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D_2 dopamine receptors enable Δ^9-tetrahydrocannabinol induced memory impairment and reduction of hippocampal extracellular acetylcholine concentration

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1 The systemic administration of Δ^9-tetrahydrocannabinol (2.5–7.5 mg kg^{-1}) reduced hippocampal extracellular acetylcholine concentration and impaired working memory in rats.

2 Both effects were antagonized not only by the CB_1 cannabinoid receptor antagonist SR141716A (0.5 mg kg^{-1}, i.p.) but also unexpectedly by the D_2 dopamine receptor antagonist S(−)-sulpiride (5, 10 and 25 mg kg^{-1}, i.p.). Conversely, Δ^9-tetrahydrocannabinol-induced memory impairment and inhibition of hippocampal extracellular acetylcholine concentration were potentiated by the subcutaneous administration of the D_2 dopamine receptor agonist (−)-quinpirole (25 and 500 μg kg^{-1}). The inhibition of hippocampal extracellular acetylcholine concentration and working memory produced by the combination of (−)-quinpirole and Δ^9-tetrahydrocannabinol was suppressed by either SR141716A or S(−)-sulpiride.

3 Our findings suggest that impairment of working memory and inhibition of hippocampal extracellular acetylcholine concentration are mediated by the concomitant activation of D_2 dopamine and CB_1 cannabinoid receptors, and that D_2 dopamine receptor antagonists may be useful in the treatment of the cognitive deficits induced by marijuana.

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Abbreviations: A77636 (−)-(1R, 3S)-3-adamantyl-1-(aminomethyl)-3,4-dihydro-5,6-dihydroxy-1H-2-benzopyran hydrochloride; CP55,940 [1α,2β-(R)5α]-(−)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropil)cyclohexyl]phenol, HA966 3-amino-1-hydroxy-2-pyrrolidone; SCH23390 R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SKF81297 R(+)-6-Chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide; SR141716A N-(piperidine-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride

Introduction

The effect of *Cannabis sativa* on cognitive functions is one of the most controversial debated questions pertaining to the consequences of marijuana use on health (Hall & Solowij, 1998; Thomas, 1993). While there is no question concerning the cognitive deficits present during marijuana intoxication, there is some doubt whether or not permanent memory deficits occur after chronic use (Thomas, 1993). Indeed, it has been argued that the cognitive deficits observed in heavy chronic marijuana users may represent antecedents, concomitants, or consequences from the chronic exposure to the drug (Pope *et al*., 1995). Investigation about the influence of *cannabis sativa* on cognitive processes may help not only to resolve the contention of marijuana effects on cognition, but also to elucidate the physiological and pathophysiological role that endogenous cannabinoids play in cognitive functions.

Animal studies have shown that memory processes are impaired by the main active principle of *cannabis sativa*, Δ^9-tetrahydrocannabinol, by different synthetic CB_1 cannabinoid receptor agonists, as well as by the endogenous cannabinoids (Brodkin & Moerschbaecher, 1997; Collins *et al*., 1995; Lichtman *et al*., 1995; Lichtman & Martin, 1996' Mallet & Benninger, 1996; 1998; Stella *et al*., 1997). In particular, Lichtman & Martin (1996) have shown that cannabinoid-induced memory impairment is specifically reversed by the CB_1 cannabinoid receptor antagonist SR141716A [N-(piperidine-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride], suggesting that this effect is mediated by cannabinoid receptors. Additional studies have shown that cannabinoids impair working memory after intrahippocampal administration (Lichtman *et al*., 1995), inhibit hippocampal long-term potentiation (Collins *et al*., 1995) and reduce hippocampal extracellular acetylcholine concentration (Gifford & Ashby, 1996; Gifford *et al*., 1997; Gessa *et al*., 1997; Carta *et al*., 1998). Moreover, further evidence has suggested that the negative effects of cannabinoids on working memory are caused from dopaminergic hyperactivity in the prefrontal cortex (Jentsch *et al*., 1997). In particular, Jentsch *et al*. (1997) have shown that both Δ^9-tetrahydrocannabinol-induced increase in dopamine turnover in the prefrontal cortex and memory impairment are prevented by HA966 [3-amino-1-hydroxy-2-pyrrolidone], an inhibitor of dopamine neuronal activity (Morrow *et al*., 1997). It is widely accepted that dopaminergic modulation of neural activity in the prefrontal cortex is essential for working memory (Durstewitz *et al*., 1999; Levy & Goldman-Rakic; 1999; Seamans *et al*., 1998; Cai & Arnsten, 1997; Zahrt *et al*., 1997; Watanabe *et al*., 1997; Desimone, 1995; Williams & Goldman-Rakic, 1995; Arnsten *et al*., 1994; Sawaguchi & Goldman-Rakic, 1991). Indeed, recent evidence has demonstrated that working memory is impaired not only when prefrontal dopamine levels or D_1 dopamine receptors activity are below normal (Sawaguchi & Goldman-Rakic, 1991), but also when they are above an optimal range (Zahrt *et al*., 1997). In accord with this theory several studies involving both rats

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and monkeys have shown that stress-induced memory impairment could be prevented by the D_1 dopamine antagonist SCH23390 [R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine] (Arnsten *et al*., 1998) and by the D_2 dopamine antagonist haloperidol (Arnsten *et al*., 1998) and that these stress effects on working memory could be mimicked by the administration of high doses of the D_1 dopamine agonist A77636 [(−)-(1R,3S)-3-adamanty-1-(aminomethyl)-3,4-dihydro-5,6-dihydroxy-1H-2-benzopyran hydrochloride] or SKF81297 [R(+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzaze-pine hydrobromide] (Cai & Arnsten, 1997). Since cannabinoids activate mesocortical dopamine neurons (Diana *et al*., 1998; Gessa *et al*., 1998b), increase dopamine release (Chen *et al*., 1990a) and turnover in the prefrontal cortex (Jentsch *et al*., 1997) and since HA966 (Jentsch *et al*., 1997), a compound that inhibits the activity of dopaminergic neurons (Morrow *et al*., 1997), antagonizes the Δ^9-tetrahydrocannabinol-induced memory deficits (Jentsch *et al*, 1997), we studied the role that dopamine plays on negative effects induced by Δ^9-tetrahydrocannabinol. Specifically, the aim of this study was to examine the role of dopamine and acetylcholine during Δ^9-tetrahydrocannabinol-induced inhibition of working memory. In particular, we set out to investigate whether the inhibitory effect of Δ^9-tetrahydrocannabinol on extracellular acetylcholine concentration and working memory was modified by the blockade or the stimulation of D_2 dopamine receptors.

Methods

Animals

Sprague Dawley rats (200–250 g; Charles River, Como, Italy) were housed individually in a Plexiglass chamber (height 15, length 40 and width 15 cm) at 22±1°C with 55% humidity. Food and water were freely available and the animals were maintained under an artificial 12 h/12 h light/dark schedule with the light cycle ranging from 0800 h to 2000 h. Experiments were conducted between 0900 h and 1700 h.

Experimental procedure

The effect of Δ^9-tetrahydrocannabinol on hippocampal acetylcholine release was studied on freely moving rats by means of microdialysis, while the effect on working memory was studied in separate groups of unoperated animals trained to correctly perform a delayed alternation task in the T-maze.

Surgery and microdialysis

Implantation of the microdialysis probes was performed under general anaesthesia as previously described (Imperato *et al*., 1992). Briefly, rats were implanted with a trasversal dialysis probe (AN 69-HF, tube outer diameter 320 μm; Hospal-Dasco, Bologna, Italy), passing the hippocampi bilaterally (A = −3.2 and V = −3.6; A and V being referred to bregma and skull, respectively). Coordinates were chosen according to the atlas of Paxinos & Watson (1986). The dialysis probe had an active dialysis length of 1 cm.

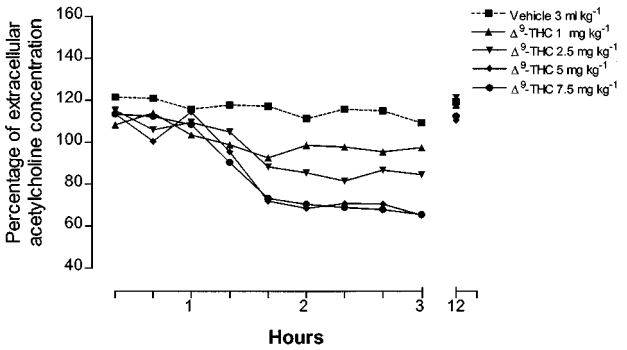

Figure 1 Time-course for the reduction of hippocampal extracellular acetylcholine concentration after Δ^9-tetrahydrocannabinol administration (ANOVA main effect Δ^9-$THC_{2.5\ mg\ kg^{-1}}$ $F_{1.16}=20.84$, $P<0.0001$; ANOVA main effect of repeated measures Δ^9-$THC_{2.5\ mg\ kg^{-1}}$ $F_{8.36}=0.82$, $P=0.59$; ANOVA main effect Δ^9-$THC_{5\ mg\ kg^{-1}}$ $F_{1.16}=20$, $P<0.0001$; ANOVA main effect of repeated measures Δ^9-$THC_{5\ mg\ kg^{-1}}$ $F_{8.36}=3.36$, $P<0.01$; ANOVA main effect Δ^9-$THC_{7.5\ mg\ kg^{-1}}$ $F_{1.16}=19.25$, $P<0.0001$; ANOVA main effect of repeated measures Δ^9-$THC_{7.5\ mg\ kg^{-1}}$ $F_{8.36}=3.54$, $P<0.01$). $P<0.05$ *vs* controls (Student-Newman-Keuls test) 80 min after Δ^9-tetrahydrocannabinol. Data are expressed as percentage (mean±s.e.m.; $n=5$) of the baseline concentration. Basal values of extracellular acetylcholine concentration, prior to drug administration, were: 1.38±0.13 fmol μl^{-1} for control group and 1.27±0.24, 1.34±0.18, 1.16±0.12 and 1.42±0.32 fmol μl^{-1} for the different groups treated with Δ^9-THC at the doses of 1, 2.5, 5 and 7.5 mg kg^{-1}, respectively. A Δ^9-tetrahydrocannabinol dose of 1 mg kg^{-1} had no significant effect on hippocampal acetylcholine concentration. s.e. values were not more than ±17.86%.

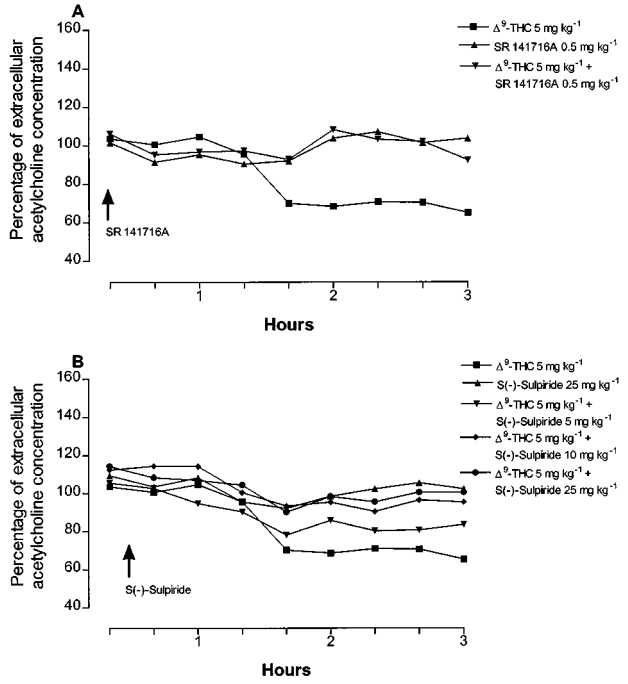

Figure 2 Time-course for the antagonism by SR141716A (A) and S(−)-sulpiride (B) of Δ^9-tetrahydrocannabinol-induced reduction of hippocampal extracellular acetylcholine concentration. (ANOVA main effect Δ^9-THC + SR 141716A $F_{1.16}=5.35$, $P<0.05$; ANOVA main effect Δ^9-THC + S(−)-sulpiride $_{5\ mg\ kg^{-1}}$ $F_{1.16}=0.42$, $P=0.52$; ANOVA main effect Δ^9-THC + S(−)-sulpiride $_{10\ mg\ kg^{-1}}$ $F_{1.16}=5.83$, $P<0.05$; ANOVA main effect Δ^9-THC + S(−)-sulpiride $_{25\ mg\ kg^{-1}}$ $F_{1.16}=6.97$, $P<0.05$). $P<0.05$ *vs* Δ^9-THC 5 mg kg^{-1} (Student-Newman-Keuls test). Data are expressed as percentage (mean± s.e.mean; $n=5$) of the baseline concentration. Basal values of extracellular acetylcholine concentration, prior to drug administration, were: 1.22±0.11, 1.32±0.18 and 1.41±0.23 for Δ^9-THC, SR 141716A and S(−)-sulpiride groups, respectively and 1.25±0.21, 1.23±0.23, 1.45±0.22 fmol μl^{-1} for the different groups treated with Δ^9-THC + S(−)-sulpiride at the doses of 5, 10 and 5 mg kg^{-1}, respectively. SR141716A and S(−)-sulpiride were given 20 and 30 min after Δ^9-tetrahydrocannabinol, respectively. SR141716A and S(−)-sulpiride given alone had no effect on acetylcholine release. s.e. values were not more than ±19.32%.

Upon completion of the experiments, each rat was sacrificed and the location of the probe verified histologically. Only data from rats with a proper location of the probe were used. Microdialysis perfusion was performed 24 h after probe implantation. The probe was perfused at a constant rate of 2 μl min^{-1} with a Ringer solution containing (mM): KCl, 4.0; NaCl, 147; $CaCl_2$, 1.5; pH 6.5. Neostigmine bromide was added at a final concentration of 10^{-7} mM in order to recover detectable concentrations of dialysate acetylcholine. Samples were collected every 20 min, corresponding to a volume of 40 μl, and were injected in a high-performance liquid chromatography (HPLC) system with electrochemical detection according to the technique described by Damsma & Westernik (1991). The detection limit for acetylcholine was 0.05 fmol per 1 μl of the sample. The average concentration of acetylcholine in the last three pre-drug samples was taken as 100% and all subsequent post-treatment values are expressed as a per cent (mean ± s.e.mean) of basal values.

Delayed alternation task in the T-maze

Working memory was evaluated in a standard T-maze made of black plexiglass and consisting of a central stem (height 10, length 40 and width 20 cm) with a start compartment (the first 20 cm of the stem) and two arms (height 10, length 60 and width 20 cm) with a wire mesh floor. The start compartment and each goal arm were separated from the distal part of the central stem by a guillotine door. Complete entrance into the goal arm was necessary in order to reach the food cup. The T-maze was located in a silent and dimly illuminated room and a weak light (25 W) was located 30 cm over the right-hand food cup, to distinguish the right from the left arm by brightness.

Training and testing of the animals were performed according to the method described previously by Murphy *et al.* (1996).

The animals were housed individually for 7 days before starting the training session in a plexiglass chamber (height

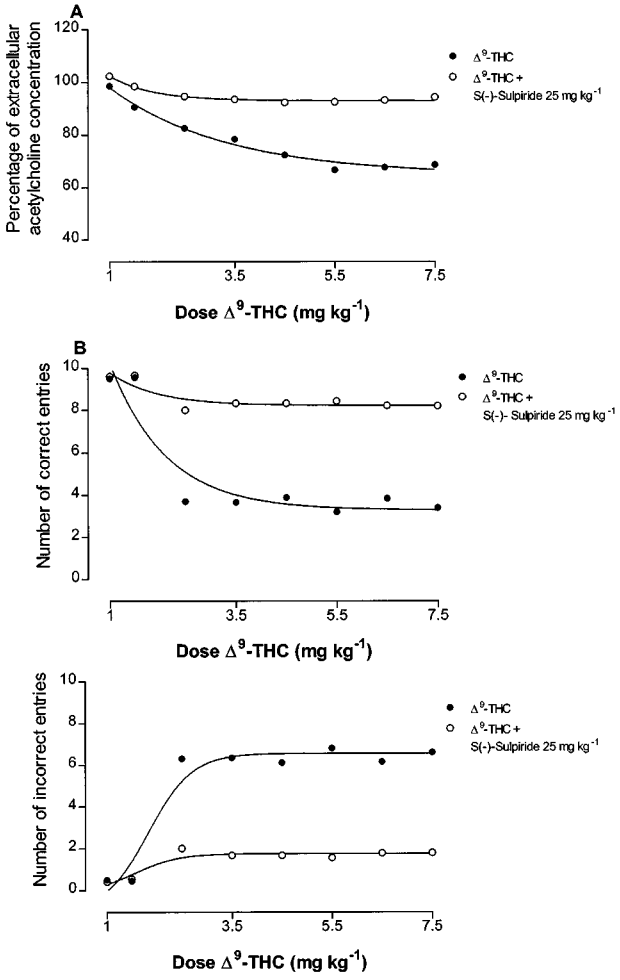

Figure 3 Effects on percentage of hippocampal extracellular acetylcholine concentration (A) (120 min after Δ^9-tetrahydrocannabinol administration) and on correct and incorrect entries in T-maze (B) (60 min after Δ^9-tetrahydrocannabinol administration) as a function of dose of Δ^9-tetrahydrocannabinol alone or in combination with S(−)-sulpiride (25 mg kg^{-1}). Each point represents the mean ± s.e.mean of five animals. s.e. values were not more than ±8.40%.

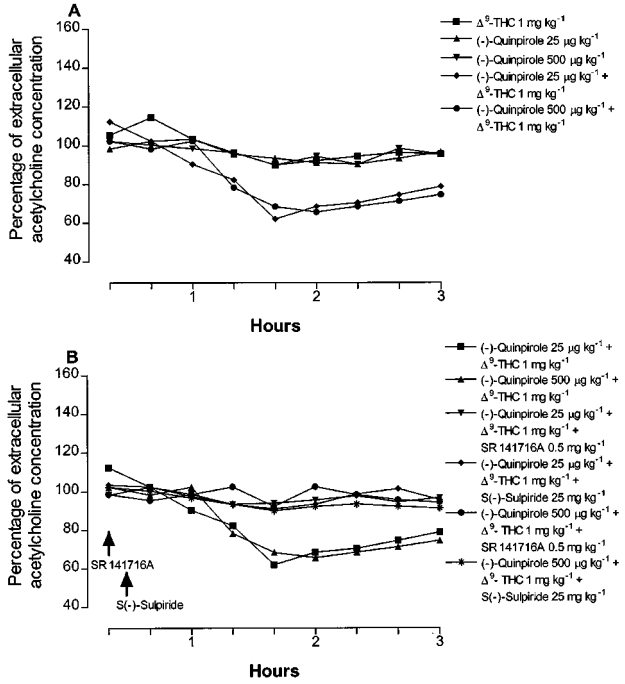

Figure 4 Time course for the potentiation by (−)-quinpirole on Δ^9-tetrahydrocannabinol-effect on hippocampal acetylcholine release (A) and reversal by SR141716A or S(−)-sulpiride (B). (ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC $F_{1.16}=7.23$, $P<0.05$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC $F_{1.16}=9.44$, $P<0.01$; ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC + SR141716A $F_{1.16}=6.65$, $P<0.05$; ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC + S(−)-sulpiride $F_{1.16}=9.32$, $P<0.01$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC + SR141716A $F_{1.16}=20.57$, $P<0.0001$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC + S(−)-sulpiride $F_{1.16}=6.63$, $P<0.01$). In (A) $P<0.05$ *vs* Δ^9-THC 1 mg kg^{-1} and in (B) $P<0.05$ *vs* (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC or (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC (Student-Newman-Keuls test). Data are expressed as percentage (mean ± s.e.mean; $n=5$) of the baseline concentration. Basal values of extracellular acetylcholine concentration, prior to drug administration, were: 1.34 ± 0.15 and 1.26 ± 0.22 fmol μl^{-1} for groups treated with (−)-quinpirole at the doses of 25 and 500 μg kg^{-1}, respectively, 1.24 ± 0.12 fmol μl^{-1} for Δ^9-THC group, 1.30 ± 0.17 and 1.43 ± 0.13 fmol μl^{-1} for groups treated with (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC and (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC, respectively, 1.15 ± 0.09 and 1.36 ± 0.18 fmol μl^{-1} for groups treated with (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC + SR141716A and (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC + S(−)-sulpiride 25 mg kg^{-1}, respectively, 1.25 ± 0.16 and 1.24 ± 0.11 fmol μl^{-1} for groups treated with (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC + SR141716A and (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC + S(−)-sulpiride, respectively. SR141716A and S(−)-sulpiride were given 20 and 30 min after Δ^9-tetrahydrocannabinol, respectively. (−)-Quinpirole and Δ^9-tetrahydrocannabinol given alone had no significant effect on acetylcholine concentration. s.e. values were not more than ±9.80%.

15, length 40 and width 25 cm) at 22±1°C and 55% humidity. To increase their motivation for food, animals were maintained at 85% of their pre-experimental body weight by feeding them a limited amount of chow following the daily session. No rat experienced weight loss during the experiment. Water was always available in the home cage.

Each training session consisted of 11 consecutive trials in which the rats had to alternate between the right and left arm of the maze in order to obtain their reward consisting of a shelled sunflower seed. During the first trial of each session, access to one of the two arms was blocked forcing the rat to enter the opposite arm. The direction of the forced trial was alternated daily. In each of the consecutive 10 trials, the food was placed in the opposite arm to that visited in the previous successful trial, with both arms being unblocked (free-choice trials). A correct trial ended with the rat eating the food. An incorrect trial ended with the rat reaching the empty food cup. If the rat did not enter an arm within 2 min, the trial was not counted and the rat was given another attempt. After each trial, the rat was removed from the goal arm and kept in the start compartment for a delay period after which the door of central stem was opened. The criteria defining a correct daily training session was nine successful trials out of 10 choice trials for 3 consecutive days. Three different delay periods were used, ranging at 2, 8, and 16 s. In the delay period of 2 s the rats' performance was stabilized at 10 correct entries (or 0 incorrect entries) in the T-maze on 10 trials, while during the delay period of 8 and 16 s performance was stabilized at nine and seven correct entries (or one and three incorrect entries) in the T-maze on 10 trials, respectively. All drugs were injected after animals had attained the above criteria. Data collected from each session were analysed in terms of number of correct and incorrect entries in the T-maze on 10 trials.

In the working memory experiments we used a cross over design. In particular, in order to reduce inter-group variables, for each different time we used the same group of animals tested after a washout period of 1 week during which they received no drug and re-established their optimal performance.

Drugs

Δ^9-Tetrahydrocannabinol (RBI, Italy) solutions were prepared from vials containing 10 mg of the drug in 1 ml of absolute ethanol. Vials were evaporated under nitrogen and the residue dissolved in two drops of Tween 80 and then diluted in saline. The specific CB_1 cannabinoid receptor antagonist SR 141617A (Sanofi Recherche, Montpellier, France) was dissolved in two drops of Tween 80 and then diluted in saline.

Δ^9-Tetrahydrocannabinol, SR 141617A and S(−)-sulpiride were administered intraperitoneally (i.p.) in a volume of 3 ml kg^{-1}, while (−)-quinpirole hydrochloride was given subcutaneously (s.c.) in a volume of 2 ml kg^{-1}. Control rats were treated with the vehicle used to dissolve the active ingredient.

Statistical analysis

Between-group comparisons were assessed by a two-way analysis of variance (ANOVA) for repeated measures. *Post-hoc* comparisons were performed by Student-Newman-Keuls tests. Statistical significance was reached at $P<0.05$.

Results

Hippocampal extracellular acetylcholine concentration

In agreement with previous results (Carta *et al*., 1998; Gessa *et al*., 1997; 1998a), the administration of Δ^9-tetrahydrocannabinol at the dose of 2.5 and 5 mg kg^{-1} reduced, in a dose-related manner, hippocampal extracellular acetylcholine concentration (Figure 1). A significant inhibition appeared at 80 min after treatment, was maximal at 120 and 180 min, and was no longer present at 12 h. A higher dose of 7.5 mg kg^{-1} produced no further reduction (Figure 1). A Δ^9-tetrahydrocannabinol dose of 1 mg kg^{-1} had no significant effect (Figure 1).

The reduction of hippocampal extracellular acetylcholine concentration induced by Δ^9-tetrahydrocannabinol (5 mg kg^{-1}) was antagonized not only, as expected, by the

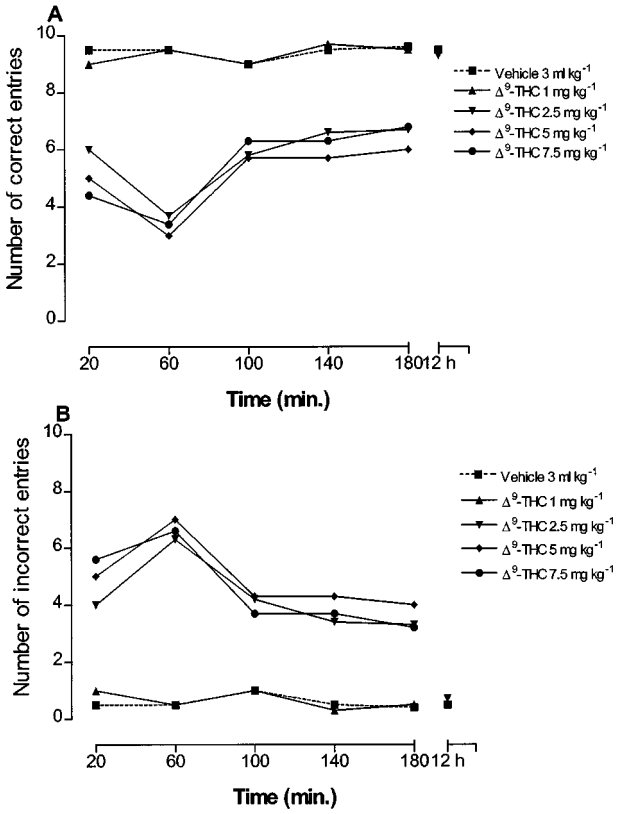

Figure 5 Time-course for the reduction of working memory after Δ^9-tetrahydrocannabinol administration (For the number of correct entries: ANOVA main effect Δ^9-$THC_{2.5\ mg\ kg^{-1}}$ $F_{1.8}=44.41$, $P<0.001$; ANOVA main effect of repeated measures Δ^9-$THC_{2.5\ mg\ kg^{-1}}$ $F_{4.20}=0.95$, $P=0.45$; ANOVA main effect Δ^9-$THC_{5\ mg\ kg^{-1}}$ $F_{1.8}=62.45$, $P<0.0001$; ANOVA main effect of repeated measures Δ^9-$THC_{5\ mg\ kg^{-1}}$ $F_{4.20}=0.93$, $P=0.46$; ANOVA main effect Δ^9-$THC_{7.5\ mg\ kg^{-1}}$ $F_{1.8}=36.62$, $P<0.001$; ANOVA main effect of repeated measures Δ^9-$THC_{7.5\ mg\ kg^{-1}}$ $F_{4.20}=1.06$, $P=0.40$; For the number of incorrect entries: ANOVA main effect Δ^9-$THC_{2.5\ mg\ kg^{-1}}$ $F_{1.8}=44.11$, $P<0.0001$; ANOVA main effect of repeated measures Δ^9-$THC_{2.5\ mg\ kg^{-1}}$ $F_{4.20}=0.95$, $P<0.45$; ANOVA main effect Δ^9-$THC_{5\ mg\ kg^{-1}}$ $F_{1.8}=58.87$, $P<0.0001$; ANOVA main effect of repeated measures Δ^9-$THC_{5\ mg\ kg^{-1}}$ $F_{4.20}=0.86$, $P=0.50$; ANOVA main effect Δ^9-$THC_{7.5\ mg\ kg^{-1}}$ $F_{1.8}=36.45$, $P<0.001$; ANOVA main effect of repeated measures Δ^9-$THC_{7.5\ mg\ kg^{-1}}$ $F_{4.20}=1.03$, $P=0.41$). Data are expressed as number of correct and incorrect entries (mean±s.e.mean; $n=5$) in T-maze on 10 trials. A Δ^9-tetrahydrocannabinol dose of 1 mg kg^{-1} had no effect on delayed alternation tasks in the T-maze. Intertrial delay was fixed at 8 s. s.e. values were not more than ±1.65%.

CB_1 cannabinoid receptor antagonist SR141716A (0.5 mg kg^{-1}), but also, dose-dependently, by the D_2 dopamine receptor antagonist S(−)-sulpiride (5, 10 and 25 mg kg^{-1}), (Figure 2). The latter also suppressed the inhibition of extracellular acetylcholine concentration induced by the effective Δ^9-tetrahydrocannabinol dose of 7.5 mg kg^{-1}, indicating a non competitive type of antagonism (Figure 3). Given alone, neither SR141716A (0.5 mg kg^{-1}) nor S(−)-sulpiride (25 mg kg^{-1}) modified hippocampal extracellular acetylcholine concentration (Figure 2). Conversely, administration of the D_2 dopamine receptor agonist (−)-quinpirole (25 μg kg^{-1}) markedly potentiated the effect of the ineffective Δ^9-tetrahydrocannabinol dose of 1 mg kg^{-1} (Figure 4). A higher dose of (−)-quinpirole (500 μg kg^{-1}) produced similar results (Figure 4). (−)-Quinpirole (25 and 500 μg kg^{-1}) given alone failed to modify extracellular acetylcholine concentration (Figure 4). Inhibition of hippocampal extracellular acetylcholine concentration induced by the combination of (−)-quinpirole (25 and 500 μg kg^{-1}) and Δ^9-tetrahydrocannabinol (1 mg kg^{-1}), was reversed by either SR 141716A (0.5 mg kg^{-1}) or S(−)-sulpiride (25 mg kg^{-1}) (Figure 4).

Working memory

In line with previous results (Jentsch *et al*., 1997), administration of Δ^9-tetrahydrocannabinol at the dose of 2.5 and 5 mg kg^{-1} impaired delayed alternation tasks in the T-maze (Figure 5). Following a dose of 2.5 mg kg^{-1}, a significant inhibition of working memory was observed at 20 min after treatment, was maximal at 60 min, persisted over 180 min and disappeared at 12 h (Figure 5). A higher dose of 7.5 mg kg^{-1} produced no further memory impairment (Figure 5). A Δ^9-tetrahydrocannabinol dose of 1 mg kg^{-1} had no effect on working memory (Figure 5). As shown in Figure 6, memory impairment induced by Δ^9-tetrahydrocannabinol (5 mg kg^{-1}) was antagonized not only, as expected, by the CB_1 cannabinoid receptor antagonist SR141716A (0.5 mg kg^{-1}) but also, unexpectedly, and dose-dependently, by the D_2 dopamine receptor antagonist S(−)-sulpiride (5, 10 and 25 mg kg^{-1}). The latter also suppressed the reduction of working memory induced by the effective Δ^9-tetrahydrocannabinol dose of 7.5 mg kg^{-1} (Figure 3). At the doses used, neither SR141716A (0.5 mg kg^{-1}) nor S(−)-sulpiride (25 mg kg^{-1}), given alone, modified working memory (Figure 6). Conversely, administration of the D_2 dopamine receptor agonist (−)-quinpirole (25 μg kg^{-1}) markedly potentiated the effect of an ineffective Δ^9-tetrahydrocannabinol dose of 1 mg kg^{-1} (Figure 7). A higher dose of (−)-quinpirole (500 μg kg^{-1}) produced similar results (Figure 7). (−)-Quinpirole (25 and 500 μg kg^{-1}) given alone failed to modify working memory (Figure 7). Finally, memory impairment produced by the combination of (−)-quinpirole (25 and 500 μg kg^{-1}) and Δ^9-tetrahydrocannabinol (1 mg kg^{-1}) was totally suppressed by either SR141716A (0.5 mg kg^{-1}) or

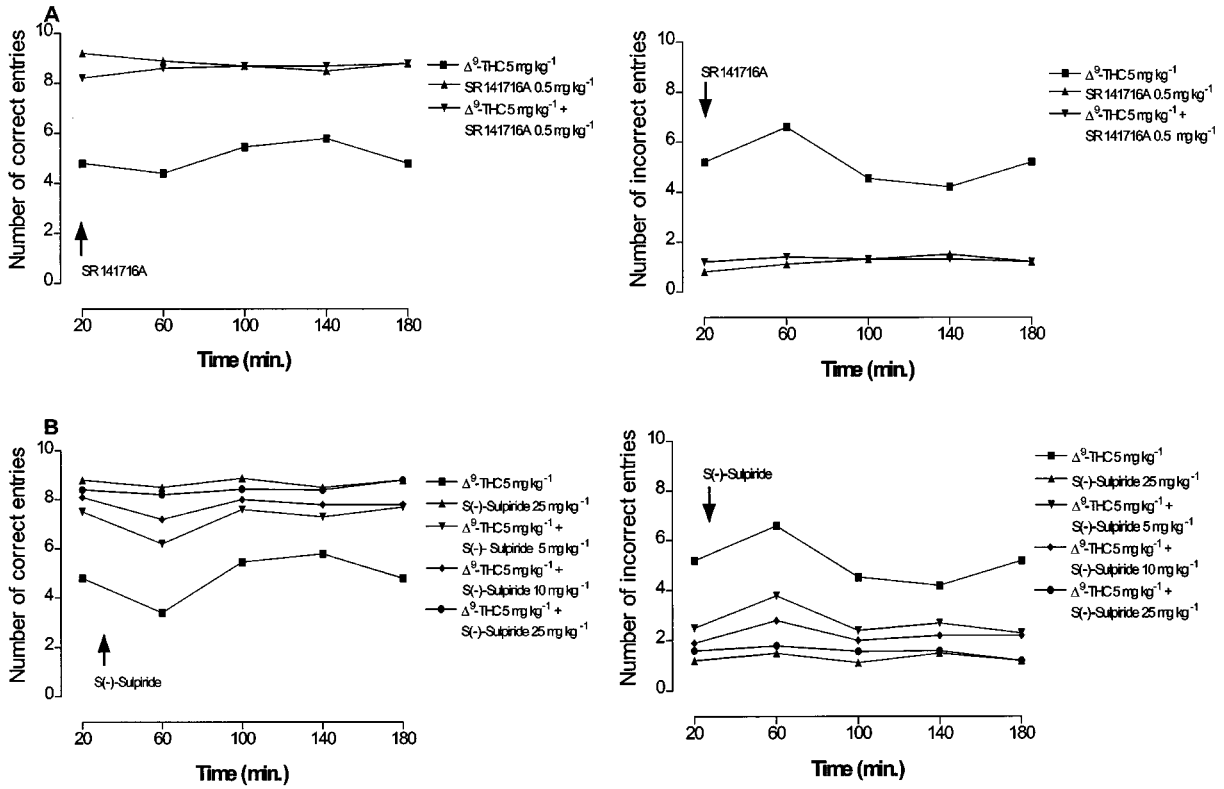

Figure 6 Time course for the antagonism by SR 141716A (A) and S(−)-sulpiride (B) of Δ^9-tetrahydrocannabinol-induced working memory impairment. (For the number of correct entries: ANOVA main effect Δ^9-THC + SR 141716A $F_{1.8} = 78.53$, $P < 0.0001$; ANOVA main effect Δ^9-THC + S(−)-sulpiride$_{5\ mg\ kg^{-1}}$ $F_{1.8} = 24.09$, $P < 0.002$; ANOVA main effect Δ^9-THC + S(−)-sulpiride$_{10\ mg\ kg^{-1}}$ $F_{1.8} = 34.41$, $P < 0.0005$; ANOVA main effect Δ^9-THC + S(−)-sulpiride$_{25\ mg\ kg^{-1}}$ $F_{1.8} = 73.14$, $P < 0.0001$; For the number of incorrect entries: ANOVA main effect Δ^9-THC + SR 141716A $F_{1.8} = 47.78$, $P < 0.0001$; ANOVA main effect Δ^9-THC + S(−)-sulpiride$_{5\ mg\ kg^{-1}}$ $F_{1.8} = 25.77$, $P < 0.0001$; ANOVA main effect Δ^9-THC + S(−)-sulpiride$_{10\ mg\ kg^{-1}}$ $F_{1.8} = 47.53$, $P < 0.0001$; ANOVA main effect Δ^9-THC + S(−)-sulpiride$_{25\ mg\ kg^{-1}}$ $F_{1.8} = 16.56$, $P < 0.004$). $P < 0.05$ *vs* Δ^9-THC 5 mg kg^{-1} (Student-Newman-Keuls test). Data are expressed as number of correct and incorrect entries (means ± s.e.mean; $n = 5$) in T-maze on 10 trials. SR141716A and S(−)-sulpiride were given 20 and 30 min after Δ^9-tetrahydrocannabinol, respectively. SR141716A and S(−)-sulpiride given alone had no effect on working memory. Intertrial delay was fixed at 8 s. s.e. values were not more than ±1.60%.

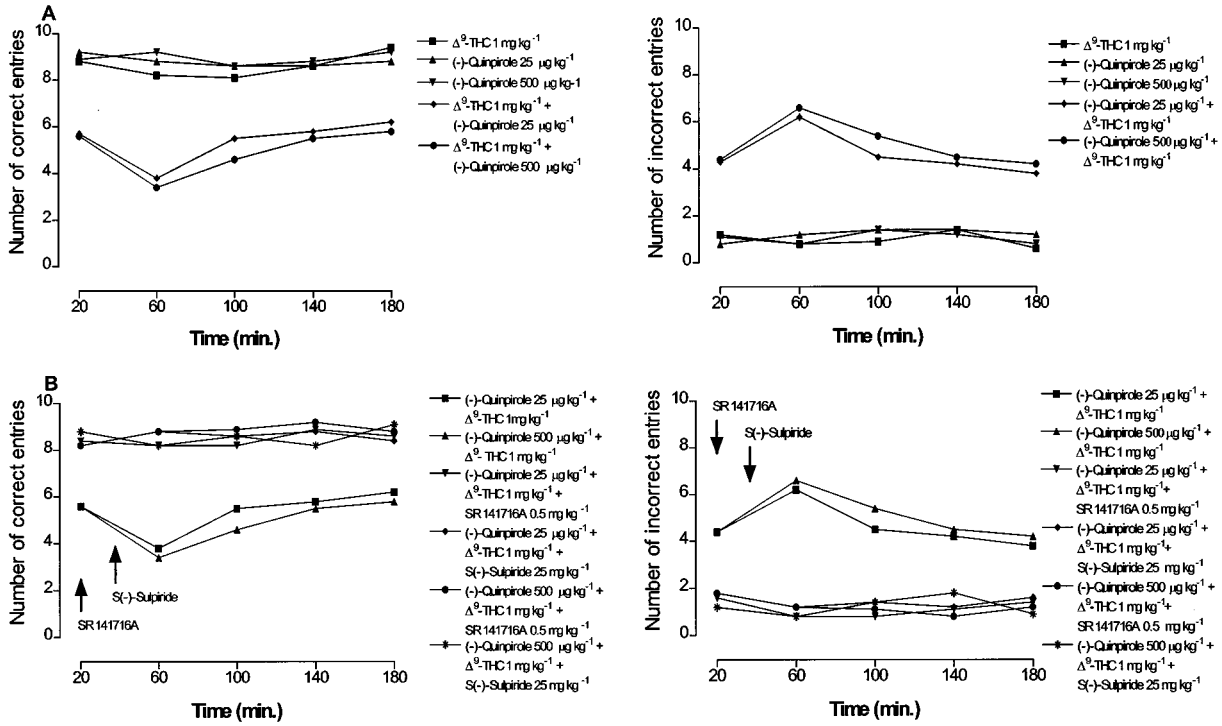

Figure 7 Time course for the potentiation by (−)-quinpirole on Δ^9-tetrahydrocannabinol effect on working memory (A) and reversal by SR 141716A or S(−)-sulpiride (B). (For number of correct entries: ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC $F_{1.8}=46.91$, $P<0.001$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC $F_{1.8}=53.75$, $P<0.0001$; ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC + SR 141716A $F_{1.8}=50.28$, $P<0.0001$; ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC + S(−)-sulpiride $F_{1.8}=55.41$, $P<0.0001$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC + SR 141716A $F_{1.8}=65.87$, $P<0.001$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC + S(−)-sulpiride $F_{1.8}=58.24$, $P<0.0001$; For number of incorrect entries: ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC $F_{1.8}=28.42$, $P<0.0001$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC $F_{1.8}=16.95$, $P<0.01$; ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC + SR 141716A $F_{1.8}=59.25$, $P<0.0001$; ANOVA main effect (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC + S(−)-sulpiride $F_{1.8}=49.43$, $P<0.0001$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC + SR 141716A $F_{1.8}=63.31$, $P<0.0001$; ANOVA main effect (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC + S(−)-sulpiride $F_{1.8}=61.47$, $P<0.0001$). In panel A $P<0.05$ *vs* Δ^9-THC 1 mg kg^{-1} and in panel B $P<0.05$ *vs* (−)-quinpirole 25 μg kg^{-1} + Δ^9-THC or (−)-quinpirole 500 μg kg^{-1} + Δ^9-THC (Student-Newman Keuls test). Data are expressed as number of correct and incorrect entries (means ± s.e.mean; $n=5$) in the T-maze on 10 trials. SR141716A and S(−)-sulpiride were given 20 and 30 min after Δ^9-tetrahydrocannabinol, respectively. (−)-Quinpirole and Δ^9-tetrahydrocannabinol given alone had no significant effect on working memory. s.e. values were not more than ±2.30%.

S(−)-sulpiride (25 mg kg^{-1}) (Figure 7). The administration of all drugs produced a significant effect on delayed response performance (Figure 8).

Discussion

These results confirm previous studies showing that Δ^9-tetrahydrocannabinol impairs working memory (Lichtman *et al*., 1995; Lichtman & Martin, 1996; Mallet & Beninger, 1996) and inhibits hippocampal extracellular acetylcholine concentration (Gessa *et al*., 1997; 1998a; Carta *et al*., 1998) through the activation of CB_1 cannabinoid receptors.

The major outcome of this study is that both Δ^9-tetrahydrocannabinol effects are antagonized by the D_2 dopamine receptor antagonist S(−)-sulpiride and potentiated by the D_2 dopamine receptor agonist (−)-quinpirole.

The results confirm previous studies indicating that Δ^9-tetrahydrocannabinol effects on the dopamine system are very complex. Several works have demonstrated that the increase in dopamine release induced by Δ^9-tetrahydrocannabinol is strain (Chen *et al*., 1991) and brain area dependent (Chen *et al*., 1993). Conversely, other studies have shown that striatal dopamine release is unaffected by Δ^9-tetrahydrocannabinol (Castaneda *et al*., 1991). However, our results indicate that memory impairment and reduction of extracellular acetylcholine concentration are produced by the concomitant activation of both the CB_1 cannabinoid and the D_2 dopamine receptors, the latter most likely being activated by endogenous dopamine released following Δ^9-tetrahydrocannabinol administration. In fact, stimulation of either receptors alone would be insufficient to produce similar effects. Our findings are also in accord with our recent results showing that analgesic (Carta *et al*., 1999) and hypothermic (Nava *et al*., 2000) effects induced by Δ^9-tetrahydrocannabinol are potentiated by (−)-quinpirole and (+)-bromocriptine and reversed by S(−)-sulpiride and S(−)-raclopride.

The mechanism underlying how the D_2 dopamine receptor stimulation enables the onset of Δ^9-tetrahydrocannabinol effects is not clear. Since CB_1 cannabinoid and D_2 dopamine receptors are both coupled to adenylate cyclase *via* a pertussis toxin-sensitive G-protein (Sibley & Monsma, 1992; Pertwee, 1997) and may be co-localized in the same brain areas (Sibley & Monsma, 1992; Matsuda *et al*., 1993), we might suppose that the concomitant activation of both receptors produce a cellular degree of cyclic AMP inhibition enough for Δ^9-tetrahydrocannabinol effects to occur. In other words, the detrimental effects of Δ^9-tetrahydrocannabinol both on working memory and hippocampal extracellular acetylcholine concentration would be due to cellular cyclic AMP reduction within definite brain areas controlling cognitive processes. In line with this hypothesis, recent studies have shown that

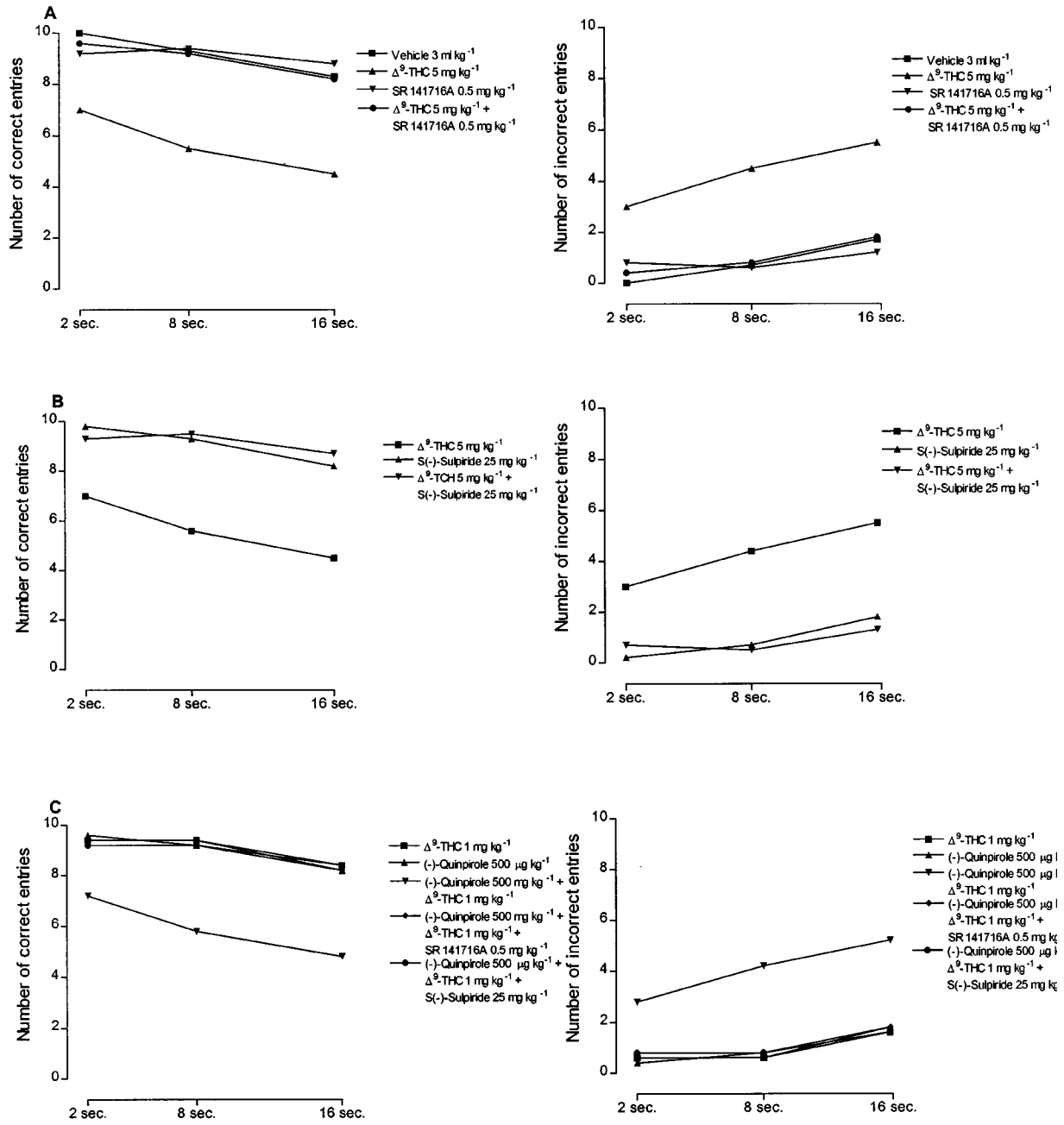

Figure 8 Effects of drugs treatment on delayed alternation tasks in T-maze for each of the three delay intervals used. The range delays were: 2, 8 and 16 s. Results represent the number of correct and incorrect entries (means ± s.e.mean; $n=5$) in the T-maze on 10 trials. Δ^9-tetrahydrocannabinol showed a significant effect on delayed response performance (For correct entries: ANOVA main effect $F_{1.4}=12.08$, $P<0.05$; For incorrect entries: ANOVA main effect $F_{1.4}=16.12$, $P<0.05$). At each delay SR141716A and S(−)-sulpiride reversed the Δ^9-tetrahydrocannabinol reduction of working memory, while (−)-quinpirole potentiated the inhibition of working memory induced by Δ^9-tetrahydrocannabinol (all statistical comparisons revealed significant with $P<0.001$). s.e. were not more than ±1.56%.

improvement of working memory function is produced in aged monkeys by perfusing the prefrontal cortex with low doses of the D_1 dopamine receptor agonists A77639 and SKF81297 (Cai & Arnsten, 1997); a treatment that should cause cellular cyclic AMP accumulation, since D_1 dopamine receptors are positively linked to adenylate cyclase (Monsma *et al.*, 1990). However, a recent observation by Glass & Felder (1997) might offer an alternative explanation for the mechanisms involved. These authors found in primary cultures of striatal neurons that the concomitant stimulation of CB_1 cannabinoid and D_2 dopamine receptors results in the accumulation of cellular cyclic AMP, in contrast to the decrease normally observed with activation of either receptors alone (Sibley & Monsma, 1992; Pertwee, 1997). In line with this hypothesis, we might suggest that *in vivo* Δ^9-tetrahydrocannabinol activating dopamine neurons (Diana *et al.*, 1998; Gessa *et al.*, 1998b) and probably releasing endogenous dopamine (Chen *et al.*, 1990a) might stimulate both CB_1 cannabinoid and D_2 dopamine receptors. The concurrent activation of both receptors might produce an accumulation of cellular cyclic AMP in neurons where these receptors are co-localized.

Our results leave the important issue concerning the correlation between extracellular acetylcholine concentration reduction and working memory impairment after Δ^9-tetrahydrocannabinol administration unresolved. The failure of physostigmine, a cholinesterase inhibitor, to improve Δ^9-tetrahydrocannabinol cognitive deficits (Lichtman & Martin, 1996) and the inability of SR141716A to prevent the

impairment of working memory induced by scopolamine (Lichtman & Martin, 1996), a muscarinic antagonist, suggest that the negative effects of cannabinoids on working memory are not directly mediated by the cholinergic system (Lichtman & Martin, 1996). Indeed, Childers & Deadwyler (1996) have shown that cannabinoids modulate conductance at a voltage-dependent K^+ channel in the hippocampus *via* a cyclic AMP dependent process without cholinergic neuronal mediation. These data suggest that cannabinoid and cholinergic systems do not affect memory through a common serial pathway. Moreover, several works have shown that cannabinoid ligands and endogenous cannabinoids can directly block the cellular processes associated with memory formation (Collins *et al*., 1995; Norwicky *et al*., 1987; Stella *et al*., 1997; Terranova *et al*., 1995). This evidence, including our results suggesting that memory loss occurred within 20 min after Δ^9-tetrahydrocannabinol administration, whereas the fall in acetylcholine concentration was observed up to 80 min after treatment, support the possibility that these two effects might be separated and controlled by different neurochemical systems, as suggested by Lichtman & Martin (1996). Conversely, the fact that the D_2 dopamine receptor antagonist, S(−)-sulpiride modified Δ^9-tetrahydrocannabinol-effects on hippocampal acetylcholine concentration and working memory might suggest that the two phenomena, although not directly correlated, may be controlled by similar mechanisms. On the other hand, several studies have demonstrated that a modulatory system such as the endogenous opiod system may control Δ^9-tetrahydrocannabinol effects on the dopamine system (Chen *et al*., 1990b; Tanda *et al*., 1997). Specifically, the opiod antagonist naloxone has been shown to prevent the increase of dopamine release induced by Δ^9-tetrahydrocannabinol in the shell of nucleus accumbens (Tanda *et al*., 1997). In light of this evidence, we may also suppose that the opiod endogenous system controls with an indirect mechanism with several steps the delayed effects of Δ^9-tetrahydrocannabinol on extracellular acetylcholine concentration. In other words, the cannabinoid and cholinergic systems may induce the inhibition of hippocampal acetylcholine concentration through the mediation of a third receptor system. This possibility may explain the delayed effects on inhibition of hippocampal acetylcholine concentration observed after Δ^9-tetrahydrocannabinol treatment. The above data, coupled with the evidence that cannabinoids inhibit the release of acetylcholine (Carta *et al*., 1999; Gessa *et al*., 1998a; Gifford *et al*., 1996; 1997), norepinephrine (Schlicker *et al*., 1997) and glutamate (Shen *et al*., 1996) from the hippocampus, suggest that the memory deficit induced by cannabinoids is a complex process involving different neurotransmitters.

The Δ^9-tetrahydrocannabinol effect on working memory is an on-off response. In fact, all effective Δ^9-tetrahydrocannabinol doses cause a similar degree of inhibition. In particular, the evidence showing that the deficit of working memory is an on-off response, coupled with results showing that the reduction of extracellular acetylcholine concentration is a dose-related response suggests that the two phenomena are not directly correlated and that several mechanisms and/or neuronal circuits could be involved in both of the inhibitory effects induced by Δ^9-tetrahydrocannabinol. Specifically, the inhibition of acetylcholine concentration could represent only one aspect of the detrimental effects induced by Δ^9-tetrahydrocannabinol, while the deficit found in working memory could be expression of a neurotransmission block into and within the hippocampus. This hypothesis could explain the on-off response observed in working memory deficit and at the same time the marked time course discrepancy between inhibition of acetylcholine concentration and loss of memory. Moreover, we may exclude that the on/off response observed in the inhibition of working memory induced by Δ^9-tetrahydrocannabinol is caused from deficits in locomotory activity or motivation. Indeed, several studies have excluded that the deficit of working memory induced by cannabinoids may be correlated to a possible reduction of food reinforcement or hunger (Lichtman *et al*., 1995; Lichtman & Martin, 1996). In fact, the sunflower seed at the end of each trial was always consumed whenever an arm was selected regardless of drug treatment. Moreover, the evidence showing that intrahippocampal administration of CP55,940 [1α,2β-(R)5α]-(−)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropil)cyclohexyl]-phenol, a synthetic cannabinoid agonist, impairs choice accuracy in radial maze without retarding the time required to complete the maze (Lichtman *et al*., 1995), indicates that the memory deficits are dissociated from locomotory activity.

The findings showing that D_2 dopamine receptor antagonists reverse, in a dose-dependent manner, hippocampal acetylcholine concentration and working memory deficit, are suggestive that D_2 dopamine receptors could exert a control on both Δ^9-tetrahydrocannabinol effects.

Previous results from our laboratory have shown that Δ^9-tetrahydrocannabinol reduces acetylcholine concentration in the prefrontal cortex as well as in the hippocampus (Gessa *et al*., 1998a) suggesting that similar mechanisms control cholinergic transmission in both areas. However, as for hippocampal extracellular acetylcholine concentration, further investigations are needed in order to clarify the correlation between acetylcholine reduction in the prefrontal cortex and memory impairment.

Irrespective of the mechanisms and brain areas involved, the finding that S(−)-sulpiride reverses Δ^9-tetrahydrocannabinol-induced amnesia suggests that D_2 dopamine receptor antagonists should be clinically tested as a potential treatment for memory deficits produced during marijuana intoxication. On the other hand, our results raise the relevant concern whether Δ^9-tetrahydrocannabinol-induced memory impairment might be potentiated by drugs of abuse, such as cocaine, amphetamine, alcohol and ecstasy which are all able to increase the release of endogenous dopamine and whether D_2 dopamine receptors play a permissive role in the pharmacological effects of cannabinoids besides cognition.

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