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Interaction between cannabinoid compounds and diazepam on anxiety-like behaviour of mice

Nima Naderi *, Abbas Haghparast, Ali Saber-Tehrani, Neguine Rezaii, Amir-Mohammad Alizadeh, Abbas Khani, Fereshteh Motamedi

Neuroscience Research Center, Shaheed Beheshti University (Medical Sciences), P.O. Box: 19615-1178, Tehran, Iran

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Abstract

Previous studies have suggested that cannabinoidergic system is involved in anxiety. However, a complete picture of cannabinoid association in the anxiety is still lacking. In the present study, we investigated the possible interaction between cannabinoidergic and GABAergic systems in the anxiety-like behaviour of mice. Intraperitoneal (i.p.) administration of the cannabinoid receptor agonist WIN55212-2 (0.25–5 mg/kg), the endocannabinoid transport inhibitor AM404 (0.25–2 mg/kg) and diazepam (0.25–8 mg/kg) dose dependently exhibited an anxiolytic effect evaluated in terms of increase in the percentage of time spent in the open arms in the elevated plus maze (EPM) test. Administration of certain fixed-ratio combinations (3:1 and 1:1) of WIN55212-2 and diazepam produced a synergistic anxiolytic effect, while the 1:3 combination produced an additive effect. In hole-board test, administration of certain ratios of WIN55212-2–diazepam combination significantly altered the animal behaviour compared to groups that received each drug alone. Co-administration of AM404 (1 and 2 mg/kg) and diazepam (0.5 mg/kg) abolished the anxiolytic effect of the former drug in EPM and the latter in hole-board test, respectively. The combination of an ineffective dose of the fatty acid amide hydrolase (FAAH) inhibitor, URB597 (0.3 mg/kg, i.p.) on anxiety-related responses with an ineffective dose of diazepam (0.25 mg/kg, i.p.) led to a synergistic effect. Co-administration of the CB1 receptor antagonist, AM251 (5 mg/kg) and an effective dose of diazepam (2 mg/kg, i.p.) attenuated diazepam-induced elevation of percentage of time spent in open arm, while lower dose of AM251 (0.5 mg/kg) failed to inhibit diazepam-induced anxiolytic effect. Taken together, the present study showed that co-administration of exogenous cannabinoids and diazepam produce additive or synergistic effect at different combinations. Moreover, it has been shown that enhancement of the function of endocannabinoids could increase the anxiolytic effect of diazepam.

Keywords: Anxiety; Cannabinoids; Diazepam; GABA; Elevated plus maze; Hole-board; Mice

1. Introduction

Several findings suggest that cannabinoid system, through the activation of cannabinoid CB1 receptors, is involved in the modulation of anxiety-related behaviour (Piomelli et al., 1998; Viveros et al., 2005; Patel and Hillard, 2006). However, the anxiety-related effects of cannabinoids remain controversial as agonists show opposite effects in different studies. Some authors suggested that the anxiolytic or anxiogenic action of cannabinoid CB1 agonists is dose-dependent. For instance, it has been shown that CP55940 and WIN55212-2, two potent cannabinoid receptor agonists, produce anxiolytic effects in mice submitted to the elevated plus maze (EPM) model of anxiety, at low doses only (Patel and Hillard, 2006). However, higher doses of these compounds could produce an anxiogenic profile (Viveros et al., 2005). Another hypothesis suggested that cannabinoid action on anxiety-like behaviour of animals is species-dependent. Based on behavioural and electrophysiological findings, Haller et al. (2007a,b) showed that WIN5521-2 reduced anxiety in mice by affecting GABA neurotransmission, whereas it increased anxiety in rats via glutamatergic mechanisms. The results of several

* Corresponding author. Tel./fax: +98 21 22431624.
E-mail address: naderi@sbmu.ac.ir (N. Naderi).

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studies are consistent with Haller's hypothesis. For instance, the natural active ingredient of cannabis plant, delta-9-tetrahydrocannabinol (THC) produced the anxiolytic effects on mice in the light–dark test (Berrendero and Maldonado, 2002). In contrast, THC was anxiogenic in adult rats submitted to the EPM and light–dark tasks (Schramm-Sapyta et al., 2007). Regarding the role of endocannabinoids on anxiety, the results are still controversial. Several studies suggest an anxiolytic role for these compounds. For instance, anxiety is increased by both the genetic disruption of the CB1 receptor and its pharmacological blockade by AM-251 or rimonabant in mice (Navarro et al., 1997; Haller et al., 2002, a,b; Uriguen et al., 2004; Patel and Hillard, 2006). It has been shown that stress may accompany the reduction in endocannabinoid levels at synapses, suggesting a tonical release of endocannabinoids under resting condition in some regions of the brain which are involved in coping with stress (Patel et al., 2004). In contrast, conflicting data has been reported using cannabinoid CB1 receptor mutant mice in the shock-probe burying test (Degroot and Nomikos, 2004). In rats, the anxiolytic effect of endocannabinoids seems to be more dominant. Pharmacological blockade of the enzyme fatty acid amide hydrolase, which is responsible for intracellular anandamide degradation, produces anxiolytic effects on adult rats tested in the elevated zero maze (Gaetani et al., 2003) and in the isolation-induced ultrasonic vocalization paradigm in rat pups (Kathuria et al., 2003). The peripheral injection of the anandamide transport inhibitor, AM404, exhibited anxiolytic-like effects in different rat models of anxiety. These effects were accompanied by an increased brain level of anandamide and were prevented by cannabinoid CB1 receptor blockade (Bortolato et al., 2006; Rutkowska et al., 2006).

Anatomical studies have shown that CB1 receptors are widely distributed in the brain structures involved in emotional control including basolateral amygdala, cortical (the entorhinal, cingulate, frontal and prefrontal) regions and the hippocampus (Breivogel and Childers, 1998; Herkenham et al., 1990). As a result of this localization, CB1 activation might have a complex pattern of influence upon neurotransmitters known to modulate anxiety (Arevalo et al., 2001; Martin et al., 2002; van der Stelt and Di Marzo, 2003). In addition, cannabinoids could activate the hypothalamic pituitary–adrenal axis which is responsible for the neuroendocrine response to stress (Weidenfeld et al., 1994). However, the exact mechanism by which cannabinoids modulate anxiety-related behaviour is not elucidated yet.

The GABAergic system, in particular $GABA_A$, has a pivotal role in the regulation of anxiety and benzodiazepines are still the most widely used anxiolytic compounds (Roy-Byrne, 2005). Electrophysiological studies have shown that endogenous cannabinoids (eCBs) can retrogradely suppress inhibitory neurotransmitter release at synapses. This type of modulation has been shown in different regions of the brain including structures involved in emotional control such as amygdala (Zhu and Lovinger 2005), prefrontal cortex (Melis et al., 2004) and hippocampus (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Involvement of GABAergic neurons in mediating cannabinoid effects on feeding behaviour has been already reported (Rahminiwati and Nishimura, 1999). However, little attention has been paid to the interaction between cannabinoid and GABAergic system to control anxiety-like behaviour. On the basis of the above evidence, the present study was designed to investigate the interaction between cannabinoidergic and GABAergic systems on anxiety-like behaviour in two models of anxiety in mice: the elevated plus maze (EPM) and hole-board test. In order to test this hypothesis, we utilized cannabinoid compounds anandamide transport inhibitor (AM 404), the cannabinoid receptor agonist (WIN55212-2), the fatty acid amide hydrolase (FAAH) inhibitor (URB597) and the cannabinoid receptor antagonist (AM251).

2. Materials and methods

2.1. Animals

The experiments were carried out on male NMRI mice (Pasteur Institute, Karaj, Iran) weighting 20–25 g. The animals were maintained at 22 °C on a 12 h light–dark cycle with food and tap water available *ad libitum*. All procedures were in accordance with the Shaheed Beheshti University of Medical Sciences Guideline for the Care and Use of Laboratory Animals and were approved by the local Research and Medical Ethics Committee.

2.2. Drugs

Diazepam, WIN55212-2, AM404 and AM251 were obtained from Sigma-Aldrich, Steinheim, Germany. The drugs except for URB597 were suspended in vehicle (80% saline, 18% dimethylsulfoxide (DMSO), 1% emulphor, 1% ethanol) and were delivered by intraperitoneal (i.p.) injection at a volume of 10 ml/kg. URB597 was dissolved in DMSO and was delivered by i.p. injection at a constant volume of 50 μl.

2.3. Elevated plus maze test

Activity and anxiety-related behaviours were assessed using the mouse elevated plus maze (EPM) test (Dawson and Tricklebank, 1995; Lister, 1987; Pellow et al., 1985). The apparatus consists of two open and two enclosed horizontal perpendicular arms (30 × 5 cm) positioned 40 cm above the floor. The junction of four arms forms a central square platform (5 × 5 cm). All drugs, either individually or in combination, were given 30 min before submitting the animal to the EPM apparatus. Each animal was placed in the central platform facing one of the open arms and allowed to explore freely for 5 min. Between each trial, the maze was thoroughly cleaned with 10% ethanol solution and afterwards by a dry cloth. The experiments were conducted under artificial laboratory illumination (fluorescent lamps, 80 lx at maze level). The sessions were recorded by a camera positioned right above the maze hanging from the ceiling. Data were obtained using Ethovision software (version 3.1), a video tracking system for automation of behavioural experiments (Noldus Information Technology, the Netherlands). During the 5 min trial, the behaviour of each mouse was recorded as: (i) the number of entries into the open or closed arms and (ii) average time spent by mouse in each of the arms. The number of entries into open arms

(OAE) and the time spent in open arms (OAT) were expressed as percentages of total entries and total test time, respectively (i.e. % OAE and %OAT). The increase in both %OAT and %OAE have been shown to be an index of lowered anxiety behaviour. The number of entries into closed arms (CAE) is an index of animal activity.

2.4. Hole-board test:

This assay was conducted in an acrylic arena with a floor of 60 cm × 30 cm. the floor consisted of 16 evenly spaced holes (3 cm in diameter) with built-in infra red sensors. The interruption of the light beam by mouse head triggers a counting device that records the number of head-dips. The apparatus was elevated to the height of 15 cm. Mice were randomly divided into groups with 8 mice per group. The groups received graded doses of diazepam or WIN55212-2 or combination of these two compounds. One group received vehicle to serve a control. 45 min after drug administration, mice were placed singly in the center of the hole-board, and during a 5-min trial the number of head-dipping and the latency to the first head-dipping were measured. At the end of each test the animal was removed and the floor was cleaned. It has been indicated that head-dipping behaviour was sensitive to changes in the emotional state of the animal, and the expression of an anxiolytic state in animals might be reflected by an increase in head-dipping behaviour (Takeda et al., 1998).

2.5. Rota-rod test

To evaluate the effect of drugs on motor coordination, the rota-rod test was carried out after EPM test. The animals were placed on rota-rod apparatus (12 rpm). Falling off the rod during the 90 s of the trial was considered as drug-induced motor impairment.

2.6. Study design

The animals were acclimatized to the experimental room for at least 2 h before performing the experiments. To minimize the confounding factors, the experiment trials were carried out in a room which had been separated from laboratory environment and all the measurements were performed automatically and without presence of investigator. Experiments were done between 1 *p*.m. and 5 *p*.m. to minimize the confounding effects of circadian rhythms.

2.7. Isobolographic analysis

One approach for assessment of the interaction between two compounds of particular pharmacological properties is the isobolographic method (Tallarida, 2000). To perform isobolographic analysis, graded doses of WIN55212-2 and diazepam were administered to groups of at least 8 mice and the percent time spent in open arms (%OAT) were measured for each group. Then, the corresponding dose–response curves for each drug were obtained. Based on the data of these curves, the ED_{50} of WIN55212-2 (i.e. the dose of compound needed to increase % OAT by 50% of maximum) and the equieffective dose of diazepam (i.e. the dose of diazepam that produced the equal effect to ED_{50} of WIN55212-2) were calculated. The ED_{50} of WIN55212-2 was plotted on the abscissa and the equieffective dose of diazepam was plotted on the ordinate. The theoretical additive effect of the two drugs (ED_{50}add) was represented by the straight line connecting the two ED_{50} points (line of additivity). For drug combinations (i.e. diazepam + WIN55212-2), different doses of drug mixtures for three fixed combination ratios of WIN55212-2 and diazepam (1:3, 1:1 and 3:1) were administered to groups of 8 animals and corresponding dose–response curve for each combination was obtained. The ED_{50} values for each combination (ED50mix) were calculated in a similar way using linear regression. If the ED_{50}mix and its confidence intervals lie on the line of additivity, the drug effects are additive (no interaction). If the points lie below this line, there is superadditivity (synergism), and if they lie above this line, there is subadditivity (antagonism). Table 1 shows the doses of diazepam and WIN55212-2 administered either alone or in combination.

2.8. Data analysis

Results were presented as mean ± SEM and were analyzed using Graph Pad Prism software (version 4, Graphpad Software

Table 1
Dose of diazepam, WIN55212-2 and fixed-ratio combinations used in the mouse elevated plus maze model

Diazepam/WIN55212-2 fixed-ratio combinations	Diazepam dose (mg/kg)	WIN55212-2 dose (mg/kg)
Diazepam alone	0.500	–
	1.000	–
	2.000	–
	4.000	–
	8.000	–
1:3	0.150	0.450
	0.200	0.600
	0.250	0.750
	0.300	0.900
	0.400	1.200
1:1	0.300	0.300
	0.400	0.400
	0.600	0.600
	0.700	0.700
	0.900	0.900
	1.200	1.200
	1.500	1.500
3:1	0.300	0.100
	0.375	0.125
	0.450	0.150
	0.600	0.200
	0.750	0.250
	0.900	0.300
	1.200	0.400
WIN55212-2 alone	–	0.250
	–	0.625
	–	1.000
	–	2.000
	–	5.000

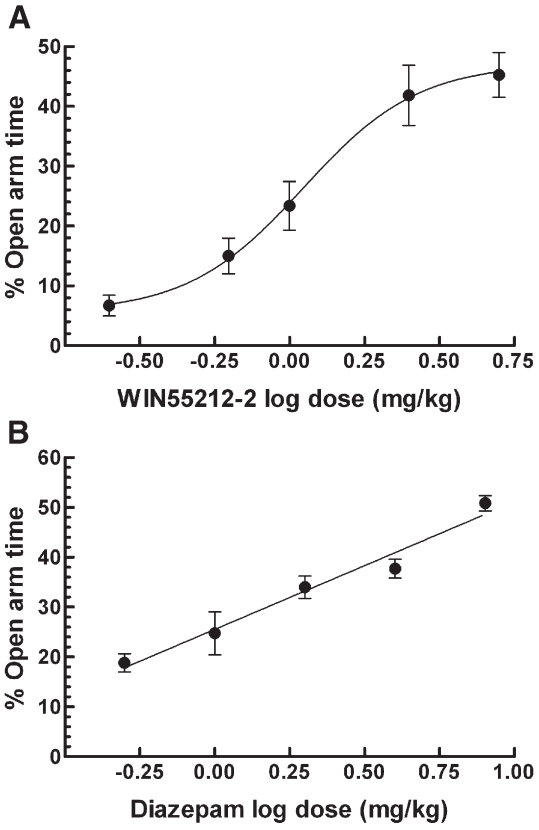

Fig. 1. Log dose–response relationship for the effect of i.p. administration of WIN55212-2 (A) and diazepam (B) on anxiety-like behaviour of mice. The *Y*-axis represents the percent time spent in open arms of the elevated plus maze model. Points represent the mean±S.E.M from 8 mice.

Inc., USA). Dose–response data for the WIN55212-2 alone and diazepam alone were analyzed using non-linear and linear regression respectively. Statistical analysis of the isobologram was performed according to Tallarida method (Tallarida, 2000). The theoretical ED_{50} value was compared with the experimental ED_{50} by Student's *t*-test to determine whether there was a statistically significant difference. Other data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Dunnett's, Newman–Keuls' or Bonferroni's test for multiple comparisons, as appropriate. *p* values less than 0.05 ($p<0.05$) were considered significant.

3. Results

3.1. Elevated plus maze test

3.1.1. Effect of WIN55212-2 and diazepam on anxiety-like behaviour

Assessment of behaviour in the elevated plus maze revealed a dose-dependent increase in %OAT for both diazepam-treated and WIN55212-2-treated mice. One-way ANOVA followed by Dunnett's test revealed a significant increase in %OAT in mice received different doses of WIN55212-2 [$F(5,42)=15.37$, $p<0.0001$] or diazepam [$F(5,36)=40.14$, $p<0.0001$] compared to vehicle treatment group. Dose–response curves for the anxiolytic effect induced by WIN55212-2 and diazepam are shown in Fig. 1A and B respectively. The ED_{50} values were summarized in Table 2. WIN55212-2 also increased %OAE at 0.625, 1 and 2.5 mg/kg [$F(5,47)=43.25$, $p<0.001$; Fig. 2A]. An increase in %OAE was also observed for diazepam at 2, 4 and 8 mg/kg compared to vehicle-treated mice [$F(6,55)=22.40$, $p<0.0001$; Fig. 3A]. Statistical analysis indicated no difference in closed arm entry (CAE) in WIN55212-2 treated [$F(7,56)=0.8433$, $p>0.05$; Fig. 2B] and diazepam-treated mice [$F(6,55)=0.8932$, $p>0.05$; Fig. 3B] compared to vehicle-treated mice.

Table 2
Effect of WIN55212-2, diazepam and their fixed-ratio combinations in the mouse elevated plus maze test

WIN55212-2/diazepam combinations	ED_{50}mix (mg/kg)	ED_{50}add (mg/kg)	*p* value
WIN55212-2 alone	1.230±0.168	–	–
Diazepam alone	0.863±0.134	–	–
1:3	1.027±0.123	0.929±0.110	>0.05
1:1	0.407±0.178 *	1.076±0.057	<0.05
3:1	0.947±0.057 *	1.109±0.047	<0.05

The ED_{50} was calculated using %OAT of each treatment group.
Data are presented as ED_{50} ±S.E.M. Statistical analysis was performed with Student's *t*-test.
* $p<0.05$ vs the respective additive group.

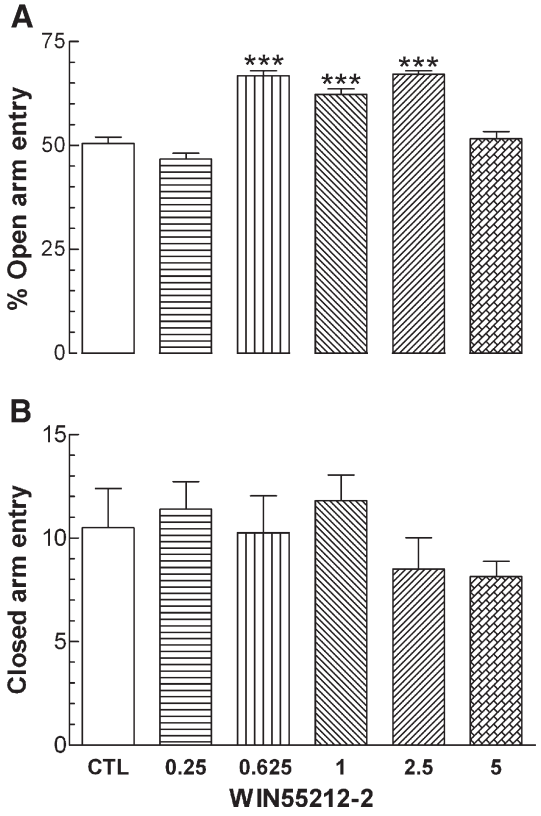

Fig. 2. Effect of the i.p. administration of different doses of WIN55212-2 on percent open arm entry (A) and closed arm entry (B) during 5 min exposure to the elevated plus maze. Bars represent the mean±S.E.M. from 8 mice. ***$p<0.001$ significantly different from control (CTL) group.

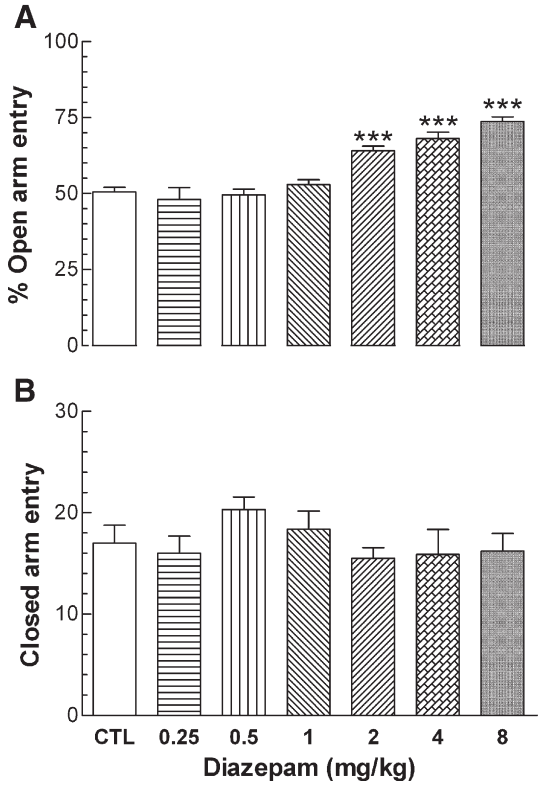

Fig. 3. Effect of the i.p. administration of different doses of diazepam on percent open arm entry (A) and closed arm entry (B) during 5 min exposure to the elevated plus maze. Bars represent the mean±S.E.M. from 8 mice. *$p<0.05$, ***$p<0.001$ significantly different from control (CTL) group.

3.1.2. Effect of the combinations of WIN55212-2 and diazepam on anxiety-like behaviour

As described before, the ED_{50} values of diazepam and WIN55212-2 was calculated from data of %OAT of the EPM test. Isobolographic analysis was performed utilizing the ED_{50} value of the cannabinoid receptor agonist WIN55212-2, plotting on the *X*-axis and the equieffective dose of the diazepam on the *Y*-axis yielded the isobologram shown in Fig. 4. The line connecting the two points on the graph demonstrates all combinations that would theoretically result in additivity. Dose ratios of 1:1, 1:3, 3:1 (WIN55212-2: diazepam) and appropriate dilutions of these dose ratios were applied to determine the experimental ED_{50}mix values for the same fixed-ratio combinations in the mouse EPM model. Based on the equieffective doses for WIN55212-2 and diazepam, the theoretically additive (ED_{50}add) values for WIN55212-2 and diazepam were calculated for the three aforementioned fixed-ratios. The dose-effect data and statistics for the isobolograms are shown in Table 2. The Student's *t*-test was performed to determine the statistically significant difference between ED_{50}mix and ED_{50}add values for each of the fixed-ratio combination. The 1:3 ratio fell upon the line of additivity, which indicates an additive reaction with this combination. The 3:1 and 1:1 combinations fell below and were significantly different from the line of additivity ($p<0.05$) indicating synergistic interactions (Fig. 4).

3.1.3. Effect of AM404 by itself or in combination with diazepam on anxiety-like behaviour

One-way ANOVA revealed a significant increase in %OAT at doses of 1 and 2 mg/kg compared to vehicle-treated mice [$F(4,39)=2.902$, $p<0.05$; Fig. 5A]. One-way ANOVA revealed no significant difference in both %OAE [$F(4,35)=2.57$, $p>0.05$; Fig. 5B] and CAE [$F(4,35)=0.83$, $p>0.05$; Fig. 5C] compared to control group. The combination of AM404 (1 and 2 mg/kg) and diazepam (0.5 mg/kg) were without effect on % OAT [$F(3,28)=1.23$, $p>0.05$; Fig. 6A], %OAE [$F(3,28)=1.45$, $p>0.05$; Fig 6B] and CAE [$F(3,28)=2.6$, $p>0.05$; Fig. 6C] compared to vehicle-treated (control) mice.

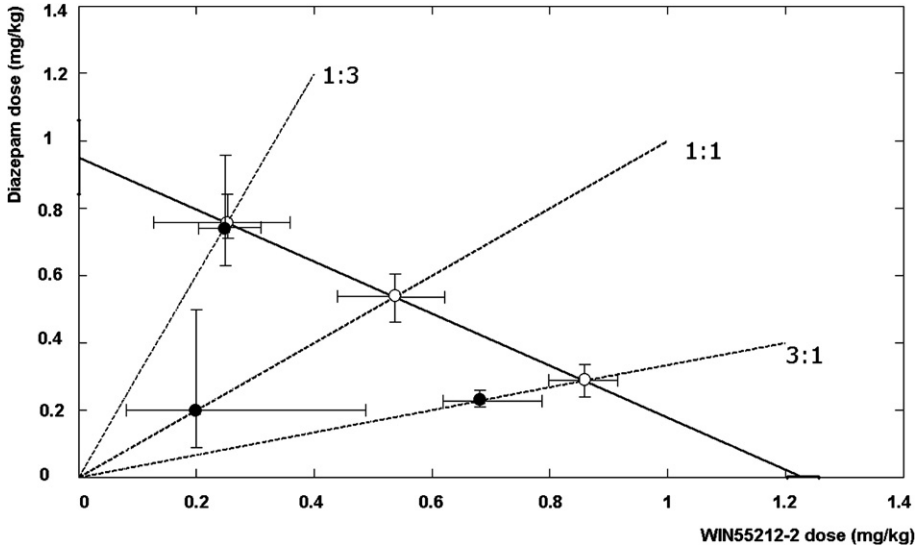

Fig. 4. Isobologram showing the interactions between WIN55212-2 and diazepam in mouse elevated plus maze test. The ED_{50} value for WIN55212-2 and the equieffective dose for diazepam (both values calculated from data of Fig. 1) are placed on the graph, on *X*- and *Y*-axes, respectively. The isobole of additivity is shown as a solid line drawn between the aforementioned values of WIN55212-2 and diazepam, which connects the *X*- and *Y*-axes. Open circles correspond to the theoretical additive ED_{50} with 95% confidence limits and filled circles correspond to the experimental ED_{50} of the mixture with 95% confidence limits. The experimental ED_{50}mix values of the mixture of WIN55212-2 and diazepam, for the fixed-ratio combinations of 1:1 and 3:1 were found to be significantly below the theoretical isoboles of additivity, indicating super-additive (synergy) interactions.

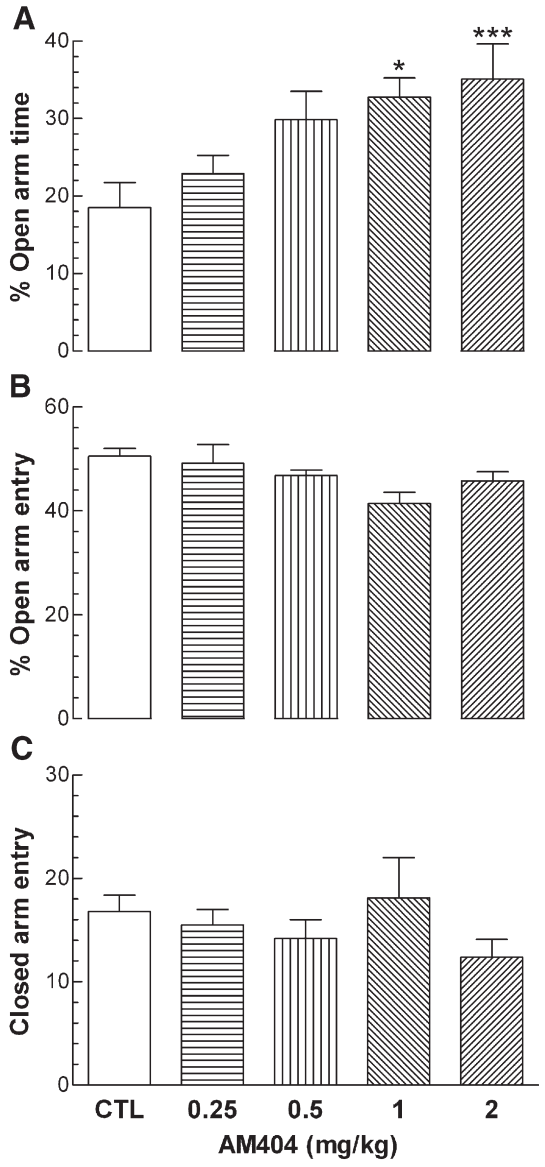

Fig. 5. Effect of the i.p. administration of different doses of AM404 on percent time spent in open arm (A) percent open arm entry (B) and closed arm entry (C) during a 5 min exposure to the elevated plus maze. Bars represent the mean±S.E.M. from 8 mice. *$p<0.05$, ***$p<0.001$ significantly different from control (CTL) group.

3.1.4. Effect of URB597 by itself or in combination with diazepam on anxiety-like behaviour

As shown in Fig. 7, two-way ANOVA revealed a significant main effect for both diazepam [$F(2,63)=23.11$, $p<0.0001$] and URB597 [$F(2,63)=14.86$, $p<0.0001$] to increase percentage time spent in open arm, while the interaction between these two factors was not significant [$F(4, 63)=2.06$, $p>0.05$]. Bonferroni post test revealed that co-administration of diazepam (0.25 mg/kg) and URB597 (0.3 mg/kg) produce significant increase in % OAT compared to control group ($p<0.01$), while mice received either URB597 (0.3 mg/kg) or diazepam (0.25 mg/kg) alone did not show significant difference in %OAT compared to control group. The combination of lower dose of URB597 (0.03 mg/kg) and diazepam (0.25 mg/kg) failed to enhance %OAT compared to control group. Other parameters (i.e. %OAE and CAE) were not significantly affected. Although administration of diazepam at 2.5 mg/kg per se produced anxiolytic effect, but, co-administration of diazepam (2.5 mg/kg) and URB597 (0.3 mg/kg) significantly increased %OAT compared to group received diazepam (2.5 mg/kg) alone ($p<0.001$).

3.1.5. Effect of AM251 alone and the interaction between AM251 and diazepam on anxiety-like behaviour

As shown in Fig. 8, AM251 (5 and 10 mg/kg) produced a significant decrease in both %OAT [$F(4,35)=3.43$, $p<0.05$)] and %OAE [$F(4,35)=2.66$, $p<0.05$] compared to control group. The lower doses of AM251 did not alter %OAT or % OAE compared to control group. There were not significant

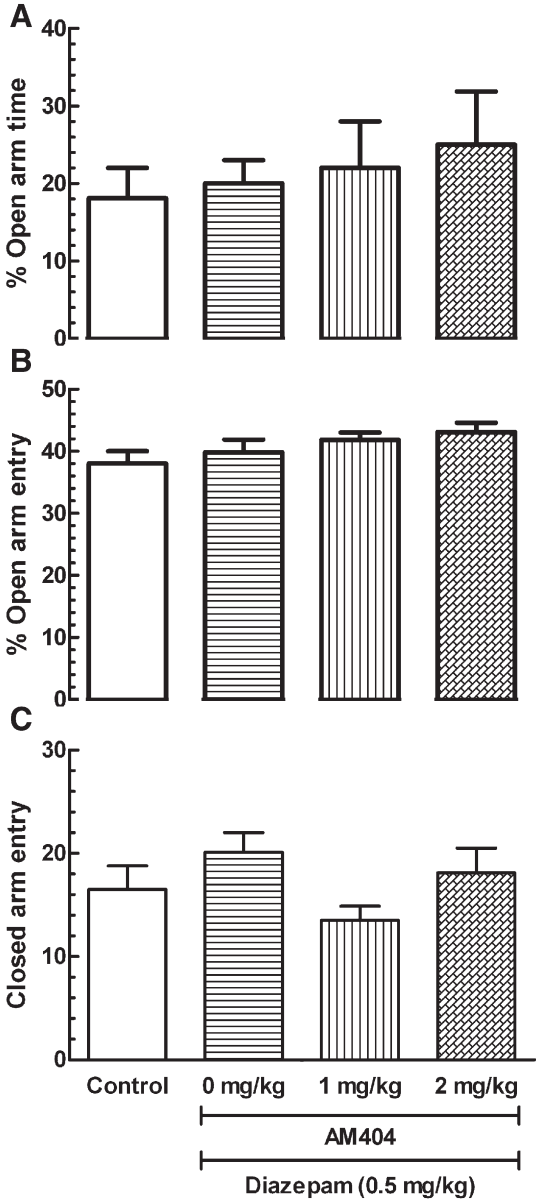

Fig. 6. Effect of the i.p. administration of diazepam (0.5 mg/kg) alone or in combination with AM404 (1 and 2 mg/kg) on percent time spent in open arm (A) percent open arm entry (B) and closed arm entry (C) during 5 min exposure to the elevated plus maze. Bars represent the mean±S.E.M. from 8 mice.

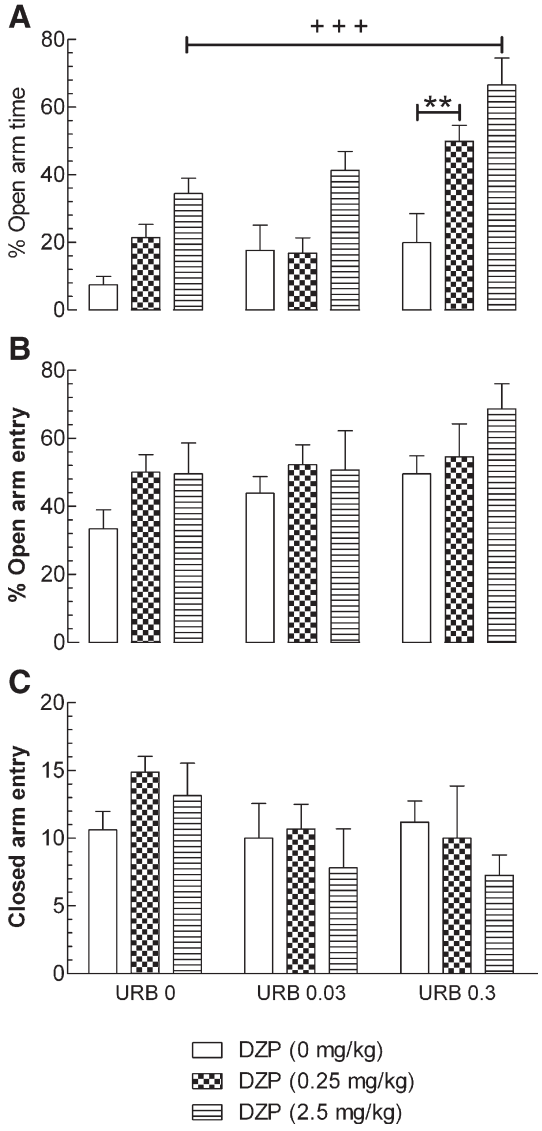

Fig. 7. Effect of the i.p. administration of URB597 (0, 0.03 and 0.3 mg/kg), diazepam (DZP, 0, 0.25 and 2.5 mg/kg) alone or in combination on percent time spent in open arm (A) percent open arm entry (B) and closed arm entry (C) during 5 min exposure to the elevated plus maze. Bars represent the mean ± S.E.M. from 8 mice. $^{**}p<0.01$ significantly different from group received URB 0.3 mg/kg alone. $^{+++}p<0.001$ significantly different from group received diazepam (DZP) 2.5 mg/kg alone.

changes in CAE among groups received various doses of AM251 and control group [$F(4,35)=0.1942$, $p>0.05$; Fig. 8C]. As shown in Fig. 9, co-administration of inactive dose of AM251 (0.5 mg/kg) and active dose of diazepam (2 mg/kg) did not change %OAT compared to mice received diazepam (2 mg/kg) alone.

However, co-administration of an effective dose of AM251 (5 mg/kg) and diazepam (2 mg/kg), significantly decreased both the percent time spent in open arms and the percent open arm entry compared to group received diazepam (2 mg/kg). In addition, %OAE significantly decreased in the group of mice received the combination of AM251 (5 mg/kg) and diazepam (1 mg/kg) compared to mice received the dose of 1 mg/kg diazepam ($p<0.01$; Fig. 9B). On the other hand, the anxiogenic action of AM251was not affected by administration of diazepam at different doses (Fig. 9).

3.2. Hole-board test

3.2.1. The effect of diazepam and WIN55212-2 alone and in combination on anxiety-like behaviour

Hole-board measures for diazepam and WIN55212-2 are summarized in Table 3. Two-way ANOVA revealed a significant interaction between diazepam and WIN55212-2 in the measurement of head-dip counts [$F(2,175)=11.83$, $p<0.001$)]. However, the main effect of diazepam alone [$F(8,175)=1.146$, $p>0.05$] or WIN55212-2 alone [$F(7, 175)=1.397$, $p>0.05$] on the head-dip count of mice was not significant. Post hoc analysis revealed an

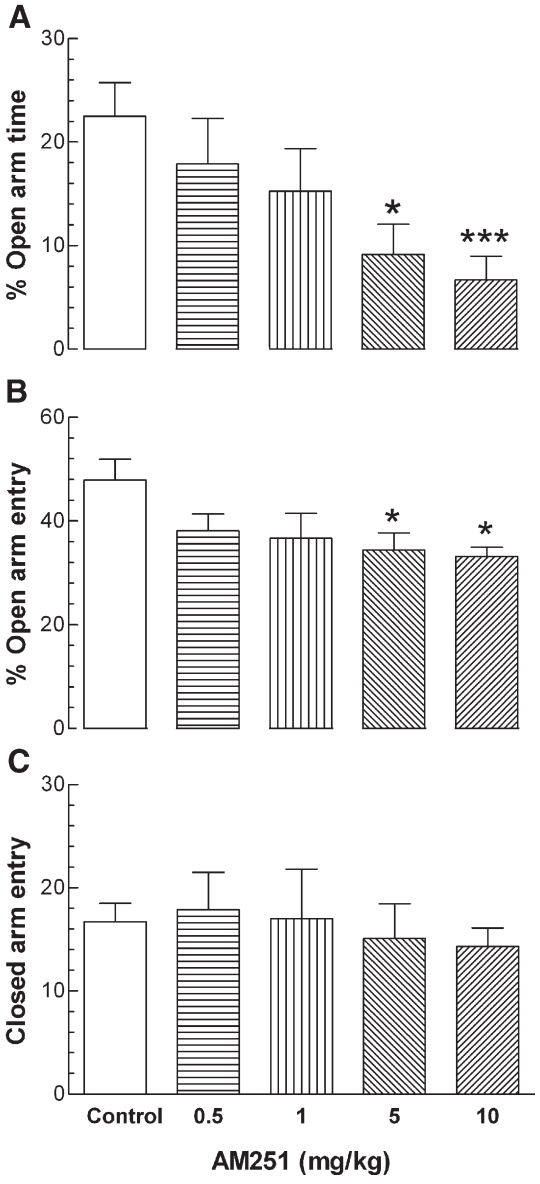

Fig. 8. Effect of the i.p. administration of different doses of AM251 on percent time spent in open arm (A) percent open arm entry (B) and closed arm entry (C) during a 5 min exposure to the elevated plus maze. Bars represent the mean ± S.E.M. from 8 mice. $^{*}p<0.05$, $^{***}p<0.001$ significantly different from control group.

increase in head-dip counts [$F(26,175)=6.85$, $p<0.001$] in mice received the fixed-ratio combinations of diazepam and WIN55212-2 compared to control group, while there was not a significant difference in groups received each drug alone compared to control group. Two-way ANOVA revealed that neither diazepam nor WIN55212-2 produced significant main effect or interaction on head-dip latency of mice in the hole-board test.

3.2.2. The effect of diazepam and AM404 in hole-board test

There was not a significant difference in head-dip count and head-dip latency in groups received AM404 (0.25, 0.5, 1 and 2 mg/kg) compared to control group (Fig. 10). Co-administration of AM404 (1 mg/kg) and diazepam (0.5 mg/kg) significantly decreased the head-dip count compared to group received diazepam (0.5 mg/kg) alone [$F(3,28)=5.41$, $p<0.01$] (Fig. 11A). No significant difference in latency of first head-dipping was observed between control and treatment groups (Figs. 10B and 11B).

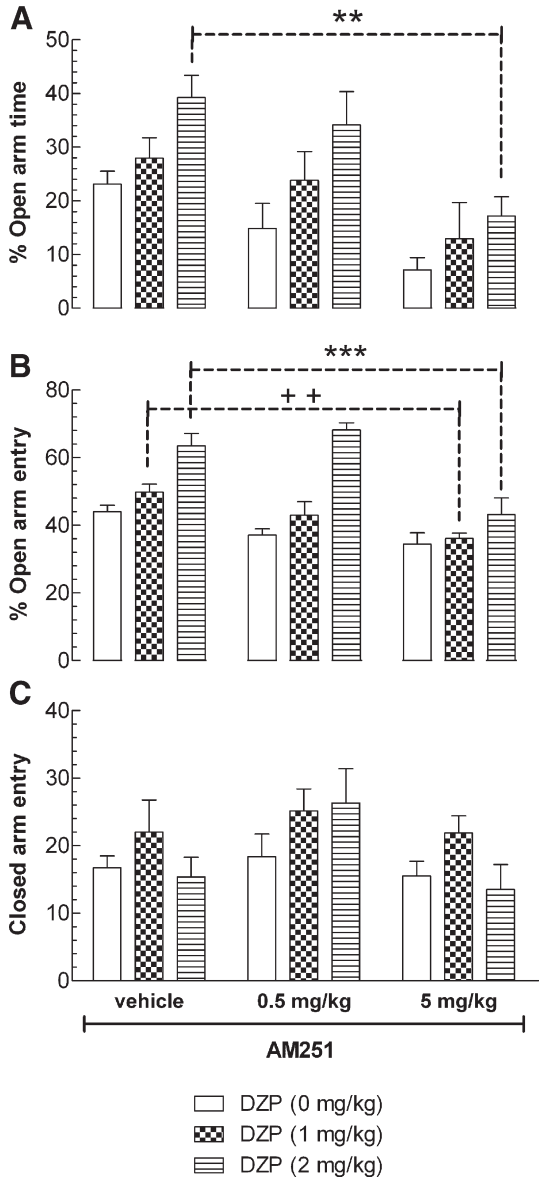

Fig. 9. Effect of the i.p. administration of AM251 (0, 0.5 and 5 mg/kg), diazepam (DZP, 0, 1 and 2 mg/kg) alone or in combination on percent time spent in open arm (A) percent open arm entry (B) and closed arm entry (C) during 5 min exposure to the elevated plus maze. Bars represent the mean±S.E.M. from 8 mice. **$p<0.01$, ***$p<0.001$ significantly different from group received diazepam (DZP) 2 mg/kg alone. $^{++}p<0.01$ significantly different from group received diazepam (DZP) 1 mg/kg alone.

Table 3
Effect of WIN55212-2, diazepam and their fixed-ratio combinations in the mouse hole-board test

Diazepam (mg/kg)	WIN55212-2 (mg/kg)	Head-dip counts	Head-dip Latency (s)
0	0	6.87±1.74	23.87±6.84
0	0.25	12.12±1.88	20.75±3.31
0	0.5	17.00±5.55	15.28±1.79
0	1	12.16±2.30	21.71±4.10
0.25	0	7.62±1.49	20.87±2.66
0.5	0	12.85±3.09	32.75±5.70
1	0	10.16±2.28	23.00±6.83
2	0	14.66±5.49	24.12±6.95
5	0	5.18±1.21	18.62±2.36
0.15	0.45	29.00±4.98	11.57±5.23
0.2	0.60	34.12±4.45	10.32±3.18
0.25	0.75	41.87±4.76	9.87±4.60
0.3	0.90	35.87±6.71	12.87±5.46
0.4	1.20	27.37±4.99	26.37±9.95
0.400	0.400	45.25±5.70	12.00±5.30
0.700	0.700	33.71±4.42	14.71±4.59
1.500	1.500	31.57±7.06	12.50±3.46
1.200	1.200	50.00±5.63	8.42±1.58
0.300	0.300	30.87±3.54	7.87±2.95
0.600	0.600	30.12±4.41	6.50±1.74
0.900	0.900	39.00±7.91	27.75±12.17
0.450	0.150	28.50±4.11	9.25±1.04
0.750	0.250	23.12±4.46	9.87±2.08
0.625	0.125	27.50±3.48	13.50±7.20
0.900	0.300	23.75±2.94	19.25±12.72
1.200	0.400	24.87±3.93	29.62±16.45
0.300	0.100	36.00±4.51	5.75±1.60
0.600	0.200	35.25±3.03	11.75±3.37

Data represent the mean±S.E.M. from 8 mice.

3.3. Rota-rod test

Analysis of the rota-rod data revealed no alteration in motor coordination of mice before and after administration of different doses of diazepam, WIN55212-2, AM404, URB597 and AM251 as well as their combinations used in this study (no fall off during 90 s time on the rod, data not shown).

4. Discussion

The results of the present study demonstrate a synergistic interaction between cannabinoid receptor agonist and diazepam. We also found that augmentation the function of endocannabinoid system can increase the anxiolytic effect of diazepam in the mouse elevated plus maze model. These findings suggest the existence of an interaction between cannabinoidergic and GABAergic systems in the modulation of anxiety-like behaviour of mice. Anxiolytic effects of cannabinoids have long

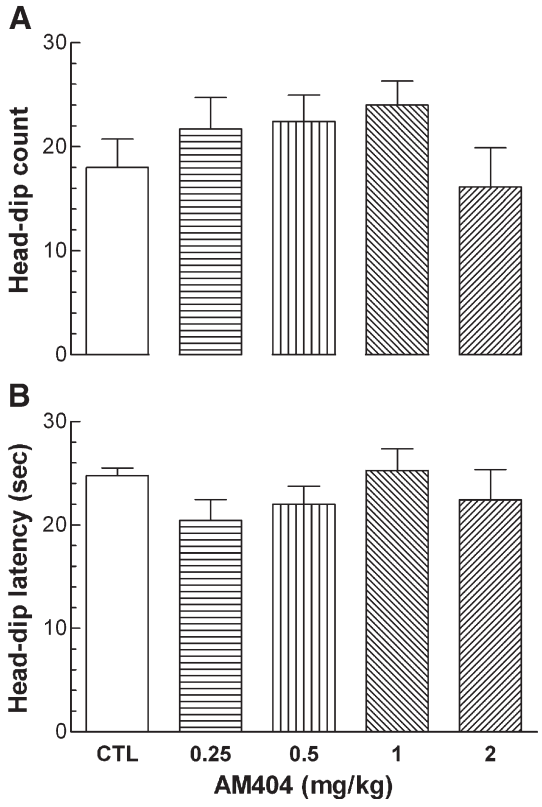

Fig. 10. Effect of the i.p. administration of different doses of AM404 on head-dip count (A) and head-dip latency (B) during a 5 min exposure to the hole-board apparatus. Bars represent the mean±S.E.M. from 8 mice.

been substantiated through a wide range of studies. Cannabinoids, by presynaptic mechanisms, modulate the release of several transmitters implicated in the control of anxiety. Stimulation of CB1 receptors with the subsequent activation of different signaling pathways is the first event underlying the effects of cannabinoids on anxious states. They suppress the outflow of glutamate in the hippocampus (Straiker and Mackie, 2005), periaqueductal grey (Vaughan et al., 2000) and amygdala (Azad et al., 2003). Cannabinoids are also inhibitory to corticolimbic release of norepinephrine, dopamine, and serotonin which are important in emotional states such as anxiety (Arevalo et al., 2001; Tzavara et al., 2003). Another important aspect of the action of cannabinoidergic system in the anxiety-like behaviour of animals is the contribution of central corticotropin releasing factor (CRF) system in the mediation of anxiogenic effects produced by certain cannabinoid receptor agonists. It has been demonstrated that GABA-CRF neurons from the central amygdala are inhibited by potent cannabinoid receptor agonist HU-210 leading to produce anxiety-like behaviour in rats (Rodriguez de Fonseca et al., 1996). It was also shown that the extracellular CRF concentration in amygdala is elevated following withdrawal induced by cannabinoid receptor antagonist rimonabant (Rodriguez de Fonseca et al., 1997). Cannabinoids, on the other hand, interfere with GABAergic transmission in the amygdala, hippocampus and prefrontal cortex. An inhibition of GABAergic activity may induce disinhibition of glutamatergic, noradrenergic and dopaminergic transmission pathways in the locus coeruleus, frontal cortex and nucleus accumbens (Arevalo et al., 2001; Muntoni et al., 2006; van der Stelt and Di Marzo, 2003). The above interactions may result in either anxiolytic or anxiogenic effects which can explain bidirectional action of cannabinoids on anxiety. However, the exact mechanism and the pathways involved in the cannabinoids anxiolytic effect are still to be unveiled. To this end, we targeted the GABA receptor family which has a pivotal role in controlling the stress conditions in human and laboratory animals.

In the present study, we investigated the possible involvement of cannabinoid receptors in diazepam-induced anxiolytic effect. We also determined the type of interaction between the cannabinoid receptor agonist and diazepam in elevated plus maze and hole-board test, which can shed light on the mechanism by which cannabinoids exert their anxiolytic profile.

Confirming the results of earlier studies (Patel and Hillard, 2006), systemic administration of WIN55212-2 individually produced anxiolytic effects by increasing both the percent time spent in the open arms and the number of entries into open arms of the EPM. Likewise, administration of certain doses of diazepam increased %OAT compared to control group. Higher doses of these drugs were not put into the dose–response curves because of the motor side effects (impairment of motor coordination) that preclude efficient determination of the anxiety-like behaviour in mice. Data obtained from %OAT were used to determine an ED_{50} for anxiolytic effect of these compounds. Because of poor dose–response relationship, we could not establish an ED_{50} considering %OAE in mice received

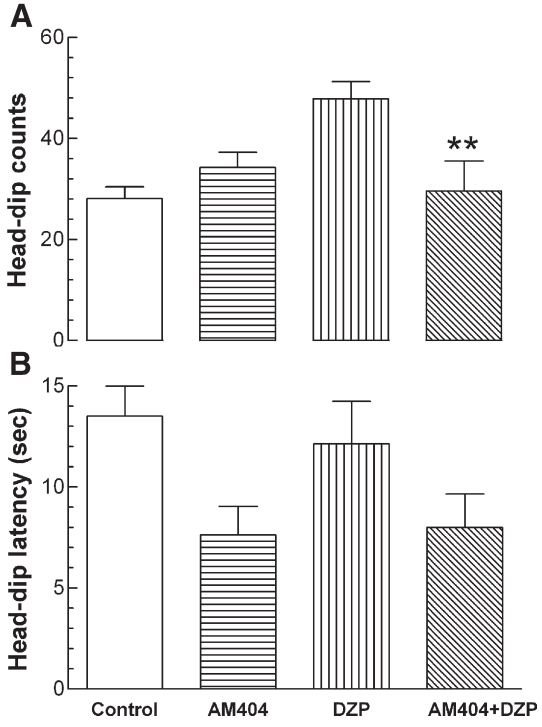

Fig. 11. Effect of the i.p. administration of diazepam (DZP, 0.5 mg/kg) alone or in combination with AM404 (1 mg/kg) on head-dip count (A) and head-dip latency (B) during 5 min exposure to hole-board apparatus. Bars represent the mean±S.E.M. from 8 mice. **$p<0.01$ significantly different from diazepam (DZP) group.

diazepam or WIN55212-2. When administered in combination, exogenous cannabinoid receptor agonist WIN55212-2 potentiated diazepam-evoked anxiolytic effect in the mouse EPM model of anxiety. This enhancement was synergistic, i.e. greater-than-additive, in certain ratios. While The 1:1 and 3:1 combinations (WIN55212-2: diazepam) produced anxiolytic effect in a synergistic manner, the 1:3 combination produced anxiolytic effect in an additive manner. In contrast to our results, Onaivi et al. (1990) reported rather a different interaction between diazepam and another cannabinoid compound, delta 9-THC, in which diazepam blocked the effect of delta 9-THC in the elevated plus maze. Delta 9-THC, the major psychoactive component of marijuana, having partial agonist activity at both CB1 and CB2 cannabinoid receptors. Therefore, its pharmacological effects appear to be strongly influenced by the dose of administration, the expression level and signaling efficiency of cannabinoid receptors and by ongoing endogenous cannabinoid release (Pertwee, 2007). In the present study, we used the full agonist of cannabinoid receptors which could more specifically illustrate the interaction between cannabinoidergic and GABAergic systems in modulation of anxiety. To confirm the results of EPM test, we assessed the interaction between cannabinoids and diazepam on anxiety-like behaviour of mice using the hole-board test. Administration of various doses of diazepam did not change the head-dip counts compared to control group. This finding was consistent with some of the previous studies indicating that diazepam did not alter the head-dip count in mouse hole-board test (Chen et al., 2006; Kliethermes and Crabbe, 2006). Likewise, mice received the various doses of WIN55212-2 did not show changes in head-dip count compared to control group, although a previous study has shown that THC could increase the head-dip count in mice compared to control group (Boucher et al., 2007). The combination of diazepam and WIN55212-2 significantly increased the head-dip count compared to control group. Head dipping on a hole-board is used as an indicator of exploratory tendencies in rodent studies. Takeda et al. indicated head-dipping behaviour was sensitive to changes in the emotional state of the animal, and suggested that the expression of an anxiolytic state in animals might be reflected by an increase in head-dipping behaviour (Takeda et al., 1998). Based on this finding, it could be suggested that co-administration of WIN55212-2 and diazepam produced a synergistic effect in alteration the emotional state of mice and this type of anxiolytic effect could not be observed when using each drug alone. The data could suggest that the synergistic anxiolytic effect between diazepam and WIN55212-2 was possibly due to an interaction between $GABA_A$ and cannabinoid receptors.

What we have discussed thus far was resulted from the administration of exogenous cannabinoids. To become still closer to the essence of this interaction inside the brain, we augmented the effect of endocannabinoids by administration of AM404, a well-documented cannabinoid reuptake inhibitor (Beltramo et al., 1997). When administered individually, AM404 produced a slight but significant anxiolytic effect in the EPM test, supporting the existence of an endogenous cannabinoid system that exerts an anxiolytic tone via CB1 receptor activation. These data concur with previous studies, which have reported that systemic administration of cannabinoid reuptake inhibitor exerted anxiolytic-like effect in different models of anxiety (Bortolato et al., 2006; Kathuria et al., 2003; Patel et al., 2002; Patel and Hillard, 2006). However, in the hole-board test, administration of AM404 did not change the head-dip count or head-dip latency compared to control group. Co-administration of inactive dose of diazepam and active doses of AM404 did not produce synergistic anxiolytic effects in the EPM test. The doses of AM404 and diazepam were carefully chosen to mimic the 3:1 combination ratio of WIN55212-2 and diazepam. i.e. the dose of WIN55212-2 which showed approximately a similar effect on EPM to that of AM404 (the equieffective dose of WIN55212-2) was three times higher than the dose of diazepam. While we expected a synergistic effect from this combination, the result of the experiment did not uphold this prediction. Co-administration of non-anxiolytic dose of diazepam and active doses of AM404 not only did not produce synergistic anxiolytic effects, but also diminished the anxiolytic effect of AM404 compared to when administered individually at the same dose. Interestingly, this drug combination attenuated the diazepam-induced increase of the head-dip count in hole-board test. Several studies indicated that certain anadamide transport inhibitors (e.g. AM404) are structurally similar to the vanilloid receptor agonists such as capsaicin and may also activate vanilloid receptors (De Petrocellis et al., 2000; Zygmunt et al., 2000). It has also been shown that AM404 is partial agonist for the transient receptor potential vanilloid type 1 channel (TRPV1) when compared with capsaicin (Roberts et al., 2002). Recent studies indicated the role of TRPV1 receptor in the anxiety-like behaviour of mice. It has been shown that the TRPV1 knock-out mice show less anxiety-related behaviour in the light–dark test and in the elevated plus maze than their wild-type littermates which indicate an anxiogenic activity for TRPV1 receptor (Marsch et al., 2007). Taken together, it could be suggested that the Overlap between the ligand recognition properties of the anandamide transporter and the capsaicin-like activity of AM404 may result in paradoxical effects in reducing anxiety-like behaviour of mice compared to exogenous cannabinoid receptor agonist WIN55212-2. Another reason to explain the controversy over the effects of WIN55212-2 and AM404 is that there might be other receptorial systems involve in neuropsychological effects of cannabinoids. For example, the orphan G-protein coupled receptor (GPCR) GPR55 which is expressed in several tissues including some brain regions involved in anxiety (Sawzdargo et al., 1999). It has been reported that several endocannabinoids (including anandamide and 2-arachidonoyl glycerol) bind to GPR55 and are potent stimulants, where as some exogenous cannabinoids including WIN55212-2 exhibited weak receptor stimulation (Drmota et al., 2004). Moreover, WIN55212-2 may induce its behavioural effect by binding to other brain-expressed GPCR, known by some as a CB3 receptor (Howlett et al., 2002).

An alternative approach to enhance the availability of endocannabinoids at synapses is the inhibition of enzymes responsible for degradation of these ligands, of which fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase are the

most important (McKinney and Cravatt, 2005). Specific inhibitors of FAAH (e.g. URB597) have been developed that significantly increase the levels of anandamide, thereby potentiating the effects of anadamide at synapses (Kathuria et al., 2003). URB597 was used to investigate whether augmentation of cannabinergic tone might be therapeutically beneficial in the anxiolytic effect of diazepam. Co-administration of URB597 and diazepam significantly enhanced the percentage time spent in open arm in the EPM model suggesting that endogenous cannabinoid are involved in the anxiolytic effects of diazepam. Previously, it has been shown that FAAH inhibitors attenuate anxiety via an increased activation of CB1 receptors (Kathuria et al., 2003; Moreira et al., in press; Patel and Hillard, 2006). However, anandamide is a neuromodulator that may bind to other site in the brain, implying that alternative mechanisms, apart from the activation of CB1 receptors may be involved in its action. For example, the TRPV1 is activated by anandamide and located in several brain region related to emotions. Therefore, increasing the endogenous levels of anandamide may induce effects that are not only CB1-mediated, but also dependent on other receptors. AM404, as a partial agonist of TPRV1 receptor, decreased the anxiolytic effect of diazepam. On the other hand, augmentation of anadamide effect on TRPV1 receptor by URB597 increased the anxiolytic effect of diazepam. Therefore, it is likely that elevated levels of endocannabinoids acting via TRPV1 receptors are partly responsible for the decreased anxiety in mice submitted to models of anxiety. These findings could suggest a possible different mechanism of action for the exogenous and endogenous cannabinoids in some of the aspects of their anxiolytic effect. Although the exact mechanism of anxiolytic action of cannabinoids still can not be explained completely, but at least it could be suggested that cannabinoids act differently in physiologic (endocannabinoids) and pharmacologic (administration of exogenous cannabinoid receptor ligands) conditions.

To further unfold the mechanism behind the involvement of cannabinoid CB1 receptor in diazepam-induced anxiolytic effect, the cannabinoid CB1 antagonist, AM251, was co-administered with diazepam. Administered individually, AM251 exerts an anxiogenic effect. This finding is in keeping with previous reports (Navarro et al., 1997; Patel and Hillard, 2006). Co-administration of an effective dose of AM251 and diazepam attenuated the anxiolytic effect of diazepam in EPM test. It was likely because of anxiogenic effect of AM251 per se, so that the overall effect obtained from co-administration of these two compounds decreases the anxiolytic effect of diazepam. To minimize the anxiogenic effect of AM251, an active dose of diazepam was co-administered with a dose of AM251 which was without effect on anxiety. But, this drug combination did not alter the anxiolytic effect of diazepam in the EPM test. Likewise, administration of various doses of diazepam did not alter the anxiogenic effect of cannabinoid receptor antagonist.

5. Conclusion

Taken together, the present study showed that co-administration of exogenous cannabinoids and diazepam produce additive or synergistic effect at different combinations. Besides, the results support the hypothesis of the involvement of cannabinoidergic system in the modulation of anxiolytic effect of diazepam and also suggest that anandamide hydrolysis inhibitors might be potential anxiolytic drugs. Although using direct cannabinoid receptor agonist in the treatment of anxiety is not appropriate due to their psychological adverse effects, however, drugs with indirect action on endocannabinoid system may be more acceptable. Therefore, it could be suggested that enhancement the function of endocannabinoids (e.g. by using URB597) in combination with putative anxiolytic drugs (e.g. diazepam) may offer novel therapeutic approaches for treatment of anxiety.

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