonstrated reliable performance after vehicle (VEH) administration. Methanandamide (Meth) disrupted this performance in $CB_1^{+/+}$, but not $CB_1^{-/-}$ mice. Asterisks represent significant differences between first and second trials (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

evidence demonstrating that the mnemonic deficits produced by Δ^9-THC, WIN 55,212-2, and methanandamide are mediated by a CB_1 receptor mechanism of action. First, the rank order of their potencies for disrupting working memory performance is consistent with their binding affinities at CB_1 receptors (Breivogel and Childers, 2000). Second, SR 141716A significantly blocked the effects of maximally effective doses of methanandamide and WIN 55,212-2. Although SR 141716A failed to block the effects of a maximally effective dose of Δ^9-THC, it should be noted that subjects failed to improve performance from trials 1 to 2 when given Δ^9-THC alone, but did show significant improvement between trials when pretreated with SR 141716A before Δ^9-THC. Finally, these same maximally effective doses of each agonist were completely inactive in $CB_1^{-/-}$ mice.

One issue that merits some discussion is the degree to which exogenous cannabinoids such as those used in the present study reflect the function of endocannabinoids. Although it seems clear that the memory-disruptive effects of these exogenous agents are CB_1 receptor-mediated, it should be cautioned that this does not imply that they directly mimic the actions of endocannabinoids. In contrast to the long half-life of exogenous cannabinoids (Lemberger et al., 1972), anandamide is rapidly metabolized within minutes (Willoughby et al., 1997), and the functional consequences of this distinction have yet to be determined. The use of mice lacking fatty acid amide hydrolase (Cravatt et al., 2001), the enzyme primarily responsible for the degradation of anandamide, may provide some answers to this question. Notably, these mice possess dramatically elevated anandamide brain levels and exhibit robust CB_1 receptor-mediated responses after exogenous anandamide administration. Nonetheless, the present data suggest that although exogenously applied cannabinoids impair working memory, endocannabinoids play a role in the extinction or forgetting of memories that are no longer relevant.

The abundant and widespread distribution of the CB_1 receptor and endocannabinoids in the central nervous system (Herkenham et al., 1991; Di Marzo et al., 2000) suggests that many aspects of complex processes such as learning and memory could be influenced by an endocannabinoid neuromodulatory system. The results of the present study provide support for a specific role of this system in facilitating the extinction and/or forgetting of previously learned behaviors. This interpretation is consistent with previous suggestions that the endocannabinoid system may play a role in "active forgetting" processes (Terranova et al., 1996). One prediction based on such a role is that depending on the paradigm used, inhibiting cannabinoid receptors could involve the apparent enhancement or the disruption of learning. Additionally, many of the deficits observed after CB_1 receptor activation by exogenously applied cannabinoids may be the result of overstimulation of this natural process.

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