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## EFFECTS OF THE CANNABINOID RECEPTOR LIGANDS ON ANXIETY-RELATED EFFECTS OF D-AMPHETAMINE AND NICOTINE IN THE MOUSE ELEVATED PLUS MAZE TEST

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The purpose of the experiments was to examine the anxiety-related effects of d-amphetamine and nicotine, and the possible involvement of the endocannabinoid system. D-amphetamine (2 mg/kg, *ip*) was administered acutely or daily for 8 days. On the 9th day, mice were challenged with d-amphetamine (2 mg/kg, *ip*) or nicotine (0.1 mg/kg, *sc*), and were tested in the elevated plus maze. Additionally, a distinct group of mice was pretreated with an acute (0.1 mg/kg, *sc*) or subchronic nicotine (6 days), and subjected to nicotine (0.1 mg/kg, *sc*) or d-amphetamine (2 mg/kg, *ip*) challenge on the 7th day. The cannabinoid receptor ligands, WIN 55,212-2, a non-selective cannabinoid receptor agonist (0.25; 0.5 and 1 mg/kg, *ip*) and rimonabant, a CB1 cannabinoid receptor antagonist (0.25; 0.5; 1 and 2 mg/kg, *ip*) were injected prior to each injection of saline or acute and subchronic d-amphetamine or nicotine. We observed that acute anxiogenic and subchronic anxiolytic effects of both psychostimulants as well as the development of full cross-tolerance to their anxiogenic effects were dose-dependently blunted by ineffective doses of WIN 55,212-2 (0.25 and 0.5 mg/kg) and rimonabant (0.5 and 1 mg/kg). These results provide evidence that the endogenous cannabinoid system is involved in the anxiety-related responses to d-amphetamine and/or nicotine.

**Keywords:** *nicotine, d-amphetamine, anxiety, elevated plus maze, cannabinoids, mice*

### INTRODUCTION

Both in humans and rodents, anxiety seems to be an important factor for the establishment and maintenance of physical dependence and withdrawal syndrome of many drugs of abuse, including nicotine and amphetamine. According to smokers' accounts, smoking may have anxiolytic effects (1) but also, despite the subjective feelings, they display higher level of anxiety-related profile than nonsmokers and smokers who quit (2). Among amphetamine abusers, psychiatric disorders, such as anxiety, panic attacks and mania are commonly reported (3), especially among first time amphetamine users (4). Withdrawal from both nicotine and amphetamine has been also associated with states of increased anxiety and dysphoria in human addicts. Anxiolysis after prolonged administration can be one of the main effects underlying psychostimulant dependence and drug relapse.

Psychostimulant drugs and cannabis which share some biological actions, are among the most widely abused drugs taken frequently in combination, and therefore the study of their functional interactions is of special interest. Nicotine, the main psychoactive component in tobacco smoke that initiates and sustains tobacco addiction, acts at the neuronal nicotinic acetylcholine receptors (nAChRs) highly distributed in the central nervous system (CNS), mainly at the pre-synaptic level, and promotes the release of several neurotransmitters, such as acetylcholine, dopamine, noradrenaline, serotonin and  $\gamma$ -aminobutyric acid (GABA) (5). Cannabinoids exert their action by

the activation of metabotropic cannabinoid (CB) receptors, followed by an inhibition of adenylyl cyclase activity (6). CB1 receptors are localized mainly in the CNS, including basal ganglia, cerebellum, hippocampus and cerebral cortex (7). A possible neurobiological substrate of the interactions between nicotine and cannabinoids is the overlapping distribution of CB1 receptors and nAChRs in brain regions including prefrontal cortex, limbic areas, hippocampus and amygdala that are involved in the regulation of emotional responses, cognition and drug abuse (8, 9). Additionally, early studies in rodents indicated the existence of functional interactions between nicotine and cannabinoid receptor agonists in the modulation of behavioral and physiological responses including anxiety-like behavior (10, 11). Also biochemical studies support the existence of interaction between nicotine and cannabinoids, as co-administration of nicotine and delta9-tetrahydrocannabinol (THC) potentiated the enhancement of c-Fos immunoreactivity in brain regions implicated in emotional and/or motivational responses (12).

Some studies have also reported a pharmacological interaction between cannabinoids and amphetamine derivatives. It is well established that amphetamine increases dopamine, noradrenaline and serotonin neurotransmission by acting on monoamine transporters, causing an increase in the cytoplasmic levels of monoamines and leading to an increase in their release from the terminals (13). Recent reports also indicate that, besides its addictive properties, amphetamine can influence the immune functions as a potent immunosuppressor causing an

enhancement of cytotoxic activity of natural killer cells *via*  $\beta$ -adrenergic mechanism (14). Despite the different mechanisms of action, both cannabis and amphetamine modulate common physiological processes such as locomotion, body temperature, anxiety and reward (15). Accordingly, THC administration prevents hypolocomotion and anxiety-like responses produced by 3,4-methylenedioxyamphetamine (MDMA, ecstasy) in rodents (16). However, the consequences of d-amphetamine and CB1 receptor ligands on anxiety-related effects have not been investigated yet.

It has been well documented that nicotine and other psychostimulant drugs (amphetamine and cocaine) are widely abused together for their psychomotor stimulant and rewarding properties. Thus, a possible cross-action between these substances could be expected. It is a major concern because tobacco is highly addictive and has also been linked to illicit drug use (17, 18). Therefore, controversies over nicotine and d-amphetamine anxiety-related effects in humans and in animal models can be still encountered in the literature.

Generally, there is a good evidence for the neuropharmacological and neuroanatomical parallels between rodent emotionality and human anxiety. Although behavioral and pharmacological effects and current association of cannabinoids and psychostimulants in humans are well known, the behavioral consequences of their interactions are still poorly documented in animal models (19). The present study was designed to analyze the consequence of combined treatments with CB1 cannabinoid receptor ligands and acute or subchronic nicotine or d-amphetamine administration in the anxiety-related effects in mice, including crossover effects. To this aim, we used the elevated plus maze (EPM) test, a model based on the natural aversion of rodents to height and open spaces, validated for the evaluation of anxiety in rodents (20, 21). The results of the present studies are discussed in context of influence of the nicotine and d-amphetamine treatment on anxiety-related responses, and of the possible mechanisms for the functional interactions between cannabinoids and psychostimulant drugs in relation to anxiety state. Functional addiction-related interactions between nicotine, d-amphetamine and cannabinoids are of special interest in the context of polydrug abuse phenomenon.

## MATERIALS AND METHODS

### *Animals*

The experiments were carried out on naive male Swiss mice (Farm of Laboratory Animals, Warszawa, Poland) weighing 20-25g at the beginning of the experiments. The animals were maintained under standard laboratory conditions (12-h light/dark cycle, room temperature  $21 \pm 1^\circ\text{C}$ ) with free access to tap water and laboratory chow (Bacutil, Motycz, Poland), and were adapted to the laboratory conditions for at least one week. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Each experimental group consisted of 8-10 animals. All experiments were carried out according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and to the European Community Council Directive for Care and Use of Laboratory Animals of 24 November 1986 (86/609/EEC), and approved by the local ethics committee.

### *Drugs*

The compounds tested were: (-)-nicotine hydrogen tartrate (Sigma, St. Louis, MO, USA), d-amphetamine sulphate (Sigma, St. Louis, MO, USA), WIN 55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-

3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanonone mesylate; Tocris Cookson, Bristol, UK), and rimonabant (kindly gifted by Sanofi-Synthelabo, Montpellier, France). Nicotine and d-amphetamine were dissolved in saline (0.9% NaCl) and refers to the salt form. WIN 55,212-2 and rimonabant were suspended in one drop of 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) and diluted in saline (0.9% NaCl). Fresh drug solutions were prepared on each day of experimentation. All agents were administered intraperitoneally (*ip*) or subcutaneously (*sc*) in a volume of 10 ml/kg. Control groups received vehicle injections at the same volume and by the same route.

### *The EPM procedure*

Anxiety responses were measured in the EPM test. The procedure was similar to the method of Lister (21). The experimental apparatus is shaped like a "plus" sign and consists of a central platform ( $5 \times 5$  cm), two open arms ( $30 \times 5$  cm) and two equal-sized closed ( $30 \times 5 \times 15$  cm) arms opposite to each other. The maze is made of dark Plexiglas, elevated to a height of 50 cm above the floor and illuminated by dim light. The test consisted of placing a mouse in the central platform facing an enclosed arm and allowing it to freely explore the maze for 5 min. Entry into one arm was defined as the animal placing all four paws past the line dividing the central square from the open arms. The test arena was wiped with a damp cloth after each trial. The number of entries into the open and closed arms and the time spent in open arms were measured by an observer blind to the drug treatment. Anxiolytic activity was indicated by increases in time spent in open arms or in number of open arms entries; anxiogenic effects are characterized by decreases in these measures. The percentage of time spent on the open arms was calculated, as was the percentage number of open arm entries. Additionally, the number of entries into the closed arms was recorded as an indicator of motor activity of animals in this test.

### *Treatment*

Experimental procedures and doses used have been chosen accordingly to recent data already published (19, 20, 22-24). During acute treatment, the animals were allocated to the following drug groups: nicotine (0.1 mg/kg, *sc*), d-amphetamine (2 mg/kg, *ip*) or saline, and mice of each group were tested 30 min after injection. The exploratory behavior in the maze was recorded for 5 min.

In the second set of experiments, animals were randomly allocated to receive 8 days of *ip* injections of d-amphetamine (2 mg/kg) or saline. On the ninth day, these animals were subjected to d-amphetamine (2 mg/kg, *ip*), nicotine (0.1 mg/kg, *sc*) or saline (for a control group), and were tested 30 min after this last injection. Additionally, another group of animals was randomly allocated to receive 6 days of *sc* injections of nicotine (0.1 mg/kg) or saline. On the seventh day, the distinct groups of animals were treated with nicotine (0.1 mg/kg, *sc*), d-amphetamine (2 mg/kg, *ip*) or saline (for a control group), and were tested 30 min after the last injection. Both experimental procedures were performed in order to see if tolerance and cross-tolerance to the anxiogenic effect of d-amphetamine and nicotine developed after the longer pretreatment period.

The next experiment was designed to investigate whether cannabinoid receptor ligand application modifies the influence of amphetamine and nicotine on anxiety level as well as the development of cross-tolerance. For this purpose, distinct groups of mice were injected with WIN 55,212-2 (0.25, 0.5 and 1.0 mg/kg, *ip*), rimonabant (0.25, 0.5, 1.0 and 2.0 mg/kg, *ip*) or saline, 15 min before saline or an acute or every chronic d-

amphetamine, nicotine or saline injection. On the test day, these mice were challenged with 2 mg/kg d-amphetamine, 0.1 mg/kg nicotine or saline, as described above, and their exploratory behavior in the maze was recorded 5 min after injection.

#### Statistical analysis

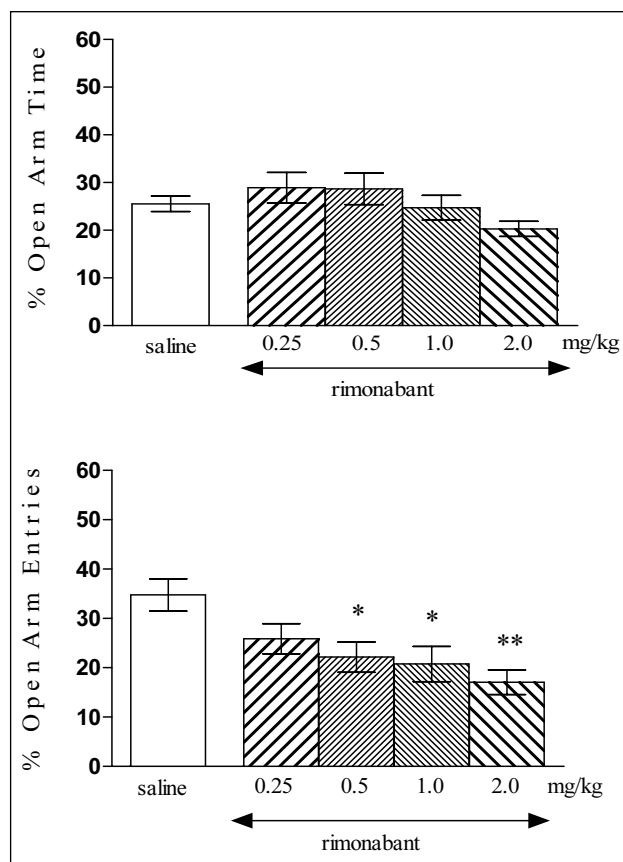
The data are expressed as the means  $\pm$  standard error of the mean (S.E.M). The statistical analyses were performed using one-way analysis of variance (ANOVA). Post-hoc comparison of means was carried out with the Tukey test for multiple comparisons, when appropriate. The confidence limit of  $p < 0.05$  was considered statistically significant.

### RESULTS

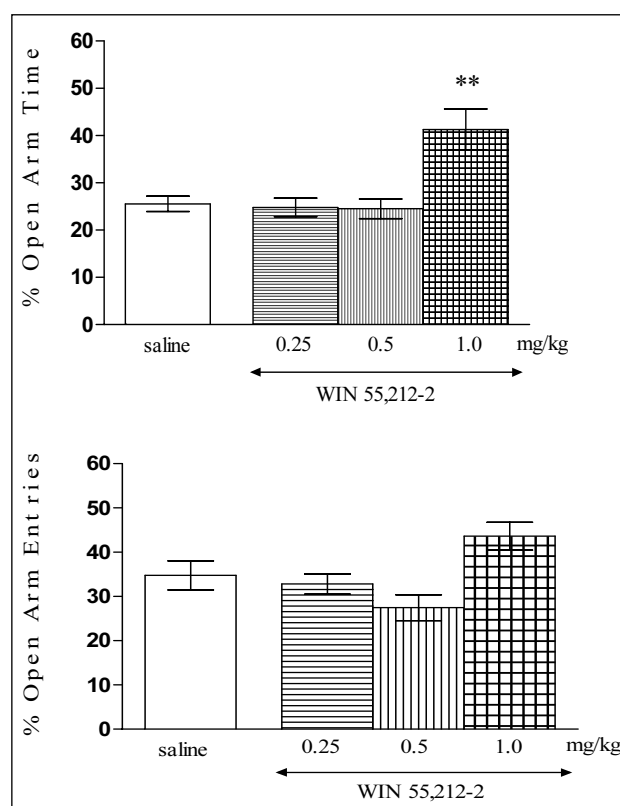
It can be seen in *Fig. 1* that rimonabant, a CB1 cannabinoid receptor antagonist, at any doses tested (0.25; 0.5; 1 and 2 mg/kg, *ip*) did not change the percentage of time spent on the open arms of the EPM but, at the doses of 0.5, 1 and 2 mg/kg it decreased the percentage of open arm entries ( $p < 0.05$  for 0.5 and 1 mg/kg;  $p < 0.01$  for 2 mg/kg, Tukey test). Concerning WIN 55,212-2 a CB1 receptor agonist, only at the highest dose of 1 mg/kg it increased the percentage of open arm time ( $p < 0.01$ ; *Fig. 2*) causing a slight anxiolytic effect. The inactive doses of 0.5 and 1 mg/kg of rimonabant and 0.25 and 0.5 mg/kg of WIN 55,212-2 have been chosen for all subsequent tests. Moreover, at

these doses both compounds did not change the performance of mice after subchronic administration (not shown).

It can be seen from *Fig. 3* that in the control saline-treated animals an acute *ip* dose of d-amphetamine (2 mg/kg) significantly decreased the percentage of time spent on the open arms ( $p < 0.001$ ) as well as the percentage of open arm entries ( $p < 0.05$ ), indicating an anxiogenic effect. *Fig. 3* also shows the influence of pretreatment with CB1 receptor ligands on acute d-amphetamine-induced changes (ANOVA on the percentage of time spent in the open arms:  $F_{5,48} = 16.696$ ,  $p < 0.0001$ ; ANOVA on the percentage of open arm entries:  $F_{5,48} = 6.707$ ,  $p < 0.0001$ ). The post-hoc Tukey test indicated that pretreatment with rimonabant (0.5 and 1.0 mg/kg) and WIN 55,212-2 (0.25 and 0.5 mg/kg) significantly reversed the anxiogenic-like effect of acute d-amphetamine (2 mg/kg) revealed as the increase in the percentage of time spent on open arms ( $p < 0.001$ ) and in the percentage of open arm entries ( $p < 0.05$  for both doses of rimonabant;  $p < 0.01$  for 0.5 mg/kg of WIN 55,212-2 and  $p < 0.001$  for 0.25 mg/kg of WIN 55,212-2) as compared with amphetamine-pretreated control group (*Fig. 3*). Similarly, *Fig. 4* shows that in the control saline-treated animals an acute *sc* dose of nicotine (0.1 mg/kg) significantly decreased the percentage of time spent on the open arms ( $p < 0.001$ ) as well as the percentage of open arm entries ( $p < 0.001$ ), also indicating an anxiogenic effect. *Fig. 4* also shows the influence of pretreatment with CB1 receptor ligands on acute nicotine-induced changes (ANOVA on the percentage of time spent in the open arms:  $F_{5,48} = 40.325$ ,  $p < 0.0001$ ; ANOVA on the percentage of open arm entries:  $F_{5,48} = 14.513$ ,  $p < 0.0001$ ). The post-hoc Tukey test indicated that pretreatment with rimonabant (0.5 and 1.0 mg/kg) and WIN



*Fig. 1.* Mean ( $\pm$  S.E.M.) percentage time spent in open arms and percentage open arm entries in the EPM test in mice, 30 min after an acute *ip* injection of cannabinoid receptor antagonist rimonabant (0.25; 0.5; 1 and 2 mg/kg) or saline;  $n = 8-10$ ; \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. saline control group, Tukey test.



*Fig. 2.* Mean ( $\pm$  S.E.M.) percentage time spent in open arms and percentage open arm entries in the EPM test in mice, 30 min after an acute *ip* injection of cannabinoid receptor agonist WIN 55,212-2 (0.25; 0.5 and 1 mg/kg) or saline;  $n = 8-10$ ; \*\*  $p < 0.01$  vs. saline control group, Tukey test.

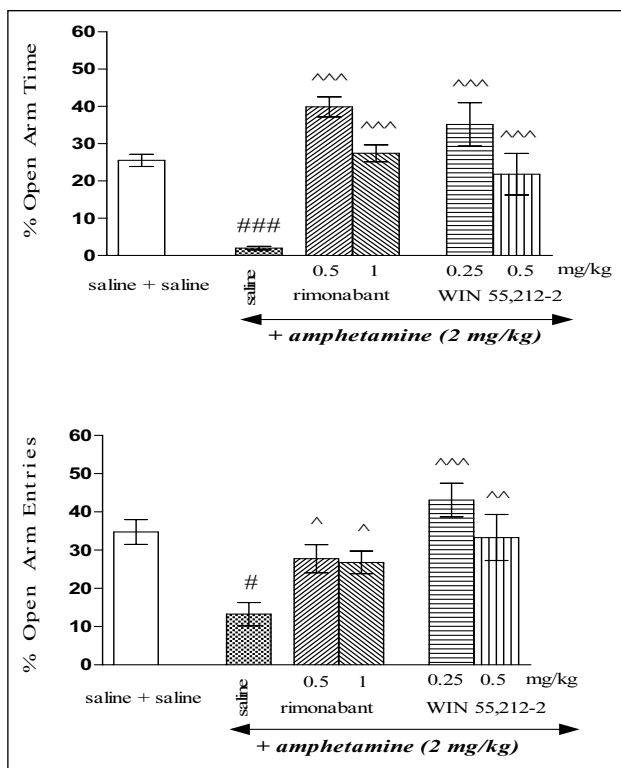


Fig. 3. Influence of cannabinoid receptor ligands: antagonist-rimonabant or agonist - WIN 55,212-2 on the anxiogenic effect of an acute d-amphetamine injection. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered 15 min prior to d-amphetamine (2 mg/kg, *ip*) or saline injection, and tested 30 min later in the EPM test in mice; n=8-10; #  $p < 0.05$  and ###  $p < 0.001$  vs. saline control group; ^  $p < 0.05$ ; ^^  $p < 0.01$  and ^^ ^  $p < 0.001$  vs. amphetamine-treated group, Tukey test.

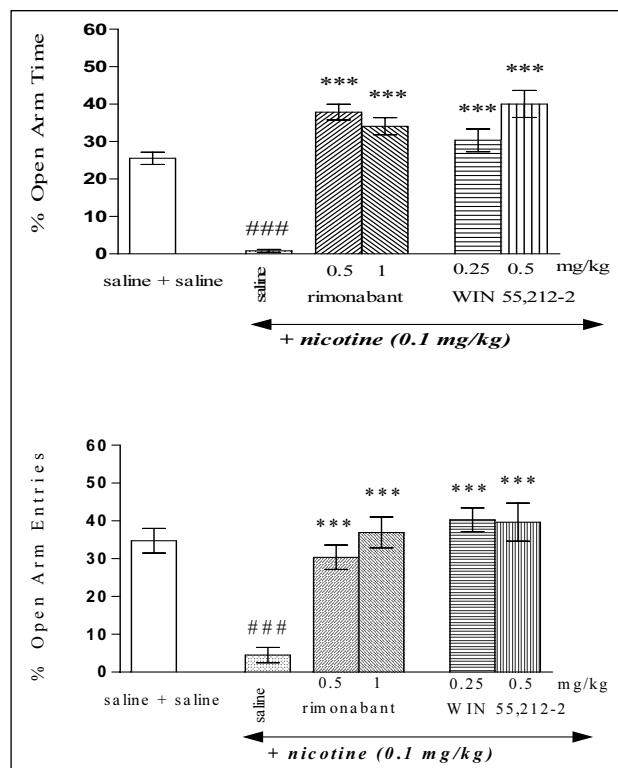


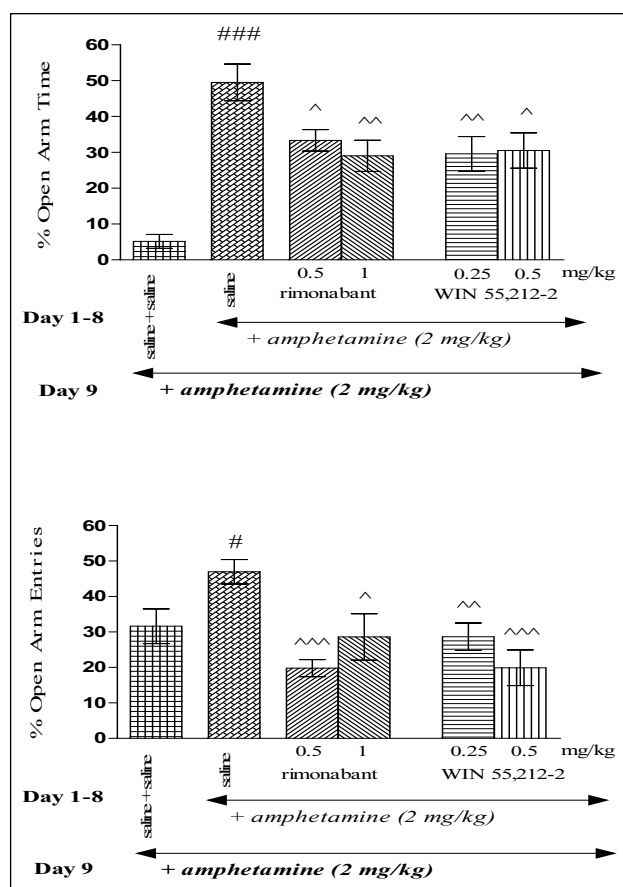
Fig. 4. Influence of cannabinoid receptor ligands: antagonist - rimonabant or agonist - WIN 55,212-2 on the anxiogenic effect of an acute nicotine injection. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered 15 min prior to nicotine (0.1 mg/kg, *sc*) or saline injection, and tested 30 min later in the EPM test in mice; n=8-10; ###  $p < 0.001$  vs. saline control group; \*\*\*  $p < 0.001$  vs. nicotine-treated group, Tukey test.

55,212-2 (0.25 and 0.5 mg/kg) significantly reversed the anxiogenic-like effect of acute nicotine (0.1 mg/kg) revealed as the increase in the percentage of time spent on open arms ( $p < 0.001$ ) and in the percentage of open arm entries ( $p < 0.001$ ) as compared with nicotine-pretreated control group (Fig. 4).

In the next experiment, the animals tested 30 min after the ninth daily injection of d-amphetamine (2 mg/kg), showed a significantly increased time in the open arms compared with the acute amphetamine group ( $p < 0.001$ , Fig. 5), as well as an increased number of entries to these arms compared with acute amphetamine group ( $p < 0.05$ , Fig. 5), suggesting that tolerance developed to the anxiogenic effect of d-amphetamine. Fig. 5 also shows the influence of CB1 receptor ligand pretreatment on subchronic d-amphetamine-induced changes (ANOVA on the percentage of time spent in the open arms:  $F_{5,43}=11.609$ ,  $p < 0.0001$ ; ANOVA on the percentage of open arm entries:  $F_{5,43}=5.884$ ,  $p = 0.0004$ ). The post-hoc Tukey test indicated that pretreatment with rimonabant (0.5 and 1.0 mg/kg) and WIN 55,212-2 (0.25 and 0.5 mg/kg) significantly reversed the anxiolytic-like effect of subchronic d-amphetamine (2 mg/kg) revealed as the decrease in the percentage of time spent on open arms ( $p < 0.05$  for 0.5 mg/kg of rimonabant and WIN 55,212-2;  $p < 0.01$  for 1 mg/kg of rimonabant and 0.25 mg/kg of WIN 55,212-2; Fig. 5) as well as in the percentage of open arm entries ( $p < 0.05$  for rimonabant 1 mg/kg,  $p < 0.01$  for 0.25 mg/kg of WIN 55,212-2 and  $p < 0.001$  for 0.5 mg/kg of rimonabant and WIN 55,212-2) as compared with amphetamine-pretreated control group (Fig. 5). In the next experiment, the animals tested 30 min

after the seventh daily injection of nicotine (0.1 mg/kg), showed a significantly increased time in the open arms compared with the acute nicotine group ( $p < 0.001$ , Fig. 6), as well as an increased number of entries to these arms compared with acute nicotine group ( $p < 0.05$ , Fig. 6), suggesting that tolerance developed to the anxiogenic effect of nicotine. Moreover, pretreatment with the CB1 receptor ligands before every daily injection of subchronic nicotine (0.1 mg/kg) also influenced the development of tolerance (ANOVA on the percentage of time spent in the open arms:  $F_{5,43}=17.356$ ,  $p < 0.0001$ ; ANOVA on the percentage of open arm entries:  $F_{5,43}=4.656$ ,  $p = 0.0017$ ). Actually, rimonabant (0.5 and 1.0 mg/kg) and WIN 55,212-2 (0.25 and 0.5 mg/kg) completely abolished the anxiolytic-like effect of subchronic nicotine, revealed as the decrease in the percentage of time spent on open arms ( $p < 0.05$ ) and, only in case of both doses of rimonabant, the decrease in the percentage of open arm entries ( $p < 0.05$ ) as compared with nicotine-pretreated control group (Fig. 6).

Finally, an amphetamine challenge (2 mg/kg, *ip*) provoked an anxiolytic action in nicotine-pretreated mice (0.1 mg/kg) ( $p < 0.001$  for the percentage of time spent on the open arms, Fig. 7). Similarly, nicotine challenge dose (0.1 mg/kg, *sc*) also resulted in an anxiolytic effect in amphetamine-treated mice ( $p < 0.001$  for the percentage of time spent on the open arms;  $p < 0.01$  for the percentage of open arm entries, Fig. 8). These effects suggest the development of full cross-tolerance between d-amphetamine and nicotine to their anxiogenic action under the present experimental conditions. Fig. 7 shows that pretreatment

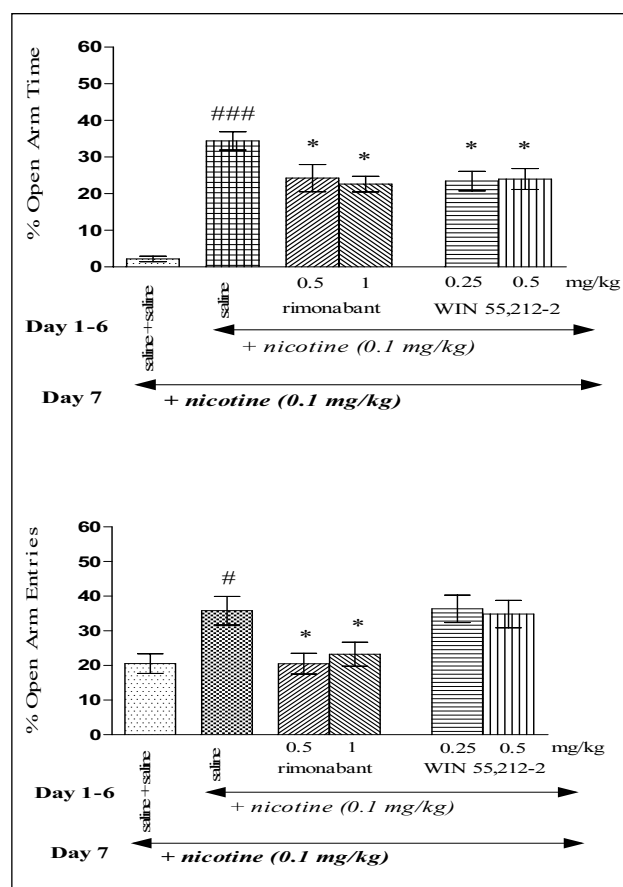


**Fig. 5.** Influence of cannabinoid receptor ligands: antagonist - rimonabant or agonist - WIN 55,212-2 on the development of tolerance to the anxiogenic effect of subchronic d-amphetamine in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered for eight days, 15 min prior to each daily amphetamine (2 mg/kg, *ip*) or saline injection, and tested on the ninth day 30 min after amphetamine (2 mg/kg, *ip*) challenge injection;  $n=8-9$ ; #  $p<0.05$  and ###  $p<0.001$  vs. saline treated and d-amphetamine-challenged group; ^  $p<0.05$ ; ^^  $p<0.01$  and ^^  $p<0.001$  vs. amphetamine-treated and amphetamine-challenged group, Tukey test.

**Table 1.** Mean ( $\pm$  S.E.M.) of closed arm entries in the EPM test in mice, 30 min after an acute *ip* injection of cannabinoid receptor ligands: antagonist - rimonabant (0.25; 0.5; 1 and 2 mg/kg), agonist - WIN 55,212-2 (0.25; 0.5 and 1 mg/kg, *ip*) or saline;  $n=8-10$

Treatment	Closed arm entries
saline	13.10 $\pm$ 0.96
rimonabant (0.25 mg/kg)	12.62 $\pm$ 1.22
rimonabant (0.5 mg/kg)	12.00 $\pm$ 0.62
rimonabant (1 mg/kg)	11.12 $\pm$ 1.72
rimonabant (2 mg/kg)	10.62 $\pm$ 0.98
WIN 55,212-2 (0.25 mg/kg)	12.50 $\pm$ 1.14
WIN 55,212-2 (0.5 mg/kg)	12.70 $\pm$ 0.94
WIN 55,212-2 (1 mg/kg)	10.40 $\pm$ 1.27

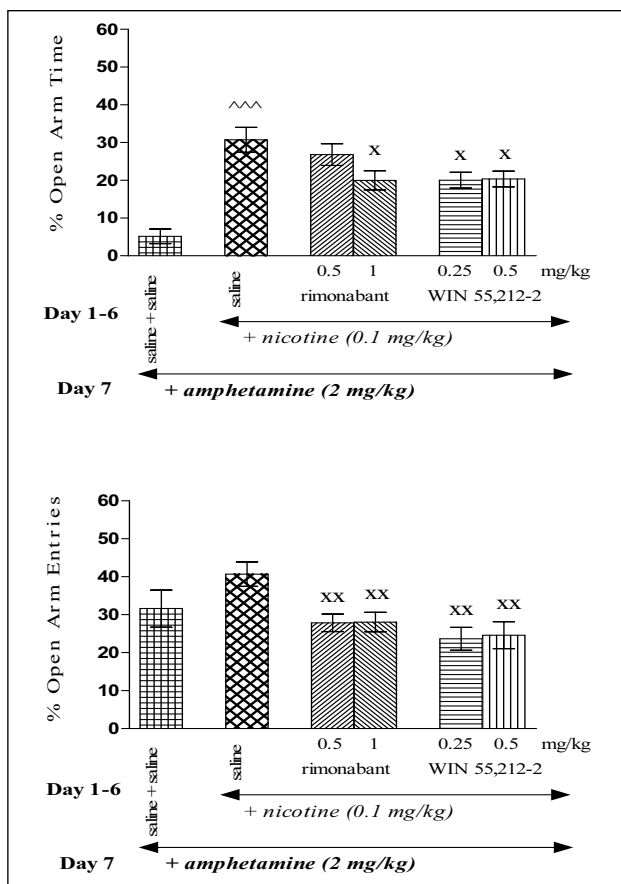
with the CB1 receptor ligands before every daily injection of subchronic nicotine (0.1 mg/kg, 6 days) influenced the anxiety-related behavior in the response to a d-amphetamine challenge (ANOVA on the percentage of time spent in the open arms:



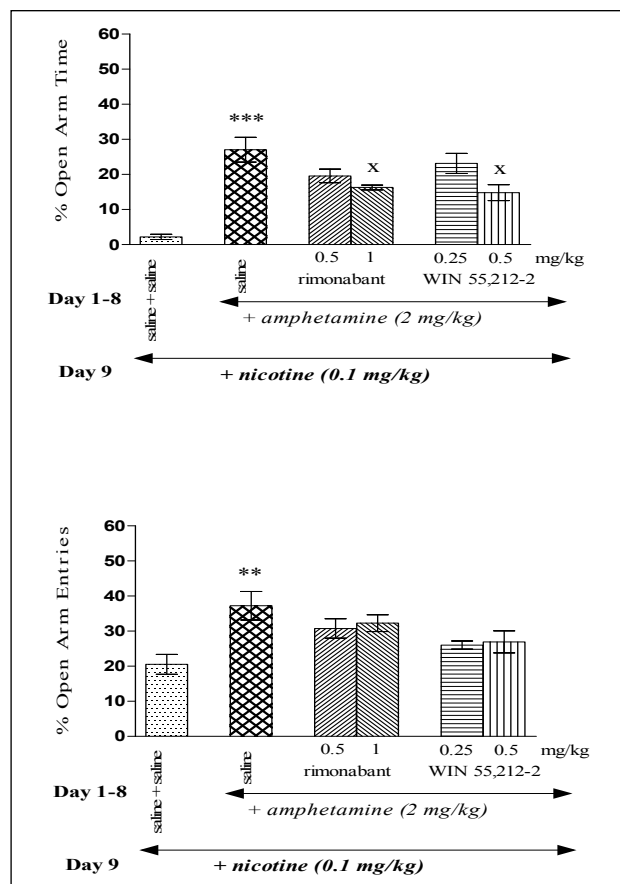
**Fig. 6.** Influence of cannabinoid receptor ligands: antagonist - rimonabant or agonist - WIN 55,212-2 on the development of tolerance to the anxiogenic effect of subchronic nicotine in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered for six days, 15 min prior to each daily nicotine (0.1 mg/kg, *sc*) or saline injection, and tested on the seventh day 30 min after nicotine (0.1 mg/kg, *sc*) challenge injection;  $n=8-10$ ; #  $p<0.05$  and ###  $p<0.001$  vs. saline-treated and nicotine-challenged group; \*  $p<0.05$  vs. nicotine-treated and nicotine-challenged group, Tukey test.

$F_{5,46}=11.311$ ,  $p<0.0001$ ; ANOVA on the percentage of open arm entries:  $F_{5,46}=3.682$ ,  $p=0.0069$ ). Actually, rimonabant (1 mg/kg) and WIN 55,212-2 (0.25 and 0.5 mg/kg) completely abolished the anxiolytic-like effect in mice subjected to chronic nicotine and challenged with d-amphetamine revealed as the decrease in the percentage of time spent on open arms ( $p<0.05$  for open arm time and  $p<0.01$  for open arm entries). However, at the dose of 0.5 mg/kg rimonabant also provoked a decrease in the percentage of open arm entries ( $p<0.01$ , Tukey test) (Fig. 7). Results of the last experiment show that pretreatment with the CB1 receptor ligands before every daily injection of chronic d-amphetamine (2 mg/kg, 8 days) influenced the anxiety-related behavior in the response to a nicotine challenge (ANOVA on the percentage of time spent in the open arms:  $F_{5,38}=13.803$ ,  $p<0.0001$ ; ANOVA on the percentage of open arm entries:  $F_{5,38}=4.137$ ,  $p=0.0043$ ). Actually, rimonabant (1 mg/kg) and WIN 55,212-2 (0.5 mg/kg) abolished the anxiolytic-like effect in mice subjected to chronic d-amphetamine and challenged with nicotine revealed as the decrease in the percentage of time spent on open arms ( $p<0.05$ , Tukey test) (Fig. 8).

Moreover, all compounds tested, alone or in combinations, given acutely or repeatedly at the doses used, did not provoke



**Fig. 7.** Influence of cannabinoid receptor ligands: antagonist - rimonabant or agonist - WIN 55,212-2 on the development of cross-tolerance between nicotine and d-amphetamine to their anxiogenic action in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered for six days, 15 min prior to each daily nicotine (0.1 mg/kg, *sc*) or saline injection, and tested on the seventh day 30 min after amphetamine (2 mg/kg, *ip*) challenge injection;  $n=8-9$ ; <sup>^^^</sup>  $p<0.001$  vs. saline-treated and amphetamine-challenged group; <sup>x</sup>  $p<0.05$  and <sup>xx</sup>  $p<0.01$  vs. nicotine-treated and amphetamine-challenged group, Tukey test.



**Fig. 8.** Influence of cannabinoid receptor ligands: antagonist - rimonabant or agonist - WIN 55,212-2 on the development of cross-tolerance between d-amphetamine and nicotine to their anxiogenic action in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered for eight days, 15 min prior to each daily amphetamine (2 mg/kg, *ip*) or saline injection, and tested on the ninth day 30 min after nicotine (0.1 mg/kg, *sc*) challenge injection;  $n=8-9$ ; <sup>\*\*</sup>  $p<0.01$  and <sup>\*\*\*</sup>  $p<0.001$  vs. saline treated and nicotine-challenged group; <sup>x</sup>  $p<0.05$  vs. amphetamine-treated and nicotine-challenged group, Tukey test.

any changes in number of closed arm entries in the EPM test (Tables 1-6). Thus, except for two groups: rimonabant (0.5 mg/kg) + d-amphetamine (2 mg/kg) in animals challenged with d-amphetamine (Table 3) and WIN 55,212-2 (0.5 mg/kg) + nicotine (0.1 mg/kg) in animals challenged with d-amphetamine (Table 5), these substances did not change locomotor activity of animals in this paradigm.

## DISCUSSION

Anxiety is among the most frequently observed psychiatric effects associated with psychostimulant addiction, including tobacco smoking, which also appears following withdrawal symptoms. Our results have further shown that in the EPM, a single injection of a low dose of d-amphetamine or nicotine had a significant anxiogenic effect in mice. Moreover, tolerance developed rapidly to this effect when these anxiogenic doses of both psychostimulant drugs were administered repeatedly. Moreover, a full cross-tolerance developed between d-amphetamine and nicotine confirming that a common neuronal mechanism can influence the anxiety-related actions of both

drugs. Furthermore, major objective of the present study was to examine the effects of CB cannabinoid receptor agonist and antagonist against the nicotine- and d-amphetamine-induced anxiety-related behavioral symptoms. It has been revealed that both, WIN 55,212-2, the non-selective CB receptor agonist and rimonabant, the CB1 receptor antagonist, at the doses non-effective in the test used, prevented nicotine- and d-amphetamine-induced acute anxiogenic and subchronic anxiolytic effects in mice. Additionally, it has been revealed that in mice chronically subjected to d-amphetamine and challenged with nicotine as well as in mice chronically treated with nicotine and subsequently challenged with d-amphetamine, both CB receptor ligands used abolished the development of the cross-tolerance to the anxiogenic action of both psychostimulants. These findings are in keeping with our previous reports showing that both WIN 55,212-2 and AM 251, another CB1 receptor antagonist, abolished memory-related effects of nicotine in mice (19). The results of this study provide pharmacological evidence that the endogenous cannabinoid system participates in the responses induced by nicotine and/or d-amphetamine on anxiety-like behavior.

The emotional impact of drugs may be important for the initiation and maintenance of drug-taking behavior, as the

Table 2. Mean number ( $\pm$  S.E.M.) of closed arm entries in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered 15 min prior to nicotine (0.1 mg/kg, *sc*), d-amphetamine (2 mg/kg, *ip*) or saline injection, and tested 30 min later in the EPM test; n=8-10

<b>Treatment</b>	<b>Closed arm entries</b>
saline + saline	13.10 $\pm$ 0.96
saline + nicotine (0.1 mg/kg)	12.33 $\pm$ 2.35
rimonabant (0.5 mg/kg) + nicotine (0.1 mg/kg)	13.10 $\pm$ 0.97
rimonabant (1 mg/kg) + nicotine (0.1 mg/kg)	10.90 $\pm$ 1.07
WIN 55,212-2 (0.25 mg/kg) + nicotine (0.1 mg/kg)	8.50 $\pm$ 0.73
WIN 55,212-2 (0.5 mg/kg) + nicotine (0.1 mg/kg)	11.28 $\pm$ 2.03
saline + amphetamine (2 mg/kg)	15.44 $\pm$ 1.80
rimonabant (0.5 mg/kg) + amphetamine (2 mg/kg)	14.20 $\pm$ 0.75
rimonabant (1 mg/kg) + amphetamine (2 mg/kg)	14.40 $\pm$ 0.94
WIN 55,212-2 (0.25 mg/kg) + amphetamine (2 mg/kg)	11.87 $\pm$ 2.40
WIN 55,212-2 (0.5 mg/kg) + amphetamine (2 mg/kg)	11.71 $\pm$ 2.02

Table 3. Mean number ( $\pm$ S.E.M.) of closed arm entries in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered for eight days, 15 min prior to each daily amphetamine (2 mg/kg, *ip*) or saline injection, and tested on the ninth day 30 min after amphetamine (2 mg/kg, *ip*) challenge injection; n=8-9;

<b>Treatment day 1-8</b>	<b>Treatment day 9</b>	<b>Closed arm entries</b>
saline + saline	amphetamine (2 mg/kg)	11.87 $\pm$ 3.04
saline + amphetamine (2 mg/kg)	amphetamine (2 mg/kg)	13.23 $\pm$ 2.40
rimonabant (0.5 mg/kg) + amphetamine (2 mg/kg)	amphetamine (2 mg/kg)	23.50 $\pm$ 2.08 *
rimonabant (1 mg/kg) + amphetamine (2 mg/kg)	amphetamine (2 mg/kg)	15.88 $\pm$ 2.08
WIN 55,212-2 (0.25 mg/kg) + amphetamine (2 mg/kg)	amphetamine (2 mg/kg)	15.12 $\pm$ 1.42
WIN 55,212-2 (0.5 mg/kg) + amphetamine (2 mg/kg)	amphetamine (2 mg/kg)	16.42 $\pm$ 2.82

\*  $p < 0.05$  vs. amphetamine-treated and amphetamine-challenged group, Tukey test

Table 4. Mean number ( $\pm$ S.E.M.) of closed arm entries in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered for six days, 15 min prior to each daily nicotine (0.1 mg/kg, *sc*) or saline injection, and tested on the seventh day 30 min after nicotine (0.1 mg/kg, *sc*) challenge injection; n=8-10

<b>Treatment day 1-6</b>	<b>Treatment day 7</b>	<b>Closed arm entries</b>
saline + saline	nicotine (0.1 mg/kg)	10.25 $\pm$ 0.79
saline + nicotine (0.1 mg/kg)	nicotine (0.1 mg/kg)	12.60 $\pm$ 1.28
rimonabant (0.5 mg/kg) + nicotine (0.1 mg/kg)	nicotine (0.1 mg/kg)	11.25 $\pm$ 0.45
rimonabant (1 mg/kg) + nicotine (0.1 mg/kg)	nicotine (0.1 mg/kg)	11.50 $\pm$ 0.63
WIN 55,212-2 (0.25 mg/kg) + nicotine (0.1 mg/kg)	nicotine (0.1 mg/kg)	16.55 $\pm$ 1.30
WIN 55,212-2 (0.5 mg/kg) + nicotine (0.1 mg/kg)	nicotine (0.1 mg/kg)	9.71 $\pm$ 1.58

reduction of anxiety might be a further mechanism reinforcing dependence. Several findings suggest that cannabinoid system, through the activation of cannabinoid CB1 receptors, is involved in the modulation of anxiety-related behavior (9, 25). There is a general consensus that the effects of cannabinoid agonists on anxiety seem to be biphasic with low doses being anxiolytic and high doses possibly anxiogenic (9, 26, 27). It is worth mentioning that, besides anxiety-related action, activation of the endocannabinoid system also induced antidepressant-like effects, and CB1 receptor agonists have been shown to enhance the anti-immobility effects of antidepressant drugs in the forced swimming test in rats (28). Concerning CB1 receptor blockade, an acute administration of high doses of rimonabant as well as the genetic

disruption of CB1 receptors induced anxiogenic-like responses in rodents (29, 30). In our study, rimonabant, at any dose tested, did not provoke any changes in the percentage of time spent on the open arms, while WIN 55,212-2, only at the highest dose, caused an increase in the percentage of open arm time (*i.e.* an anxiolytic effect). However, the exact mechanism by which cannabinoids modulate anxiety-related behavior is not elucidated yet. Considering neuronal circuitries underlying these behavioral responses, the prefrontal cortex (PFC), hippocampus and the amygdala are the main cerebral regions involved. These structures belong to the emotional circuit and contain high levels of CB1 receptors (7). In this context, a recent study (31) has revealed that low doses of methanandamide, the metabolically stable analog of

Table 5. Mean number ( $\pm$  S.E.M.) of closed arm entries in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered for six days, 15 min prior to each daily nicotine (0.1 mg/kg, *sc*) or saline injection, and tested on the seventh day 30 min after amphetamine (2 mg/kg, *ip*) challenge injection;  $n=8-9$ ; \*  $p<0.05$  vs. nicotine-treated and amphetamine-challenged group, Tukey test

<i>Treatment day 1-6</i>	<i>Treatment day 7</i>	<i>Closed arm entries</i>
saline + saline	amphetamine (2 mg/kg)	11.87 $\pm$ 3.04
saline + nicotine (0.1 mg/kg)	amphetamine (2 mg/kg)	10.23 $\pm$ 0.52
rimonabant (0.5 mg/kg) + nicotine (0.1 mg/kg)	amphetamine (2 mg/kg)	11.44 $\pm$ 1.42
rimonabant (1 mg/kg) + nicotine (0.1 mg/kg)	amphetamine (2 mg/kg)	13.55 $\pm$ 1.58
WIN 55,212-2 (0.25 mg/kg) + nicotine (0.1 mg/kg)	amphetamine (2 mg/kg)	11.25 $\pm$ 0.77
WIN 55,212-2 (0.5 mg/kg) + nicotine (0.1 mg/kg)	amphetamine (2 mg/kg)	17.75 $\pm$ 3.94 *

Table 6. Mean number ( $\pm$  S.E.M.) of closed arm entries in the EPM test in mice. Rimonabant (0.5 and 1 mg/kg, *ip*), WIN 55,212-2 (0.25 and 0.5 mg/kg, *ip*) or saline were administered for eight days, 15 min prior to each daily amphetamine (2 mg/kg, *ip*) or saline injection, and tested on the ninth day 30 min after nicotine (0.1 mg/kg, *sc*) challenge injection;  $n=8-9$

<i>Treatment day 1-8</i>	<i>Treatment day 9</i>	<i>Closed arm entries</i>
saline + saline	nicotine (0.1 mg/kg)	10.25 $\pm$ 0.79
saline + amphetamine (2 mg/kg)	nicotine (0.1 mg/kg)	11.37 $\pm$ 1.97
rimonabant (0.5 mg/kg) + amphetamine (2 mg/kg)	nicotine (0.1 mg/kg)	12.31 $\pm$ 0.90
rimonabant (1 mg/kg) + amphetamine (2 mg/kg)	nicotine (0.1 mg/kg)	12.00 $\pm$ 0.89
WIN 55,212-2 (0.25 mg/kg) + amphetamine (2 mg/kg)	nicotine (0.1 mg/kg)	11.75 $\pm$ 0.52
WIN 55,212-2 (0.5 mg/kg) + amphetamine (2 mg/kg)	nicotine (0.1 mg/kg)	12.20 $\pm$ 1.77

endogenous CB1 agonist anandamide (AEA), injected into the PFC, caused an anxiolytic effect in rats, whereas higher doses induced anxiety-like behavior. When AEA level in the PFC were increased by microinjection of a selective inhibitor of fatty acid amide hydrolase, the authors observed an anxiolytic response at low, but not at high doses, whereas a decrease of AEA levels in the PFC produced an anxiogenic effect. These findings support a role for physiological increases in AEA in the PFC in the development of anxiolytic effects, while more marked increases or decreases of AEA might lead to an anxiogenic response. It has been also revealed that low doses of THC microinjected into the PFC and ventral hippocampus, but not into basolateral amygdala induced an anxiolytic response measured in rat EPM test, whilst higher doses switched an anxiogenic profile (32).

Cannabinoids are frequently abused not only alone, but also in combination with other drugs of abuse like nicotine, ethanol, opioids, cocaine and amphetamine. Recently, pharmacological evidence points to the hypothesis that the endocannabinoid system might influence the direct reinforcing effect of these drugs (33-35). Little attention has been paid to the interaction between cannabinoid and psychostimulants to control anxiety. Starting from this background, the objectives of the present experiment were to further unfold the involvement of cannabinoid system in nicotine- and d-amphetamine-induced anxiety-related effects.

Concerning nicotine, in animal models this drug affects anxiety in different ways. In rodents, it has been shown that nicotine can be anxiogenic, anxiolytic or have no effect on anxiety level (22, 24, 36). It is well known that neuronal nAChRs are implicated in learning and memory processes, reward, antinociception and anxiety (37), and their activation by nicotine enhances release of

many neurotransmitters (*e.g.* acetylcholine itself, GABA, monoamines and glutamate) (5). With respect to this primary target of nicotine action, the contribution of alpha4beta2 and alpha7 subunit of the nAChRs, especially in the dorsal hippocampus, was suggested for its anxiogenic effects of after an acute administration, and appears to involve stimulation of postsynaptic hippocampal 5-HT<sub>1A</sub> receptors (38). This effect may be also accompanied by the increased secretion of corticosteroids through the activation of the hypothalamic-pituitary-adrenal axis (39). Furthermore, because of the overlapped distribution of nAChRs on dopaminergic pathways, the primary target of addictive psychostimulants, and the similarity of their central effects, nicotine is thought to play a role as a trigger for other abused drugs, including amphetamine (40).

Besides the functional interactions of nicotine with brain nAChRs, other different mechanisms mediating anxiety-related behaviors can be predicted, including cannabinoid, GABAergic, serotonergic, and monoaminergic (7). Among them, interactions between brain cannabinoid (CB1) receptors and nAChRs as well as behavioral interactions between cannabinoids and nicotine have been demonstrated (27, 34, 41). Accordingly, co-administration of sub-threshold doses of THC and nicotine produced an anxiolytic effect in animals (12), while rimonabant attenuated nicotine-induced anxiolytic effects and increased the anxiogenic response in the EPM test (10). The recent results also suggested that nicotine exposure would be able to enhance endocannabinoid concentration in the brain structures involved in the control of emotional responses (10). However, the pharmacological activation of cannabinoid receptors by an administration of THC or WIN 55,212-2 did not enhance nicotine anxiolytic-like effects, suggesting that this physiological

interaction can not be potentiated by the simultaneous pharmacological activation of both systems (9, 10). On the other hand, the blockade of CB1 receptors by cannabinoid receptor antagonists, such as rimonabant and AM 251, may elevate the basal level of acetylcholine in some brain areas (42). In our experiments we observed that the activity of CB1 cannabinoid receptors is required to induce anxiety-related effects of nicotine administration since this response was abolished by rimonabant.

Unlike CB receptor antagonists, interactions of the synthetic cannabinoid receptor agonists with nicotine behavior have not been thoroughly investigated. Thus, in the present study, also the pharmacological activation of cannabinoid receptors by an administration of WIN 55,212-2 attenuated both anxiogenic and anxiolytic nicotine effects, and this effect confirms our recent data concerning mnemonic effect of nicotine (19). In order to further explain the reasons for such conflicting results, the blockade of a basal tonic stimulation of the cannabinoid system by their endogenous ligands can be pointed out as a possible mechanism (10, 19). Stimulation of CB1 receptors by WIN 55,212-2 can mimic the effects induced by nicotine on the endocannabinoid system, and counteract nicotine responses by this mechanism. It is important to note that WIN 55,212-2 is a non-specific CB receptor agonist, and its alternative mechanism of action is through an interaction with both CB1 cannabinoid receptors in the brain and CB2 peripheral cannabinoid receptors (43). Accordingly, it has been revealed that WIN 55,212-2-evoked [<sup>3</sup>H]dopamine release was not inhibited by the CB1 cannabinoid receptor agonist AM 251, indicating that this effect is not mediated by CB1 receptors (44). The main problem with the lack of convergence of the data may also lie in the lack of selectivity of the cannabinoid receptor ligands, the known inverse agonistic properties of most cannabinoid receptor antagonists or the involvement of different (*i.e.* CB1 and non-CB1) receptor subtypes, as already stated (6, 45). We may propose that both WIN 55,212-2 and rimonabant mediate dopaminergic and cholinergic neurons through an indirect modulatory effect, such as alteration in endocannabinoid regulatory system, and this effect can explain similar effects of CB receptor agonist and antagonist on nicotine effect observed in the present study.

There are several possible reasons for the frequent association between cannabis and amphetamine (or ecstasy) in humans, such as attenuation of the negative side effects associated to amphetamine consumption (46). Despite the different mechanisms of action (cannabinoid receptors or monoamine transporters, respectively) both cannabinoids and amphetamine modulate common physiological processes such as locomotor activity, body temperature, reward as well as anxiety-related responses (15). Accordingly, THC administration prevented hyperthermia, hyperlocomotion and anxiety-like effects of MDMA in rodents (16). In turn, MDMA could reduce the severity of THC withdrawal syndrome by attenuating somatic signs (15). In our present study, similarly to nicotine, we have revealed that both acute anxiogenic and subchronic anxiolytic effects of d-amphetamine were diminished by CB receptor agonist WIN 55,212-2 and CB1 receptor antagonist rimonabant. Our working hypothesis is that amphetamine and cannabinoid receptor ligands share some common sites of action that are probably related to CB1 receptors in the brain. With regard to the known inhibitory effects of cannabinoid agonists on the release of several neurotransmitters, including dopamine, glutamate and GABA, it is possible that rimonabant blocks the disinhibitory action of an endocannabinoid tone on GABAergic neurons or, alternatively, on the excitatory glutamatergic input to the GABA-containing neurons in the dopamine mesocorticolimbic pathway (3, 45, 47). Finally, in our study, the development of full crossover effects between nicotine and d-amphetamine may

suggest that their behavioral anxiety-related effects observed in the EPM are controlled by the same neural pathways, probably cannabinoid-dependent.

The mechanisms by which nicotine and amphetamine modify anxiety-related behavior have not yet been completely elucidated. The aim of the present study was to investigate the possible involvement of the endocannabinoid system in the anxiolytic- and anxiogenic-like responses induced by both psychostimulant drugs in the EPM in mice. Interestingly, both WIN 55,212-2, a CB cannabinoid receptor agonist and rimonabant, a CB1 cannabinoid receptor antagonist, blocked an anxiogenic effect of acute nicotine and d-amphetamine as well as an anxiolytic effect of subchronic nicotine and d-amphetamine injections including reciprocal cross-effects between the two drugs. Our results provide the pharmacological evidence for the specific involvement of endocannabinoid system in mediating the effects induced by nicotine and/or d-amphetamine on anxiety-like behavior, and can suggest new findings to support the use of cannabinoid ligands in the treatment of psychostimulant addiction.

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