Pharmacological Reports
2006, 58, 200–206
ISSN 1734-1140

Effects of cannabinoids on the anxiety-like response in mice

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Abstract:
Several pieces of anatomical, biochemical and pharmacological evidence indicate that the endocannabinoid system *via* CB_1 receptors is implicated in the control of emotional behavior. However, previous studies have reported unclear and contradictory results concerning the role of cannabinoids in anxiety. The aim of the present study was to examine the influence of the cannabinoid agonist WIN 55,212-2 (1 and 5 mg/kg), the CB_1 antagonist AM 281 (1, 2 and 4 mg/kg), the inhibitor of anandamide hydrolysis $AACOCF_3$ (1 and 4 mg/kg) and the inhibitor of anandamide transporter AM 404 (1 and 4 mg/kg) on the anxiety-like response in mice in the light/dark box test. WIN 55,212-2 (5 mg/kg) induced the anxiogenic-like effect accompanied by motor inhibition, $AACOCF_3$ (4 mg/kg) induced the selective anxiolytic-like effect, whereas AM 404 and AM 281 were without effect. Pretreatment with AM 281 (2 mg/kg) blocked the anxiogenic-like and sedative responses induced by WIN 55, 212-2, as well as the anxiolytic-like effect of $AACOCF_3$. These results support the hypothesis that the endocannabinoid system is involved in the regulation of anxiety-like behavior, and also suggest that the inhibitors of anandamide hydrolysis might be potential anxiolytic drugs.

Key words:
cannabinoid, CB_1 receptor, light/dark box test, anxiety, WIN 55,212-2, AM 281, AM 404, $AACOCF_3$

Abbreviations: 2-AG – arachidonoylglycerol, Δ^9-THC – Δ^9-tetrahydrocannabinol, CNS – central nervous system.

Introduction

Cannabis and cannabinoids exert many of their biological functions by two types of cannabinoids receptors: CB_1, identified in 1988 and subsequently cloned in 1990; and CB_2, cloned in 1993. CB_1 receptors are located mostly in the central nervous system (CNS) and also expressed on peripheral neurones. In contrast, CB_2 receptors are predominantly confined to the periphery and are involved in the immunoregulatory effect of cannabinoids [3, 5, 18, 30, 33]. Both receptors inhibit cAMP formation *via* $G_{i/o}$ proteins, and activate mitogen-activated-protein kinase [5, 6]. In addition, CB_1 receptor stimulation activates ion channels such as A-type and inwardly rectifying potassium channels, and inhibit voltage sensitive N-type and P/Q-type Ca^{2+} channels [14, 24]. New data suggest possible existence of a third cannabinoid receptor "CBx" located in the brain. It is sensitive to WIN 55,212-2 (a CB_1 and CB_2 receptor agonist), anandamide and SR141716A (a selective CB_1 antagonist), but not to Δ^9-tetrahydrocannabinol (Δ^9-THC) [4]. Five endocannabinoids have been described so far: N-arachidonoylethanolamine (anandamide), 2-arachidonoylglycerol (2-AG), 2-arachidonylglyceryl ether (noladin), O-arachidonoylethanolamine (virodhamine) and N-arachidonoyldopamine [10, 17, 19, 32, 34, 36]. Anandamide and 2-AG are thought to act as retro-

grade synaptic messengers in the CNS. By activating presynaptic CB_1 receptors, they can cause inhibition of both excitatory and inhibitory neurotransmitter release [9, 23, 35, 38]. Both endocannabinoids are deactivated through a two-step process consisting of transportation into cells followed by intracellular hydrolysis [8, 13, 39].

Anatomical studies have shown that CB_1 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 [3, 18]. As a result of this localization, CB_1 activation might have a complex pattern of influence upon neurotransmitters known to modulate anxiety [1, 27, 28, 38]. In addition, cannabinoids could activate the hypothalamic-pituitary-adrenal axis which is responsible for the neuroendocrine response to stress [40].

These data suggest that the cannabinoid system participates in regulating emotional response. However, retrospective studies in cannabis users, clinical trials, as well as animal experiments have reported unclear and contradictory results concerning the role of CB_1 receptors in anxiety. In humans, the drug produced various effects, which can range from relaxation and euphoria to anxiety and acute panic disorders [5]. Similarly, in animals, Δ^9-THC and other cannabinoid agonists can exert both anxiolytic-like and anxiogenic-like responses depending on a set of variables such as drug dose, genetic background and environmental context [2, 12, 15, 16, 31].

The aim of this investigation was to study the influence of modulation of the cannabinoid system activity produced by the CB_1 and CB_2 receptor agonist WIN 55,212-2, the inhibitor of anandamide hydrolysis $AACOCF_3$, the anandamide transporter inhibitor AM 404 and the CB_1 receptor antagonist AM 281 on the anxiety-like response. We used the light/dark box test which has been validated for the evaluation of anxiety in rodents, and the open-field test for an additional evaluation of motor activity.

Materials and Methods

Animals

The studies were carried out on male BALB/c mice weighing 18–24 g (purchased from a licensed breeder). The animals were kept in a room at a temperature of 20 ± 2°C under 12/12 h light/dark cycle (lights on at 7 a.m.), with food and water freely available. Treatment of laboratory animals used in the present study was in full compliance with the respective Polish and European regulations, and was approved by the Local Ethics Committee.

Chemicals

(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate (WIN 55,212-2 mesylate, Tocris, UK), 1,1,1-trifluoro-6Z,9Z,12Z,15Z- heneicosatetraen-2-one ($AACOCF_3$, Tocris, UK), *N*-(4-hydroxyphenyl)-5Z,8Z,11Z-eicosatetraenamide (AM 404, Tocris, UK), 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM 281, Tocris, UK), cremophor EL (Sigma, Germany), dimethyl sulfoxide (DMSO, Sigma, Germany). WIN 55,212-2 and AM 404 were dissolved in a mixture of ethanol: cremophor EL: saline (1:1:18), AM 281 was dissolved in 4% DMSO and $AACOCF_3$ was diluted with cremophor EL : saline (1:18). All compounds were given intraperitoneally (*ip*) in a volume of 10 ml/kg, 30 min before tests. Control groups received the same volume of the corresponding vehicle.

Light/dark box test

Light/dark box test was performed according to the method described by Costall et al. [7]. Each animal was placed individually in the centre of the white area and video-recorded over a 5-min period (the operator withdrew from the room). Four behavior parameters were noted: a) the time spent in the white area, b) the number of transitions between the two compartments, c) the number of exploratory rearings and d) the number of line crossings in the white and black areas.

Open-field test

Locomotor exploratory activity was measured in the open field, which was a square black floor measuring 75 × 75 cm divided into 25 equal squares and surrounded by 20 cm high walls. A single mice was placed in the centre of the floor and allowed to explore. Sector line crossings (ambulations) and rearings were observed and recorded for 5 min. The only source of light in the testing room was a 70-W bulb placed directly above the box.

Statistics

The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test as a *post-hoc* test. The accepted level of significance was $p < 0.05$.

Results

Light/dark box test

WIN 55,212-2 significantly reduced the time spent [$F(2,27) = 5.44$, $p < 0.02$] and the number of line crossings in the white section [$F(2,27) = 4.12$, $p < 0.05$]. *Post-hoc* comparisons revealed that both decreases were solely caused by the 5 mg/kg dose (Fig. 1).

AM 281 failed to significantly alter the time spent [$F(3,32) = 0.70$, $p < 0.56$] in the white section but decreased the number of line crossings in the white section [$F(3,32) = 2.91$, $p < 0.05$], the number of line crossings [$F(3,32) = 5.29$, $p < 0.01$] and rearings [$F(3,32) = 5.50$, $p < 0.01$] in the black section, as well as the number of transitions between the two compartments [$F(3,32) = 5.94$, $p < 0.01$]. *Post hoc* comparisons showed that the highest dose, i.e. 4 mg/kg, had the strongest influence on parameters measuring the motor activity of mice, while the influence of the middle dose, i.e. 2 mg/kg, was the weakest (Fig. 2). The latter dose was used in the interaction study.

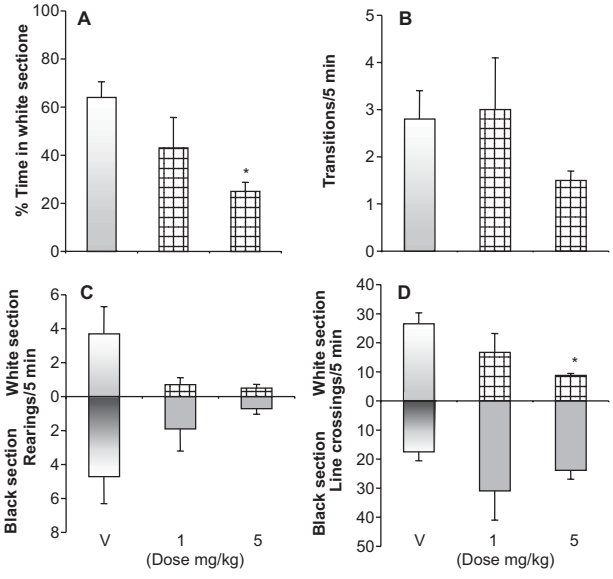

Fig. 1. The effect of WIN 55,212-2 (1 and 5 mg/kg) on the behavior of mice in the light/dark box test. Each histogram represents the means ± SEM of percent time spent in the white section (**A**), the transitions between the two compartments (**B**), rearing behavior (**C**) and line crossings (**D**); n = 8–10. Statistical analyses were performed using one-way ANOVA followed by *post hoc* comparisons using Dunnett's test. * $p < 0.05$ compared to the control (V; vehicle, ethanol: cremophor EL: saline (1:1:18))

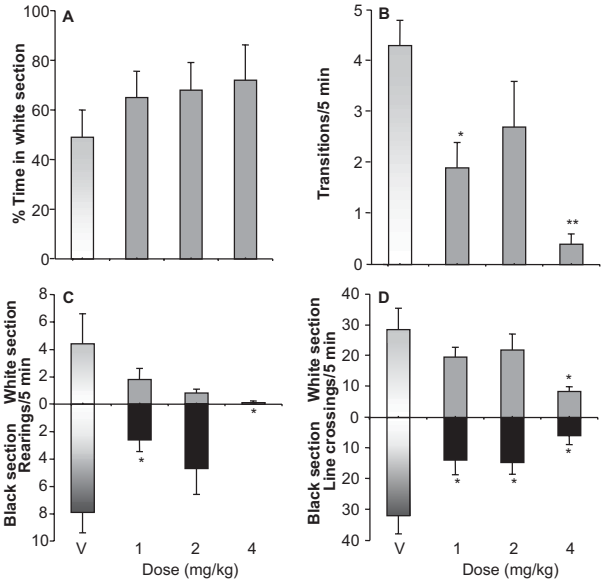

Fig. 2. The effect of AM 281 (1, 2 and 4 mg/kg) on the behavior of mice in the light/dark box test. Each histogram represents the means ± SEM of percent time spent in the white section (**A**), the transitions between the two compartments (**B**), rearing behavior (**C**) and line crossings (**D**); n = 8–10. Statistical analyses were performed as described in Figure 1. * $p < 0.05$, ** $p < 0.001$ compared to the control (V; vehicle, 4% DMSO)

The effects of AM 404 and $AACOCF_3$ in the light/dark box test are depicted in Figure 3. One-way ANOVA revealed significant effect of treatment on the time spent [$F(4,39) = 2.63$, $p < 0.05$] and the number of line crossings [$F(4,39) = 8.77$, $p < 0.0001$] in the white section, as well as on the total number of transitions between the two compartments [$F(4,39) = 4.01$, $p < 0.01$]. *Post hoc* test showed that treatment with 4 mg/kg of $AACOCF_3$ significantly increased the time spent and the number of line crossings in the white compartment, while AM 404 at the same dose did not significantly alter these parameters. Both compounds given at higher doses (4 mg/kg) significantly reduced the number of transitions between the two compartments as compared with the control group.

The impact of AM 281 (2 mg/kg) on the effects of WIN 55,212-2 (5 mg/kg) and $AACOCF_3$ (4 mg/kg) in the light/dark box test is depicted in Figure 4. One-way ANOVA revealed significant effect of treatment on

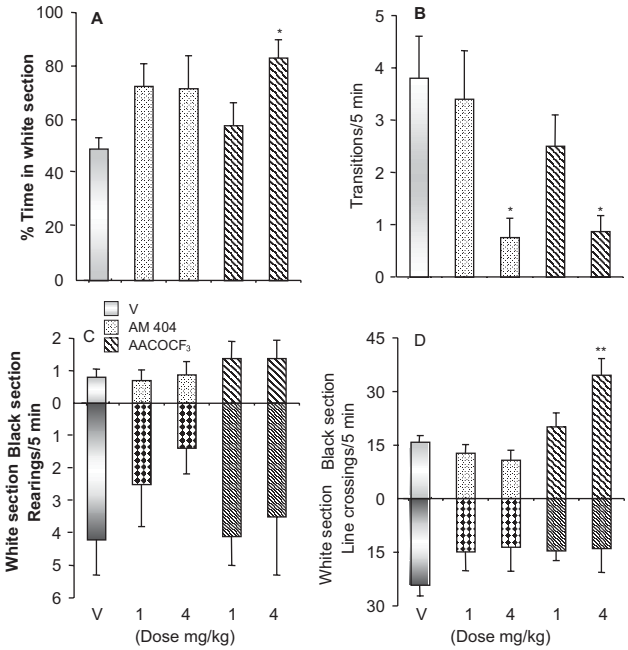

Fig. 3. Effects of AM 404 (1 and 4 mg/kg) and $AACOCF_3$ (1 and 4 mg/kg) on the behavior of mice in the light/dark box test. Each histogram represents the means ± SEM of percent time spent in the white section (**A**), the transitions between the two compartments (**B**), rearing behavior (**C**) and line crossings (**D**); n = 8–10. Statistical analyses were performed as described in Figure 1. * $p < 0.05$, ** $p < 0.001$ compared to the control (V; vehicle, ethanol: cremophor EL : saline (1:1:18))

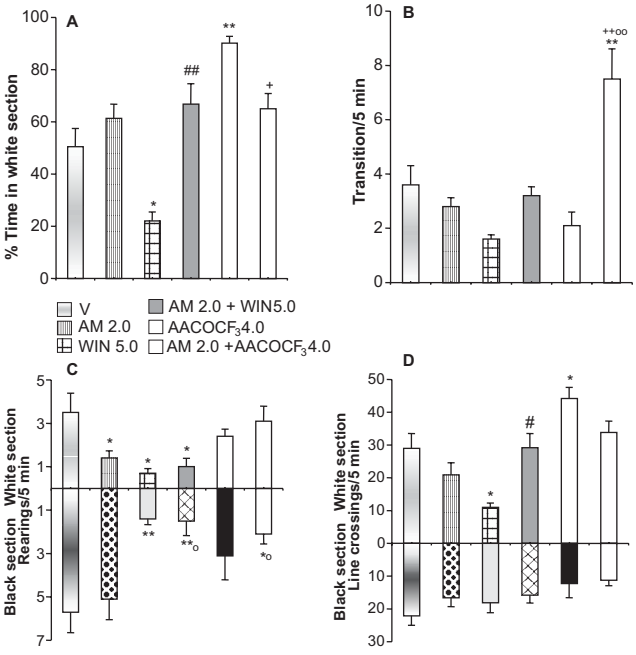

Fig. 4. The impact of AM 281 on the anxiogenic-like effect of WIN 55,212-2 and the anxiolytic-like effect of $AACOCF_3$ observed in the light/dark box test. Each histogram represents the means ± SEM of percent time spent in the white section (**A**), the transitions between the two compartments (**B**), rearing behavior (**C**) and line crossings (**D**). Each group received two injections. Control animals received a mixture of ethanol: cremophor EL: saline (1:1:18) and 4 % DMSO. WIN 55,212-2- and $AACOCF_3$-treated mice received an injection of either AM 281 or 4% DMSO. AM 281-treated mice received a mixture of ethanol: cremophor EL: saline (1:1:18). V = vehicle, AM 2.0 = AM 281 2 mg/kg, WIN 5.0 = WIN 55,212-2 5 mg/kg, $AACOCF_3$ 4.0 = $AACOCF_3$ 4 mg/kg; n = 10. Statistical analyses were performed as described in Figure 1. * $p < 0.05$, ** $p < 0.001$ compared to the control (V); $^{o}p < 0.05$; $^{oo}p < 0.001$ compared to AM 281; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ compared to WIN 55,212-2; $^{+}p < 0.05$, $^{++}p < 0.001$ compared to $AACOCF_3$

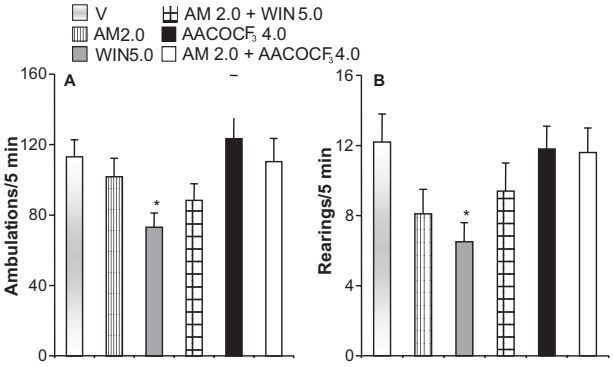

Fig. 5. Effects of WIN 55,212-2 and $AACOCF_3$ given alone or in combination with AM 281 on ambulations (**A**) and rearings (**B**) in the open-field test. V = vehicle, AM 2.0 = AM 281 2 mg/kg, WIN 5.0 = WIN 55,212-2 5 mg/kg, $AACOCF_3$ 4.0 = $AACOCF_3$ 4 mg/kg. Each group received two injections as described in Figure 4. Results are presented as the means ± SEM; n = 10. Statistical analyses were performed as described in Figure 1. * $p < 0.05$ compared to the control

the time spent [$F(5,54) = 16.05$, $p < 0.0001$), the number of line crossings [$F(5,54) = 9.61$, $p < 0.0001$] and rearings [$F(5,54) = 4.67$, $p < 0.01$] in the white section, the number of rearings [$F(5,54) = 5.47$, $p < 0.001$] in the black section, as well as the number of transitions between the two compartments [$F(5,54) = 11.76$, $p < 0.0001$]. *Post-hoc* comparisons showed that WIN 55,212-2 significantly decreased the time spent and the number of line crossings in the white compartment, as well as the number of rearings in both the compartments. In contrast, $AACOCF_3$ produced the opposite response, i.e. a significant increase in the time spent and the number of line crossings in the white compartment. The pretreatment with AM 281 reversed the suppressing effect of WIN 55,212-2 on both the time spent and the number of line crossings in the white section. AM 281 also antagonized both effects of $AACOCF_3$, i.e. the increase in the time spent and the number of line crossings in the white area.

Open field test

These experiments were performed to assess whether a change in locomotor behavior was underlying the anxiogenic-like effect of WIN 55,212-2 or the anxiolytic-like effect of $AACOCF_3$ observed in the light/dark box test. Therefore, in accordance with the test results presented above both compounds, alone and in combination with AM 281, were administered at higher doses only, i.e. WIN 55,212-2 was given at a dose of 5 mg/kg, and $AACOCF_3$ at a dose of 4 mg/kg.

The resultant analysis showed that there was a significant effect of treatment on both ambulations [$F(5,54) = 2.99$, $p < 0.02$] and rearings [$F(5,54) = 2.67$, $p < 0.05$]. *Post-hoc* comparisons revealed that these parameters were decreased by WIN 55,212-2 only. In both cases its inhibiting effect was antagonized by AM 281. AM 281 and $AACOCF_3$ administered either alone or in combination did not affect the locomotor exploratory activity (Fig. 5).

Discussion

Anxiety-like responses have been evaluated using the light/dark box, a model of anxiety in which mice are exposed to a conflict represented by the novelty and aversive characteristics of lit compartment of the box. An increased exploratory activity in brightly lit environment measured as a rearing behavior, line crossings and the time spent in the white section was an index of anxiolytic action [7].

Among the examined compounds modulating the cannabinoid system activity, only WIN 55,212-2, the CB_1 and CB_2 receptor agonist, and the anandamide hydrolysis inhibitor $AACOCF_3$ can modify the anxiety-like behavior. WIN 55,212-2 reduced exploratory activity in the lit compartment, suggesting the anxiogenic response. $AACOCF_3$ evoked just an opposite effect, i.e. it increased the exploratory activity in the lit compartment. Both compounds were active only at higher tested doses. In the open field test, WIN 55,212-2 decreased the exploratory motor activity, whereas $AACOCF_3$ was without effect. The profile of action of $AACOCF_3$ indicates the selective axiolytic-like effect. The anxiolytic-like effect of WIN 55,212-2 was accompanied by a decrease in the motor activity. Therefore, due to the possibility of interaction between those two effects an unambiguous evaluation thereof is difficult.

The obtained results correspond to the results of previous tests related to the influence of other cannabinoid agonists on the anxiogenic response. In several models of anxiety (the elevated plus-maze, the defensive withdrawal and the light/dark box tests), anxiogenic-like effects of THC, as well as those of nabilone, HU-210 and CP 55,940 were revealed. These effects were observed after the application of high doses; the low ones, on the contrary, usually

evoked an anxiolytic effect [12, 20, 26, 31]. Like in the present examination, a decreased motility was observed after higher doses [20].

Both effects of WIN 55, 212-2, i.e. the anxiogenic-like and the sedative one were blocked by AM 281, the CB_1 receptor antagonist, which suggests a stimulation of the CB_1 receptor.

AM 281 did not influence anxiety reaction in the dark/light box test when given alone. This result differs considerably from the one obtained during examinations of another antagonist of the CB_1 receptor, SR 141716. Similarly to CB_1 agonists, it evoked both anxiogenic [29] and anxiolytic effects [15]. Simultaneously, it was proved that SR 141716, at a non-anxiogenic dose, abolished anxiolytic-like [2] and sedative effects of cannabinoid agonists [20].

Up to the present, the surveys' results have pointed that anandamide hydrolysis inhibition reduces anxiety [11]. Studies of Kathuria et al. [22] showed anxiolytic actions of two FAAH inhibitors, URB 532 and URB 597 in the elevated zero maze and the isolation-induced ultrasonic emission models. These effects were observed together with the increased brain levels of anandamide, and were prevented by CB_1 receptor blockade. This observation, as well as the fact that CB_1 knockout mice exhibit an increase in the basal level of anxiety, seem to confirm the hypothesis that endocannabinoid system is activated as a response to anxiogenic situation and this very activation may be a part of a negative feedback system that limits anxiety [11, 15, 38].

In the presented paper, the inhibitor of anandamide hydrolysis $AACOCF_3$ also inhibited the anxiety reaction, and this effect was blocked by the co-admininistration of AM 281. In contrast, AM 404, an inhibitor of anandamide cellular uptake did not elicit anxiolytic effect. Perhaps lack of activity of AM 404 is strictly connected with the fact that it also activates a vanilloid 1 receptor (VR1). Agonists of this receptor have recently been found to exert anxiety effects [21]. Besides, it is known that, depending on concentration, anandamide exerts bidirectional activity which is explained by e.g. the ability to stimulate two different types of G protein: $G_{i/o}$ and G_s [37].

Cannabinoids, probably by presynaptic mechanisms, modulate the release of several transmitters implicated in the control of anxiety. They suppress the outflow of glutamate in the hippocampus, periaqueductal grey (PAG) and amygdala. Cannabinoids are inhibitory to corticolimbic release of norepinephrine, dopamine, serotonin and anxiogenic neuropeptides corticotropin-releasing factor (CRF) and cholecystokinin (CCK). On the other hand, they also interfere with GABAergic transmission in the amygdala, hippocampus and prefrontal cortex. An inhibition of GABAergic activity may induce disinhibition of glutamatergic and dopaminergic transmission pathways in the frontal cortex and nucleus accumbens [1, 23, 28, 38]. Besides, a number of studies have shown an involvement of μ- and δ-opioid receptors in regulating emotional response induced by cannabinoids [2, 25, 26]. The above interactions may result in either anti- or pro-anxiety effects which can explain bidirectional action of cannabinoids on anxiety.

Summing up, the presented results support the hypothesis that the endocannabinoid system is involved in the anxiety-like behavior regulation and also suggest that anandamide hydrolysis inhibitors might be potential anxiolytic drugs.

Acknowledgment:
This study was supported by Wrocław Medical University, grant No. 459.

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Received:
March 9, 2005; in revised form: December 16, 2005.