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Role of different brain structures in the behavioural expression of WIN 55,212-2 withdrawal in mice

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1 We have evaluated several responses induced by the cannabinoid agonist WIN 55,212-2 related to its addictive properties, including rewarding effects and the development of physical dependence in mice. Moreover, we have studied the specific involvement of several brain regions with high density of CB1 cannabinoid receptors, such as striatum, hippocampus, amygdala and cerebellum, in the behavioural expression of SR 141716A-precipitated WIN 55,212-2 withdrawal.

2 The systemic administration of the CB1 receptor antagonist SR 141716A (10 mg kg^{-1}, s.c.) precipitated behavioural signs of withdrawal in mice chronically treated with WIN 55,212-2 (1 and 2 mg kg^{-1}, intraperitoneal (i.p.)), revealing the development of physical dependence.

3 The microinjection of SR 141716A (1.5 and 3 μg) into the cerebellum induced severe manifestations of abstinence in mice dependent on WIN 55,212-2 (1 mg kg^{-1}, i.p.). Out of 10 signs evaluated, seven were statistically significant: wet dog shakes, body tremor, paw tremor, piloerection, mastication, genital licks and sniffing. When the cannabinoid antagonist was administered into the hippocampus and the amygdala, a moderate but significant withdrawal syndrome was also observed. However, no signs of abstinence were induced when SR 141716A was microinjected into the striatum.

4 WIN 55,212-2 produced rewarding effects in the place-conditioning paradigm in mice pre-exposed to a priming injection of the drug.

5 These results show a reliable behavioural model to reveal rewarding effects and physical dependence induced by the repeated administration of WIN 55,212-2 in mice. The cerebellum and to a lesser extent the hippocampus and the amygdala participate in the behavioural expression of cannabinoid withdrawal.

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Abbreviations: AC, adenylyl cyclase; CNS, central nervous system; CP 55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol; DMSO, dimethylsulphoxide; HU 210, *R*(−)-7-hydroxy-delta(6)-tetra-hydrocannabinol-dimethylheptyl; IP3, inositol triphosphate; MAPKs, mitogen-activated protein kinases; SR 141716A, (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4,-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxyamidehydrochloride); THC, delta9-tetrahydrocannabinol; WIN 55,212-2, ((*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]1-naphthyl)methanone)

Introduction

Molecular cloning of membrane receptors for *Cannabis Sativa* derivatives was a key step in the understanding of cannabinoid pharmacology. Two types of cannabinoid receptors have been identified so far: the CB1 receptor, mainly located in the central nervous system (CNS) (Matsuda *et al*., 1990), and the CB2 receptor, which has a predominant peripheral distribution in immune cells (Munro *et al*., 1993). Both receptor subtypes are coupled to $G_{i/o}$ proteins and their activation induces intracellular signalling events including inhibition of adenylyl cyclase (AC) activity, activation of mitogen-activated protein kinases (MAPKs), and changes in Ca^{2+} and K^+ currents (Wilson & Nicoll, 2002; Piomelli, 2003). Several natural (delta9-tetrahydrocannabinol, THC) and synthetic agonists (CP 55,940, HU 210, WIN 55,212-2) as well as endogenous ligands (anandamide, 2-arachidonyl glycerol, noladin ether and palmitoylethanolamide) that bind to cannabinoid receptors have been identified (Fride & Mechoulam, 2003). These compounds exhibit differences in their affinities and intrinsic activities for CB1 and CB2 receptors (Breivogel *et al*., 1998; Griffin *et al*., 1998). In this sense, WIN 55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzo xazin-6-yl]1-naphthyl)methanone), the most typical aminoalkylindole cannabinoid agonist, reveals a higher intrinsic activity at CB1 receptors than the natural agonist THC (Breivogel *et al*., 1998; Griffin *et al*., 1998; Howlett *et al*., 2002). Previous studies have shown that CB1 receptors mediate the main effects of cannabinoids in the CNS (Ledent *et al*., 1999). Thus, CB1 receptors are responsible for the effects of cannabinoids on nociceptive transmission, motor function, learning and memory processes, and drug addiction (Ameri, 1999; Ledent *et al*., 1999; Zimmer *et al*., 1999; Maldonado & Rodríguez de Fonseca, 2002; Martin *et al*., 2002). Behavioural responses related to the addictive properties of cannabinoids,

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including rewarding effects and the development of physical dependence, have been previously investigated. Rewarding effects of cannabinoids have been revealed in several behavioural paradigms such as conditioned place preference and self-administration procedures, although different results have been reported mainly depending on the experimental designs used (Takahashi & Singer, 1979; Sañudo-Peña *et al*., 1997; Martellotta *et al*., 1998; Cheer *et al*., 2000; Valjent & Maldonado, 2000; Braida *et al*., 2001; Fattore *et al*., 2001; Navarro *et al*., 2001; Justinova *et al*., 2003). Several studies have shown that the administration of SR 141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4,-dichlorophenyl)-4-methyl-1-*H*-pyrazole-3-carboxyamidehydrochloride), a selective CB1 receptor antagonist, precipitated a withdrawal syndrome in animals chronically treated with cannabinoids (Tsou *et al*., 1995; Hutcheson *et al*., 1998; Rubino *et al*., 1998). In rodents, this withdrawal syndrome is characterized by the presence of somatic signs such as wet dog shakes, head shakes, facial rubbing, front paw tremor, body tremor, ataxia, hunched posture, ptosis, piloerection, mastication, hypolocomotion and scratching, and the absence of vegetative manifestations. Additionally, several biochemical changes were also observed during cannabinoid withdrawal such as a compensatory upregulation in the AC pathway that selectively occurs in the cerebellum (Hutcheson *et al*., 1998; Tzavara *et al*., 2000), an enhancement in the release of corticotrophin-releasing factor (CRF) and c-Fos inmunoreactivity in the amygdala (Rodríguez de Fonseca *et al*., 1997), and a reduced dopaminergic transmission in the ventral striatum (Diana *et al*., 1998). Although these previous studies pointed out a role of several brain structures in cannabinoid withdrawal, the anatomical sites that mediate the somatic manifestations of physical dependence have not been identified.

Here, we have further investigated several pharmacological responses of WIN 55,212-2 related to its addictive properties. We have evaluated the rewarding properties of WIN 55,212-2 in the place-conditioning paradigm. We have developed a model of WIN 55,212-2 physical dependence in mice in order to investigate the neuroanatomical regions involved in the manifestation of somatic signs of cannabinoid withdrawal, with particular interest in those regions with high CB1 receptor density. For this purpose, the CB1 antagonist SR 141716A was directly microinjected into the striatum, hippocampus, amygdala and cerebellum of mice chronically treated with the cannabinoid agonist WIN 55,212-2.

Methods

Animals

Male CD1 mice (Charles River, France) weighing 26–30 g at the start of the study were housed grouped (five per cage) in a temperature ($21 \pm 1^{\circ}C$) and humidity ($55 \pm 10\%$) controlled room with a 12 h light–dark cycle (light between 0800 and 2000). Food and water were available *ad libitum*. Mice were habituated to their new environment for 1 week after arrival, before starting the experimental procedure. Behavioural tests and animal care were conducted in accordance with the Guidelines of The European Communities Council Directive 86/609/EEC regulating animal research and were approved by the Local Ethical Committee (CEEA-IMAS-UPF). The observer was blind to the treatment in all the experiments.

Place preference paradigm

An unbiased place-conditioning procedure was used to evaluate the rewarding properties of WIN 55,212-2, as previously reported (Matthes *et al*., 1996; Maldonado *et al*., 1997). During the preconditioning phase, drug-naive mice had free access to explore both compartments of the conditioning apparatus during 20 min. No initial place preference or aversion for the different compartments was observed. The conditioning phase consisted of five pairings with WIN 55,212-2 (0.1 and 1 mg kg^{-1}, intraperitoneal (i.p.)) (days 1, 3, 5, 7 and 9) and five pairings with vehicle (days 2, 4, 6, 8 and 10) during a 45 min conditioning time. Treatments were counterbalanced as closely as possible between compartments. Control animals received vehicle every day. The test phase was conducted exactly as the preconditioning one, that is, free access to both compartments during 20 min. In order to exclude the possible dysphoric effect of the first drug administration, two groups of mice received a single injection (priming) of WIN 55,212-2 (0.1 and 1 mg kg^{-1} respectively, i.p.) in their home cages 24 h before starting the conditioning procedure, as previously reported for THC place-conditioning experiments (Valjent & Maldonado, 2000). The time in the central area was proportionally shared and added to the time value of each compartment, as previously reported (Matthes *et al*., 1996; Valverde *et al*., 1996). A place-conditioning score was calculated for each mouse as the difference between the time spent in the drug-paired compartment during the testing and preconditioning phases.

Physical dependence induced by WIN 55,212-2 and evaluation of withdrawal syndrome after systemic administration of SR 141716A

The CB1 cannabinoid receptor agonist WIN 55,212-2 (1 and 2 mg kg^{-1}, i.p.) or its vehicle was administered twice daily during 5 days (0900 and 2000) in order to induce physical dependence in mice ($n = 10$–12 per group). On day six, mice only received the morning injection and 2 h later mice were placed in a circular plastic observation area for a 15 min period. At the end of this period, the CB1 cannabinoid receptor antagonist SR 141716A (10 mg kg^{-1}, s.c.) was administered to precipitate the withdrawal syndrome. The following abstinence signs were evaluated, 15 min before and 30 min after SR 141716A injection: wet dog shake, ptosis, body tremor, ataxia, front paw tremor, piloerection, hunched posture, mastication, genital lick, cramp and sniffing. The number of wet dog shakes, front paw tremors, sniffings and cramps was counted. Ptosis, body tremor, ataxia, piloerection, hunched posture, mastication and genital licks were scored 1 for appearance and 0 for nonappearance within each 5 min time. A quantitative value was calculated in each animal for the different checked signs by adding the scores obtained in each 5 min period. A global withdrawal score, ranging from 0 to 100, was calculated for each animal by giving to each individual sign a relative weight, as previously reported (Valverde *et al*., 2000).

Physical dependence induced by WIN 55,212-2 and evaluation of withdrawal syndrome after intracerebral administration of SR 141716A

In this set of experiments, the participation of several brain structures in the somatic manifestations of WIN 55,212-2 withdrawal was examined. For this purpose, the CB1 cannabinoid receptor antagonist SR 141716A (0.75, 1.5 and 3 μg per mouse) was directly administered into the brain of WIN 55,212-2-dependent mice (1 mg kg^{-1}, i.p., twice daily, 5 days). Mice were first anaesthetized with a ketamine/xylazine mixture and subsequently mounted in a stereotaxic frame (KOPF Instruments, Tujunga, CA, U.S.A.). They were implanted with guide cannuli (7 mm long, 30 gauge) into different brain regions: third ventricle (i.c.v.), striatum, hippocampus, amygdala and cerebellum. The coordinates (expressed in mm) were taken from bregma and the skull surface according to the stereotaxic atlas (Paxinos & Franklin, 1997). Mice were implanted unilaterally into the third ventricle (AP: −0.5; L: 0; H: −3) ($n=17$) and cerebellum (AP: −7.0; L: 0; H: −1.7) ($n=19$), and bilaterally into the striatum (AP: +0.38; L: ±2.0; H: −4.0) ($n=19$), hippocampus (AP: −1.7; ML: ±1.75, DV: −2.1) ($n=10$) and amygdala (AP: −1.46; ML: ±2.80, DV: −4.80) ($n=13$). Cannuli were subsequently fixed to the skull with dental cement. After surgery, mice were individually housed for recovering during 4 days. After this period, mice were chronically treated with WIN 55,212-2 (1 mg kg^{-1}, i.p., twice daily, 5 days), as described above. Following the induction of WIN 55,212-2 dependence, a withdrawal syndrome was precipitated by the administration of different doses of SR (0.75, 1.5 and 3 μg). At 2 h after each morning injection of WIN 55,212-2, the withdrawal syndrome was precipitated by the administration of SR 141716A or its vehicle (first) through the cannuli in an injection volume of 2 μl (animals implanted bilaterally received 1 μl through each cannula). Each animal received the different doses of SR 141716A in consecutive days by using a Latin Square design. In order to verify that the withdrawal syndrome was not conditioned to the experimental context, the last day, mice received the morning injection of WIN 55,212-2, and 2 h later they were observed for withdrawal signs after the intracerebral administration of vehicle (second) (Figure 4, Table 1). During the different days of withdrawal syndrome evaluation, mice received the chronic WIN 55,212-2 treatment (1 mg kg^{-1}, i.p., twice daily).

After completion of the experimental sequence, the histological verification of the cannuli was performed. Mice were killed, and the brains were removed and frozen. Coronal sections (40 μm) of the brain were cut on a cryostat at −26°C and the site of injection was compared to standard stereotaxic plates (Paxinos & Franklin, 1997) (Figure 3). Data from mice with wrong location of the cannuli were removed from the study.

Table 1 WIN 55,212-2 withdrawal precipitated by intracerebral administration of SR141716A

	Wet dog shakes	*Ptosis*	*Body tremor*	*Paw tremor*	*Piloerection*	*Hundred posture*	*Mastication*	*Genital licks*	*Sniffing*	*GWS*
i.c.v.										
Veh (first)	1.88±0.38	0.00±0.00	0.00±0.00	4.29±0.67	0.00±0.00	0.00±0.00	0.18±0.10	0.29±0.14	0.00±0.00	2.89±0.42
SR 1.5	2.47±0.67	1.06±0.33[a]	0.53±0.23	5.00±1.65	1.82±0.44[b]	0.00±0.00	1.00±0.28[a]	0.88±0.24	0.00±0.00	7.78±0.91[b]
SR 3	2.35±0.59	1.41±0.51[b]	2.65±0.59[b]	6.18±0.98	3.06±0.56[b]	0.12±0.12	1.41±0.33[b]	0.47±0.15	0.00±0.00	11.62±1.20[b]
Veh (second)	1.41±0.33	0.00±0.00	0.00±0.00	4.18±0.90	0.24±0.18	0.00±0.00	0.06±0.06	0.47±0.17	0.00±0.00	2.92±0.46
Cerebellum										
Veh (first)	2.06±0.42	0.00±0.00	0.12±0.12	7.00±1.06	0.18±0.18	0.06±0.06	0.18±0.13	0.53±0.12	0.12±0.08	5.12±0.77
SR 0.75	2.68±0.45	0.05±0.05	0.00±0.00	3.68±0.58	0.00±0.00	0.00±0.00	0.00±0.00	0.21±0.10	0.00±0.00	2.85±0.37
SR 1.5	6.25±0.80[b]	0.00±0.00	0.50±0.33	17.25±2.54[b]	0.00±0.00	0.13±0.13	1.63±0.42[b]	0.75±0.16	1.75±0.73[a]	12.80±1.52[b]
SR 3	6.13±1.61[b]	0.00±0.00	0.13±0.52[b]	24.25±3.71[b]	0.88±0.44[b]	0.13±0.13	3.63±0.53[b]	1.38±0.32[b]	4.50±1.27[b]	20.36±2.40[b]
Veh (second)	2.47±0.36	0.00±0.00	0.05±0.05	3.16±0.74	0.00±0.00	0.00±0.00	0.11±0.07	0.47±0.12	0.26±0.13	2.93±0.41
Striatum										
Veh (first)	1.42±0.32	0.26±0.15	0.00±0.00	3.21±0.94	0.00±0.00	0.00±0.00	0.11±0.07	0.42±0.14	0.05±0.05	2.67±0.54
SR 1.5	1.20±0.30	0.00±0.00[a]	0.00±0.00	1.80±0.39	0.00±0.00	0.00±0.00	0.00±0.00	0.30±0.11	0.00±0.00	1.43±0.29
SR 3	1.05±0.43	0.00±0.00[a]	0.00±0.00	2.70±0.76	0.00±0.00	0.00±0.00	0.10±0.07	0.25±0.12	0.00±0.00	2.06±0.52
Veh (second)	1.17±0.42	0.00±0.00[a]	0.00±0.00	2.33±0.65	0.00±0.00	0.00±0.00	0.00±0.00	0.44±0.15	0.00±0.00	1.80±0.41
Hippocampus										
Veh (first)	1.50±0.60	1.92±0.47	2.25±0.37	3.92±0.82	3.50±0.62	0.00±0.00	0.75±0.35	0.50±0.15	0.08±0.08	10.26±1.08
SR 1.5	2.83±0.97	2.58±0.48	3.83±0.60	7.17±2.40	3.83±0.69	0.00±0.00	1.83±0.44	1.00±0.25	0.17±0.11	15.84±1.92[a]
SR 3	5.00±1.33	2.60±0.56	3.30±0.60	10.00±1.64	5.50±0.22[a]	0.00±0.00	2.00±0.33[a]	1.00±0.30	1.20±0.00	19.61±1.31[b]
Veh (second)	2.33±0.62	1.22±0.52	0.89±0.39	6.11±1.64	2.11±0.56	0.00±0.00	0.33±0.17	0.44±0.18	0.00±0.00	7.92±1.25
Amygdala										
Veh (first)	2.46±0.68	1.31±0.41	2.00±0.52	5.15±1.30	2.69±0.61	0.08±0.08	0.31±0.17	0.15±0.10	0.15±0.10	8.99±1.18
SR 1.5	3.62±0.98	1.23±0.38	2.46±0.61	7.92±2.18	3.46±0.63	0.00±0.00	0.92±0.31	0.31±0.13	0.15±0.10	12.22±1.99
SR 3	5.54±1.29	2.00±0.57	3.15±0.63	9.85±2.40	3.85±0.55	0.31±0.13	1.54±0.30[a]	0.54±0.14	0.46±0.31	16.24±1.58[b]
Veh (second)	2.54±0.64	0.92±0.54	1.85±0.59	5.85±1.48	2.77±0.61	0.00±0.00	0.38±0.14	0.15±0.10	0.00±0.00	8.92±1.43

Individual signs of withdrawal and global withdrawal score (GWS) in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}, i.p.) after the microinjection of SR 141716A (0.75, 1.5 and 3 μg 2 μl^{-1}) into the third ventricle (i.c.v.), cerebellum, striatum, hippocampus and amygdala (see Methods). Data are expressed as mean±s.e.m.
[a]$P<0.05$ vs Veh (first) group (*post hoc* Dunnett).
[b]$P<0.01$ vs Veh (first) group (*post hoc* Dunnett).

Drugs

R-(+)-WIN 55,212-2 mesylate salt (Sigma Chemical Co., Madrid, Spain) was dissolved in a solution consisting of ethanol/cremophor EL (Sigma Chemical Co., Madrid, Spain)/distilled water (1 : 1 : 8) and was administered by i.p. route in a volume of 10 ml kg^{-1} body weight. The selective CB1 cannabinoid receptor antagonist SR 141716A was kindly provided by Sanofi (Montpellier, France). SR 141716A was dissolved in a solution consisting of ethanol/cremophor EL/distilled water (1 : 1 : 8) and was injected in a volume of 20 ml kg^{-1} body weight (s.c.). For intracerebral administration, SR 141716A was dissolved in 5% dimethylsulphoxide (DMSO) (Scharlau Chemie S.A., Barcelona, Spain) and the injection volume was 2 μl. Ketamine chlorhydrate (100 mg kg^{-1}) (Imalgène 1000®, Rhône Mérieux, Lyon, France) and xylazine hydrochloride (20 mg kg^{-1}) (Sigma Chemical Co., Madrid, Spain) were mixed and dissolved in ethanol and distilled water (1 : 9). The anaesthetic mixture was administered in an injection volume of 20 ml kg^{-1} body weight (i.p.).

Statistical analysis

Results from the conditioned place preference paradigm were analysed using a two-way ANOVA with priming and treatment as factors between subjects followed by one-way ANOVAs and the Dunnett test as *post hoc* analysis. Data from physical dependence studies were analysed by using a one-way ANOVA between subjects followed by the Dunnett test after significant main effects. The level of significance was $P<0.05$ in all experiments.

Results

WIN 55,212-2-induced conditioned place preference

The motivational responses induced by WIN 55,212-2 (0.1 and 1 mg kg^{-1}) administration were explored by using the place-conditioning paradigm. As shown in Figure 1, WIN 55,212-2 (0.1 mg kg^{-1}) induced rewarding effects only when animals were pretreated with a priming injection of the drug. Such a priming injection (WIN 55,212-2 at the dose of 0.1 mg kg^{-1}, i.p.) was given 24 h before the first conditioning session. In contrast, animals not receiving the WIN 55,212-2 pre-exposure did not exhibit any rewarding response in the place-conditioning paradigm. Thus, two-way ANOVA showed no treatment effect ($F(2,47)=3.031$; $P=0.058$), no priming effect ($F(1,47)=2.193$; NS), but a significant interaction between these two factors ($F(1,47)=4.888$; $P<0.05$). Subsequent, one-way ANOVA and *post hoc* analysis showed a significant preference for the compartment associated with WIN 55,212-2 in the group of mice receiving the dose of 0.1 mg kg^{-1} and preinjected with the same dose of this CB1 agonist, when compared to control mice ($P<0.01$) (Figure 1).

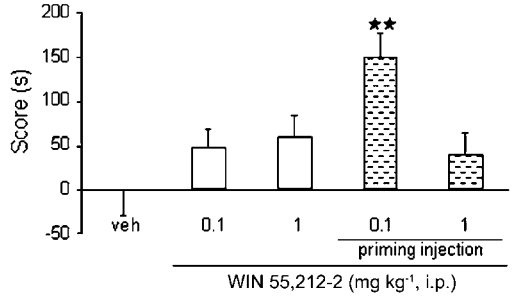

Figure 1 Rewarding properties of WIN 55,212-2 (0.1 and 1 mg kg^{-1}, i.p.) in the place-conditioning paradigm. Vertical axis represents the place preference score, calculated as the time spent in the conditioned compartment on the testing day minus the time in the same compartment on the preconditioning day. Data are expressed as mean ± s.e.m. ($n=10$–11 per group). ★★$P<0.01$, WIN 55,212-2-treated vs vehicle group (*post hoc* Dunnett).

WIN 55,212-2 withdrawal precipitated by systemic administration of SR 141716A

Mice were chronically treated with WIN 55,212-2 (1 and 2 mg kg^{-1}) twice daily during 5 days to induce physical dependence. On day six, a withdrawal syndrome was precipitated by the systemic administration of SR 141716A (10 mg kg^{-1}). Thus, SR 141716A challenge precipitated a variety of somatic signs of withdrawal in mice chronically treated with WIN 55,212-2 (Figure 2). One-way ANOVA revealed a significant effect of WIN 55,212-2 treatment for ataxia ($F(2,33)=18.066$; $P<0.01$), hunched posture ($F(2,33)=33.577$; $P<0.01$), mastication ($F(2,33)=36.175$; $P<0.01$), paw tremor ($F(2,33)=16.146$; $P<0.01$), piloerection ($F(2,33)=5.299$; $P<0.05$), body tremor ($F(2,33)=7.054$; $P<0.01$) and wet dog shakes ($F(2,33)=10.285$; $P<0.01$). The global withdrawal score calculated for each experimental group revealed a severe degree of abstinence in WIN 55,212-2-dependent mice ($F(2,33)=58.711$; $P<0.01$). Taking into account that the same severity of abstinence was obtained after the chronic treatment with both doses of WIN 55,212-2 (1 and 2 mg kg^{-1}), the lowest dose, 1 mg kg^{-1}, was chosen for the following experiments.

WIN 55,212-2 withdrawal following intracerebral administration of the CB1 receptor antagonist SR 141716A

Neuroanatomical distribution of the final injection site in each brain structure in the different animals is shown in Figure 3. The use of a within-subject design for the evaluation of cannabinoid withdrawal was validated since there were no significant differences between the symptoms observed after the first and second vehicle administrations (Figure 4, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the third ventricle

Intracerebroventricular (i.c.v.) administration of the CB1 receptor antagonist SR 141716A (1.5 and 3 μg) precipitated the expression of behavioural signs of withdrawal in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}). One-way ANOVA showed a significant effect for ptosis ($F(3,67)=5.700$; $P<0.01$), body tremor ($F(3,67)=15.692$; $P<0.01$), piloerection ($F(3,67)=15.325$; $P<0.01$) and mastication ($F(3,67)=8.333$; $P<0.01$). For the global withdrawal score, one-way ANOVA exhibited a significant response ($F(3,67)=26.780$; $P<0.01$) and *post hoc* analysis revealed a

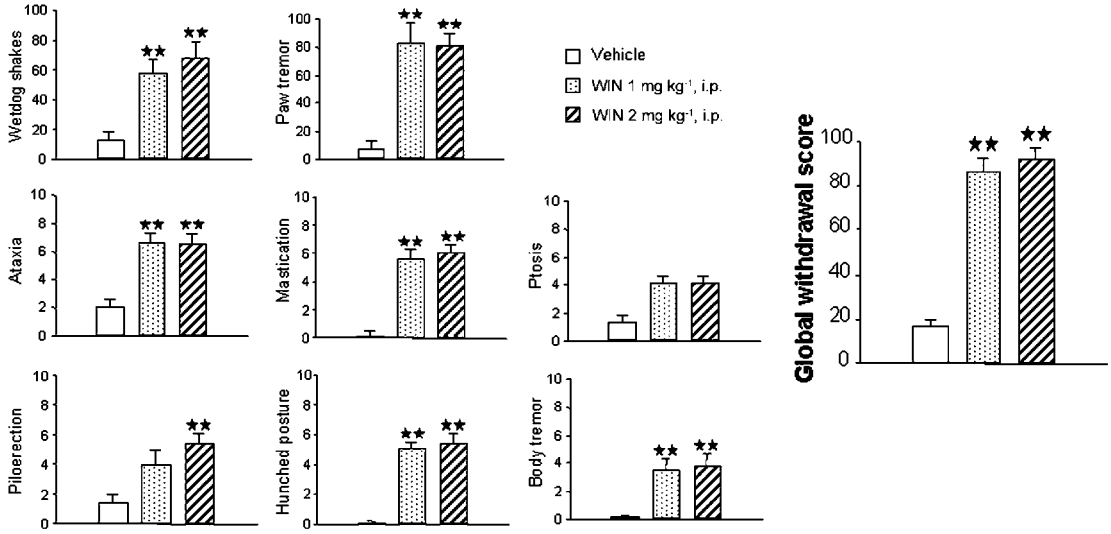

Figure 2 WIN 55,212-2 withdrawal in mice precipitated by systemic SR 141716A administration. Abstinence was precipitated by the administration of SR 141716A (10 mg kg^{-1}, s.c.) in mice receiving a chronic administration of WIN 55,212-2 (1 and 2 mg kg^{-1} i.p., twice daily, 5 days). Counted (wet dog shakes and paw tremor) and checked (ptosis, ataxia, mastication, body tremor, piloerection and hunched posture) signs of withdrawal were observed during 30 min after SR 141716A administration. The global withdrawal score was calculated by giving a relative weight for each individual sign (see Methods for details). Data are expressed as mean ± s.e.m. ($n = 10$–12 per group). ★★$P < 0.01$, WIN 55,212-2-treated vs vehicle group (*post hoc* Dunnett).

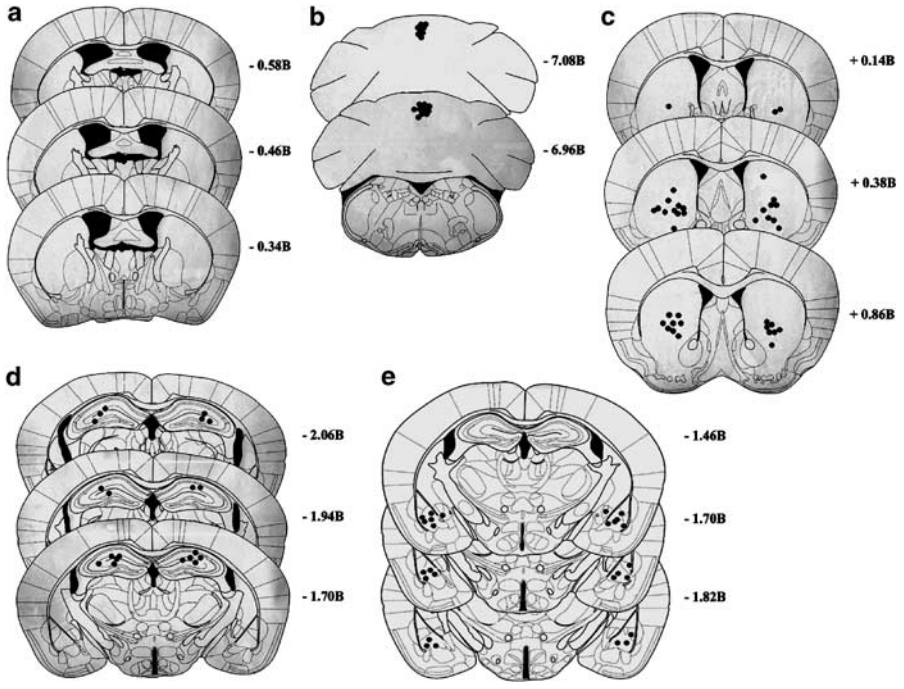

Figure 3 Representative coronal sections of the final site of injection of SR 141716A in WIN 55,212-2-dependent mice based on the atlas (Paxinos & Franklin, 1997). Grey points correspond to the localization of the sites of injection in (a) the third ventricle, (b) cerebellum, (c) striatum, (d) hippocampus and (e) amygdala. Values on the right of each section refer to the distance from Bregma.

significant effect of SR 141716A administration at both doses used, 1.5 and 3 μg ($P < 0.01$) (Figure 4a, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the cerebellum

Cerebellar administration of the CB1 receptor antagonist SR 141716A (0.75, 1.5 and 3 μg) precipitated several somatic signs of withdrawal in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}). One-way ANOVA demonstrated a significant effect for seven out of 10 signs analysed: wet dog shakes ($F(4,70) = 8.618$; $P < 0.01$), body tremor ($F(4,70) = 5.679$; $P < 0.01$), paw tremor ($F(4,70) = 34.249$; $P < 0.01$), piloerection ($F(4,70) = 4.474$; $P < 0.01$), mastication ($F(4,70) = 49.421$; $P < 0.01$), genital licks ($F(4,70) = 6.829$; $P < 0.01$) and sniffing ($F(4,70) = 18.494$; $P < 0.01$). One-way ANOVA for the global

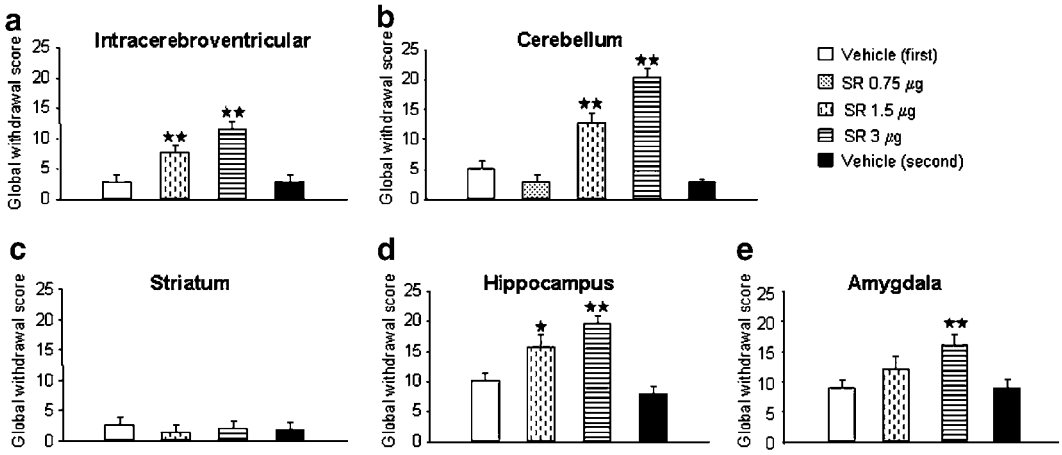

Figure 4 Global withdrawal score after microinjection of SR 141716A into several brain areas of mice chronically treated with WIN 55,212-2. SR 141716A (0.75, 1.5 and 3 μg) or its vehicle was microinjected into (a) the third ventricle, (b) cerebellum, (c) striatum, (d) hippocampus and (e) amygdala of WIN 55,212-2-dependent mice (1 mg kg^{-1}, twice daily, 5 days) by using a Latin Square design. Data are expressed as mean $\pm$ s.e.m. ($n = 10$–19). $^{\star}P<0.05$; $^{\star\star}P<0.01$, SR 141716A-administered vs vehicle (first) group (*post hoc* Dunnett).

withdrawal score also revealed a significant response ($F(4,70) = 54.985$; $P<0.01$), and subsequent *post hoc* analysis showed a significant effect of SR 141716A administration at the doses of 1.5 and 3 μg ($P<0.01$) (Figure 4b, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the striatum

Microinjection of SR 141716A (1.5 and 3 μg) in the striatum did not induce any behavioural manifestation of withdrawal in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}) (Figure 4c, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the hippocampus

Microinjection of SR 141716A (1.5 and 3 μg) in the hippocampus precipitated a significant expression of several somatic signs of withdrawal in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}). One-way ANOVA revealed a significant effect for body tremor ($F(3,42) = 6.071$; $P<0.01$), piloerection ($F(3,42) = 5.090$; $P<0.01$) and mastication ($F(3,42) = 4.887$; $P<0.01$). For the global withdrawal score, one-way ANOVA revealed a significant effect of SR 141716A ($F(3,42) = 12.130$; $P<0.01$), and subsequent *post hoc* analysis demonstrated a significant effect for both doses used, 1.5 μg ($P<0.05$) and 3 μg ($P<0.01$) (Figure 4d, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the amygdala

SR 141716A (1.5 and 3 μg) injection into the amygdala elicited a withdrawal syndrome with a significant expression of mastication ($F(3,51) = 2.903$; $P<0.05$). Although other signs of withdrawal were not significantly observed, one-way ANOVA for the global withdrawal score also revealed a significant effect of SR 141716A ($F(3,51) = 4.855$; $P<0.01$), and subsequent *post hoc* analysis demonstrated a significant effect with the highest dose used, 3 μg ($P<0.01$) (Figure 4e, Table 1).

Discussion

In this study we have evaluated the ability of the cannabinoid agonist WIN 55,212-2 to produce rewarding effects in the place-conditioning paradigm and the somatic expression of WIN 55,212-2 withdrawal. Furthermore, we have investigated the participation of different brain structures in the behavioural expression of WIN 55,212-2 withdrawal. We show that WIN 55,212-2 induced rewarding effects in the place-conditioning paradigm. This response was only observed with the lowest dose tested and when mice received a priming injection of the drug. In agreement with our results, THC induced a conditioned place preference only when mice received a previous priming THC exposure (Valjent & Maldonado, 2000). In the present study and in the previous report, mice received the first cannabinoid administration in their home cages, and thus the negative consequences of the first exposure to the drug cannot be associated with the contextual cues of the conditioning apparatus.

SR 141716A challenge precipitated several somatic signs of withdrawal in mice chronically treated with WIN 55,212-2. These signs included wet dog shakes, paw tremor, ataxia, mastication, body tremor, piloerection and hunched posture, and are similar to those reported during the withdrawal syndrome to other cannabinoid agonists in rodents such as THC (Hutcheson *et al*., 1998; Ledent *et al*., 1999; Lichtman *et al*., 2001; Ghozland *et al*., 2002b; Maldonado, 2002; Castañé *et al*., 2003). The administration of SR 141716A in vehicle-treated mice precipitated a slight presence of some somatic signs similar to those observed during cannabinoid withdrawal, suggesting an intrinsic activity of this compound as previously reported (Hutcheson *et al*., 1998; Rubino *et al*., 1998). The main focus of our study was to investigate the involvement of several brain structures containing a high

density of CB1 cannabinoid receptors and related to cannabinoid behavioural effects, such as the striatum, hippocampus, amygdala and cerebellum (Herkenham *et al*., 1990), in the behavioural expression of cannabinoid withdrawal. We also investigated the consequences of i.c.v. SR 141716A administration in WIN 55,212-2-dependent mice as a positive control for the other intracerebral administrations, considering that the ventricle allows the diffusion of the drug in the whole brain. We show that the microinjection of the CB1 cannabinoid receptor antagonist SR 141716A into the third ventricle, cerebellum, hippocampus and amygdala but not into the striatum precipitated somatic signs of cannabinoid withdrawal in mice chronically treated with WIN 55,212-2. The severity of WIN 55,212-2 abstinence was dependent on the site of injection of the cannabinoid antagonist. The microinjection of SR 141716A in the third ventricle precipitated a withdrawal syndrome in WIN 55,212-2-dependent mice, which was qualitatively and quantitatively different from the one observed after systemic administration of the antagonist. Several signs reported during systemic SR 141716A administration such as wet dog shakes, paw tremor and hunched posture did not appear statistically significant after i.c.v. SR 141716A administration and the intensity of the withdrawal was lower. These differences may be due to the use of higher doses of antagonist when administered systemically. After the microinjection of SR 141716A into the several brain structures evaluated, the highest severity of cannabinoid withdrawal was observed when SR 141716A was microinjected in the cerebellum. A total of seven out of 10 signs evaluated (wet dog shakes, body tremor, paw tremor, piloerection, mastication, genital licks and sniffing) were statistically significant and the global withdrawal score reached the highest value. After the microinjection of SR 141716A in the hippocampus and amygdala, the global withdrawal score achieved significant values but in these brain areas the basal withdrawal score obtained after vehicle administration was also elevated, suggesting that the involvement of the hippocampus and amygdala in the control of emotional responses could induce a higher behavioural reactivity to the microinjection procedure. However, no signs of cannabinoid withdrawal were observed when the CB1 antagonist was administered in the striatum.

In agreement with the high severity of cannabinoid withdrawal after SR 141716A microinjection in the cerebellum, previous studies have also suggested a crucial role of this structure in the somatic expression of cannabinoid withdrawal (Hutcheson *et al*., 1998; Tzavara *et al*., 2000; Ghozland *et al*., 2002a). Thus, cannabinoid withdrawal syndrome was associated to a compensatory increase of AC activity (Hutcheson *et al*., 1998) and a downstream activation of the protein kinase A (PKA) (Tzavara *et al*., 2000) in this brain area, which was not observed in other brain structures also containing CB1 receptors. The cerebellar granule neurons have been reported to be involved in the adaptive changes induced in this structure by chronic cannabinoid administration and withdrawal (Ghozland *et al*., 2002a). All these previous studies used the natural cannabinoid agonist THC, whereas no data have been yet published about the adaptive changes produced by the synthetic agonist WIN 55,212-2.

Our study indicates that the striatum is not involved in the somatic expression of cannabinoid abstinence. This is in agreement with previous results showing that AC activity in the striatum was unaffected during SR 141716A-precipitated withdrawal (Hutcheson *et al*., 1998). However, chronic CP-55,940 administration induced a desensitization of the CB1 cannabinoid receptors and the transduction system ($G_{\alpha s}$ and $G_{\alpha i}$) coupled to CB1 receptors in the rat striatum, which recovered during withdrawal, and might be part of the molecular mechanisms underlying cannabinoid dependence (Rubino *et al*., 1998). Interestingly, we also show in our study that the hippocampus, a brain structure mainly related to learning and memory processes, participates in the behavioural expression of cannabinoid withdrawal syndrome since a moderate withdrawal was observed after microinjection of the cannabinoid antagonist (significant manifestation of three withdrawal signs: body tremor, piloerection and mastication). Learning and memory processes have been suggested to play an important role in opiate addiction (Fan *et al*., 1999; Lu *et al*., 2000). However, no compensatory changes in the cAMP pathway into the hippocampus has been previously reported after cannabinoid withdrawal (Hutcheson *et al*., 1998; Tzavara *et al*., 2000). Therefore, the role of this brain area in cannabinoid abstinence might be mediated through adaptive changes in other intracellular signalling mediators. Indeed, the stimulation of cannabinoid receptors modulates several intracellular signalling systems including MAP kinases, IP3 and cationic channels (Piomelli, 2003), and it has been recently reported that cannabinoids induce long-term alterations in the hippocampus through the activation of the ERK pathway (Derkinderen *et al*., 2003). These long-term ERK changes may be important for the effects of cannabinoids in the context of drug abuse. Furthermore, a recent study showed significant alterations in the hippocampal cannabinoid receptors associated to the behavioural withdrawal syndrome precipitated by SR 141716A in THC-dependent rats, which could be related to cannabinoid tolerance and dependence (Breivogel *et al*., 2003).

Finally, a mild withdrawal syndrome was induced when the cannabinoid antagonist was administered in the amygdala, and only one withdrawal sign, mastication, significantly appeared. Interestingly, the administration of the opiate antagonist methylnaloxonium in the amygdala of morphine-dependent rats also resulted in a mild withdrawal syndrome, being mastication one of the most sensitive signs (Maldonado *et al*., 1992). Recent studies have related the amygdala and other limbic structures to the aversive/dysphoric stimulus associated to withdrawal from drugs of abuse (Koob, 2003). Thus, cannabinoid withdrawal is accompanied by a marked increase in extracellular CRF and enhanced c-Fos immunoreactivity in the central nucleus of the amygdala, which could be related to the dysphoric aspects of cannabinoid abstinence (Rodriguez de Fonseca *et al*., 1997). Nevertheless, further studies are needed to clarify which are the mechanisms responsible for the implication of the amygdala in the somatic expression of cannabinoid withdrawal.

Taken together, our findings show that WIN 55,212-2 induces rewarding effects and physical dependence in mice. Moreover, the cerebellum plays a crucial role in the behavioural expression of cannabinoid abstinence, but other brain structures such as the hippocampus and the amygdala also play a significant role. Therefore, the neuroanatomical substrate of cannabinoid somatic abstinence is not restricted to a single brain structure, and several brain areas seem to be required for the complete expression of cannabinoid withdrawal.

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