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ORIGINAL INVESTIGATION

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Δ^9-THC-induced cognitive deficits in mice are reversed by the $GABA_A$ antagonist bicuculline

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Abstract *Rationale:* The results of recent in vitro studies have underscored the important role that activation of CB_1 receptors has on GABAergic activity in brain areas associated with memory. *Objectives:* The primary purpose of this study was to test the hypothesis that the memory disruptive effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) in vivo are mediated through GABAergic systems. Conversely, we also evaluated whether blocking CB_1 receptor signaling would alter memory deficits elicited by GABA agonists. *Methods:* The $GABA_A$ antagonist bicuculline and $GABA_B$ antagonist CGP 36742 were evaluated for their ability to ameliorate Δ^9-THC-induced deficits in a mouse working memory Morris water maze task. Mice were also assessed in a T-maze task, as well as non-cognitive behavioral assays. Additionally, the effects of $GABA_A$ and $GABA_B$ agonists were assessed in either CB_1 (−/−) mice or wild type mice treated with the CB_1 antagonist SR 141716. *Results:* Memory deficits resulting from 10 mg/kg Δ^9-THC in the Morris water maze were completely reversed by bicuculline, though unaffected by CGP 36742. Bicuculline also blocked the disruptive effects of Δ^9-THC in the T-maze, but failed to alter non-mnemonic effects of Δ^9-THC. Although CB_1 (−/−) mice exhibited supersensitivity to muscimol-induced water maze deficits compared with wild type control mice, muscimol elicited virtually identical effects in SR 141716-treated and vehicle-treated wild type mice. *Conclusions:* This is the first demonstration of which we are aware showing that $GABA_A$ receptors may play a necessary role in Δ^9-THC-induced memory impairment in whole animals.

Keywords Cannabinoid · GABA · GABAergic · Working memory · Bicuculline · Morris water maze · THC · SR 141716 · CB1 receptor

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Introduction

The disruptive effects of marijuana consumption as well as administration of its primary psychoactive ingredient Δ^9-tetrahydrocannabinol (Δ^9-THC) on learning and memory are well documented. In recent years, great strides have been made towards understanding the biochemical basis for these effects. For example, several different lines of investigation have demonstrated that the mnemonic deficits caused by Δ^9-THC and other cannabinoid agonists are mediated via their activity at CB_1 receptors. The receptor antagonist for this receptor SR 141716 blocks cannabinoid-induced memory impairment (Lichtman and Martin 1996; Mallet and Beninger 1998; Varvel et al. 2001). Furthermore, cannabinoid agonists fail to impair working memory in CB_1 (−/−) mice, as evaluated in the Morris water maze (Varvel and Lichtman 2002).

Likely target sites for cannabinoid-induced memory impairment include the hippocampus and prefrontal cortex. Notably, CB_1 receptors as well as the endogenous cannabinoids anandamide and 2-AG are present at high concentrations in these and other forebrain areas associated with memory. Localization studies revealed that in the hippocampus CB_1 receptors are located almost exclusively presynaptically, the vast majority being located on the axon terminals of a subset of GABAergic interneurons (Katona et al. 1999; Marsicano and Lutz 1999; Tsou et al. 1999). Indeed, there appears to be a functional link between endocannabinoid and GABAergic systems. Activation of CB_1 receptors in vitro has been shown to modulate GABA release in primary pyramidal neurons of the hippocampus (Hajos et al. 2000; Hoffman and Lupica 2000; Irving et al. 2000; Katona et al. 1999). Similar processes have recently been demonstrated in vivo, as both Δ^9-THC and WIN 55,212-2 have been shown to decrease levels of GABA in the cortex of awake rats (Ferraro et al. 2001; Pistis et al. 2002). These studies taken together suggest that cannabinoids may produce some of their physiological effects, such as those on learning and memory, through specific GABAergic pathways.

The majority of published reports examining functional links between the cannabinoid and GABAergic systems on behavior comes from the laboratory of Pertwee and colleagues. They found that facilitators of GABA transmission potentiate the cataleptic effects of Δ^9-THC, and GABA antagonists block this interaction (Pertwee et al. 1988, 1991). In addition, the cataleptic effects of muscimol injected into the globus pallidus can be potentiated by Δ^9-THC as well as by anandamide (Pertwee et al. 1991; Wickens and Pertwee 1993). Similarly, unilateral coadministration of muscimol and Δ^9-THC potentiated circling behavior. These findings may be related to the observation that Δ^9-THC can inhibit the reuptake of GABA in globus pallidus (Maneuf et al. 1996) and striatonigral neurons (Romero et al. 1998), effectively increasing GABAergic transmission. There is also evidence that a similar process may occur in hippocampal neurons, as Δ^9-THC has been shown to potentiate the depolarizing effects of GABA in grease-gap preparations of hippocampal slices (Coull et al. 1997), and the hyperpolarizing effects of WIN 55,212 in hippocampal slices were blocked by bicuculline (Kirby et al. 2000). In contrast, there are no published reports of which we are aware that have examined potential functional links between cannabinoids and the GABAergic system on memory in the whole animal.

The primary purpose of the present study was to test the hypothesis that Δ^9-THC-induced memory impairment is mediated through GABAergic pathways. To this end, we evaluated whether the $GABA_A$ receptor antagonist bicuculline as wells as the $GABA_B$ receptor antagonist CGP 36742 would ameliorate the disruptive effects of Δ^9-THC in a working memory Morris water maze task. As bicuculline was found to block Δ^9-THC-induced performance deficits in the Morris water maze, we evaluated whether it would also block Δ^9-THC's disruptive effects in a second memory task, the alternating T-maze assay. Additionally, we evaluated the specificity of this interaction, by assessing whether bicuculline would decrease non-mnemonic pharmacological effects of Δ^9-THC. Mice were given the appropriate drugs and assessed in the cannabinoid "tetrad" assay for the assessment of hypomotility, analgesia, catalepsy, and hypothermia. Finally, we evaluated whether CB_1 receptors play an important role in mediating the disruptive effects of GABA agonists in the working memory Morris water maze task. Specifically, the memory disruptive effects of the $GABA_A$ receptor agonist muscimol as well as the $GABA_B$ receptor agonist baclofen were assessed in either CB_1 (−/−) mice or SR 141716-treated wild type mice.

Materials and methods

Subjects

Male C57BL/6 mice (Jackson Laboratory) and male CB_1 (−/−) and CB_1 (+/+) littermates (Varvel and Lichtman 2002) were housed in a temperature-controlled (20–22°C) environment, with a 12-h light/dark cycle. A total of 68 mice were employed in this study. Food and water were available ad libitum in their home cages. The mice involved in the T-maze experiments were restricted to 85% of their free-feeding weights (23–28 g). The Institutional Animal Care and Use Committee at Virginia Commonwealth University approved all experiments.

Drugs

Δ^9-THC and SR 141716 were provided by the National Institute on Drug Abuse (Bethesda, Md., USA), and were dissolved in a 1:1 mixture of absolute ethanol and alkamuls-620 (Rhone-Poulenc, Princeton, N.J., USA) and diluted with saline to a final ratio of 1:1:18 (ethanol/alkamuls/saline). A single dose of 10 mg/kg Δ^9-THC was used in the Morris water maze experiments because we have previously found that this dose reliably produces a maximally disruptive effect in the working memory model without affecting such non-mnemonic measures as swim speed or thigmotaxia (Varvel et al. 2001; Varvel and Lichtman 2002). Moreover, this dose does not disrupt performance in a cued version of the task (in which the location of the platform is explicitly marked) or in a reference memory task with similar motor and motivational requirements. A dose of 3.0 mg/kg SR141716A was used, as we have previously found that this dose effectively blocked the pharmacological effects of cannabinoid receptor agonists in the Morris water maze (Varvel et al. 2001).

CGP 36742 was provided by Novartis (Basel, Switzerland), while muscimol, (+)-bicuculline, and (RS)-baclofen were purchased from Tocris (Ellisville, Mo., USA). Each of the GABA compounds was diluted with saline. In order to verify the GABA receptor subtype selectivity and identify appropriate doses of bicuculline and CGP 36742 in the Morris water maze, a preliminary experiment was conducted in which each antagonist was given in combination with muscimol (0.5 mg/kg) and baclofen (4 mg/kg), selective agonists of $GABA_A$ and $GABA_B$ receptors, respectively. The results of this pilot study (data not shown) indicated that, as expected, 1 mg/kg bicuculline and 30 mg/kg CGP 36742 are sufficient to antagonize the disruptive effects of $GABA_A$ and $GABA_B$ agonists, respectively. Consequently, these doses were employed in the Δ^9-THC experiments.

All injections were administered subcutaneously in an injection volume of 1 ml/0.1 kg and given 30 min prior to testing, unless otherwise indicated. Double injections were performed in rapid succession in a counterbalanced manner across test days.

Apparatus

The water maze consisted of a large circular galvanized steel pool (1.8 m diameter, 0.6 m height) filled with water (22°C) 1 cm above a white platform (10 cm diameter). A

sufficient amount of white paint (Proline-Latex Flat) 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 laminated 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, Ohio, USA) was used to analyze the swim path of each subject and calculated several corresponding dependent measures.

The T-maze was constructed of black Plexiglas with a runway arm of 50 cm in length, each choice arm of 40 cm length, and all arms were 15 cm wide with walls 30 cm in height. There was a rectangular start box (25 cm×15 cm) at the beginning of the runway arm and food cups (shielded from sight by a 3 cm high barrier) were located at the terminal end of each choice arm. Apple Jacks cereal (1/4 a piece) served as the reinforcer. The maze was wiped down after each individual session with a 1:1 "Whistle" solution (water and whistle).

Procedure

Water maze Mice were initially trained to locate a hidden platform in a standard fixed platform memory acquisition task, in which the platform remained in a constant position. This acquisition phase lasted for eight sessions, each of which consisted of four trials separated by approximately 10 min. 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 were determined randomly, except that each starting point was used only once each session). After 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 and again allowed to remain on the platform for 30 s. Once all mice had learned to locate the platform location they were trained to perform a working memory task, in which the location of the platform varied from day to day. 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). In order to be eligible for testing with drug or vehicle, the subjects were required to locate the platform in less than 30 s on trials 2–4, and were required to meet this criterion on three out of their four most recent training sessions. However, on test days, only two trials were given. Drug tests were conducted once or twice per week, with at least 72 h and one training session between tests. Test sessions were otherwise identical to training sessions, with the exception that only two trials were conducted, in order to minimize the use of non-spatial strategies (i.e. egocentric, or route/taxon strategies). While the water maze experiments were essentially conducted using a within subject design, in several cases additional groups of mice were tested in order to increase the sample size to provide sufficient power for statistical analyses. Consequently, not every mouse was evaluated under every condition, and thus between subjects statistical analyses were conducted

T-maze Two acclimation sessions (2 min of free access, both arms baited with a (1/4) piece of Fruit Loops cereal) preceded the start of delayed spatial alternation training. Next, an initial "forced-choice" procedure was employed in which mice were required to enter alternate arms of the maze sequentially to obtain the reinforcer, with access to the opposite arms being blocked by a gate. Each trial commenced with the raising of the start gate. The latency to enter the goal arm was then recorded, followed by the lowering of the goal arm gate. After consuming the reinforcer the mouse was returned to the start box for 10 s before the start of the next trial while the alternate arm was baited and opened. After two or three sessions of this forced-choice procedure, mice were trained in a "free-choice" procedure which was similar to the one just described except that both arms were open, though only one was baited. After a correct choice was made (defined as completely entering the currently baited arm) the gate was lowered and the mice were allowed 15 s to consume the reinforcer before being returned to the start box. On the subsequent trial, the alternate arm was baited. Entry into an unbaited arm (i.e. an incorrect choice) resulted in the lowering of the goal box and a 15-s time out before being returned to the start box, with the baited arm remaining the same for the next trial. Each training session continued until the mice correctly alternated arms on five of six consecutive trials, with a maximum of 12 trials. Once this level of performance could be maintained for two of three consecutive sessions, mice were eligible to begin drug testing. Drug tests were performed once or twice per week, with at least 72 h between tests. Tests were executed identically to the training sessions, with the exception of the drug administration. Training sessions were conducted on non-test days to ensure stable baseline performance.

Tetrad Following vehicle or drug administration mice were evaluated in four behavioral assays reflective of cannabinoid activity as previously described (Cravatt et al. 2001). Locomotor activity was assessed 5–15 min following drug administration in which the number of photocell beam interruptions in a darkened cage was tallied by a Digiscan Animal Activity Monitor (Med Associates, Inc., St Albans, Vt., USA). At 20 min post-injection, antinociception was assessed in the warm water (52°C) tail withdrawal test, with an automatic 10 s cut-off. At 40 min post-administration, catalepsy was evaluated using

the bar test, in which the front paws of each subject were placed on a metal rod (0.75 cm diameter) that was elevated 4 cm from the surface. The forepaws of each mouse were gently placed on the raised bar, and descent latencies were recorded. At 60 min, core temperatures were recorded to the nearest 0.1°C by inserting a rectal probe connected to a telethermometer (Yellow Spring Industries, Inc., Yellow Springs, Ohio, USA) to a depth of 2.5 cm. Baseline tail withdrawal latencies and rectal temperatures were evaluated prior to the injections.

Statistical analysis

For the water maze experiments, two-way repeated measure ANOVAs were conducted analyzing the effects of drug and trial on escape latencies and path lengths. Planned comparisons were conducted on the raw escape latency and path length scores in which trials 1 and 2 were compared for each injection condition. Finally, 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 (a ratio of 0.5 indicates that path lengths of the two trials were identical, while ratios greater than 0.5 indicate the degree of improvement between the first and second trial). The ED_{50} values for disrupting the savings ratio were calculated by least squares linear regression.

Data from the T-maze and tetrad tests were analyzed using repeated measures ANOVAs. The tail withdrawal data were expressed as percent MPE (%MPE) using the following equation: %MPE=100×(post-injection withdrawal latency−pre-injection withdrawal latency)/(10 s −pre-injection latency). Hypothermia data were expressed as: post-injection rectal temperature−pre-injection rectal temperature. Differences between treatment groups were identified with one-way ANOVAs, considered significant at the $P<0.05$ level. In cases where significant treatment effects were identified, Dunnett's post hoc tests were conducted.

Results

Evaluation of GABAergic antagonists on the pharmacological effects of Δ^9-THC

As shown in Fig. 1, deficits in spatial working memory performance produced by 10 mg/kg Δ^9-THC were reversed by 1 mg/kg bicuculline, which by itself had no effects in this model. Results of a two-way ANOVA revealed significant main effects of trial [$F(1,95)=48$, $P<0.001$], and drug treatment [$F(3,95)=4.1$, $P<0.05$], though the interaction between drug and trial on escape latencies failed to achieve significance. Shown in Fig. 1a are the results of a series of planned comparisons on the escape latency data. Δ^9-THC significantly impaired performance as reflected by the lack of a significant difference between trials 1 and 2. Significant decreases in escape latency of trial 2 compared to trial 1 were found in the vehicle ($P<0.01$), bicuculline ($P<0.01$), and bicuculline + Δ^9-THC ($P<0.001$) conditions. A similar pattern of results was found with the path length data (Fig. 1b), where the interaction between drug treatment and trial was significant [$F(3,95)=2.9$, $P<0.05$]. Again, treatment with Δ^9-THC disrupted performance, as no difference was found between trials 1 and 2. Mice exhibited significant improvement following the vehicle ($P<0.01$), bicuculline ($P<0.01$), and bicuculline-Δ^9-THC ($P<0.01$) treatments. Lower doses (0.25 mg/kg and 0.5 mg/kg) of bicuculline

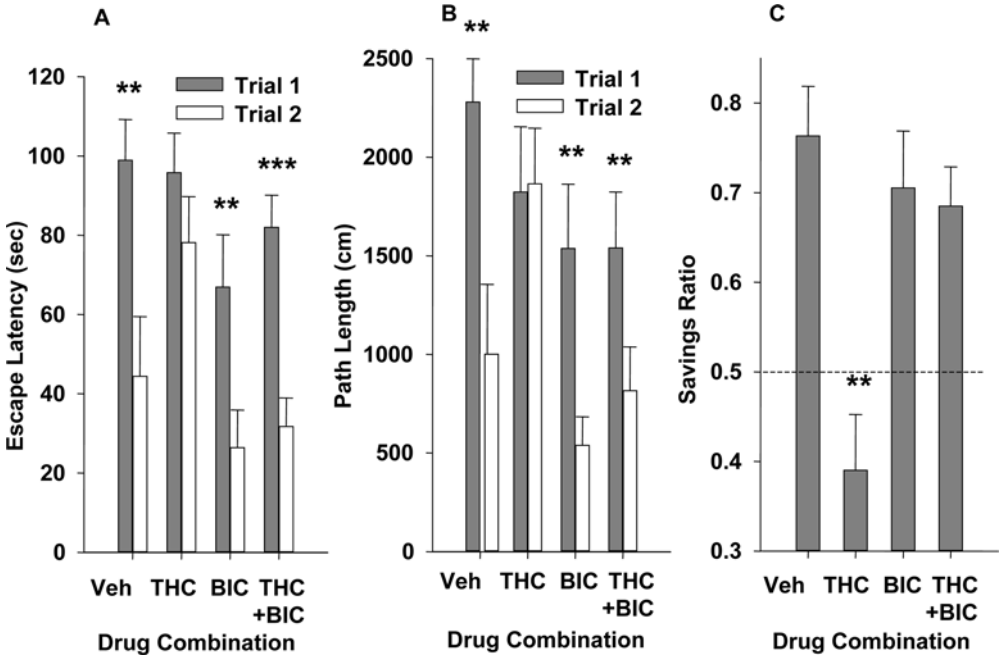

Fig. 1 Blockade of $GABA_A$ receptors reverses Δ^9-THC-induced impairments in the Morris water maze. Deficits in spatial working memory performance produced by 10 mg/kg Δ^9-THC are reversed by 1 mg/kg bicuculline (*BIC*) as assessed by escape latencies (**a**) and path length (**b**) to the hidden platform. **c** Path length data transformed into a savings ratio (path $length_1$/[path $length_1$+path $length_2$]), the stippled line reflects chance level of performance. $^{**}P<0.01$, $^{***}P<0.001$ between trials 1 and 2 (Dunn's test) for each respective condition in **a** and **b**. $^{**}P<0.01$ between drug and vehicle performance (Dunnett's test) in **c**. n=9–15 per group. Data represent means±SEM

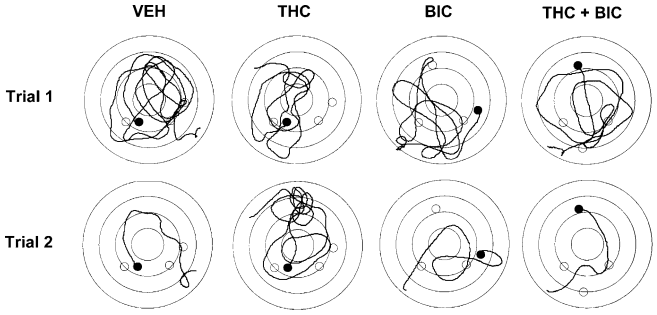

Fig. 2 Representative sample of swim path traces illustrating the impairment (i.e. lack of improvement in search strategy between trials 1 and 2) produced by 10 mg/kg Δ^9-THC (THC) and its reversal by coadministration of 1 mg/kg bicuculline (*BIC*), which by itself had no effect

failed to block the effects of Δ^9-THC and higher doses of drug (2 mg/kg) elicited seizures (data not shown). In order to make further comparisons between the different drug conditions in a normalized data set, the path length data were transformed into a saving ratio. As shown in Fig. 1c, there was a significant effect of drug treatment [$F(3,47)=4.5$, $P<0.01$], with Δ^9-THC yielding a savings ratio that was significantly below all other conditions ($P<0.05$). A representative sample of the swim traces from each drug condition during trials 1 and 2 is shown in Fig. 2. None of these treatments significantly affected swim speeds (data not shown).

Figure 3 depicts the results of a second experiment designed to determine whether the $GABA_B$ antagonist CGP 36742 would block the deficits produced by 10 mg/kg Δ^9-THC. Two-way ANOVAs revealed significant drug by trial interactions for escape latency [$F(3,75)=5.1$, $P<0.01$], and path length [$F(3,75)=8.1$, $P<0.001$]. As shown in Fig. 3a,b, mice treated with the vehicle exhibited improved performance across the two trials, as the second trial was significantly lower than the first trial for both escape latencies ($P<0.01$) and path lengths ($P<0.01$). A similar improvement was found following CGP 36742 treatment, as reflected by the escape latency ($P<0.01$) and path length ($P<0.01$) data. In contrast, Δ^9-THC treatment impaired performance, as no significant improvement was found for trial 2 compared with trial 1 for either escape latencies or path lengths. Similarly, no significant differences were found for either escape latencies or path lengths in the CGP 36742+Δ^9-THC condition, indicating that this $GABA_B$ antagonist failed to block the effects of 10 mg/kg Δ^9-THC. Further comparisons between the groups using the corresponding savings ratio data yielded a significant effect of drug treatment [$F(3,37)=10.8$, $P<0.001$]. As can be seen in Fig. 3c, CGP 36742 failed to ameliorate performance deficits caused by Δ^9-THC, as treatment with either 10 mg/kg Δ^9-THC or 10 mg/kg Δ^9-THC+30 mg/kg CGP 36742 both significantly disrupted performance compared to vehicle ($P<0.01$). Again, no treatment effects on swim speeds were observed (data not shown).

In order to assess further the ability of bicuculline to ameliorate Δ^9-THC-induced memory deficits, we examined these drugs in a second animal model of learning and memory, the alternating-choice version of the T-maze. As shown in Fig. 4a, Δ^9-THC produced a significant reduction in choice accuracy in this T-maze task [$F(3,34)=12.4$, $P<0.001$], at 3.0 mg/kg ($P<0.05$) and 10 mg/kg ($P<0.05$). A significant increase in mean latency/trial was also observed [$F(3,34)=3.0$, $P<0.05$] (Fig. 4b), with only the 10 mg/kg dose significantly differing from vehicle ($P<0.05$). This dose also suppressed response frequency (data not shown). To avoid non-mnemonic pharmacological (e.g. sensorimotor or motivational) effects of Δ^9-THC, we employed a 3 mg/kg Δ^9-THC dose in the bicuculline experiment. The data depicted in Fig. 4c yielded a significant effect of drug treatment [$F(3,35)=30.2$, $P<0.001$], with 3.0 mg/kg Δ^9-THC signifi-

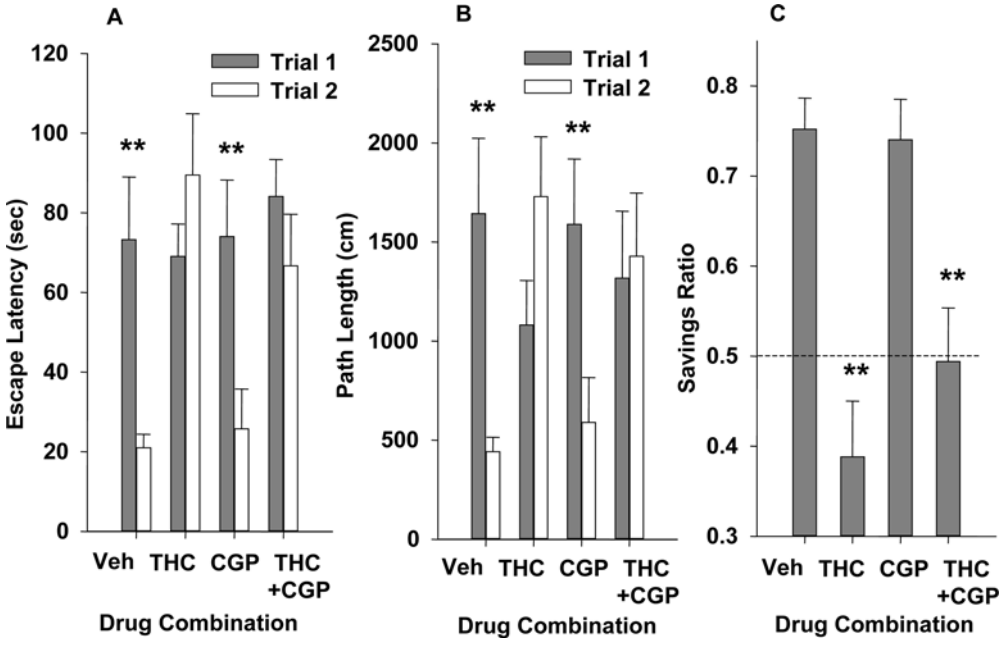

Fig. 3 Blockade of $GABA_B$ receptors fails to attenuate Δ^9-THC-induced memory impairment. Deficits in spatial working memory performance produced by 10 mg/kg Δ^9-THC are not affected by 30 mg/kg CGP 36742 (*CGP*), which by itself has no effects in this model as assessed by escape latencies (**a**) or path lengths (**b**). **c** Path length data transformed into a savings ratio (path length$_1$/[path length$_1$+path length$_2$]), the stippled line reflects chance level of performance. $^{**}P<0.01$ between trials 1 and 2 (Dunn's test) for each respective condition in **a** and **b**. $^{**}P<0.01$ between drug and vehicle performance (Dunnett's test) in **c**. n=8–11 per group. Data represent means±SEM

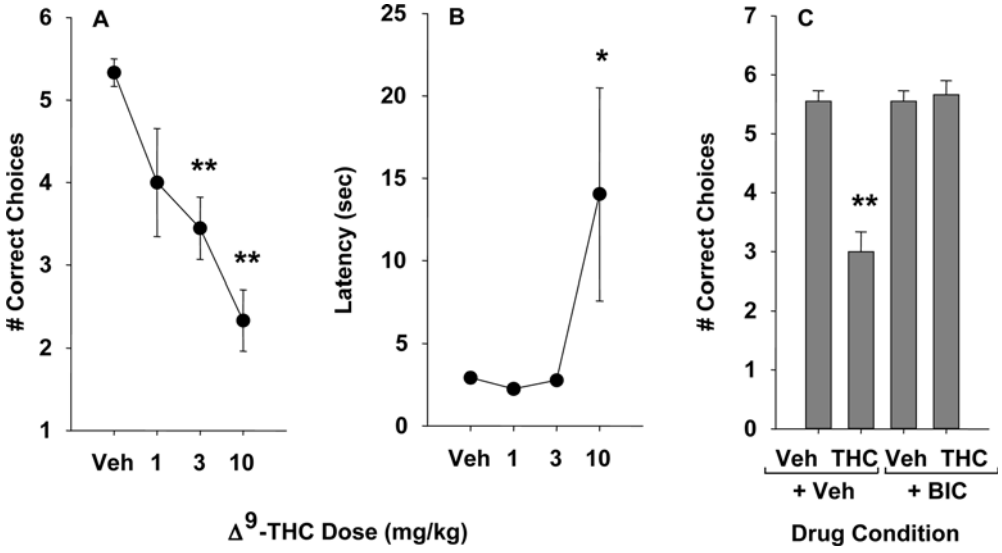

Fig. 4 Bicuculline (*BIC*) blocks Δ^9-THC-induced alternating T-maze deficits. The number of correct arm entries after varying doses of Δ^9-THC (**a**). The average latency to make a choice (**b**). The deficits produced by 3 mg/kg Δ^9-THC were completely reversed by 1 mg/kg bicuculline, which by itself had no effects in this model (**c**). *Asterisks* denote significant differences from vehicle, **P*<0.05, ***P*<0.01. *n*=9 per group. Data represent means ±SEM

cantly decreasing accuracy ($P<0.001$), and 1.0 bicuculline mg/kg completely reversing this deficit ($P<0.001$). No significant effects were found for the latency data (data not shown). Thus, bicuculline blocked Δ^9-THC-induced memory impairment in two distinct mouse models.

In the next experiment, we evaluated whether bicuculline would also normalize other Δ^9-THC-induced behaviors. Subjects were given an injection of either vehicle or bicuculline (1 mg/kg) with an injection of vehicle or Δ^9-THC (10 mg/kg or 30 mg/kg) and evaluated in the mouse tetrad assay. As shown in Fig. 5, 1.0 mg/kg bicuculline failed to antagonize any of the effects of Δ^9-THC. A significant effect of Δ^9-THC was found for analgesia [$F(2,35)=90$, $P<0.001$], with both 10 mg/kg and 30 mg/kg Δ^9-THC increasing tail withdrawal latencies compared to vehicle ($P<0.001$). No significant interaction between Δ^9-THC and bicuculline was observed (Fig. 5a). Significant effects of Δ^9-THC on hypothermia were also observed [$F(2,35)=88$, $P<0.001$], with both doses of Δ^9-THC significantly reducing body temperature (Fig. 5b). No effect of bicuculline was seen for this measure. Δ^9-THC produced a significant biphasic effect on spontaneous activity [$F(2,34)=19$, $P<0.001$], with 10 mg/kg Δ^9-THC increasing activity levels ($P=0.01$) and 30 mg/kg Δ^9-THC decreasing activity levels compared to vehicle ($P<0.05$) (Fig. 5c). Again, bicuculline failed to affect activity levels. Δ^9-THC also produced catalepsy [$F(2,35)=40$, $P<0.001$], which was significant at the 30 mg/kg dose (Fig. 5d). Similar to the other measures, no significant interactions between Δ^9-THC and bicuculline were observed. These findings show that bicuculline normalizes mnemonic deficits produced by Δ^9-THC, but not analgesic, hypothermic, and motor alterations elicited by Δ^9-THC.

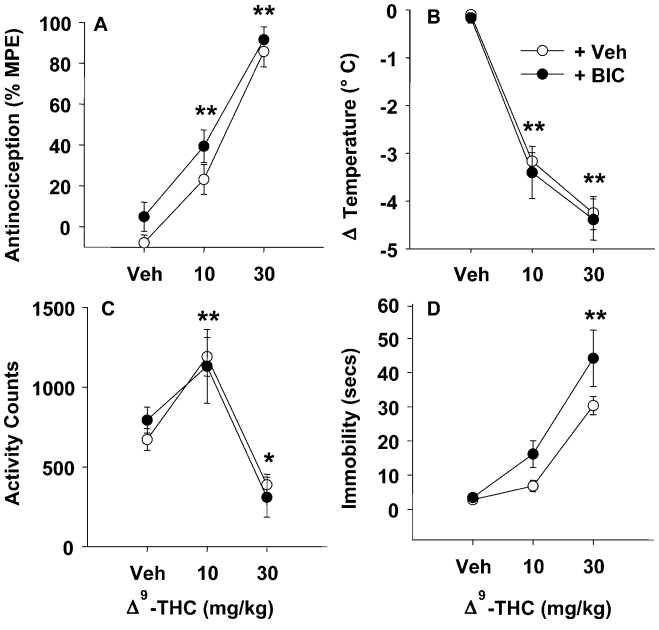

Fig. 5 Bicuculline fails to alter non-mnemonic effects of Δ^9-THC as assessed in the tetrad. Δ^9-THC produced significant effects in the tail flick (**a**), rectal temperature (**b**), spontaneous activity (**c**), and bar catalepsy (**d**) assays. However, none of the effects was blocked by coadministration of 1 mg/kg bicuculline (*BIC*). *Asterisks* denote significant (combined) differences from vehicle, ***P*<0.01. *n*=6 per group. Data represent means±SEM

Evaluation of CB_1 receptors in performance deficits elicited by GABA agonists in the Morris water maze

In the previous experiments, bicuculline prevented Δ^9-THC-induced deficits in the Morris water maze and T-maze tasks, but failed to block several non-mnemonic pharmacological effects of Δ^9-THC. This pattern of results suggests that GABA plays an important role in Δ^9-THC-induced memory impairment. The purpose of the next set of experiments was to test the converse hypothesis. Specifically we asked, do CB_1 receptors play an

important role in the disruptive effects of GABA agonists in the Morris water maze?

First, we evaluated Morris water maze performance of CB_1 (−/−) and (+/+) mice following injections of the $GABA_A$ agonist muscimol or the $GABA_B$ agonist baclofen. In order to assess whether there were any genotype differences irrespective of drug, two separate two-way ANOVAs, with the between subject factor of genotype and the within subject factor of trial, were conducted on the vehicle condition for both the escape latency (Figs 6a,c, 7a,c) and path length (Figs 6b,d, 7b,d) data. As expected, a significant main effect of trial was found for both escape latency data [$F(1,59)=76$, $P<0.001$], and path length data [$F(1,59)=75$, $P<0.001$], indicated that the mice learned the platform location. However, there were no apparent genotype effects, as neither the main effects of genotype nor the genotype by trial interactions approached statistical significance. These results indicate that under baseline conditions both CB_1 (−/−) and (+/+) mice performed equally well in the task, thus allowing comparison of each GABA agonist between the genotypes.

Surprisingly, CB_1 (−/−) mice were apparently more sensitive to the memory-disrupting effects of muscimol than their wild type littermates (Fig. 6). The assessment of different dose ranges of muscimol precluded the use of examining genotype directly in the analysis, so separate two-way ANOVAs, with drug and trial as the factors, were conducted on each genotype. Significant interactions were found between drug and trial in CB_1 (−/−) mice for both escape latency [$F(3,35)=4.6$, $P<0.05$], and path length [$F(3,35)=5.6$, $P<0.05$]. As shown in Fig. 6a,b, CB_1 (−/−) mice exhibited significant improvement between trials 1 and 2 for escape latency and path length for vehicle ($P<0.01$ for both measures) and 0.03 mg/kg muscimol ($P<0.01$ for both measures). However, no statistical differences were found between the two trials following either 0.06 mg/kg or 0.12 mg/kg muscimol, suggesting that memory was impaired. In contrast, muscimol was less potent in CB_1 (+/+) mice (Fig. 6c,d). There were main effects of drug [$F(5,93)=2.5$, $P<0.05$], and trial [$F(1,93)=32$, $P<0.001$], on escape latencies, though the drug by trial interaction on the escape latency data failed to achieve statistical significance in these mice. However, the drug by trial interaction on the path lengths was significant [$F(5,93)=2.8$, $P<0.05$]. Escape latencies and path lengths were significantly decreased across from trial 1 to trial 2 after vehicle ($P<0.01$ for both measures), 0.062 mg/kg muscimol ($P<0.01$ for both measures), and 0.12 mg/kg muscimol ($P<0.01$ for both measures), but not following either 0.25 mg/kg or 0.5 mg/kg muscimol. Analysis of the corresponding path length savings ratio data revealed significant effects of muscimol dose in both CB_1 (+/+) mice [$F(4,40)=3.2$, $P<0.05$], and CB_1 (−/−) mice [$F(3,17)=8.4$, $P<0.01$]. The ED_{50} (95% CI) values for muscimol using the savings ratio measure were 0.17 mg/kg (0.08–0.36 mg/kg) and 0.05 mg/kg (0.04–0.07 mg/kg) for the CB_1 (+/+) and CB_1 (−/−) mice, respectively (see Fig. 6e). Accordingly, muscimol was 3.5 (1.2–14.9) times more potent (95% confidence limits) in CB_1 (−/−) mice than in CB_1 (+/+) mice. Muscimol tended to decrease swim speed, though this effect failed to achieve statistical significance ($P=0.06$). However, there was no effect of genotype on this measure (data not shown).

As can be seen in Fig. 7, baclofen was equipotent in disrupting performance of both genotypes. Results from a three-way ANOVA yielded significant two-way interactions between drug dose and trial for both escape latency [$F(3,34)=3.2$, $P<0.05$], and path length [$F(3,34)=5.7$,

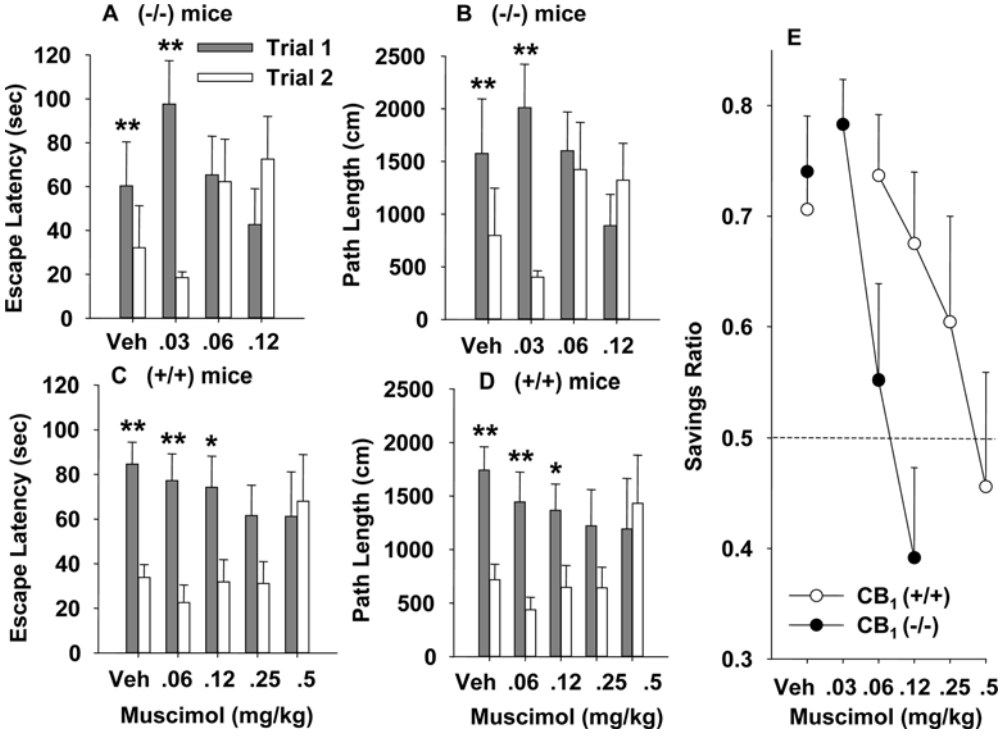

Fig. 6 CB_1 (−/−) mice exhibit an increased sensitivity to muscimol compared to wild type mice. Dose-effect determination for muscimol-induced working memory performance deficits in CB_1 (−/−) mice (**a** and **b**) and CB_1 (+/+) mice (**c** and **d**). *Asterisks* denote significant differences between escape latencies or path lengths of trials 1 and 2 (Dunn's test), $*P<0.05$, $**P<0.01$. **e** Path length data from both genotypes transformed into a savings ratio (path $length_1$/[path $length_1$+path $length_2$]). Muscimol was found to be 3.5 (1.2–14.9) times more potent in CB_1 (−/−) than in CB_1 (+/+) mice. n=5–9 per group. Data represent means±SEM

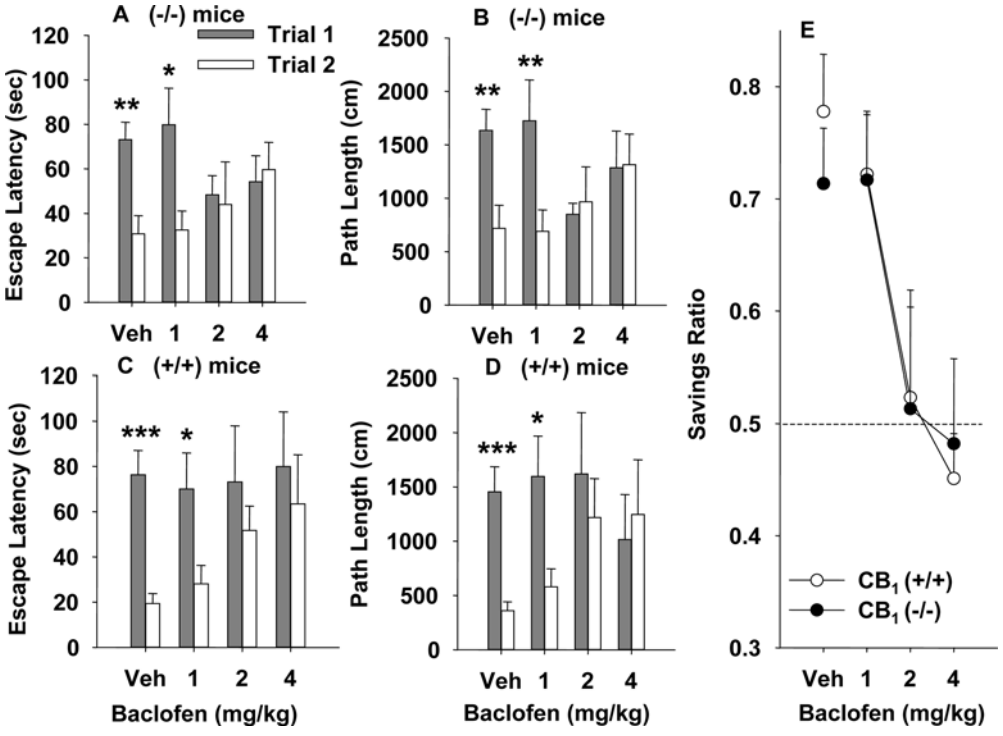

Fig. 7 Baclofen elicits equipotent effects in CB_1 (−/−) and (+/+) mice. Dose-effect determination for baclofen-induced Morris water maze working memory performance deficits in CB_1 (−/−) mice (**a** and **b**) and CB_1 (+/+) mice (**c** and **d**). *Asterisks* denote significant differences between escape latencies or path lengths of trials 1 and 2 (Dunn's test), *P<0.05, **P<0.01, ***P<0.001. **e** Path length data from both genotypes transformed into a savings ratio (path length$_1$/[path length$_1$+path length$_2$]). n=5–7 per group. Data represent means±SEM

P<0.01]. However, the effects of genotype were not significant for either dependent measure. As shown in Fig. 7a,b escape latencies and path lengths were significantly improved during trial 2 compared to trial 1 in CB_1 (−/−) mice following administration of vehicle (P<0.01 for both measures) and 1 mg/kg baclofen (P<0.05 for escape latency and P<0.01 for path length). However, no significant differences between the two trials were found following injections of 2 mg/kg or 4 mg/kg baclofen, though trial 1 performance appeared to be improved following these doses. Similarly, in the CB_1 (+/+) mice (Fig. 7c,d) the escape latencies and path lengths were significantly improved following administration of vehicle (P<0.001 for both measures) and 1 mg/kg (P<0.05

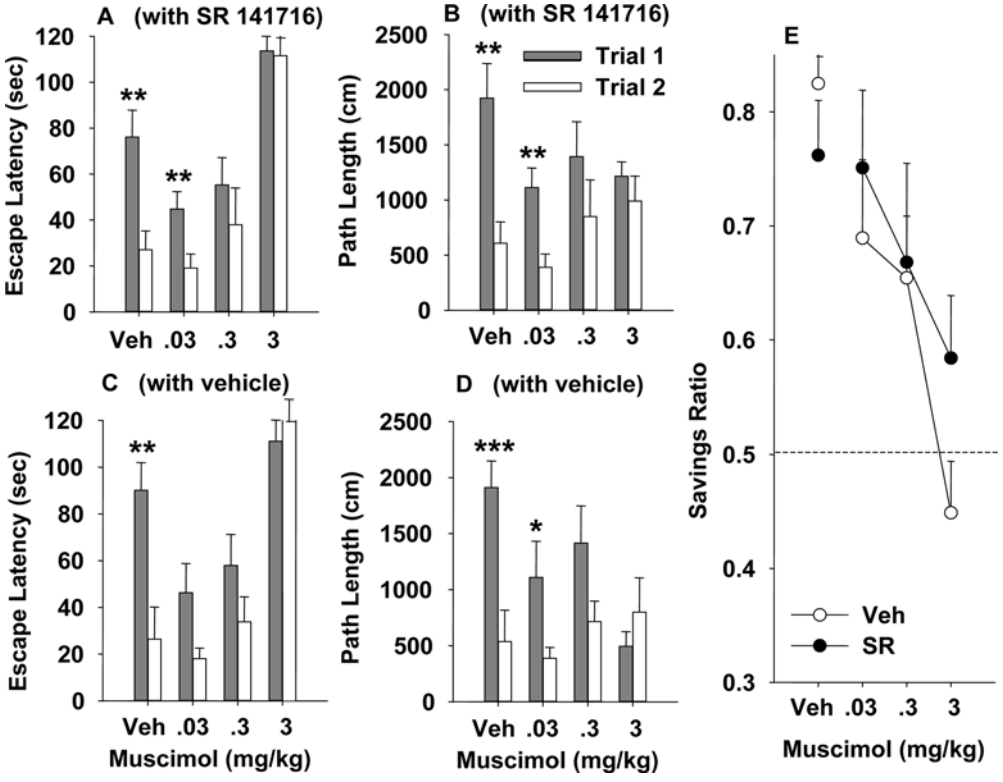

Fig. 8 SR 141716 failed to alter spatial working memory performance deficits produced by muscimol. Dose-effect determination for muscimol-induced Morris water maze working memory performance deficits in mice coadministered 3.0 mg/kg SR 141716 (**a** and **b**) and mice coadministered vehicle (**c** and **d**). *Asterisks* denote significant differences between escape latencies or path lengths of trials 1 and 2, *P<0.05, **P<0.01, ***P<0.001. **e** Path length data of both genotypes transformed into a savings ratio (path length$_1$/[path length$_1$+path length$_2$]. Note that apparent short path lengths in trials 1 and 2 of mice treated with 3 mg/kg muscimol is due to a drug-induced impairment of swimming, which left many mice just floating for the bulk of the 120 s trial. n=8 per group. Data represent means±SEM

for both measures), but not after 2 mg/kg or 4 mg/kg baclofen. Analysis of the corresponding savings ratios revealed significant effects of baclofen dose [$F(3,40)=7.9$, $P<0.001$], but no significant differences were found for genotype or the dose by genotype interaction. As represented in Fig. 7e, there was no genotype difference in baclofen potency, with the baclofen ED_{50} (95% CI) values for CB_1 (+/+) and CB_1 (−/−) mice calculated to be 1.4 (0.8–2.3) mg/kg and 1.6 (0.9–3.0) mg/kg, respectively. Average swim speeds were not affected by baclofen dose or genotype.

Because CB_1 (−/−) mice were more sensitive than CB_1 (+/+) mice to the disruptive effects of muscimol in the Morris water maze working memory task, we next evaluated whether an injection of the CB_1 receptor antagonist SR 141716 (3 mg/kg) in wild type mice would also augment the effects of muscimol. The results from the combination of muscimol and either vehicle or 3 mg/kg SR 141716 in the working memory task are presented in Fig. 8. Two separate two-way ANOVAs were initially conducted on this data set to determine whether SR 141716 altered performance by itself, in which the factors included drug condition (i.e. vehicle–vehicle versus SR-141716-vehicle) and trial. Significant main effects of trial were found for escape latency [$F(1,31)=36.8$, $P<0.001$], and path length [$F(1,31)=45.5$, $P<0.001$], though the effects of drug and the drug by trial interaction were not significant. These findings indicate that an acute injection of 3 mg/kg SR 141716 does not alter performance in the working memory Morris water maze task.

Separate three-way ANOVAs were conducted on escape latency and path length data to determine the effects of muscimol, SR 141716 cotreatment, and trial. Significant muscimol dose by trial interactions were identified for escape latency [$F(3,54)=6.1$, $P<0.01$], and path length [$F(3,54)=6.7$, $P<0.001$], indicating that the improvement between trials was negatively affected by the dose of muscimol. There were no main effects of SR 141716 cotreatment and no SR 141716 by muscimol interaction. As shown in Fig. 8a,d, mice maintained significant improvement on trial 2 compared to trial 1 in the vehicle–vehicle ($P<0.01$ for escape latency and $P<0.001$ for path length), SR 141716-vehicle ($P<0.05$ for both dependent measures), vehicle-muscimol (0.03 mg/kg, $P<0.05$ for path length), and SR 141716-muscimol (0.03 mg/kg, $P<0.01$ for both measures) conditions. In contrast no significant improvement was found after 0.3 mg/kg or 3.0 mg/kg muscimol in mice coadministered either vehicle or SR 141716. The saving ratios data revealed a significant main effect of muscimol dose [$F(3,61)=5.7$, $P<0.01$], but the main effect of SR 141716 and interaction between muscimol and SR 141716 failed to achieve significance. ED_{50} values for muscimol were found to be 0.25 (0.08–0.80) mg/kg when pretreated with vehicle, and 0.88 (0.21–3.8) mg/kg when pretreated with SR 141716, which were found by potency ratio analysis to not be different.

In contrast to the previous experiments, swim speeds were significantly depressed following 3 mg/kg muscimol, regardless of SR 141716 coadministration (data not shown). In fact, the majority of the mice given this dose of drug floated for the duration of the 120 s trials, which resulted in short path lengths, even though they never found the platform. Results from a two-way ANOVA on average swim speeds found significant effects of muscimol [$F(3,54)=92.5$, $P<0.001$], as well as for SR 141716 [$F(3,54)=22.4$, $P<0.01$], but no interaction.

Discussion

These results support the hypothesis that the activation of $GABA_A$ receptors plays a critical role in Δ^9-THC-induced memory impairment. Specifically, the $GABA_A$ antagonist bicuculline, but not the $GABA_B$ antagonist CGP 36742, prevented deficits produced by Δ^9-THC in the working memory Morris water maze task. Bicuculline also blocked Δ^9-THC-induced memory impairment in an alternation T-maze task. In contrast, bicuculline failed to block the hypomotilty, analgesic, hypothermic, and cataleptic effects of Δ^9-THC, indicating an interaction that is selective to memory. The fact that the Morris water maze and alternation T-maze tasks differ markedly with respect to motivational demands (i.e. escape versus hunger), reinforcers (i.e. platform versus food), and motor requirements (i.e. swimming versus walking and/or running) supports the hypothesis that $GABA_A$ receptors may play a necessary role in the expression of cannabinoid-induced memory impairment in these two tasks. Indeed, a compelling amount of in vitro evidence indicates that cannabinoids modulate GABAergic systems in brain areas associated with learning and memory (see Katona et al. 1999; Hajos et al. 2000; Hoffman and Lupica 2000; Ohno-Shosaku et al. 2001; Wilson et al. 2001).

Importantly, we have previously found that the memory disruptive effects of the 10 mg/kg dose of Δ^9-THC employed in the present study impairs memory in the Morris water maze through a CB_1 receptor mechanism of action (Varvel et al. 2001; Varvel and Lichtman 2002). Specifically, Δ^9-THC-induced memory impairment was completely blocked by the CB_1 receptor antagonist SR 141716 and failed to occur in CB_1 (−/−) mice. Similarly, we have previously found that the memory disruptive effects of other cannabinoid agonists (i.e. WIN 55,212-2 and methanandamide) in this task are CB_1 receptor-mediated (Varvel et al. 2001; Varvel and Lichtman 2002). Additionally, 10 mg/kg Δ^9-THC was found to elicit maximally disruptive effects in the working memory model of the Morris water maze, without affecting non-mnemonic measures, such as swim speed or thigmotaxia and does not disrupt performance in a cued version of the task (in which the location of the platform is explicitly marked) or in a reference memory retention task with similar motor and motivational requirements.

The behavioral specificity of the bicuculline blockade of Δ^9-THC-induced memory impairment observed in the

Morris water maze was evaluated by determining whether bicuculline would also alter the effects of Δ^9-THC in a T-maze task. Although both tasks are similar in that working memory is required to perform the task successfully, several key differences exist. In addition to the motor and motivational requirements described above, performance in the water maze task relies largely on processing of spatial information related to exteroceptive visual cues, while the T-maze task can be performed with purely egocentric (i.e. turn right or left) strategies. Although the use of such egocentric, or "route" strategies, in our water maze task cannot be completely excluded due to the use of the same release position on both trials, this risk is minimized by limiting each test session to two trials (see Fig. 2). It is worth noting that while significant deficits in choice-accuracy in the T-maze task were observed at both 3 mg/kg and 10 mg/kg Δ^9-THC, potentially confounding effects on motor activity (i.e. increased choice latency) were also observed at 10 mg/kg Δ^9-THC. In contrast, our previous work and the present results show that 10 mg/kg Δ^9-THC does not produce analogous "non-mnemonic" effects (i.e. on swim speeds, thigmotaxia, or first trial latencies), suggesting that the water maze model described here is less prone to these confounds.

The possibility that memory enhancing effects may have simply overshadowed Δ^9-THC-induced deficits cannot be entirely ruled out. Specifically, the procedures used here were not specifically designed to detect memory enhancement. Furthermore, bicuculline has been shown to enhance some forms of memory in certain situations. For example, bicuculline was recently reported to increase retention of a passive avoidance memory in rats when intrahippocampally administered immediately post-training (Zarrindast et al. 2002). Nonetheless, the facts that bicuculline alone had no enhancing effects on working memory performance and failed to antagonize the baclofen-induced deficits seems to argue against the explanation that an independently mediated enhancement of memory may have simply overshadowed Δ^9-THC-induced deficits.

Conversely, the well-described effects of Δ^9-THC in the cannabinoid "tetrad" (i.e. antinociception, hypothermia, hypoactivity, and catalepsy) were not blocked by bicuculline. These observations suggest that the apparent antagonistic relationship between Δ^9-THC and bicuculline may be restricted to mnemonic effects, an idea that may have implications for which brain areas are involved. Specifically, the memory deficits produced by Δ^9-THC have been associated primarily with the hippocampus (Lichtman et al. 1995; Egashira et al. 2002), while each of the components of the tetrad has been traditionally associated with other brain areas (e.g. striatum, PAG, hypothalamus). The apparent specificity of this bicuculline effect may also have important implications for attempts to limit undesirable side effects of putative cannabinoid therapeutics, raising the possibility that cognitive disruptions could be minimized without interfering with therapeutic effects.

The final goal of this study was to determine whether CB_1 receptor signaling mediates the memory disruptive effects of GABA agonists. This hypothesis was tested by evaluating the effects of the $GABA_A$ agonist muscimol and the $GABA_B$ agonist baclofen in both CB_1 (−/−) and (+/+) mice on Morris water maze performance. Unexpectedly, CB_1 (−/−) mice exhibited an increased sensitivity to the memory disruptive effects muscimol, but not baclofen, compared to CB_1 (+/+) mice. However, it should be noted that the short latencies observed during trial 1 following 2 mg/kg or 4 mg/kg baclofen may have partly resulted in the failure to observe significant improvements during trial two. Nonetheless, these findings suggest that the interaction demonstrated with Δ^9-THC and bicuculline may reflect a tonic endocannabinoid regulation of activity at $GABA_A$ synapses. Supersensitivity to $GABA_A$ stimulation would be consistent with a release from the inhibitory influence exerted by CB_1 receptors on GABA release. On the other hand, the failure to potentiate the effects of muscimol with SR 141716 suggests that increased sensitivity to $GABA_A$ stimulation in CB_1 (−/−) mice may result from a compensatory response to development without CB_1 receptors or other confounds related to the use of transgenic mice (see Mogil and Grisel 1998; Nelson and Young 1998). Alternatively, or possibly concurrently, the conflicting results may arise from issues related to receptor selectivity. Endocannabinoids are believed to inhibit hippocampal GABA release via CB_1 receptors, as well as glutamate release via a cannabinoid receptor that is distinct from CB_1, though still sensitive to SR 141716. The primary evidence for this is the finding that in CB_1 (−/−) mice the inhibitory effects of cannabinoids on GABA, but not glutamate, are abolished, while SR 141716 treatment blocks both effects (Hajos and Freund 2002). Thus, genetic deletion of the CB_1 receptor and blockade of both receptors with SR 141716 may result in a different balance of influences on principal hippocampal neurons. Nonetheless, the present results indicate that the memory disruptive effects of $GABA_A$ and $GABA_B$ agonists occur in the absence of CB_1 receptors.

In conclusion, these results suggest that $GABA_A$ receptors play a necessary role in the cognitive-impairing effects of Δ^9-THC in at least two animal models. This relationship seems to be selective to memory, since $GABA_A$ receptors do not appear to contribute to non-mnemonic effects of Δ^9-THC, as assessed in the tetrad test. Conversely, CB_1 receptors do not appear to play a necessary role for GABAergic-induced memory impairment. Characterizing the role that GABA plays in mediating the effects of exogenously administered cannabinoids as well as the endocannabinoid system may not only increase our understanding of cannabinoid modulation of memory, but also could further our understanding of memory systems in general. Moreover, this knowledge could lead to improved strategies for treating cannabis-related disorders as well as a wide variety of cognitive ailments.

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