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# *Blockade of Serotonin 2C Receptors with SB-242084 moderates reduced locomotor activity and rearing by Cannabinoid 1 Receptor antagonist AM-251*

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Short Title: Co-administration of SB-242084 and AM-251 in behavior tests of rats

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# 1. Abstract

The endocannabinoid (eCB) and serotonin (5-HT) systems have key roles in the regulation of several physiological functions like motor activity and food intake but also in the development of psychiatric disorders. Here we tested the hypothesis, whether blockade of serotonin 2C ($5\text{-HT}_{2C}$) receptors prevents the reduced locomotor activity and other behavioral effects caused by a cannabinoid 1 ($CB_1$) receptor antagonist. As a pretreatment, we administered SB-242084 (1 mg/kg, ip.), a $5\text{-HT}_{2C}$ receptor antagonist or vehicle (VEH) followed by the treatment with AM-251 (5 or 10 mg/kg, ip.), a $CB_1$ receptor antagonist or VEH. The effects of the two drugs alone or in co-administration were investigated in social interaction (SI) and elevated plus maze (EPM) tests in male Wistar rats. Our results show that AM-251 decreased the time spent with rearing in the SI test and decreased locomotor activity in EPM test. In contrast, SB-242084 produced increased locomotor activity in SI test and evoked anxiolytic-like effect in both SI and EPM tests. When applied the drugs in combination, these behavioral effects of AM-251 were moderated by SB-242084. Based on these findings we conclude that certain unwanted behavioral effects of $CB_1$ receptor antagonists could be prevented by pretreatment with $5\text{-HT}_{2C}$ receptor antagonists.

## 2. Introduction

The potential therapeutic modulation of the endocannabinoid (eCB) system and the role of cannabinoid 1 ($CB_1$) receptors in the regulation of various physiological functions have been extensively investigated in the past decade. The most promising $CB_1$ receptor antagonist drugs had been developed for the therapy of obesity and metabolic syndrome, however, these drugs have been suspended due to their psychiatric side effects, such as depressive-like symptoms, psychomotor retardation and anxiety [1]. Exploration of the eCB system is still in the focus of medical research, so, understanding the mechanism of the side effects caused by $CB_1$ receptor antagonists is sufficient to work out interventions to prevent them, which may open a way for new therapeutic application of these drugs. Regarding the development of psychiatric side effects of $CB_1$ receptor antagonists, animal studies have shown that activation or blockade of $CB_1$ receptors modulate the excitability of serotonergic neurons in dorsal raphe nucleus and influences the serotonin release [2]. Furthermore, a growing body of human studies suggests that serotonergic neurons appreciably contribute to the development of psychiatric side effects induced by $CB_1$ receptor antagonists [3]. At the same time, the key role of different 5-$HT_2$ receptor subtypes has been demonstrated in the regulation of neuronal excitability, sleep-wake cycle and also in the control of anxiety and locomotor activity [4, 5]. The link between eCB system and locomotor regulation is shown by the fact that psychomotor performance of chronic cannabis smokers during abstinence is decreased [6], presumably as a result of the down regulation of $CB_1$ receptors and eCB dysfunction in cortical areas and in basal ganglia [7, 8].

In terms of the interplay between the serotonergic and cannabinoid systems, it is important to emphasize, that the $G_{q/11}$ protein coupled 5-$HT_{2C}$ receptors and the $G_i/G_o$-linked $CB_1$ receptors are co-distributed in high density in brain regions related to mood and locomotor regulation together [9, 10]. The interaction between $CB_1$ and 5-$HT_{2C}$ receptors has been demonstrated in the regulation of appetite too. Namely, administration of the $CB_1$ receptor antagonist AM-251, either by microinjection into the nucleus accumbens or intraperitoneally, has been shown to produce hypophagia which effect was preventable with the 5-$HT_{2C}$ receptor antagonist SB-242084 [11, 12]. However, interaction between these receptors has also been postulated in the control of other physiological processes, like movement and mood regulation [13, 14].

Based on the above mentioned evidences, here we test the hypothesis if blockade of 5-$HT_{2C}$ receptors is able to prevent the reduced locomotor activity and anxiety-like effect caused by $CB_1$ receptor antagonist, similarly to their interaction on food intake. For that, we injected the

highly selective 5-$HT_{2C}$ receptor antagonist, SB 242084, as a pretreatment, before the application of AM-251 and tested the effect of the drugs using behavioral tests. We applied social interaction (SI) and elevated plus maze (EPM) tests, which apply psychological (social or environmental) stress factors, have remarkable locomotor component and were used most frequently to investigate the behavioral effects of AM-251 and SB-242084 [15].

# 3. Materials and methods

## 3.1. Animal maintenance

All animal experiments and housing conditions were carried out in accordance with the EU Directive 2010/63/EU and the National Institutes of Health "Principles of Laboratory Animal Care" (NIH Publications No. 85-23, revised 1985), as well as specific national laws (the Hungarian Governmental Regulations on animal studies 40/2013). The experiments were approved by the National Scientific Ethical Committee on Animal Experimentation. Male, experimentally naïve, Wistar rats (220-320 g) were purchased from Animal Facility (Semmelweis University, Budapest, Hungary) and kept under controlled environmental conditions (temperature at 21±1°C, 12:12 light/dark cycle). We used different animals in the SI and EPM tests. Food and water were available *ad libitum* during the whole experiment. All effort was made to reduce pain and suffering of the animals.

## 3.2. Drugs

SB-242084 (SB) [ 6-chloro-5-methyl-1-[2-(2-methylpyrid-3-yloxy)-pyrid-5-yl carbamoyl] indoline] and AM-251 (AM) [ N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4- dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] were purchased from Tocris Cookson ™ (Bristol, UK). Both compounds were dissolved in vehicle (VEH) consisted of 70% PBS (phosphate buffered saline, pH=7.4), 20% dimethylsulfoxide and 10% Tween 80. Animals were randomly assigned to the treatment groups. The applied doses of the drugs, namely 1 mg/kg for SB-242084 and 5 or 10 mg/kg for AM-251 (AM D5 and AM D10, respectively) were chosen based on our previous experiments or publications demonstrating significant behavioral effects [16, 17]. All injections were performed intraperitoneally (ip.) in 1 ml/kg volume.

## 3.3. SI test

The procedure was carried out as described earlier [18]. We established familiar conditions by creating a low-light (5 lx) and familiar arena to which rats were habituated for three days.

Each rat was tested for social interaction with an unknown test partner with similar body weight (±15 g). Both members of a pair had the same prior familiarization experience and received the same drug treatment. At the end of the test, the box was wiped thoroughly and dried. The animals were tested in random order in a darkened room for 7.5 min, in the evenly illuminated test box (60 x 60 x 40 cm). All rats were treated with one of following treatments: VEH + VEH, VEH + AM D5, SB + VEH or SB + AM D5. The second injection was given 5 min after the first one; the test was started 30 min after the second ip. injection. The behavior of the animals was recorded with a camcorder. Social interaction and locomotor activity were measured as previously described [18].

### 3.4. EPM test

The test was performed in the housing room of the rats, under artificial laboratory illumination (200 lx at maze level). The EPM apparatus (metal, painted black, arm length, 50 cm; arm width, 15 cm; central platform, 15×15 cm; closed arm walls height, 40 cm) was elevated 50 cm above the floor. The test began by placing a single rat on the central platform facing an open arm. The first 5 min of free exploration were recorded with a camcorder. At the end of the test, the maze was cleaned thoroughly and dried. All rats were treated by one of the following treatments: VEH+VEH, VEH+AM D5, VEH+AM D10, SB+VEH, SB+AM D5 or SB+AM D10. The second injection was given 10 min after the first one. The test was begun 30 min following the second treatment. Percentage of time spent in open arms (OAT %) and open/total (open plus closed) arm entries ratio (OAE %) were calculated and used as measures of anxiety. Closed arm entries (CAE) were considered as indicators of general locomotor activity.

### 3.5. Data analysis

Camcorder recordings were stored and scored offline by an observer blind to the treatments, using Observer XT 10.0 software (Noldus®, The Netherlands). Data were analyzed with STATISTICA 7.0 (Statsoft®, Tulsa, USA). To evaluate the potential interactions between the drugs, two-way analysis of variance (ANOVA) was used with the following factors: (1) pretreatment: VEH or SB, (2) treatment: VEH, AM D5 or AM D10. To test the effect of different doses of drugs, one-way ANOVA was used followed by Dunnett's *post hoc* analysis. Results are expressed as mean ± S.E.M. The results were considered statistically significant in case of $p<0.05$.

# 4. Results

## 4.1. Effects of AM-251 and SB-242084 on explorative behavior in the SI, and locomotor activity in the SI and EPM tests

In the SI test, we found a significant AM D5 effect in the rearing time and number (two-way ANOVA: $F_{1,56}$=5.436, $p$=0.0233 and $F_{1,56}$=6.418, $p$=0.0141, respectively). *Post hoc* result also showed, that AM D5 significantly reduced the rearing time (one-way ANOVA: $F_{3,56}$=2.665, $p$=0.0565), presenting decline in explorative behavior. This effect was compensated, but not thoroughly blocked by SB pretreatment (for *post hoc* results see Fig. 1, A).

As for line-crossing number, only SB showed significant effect in two-way ANOVA ($F_{1,56}$=46.68, $p$<0.0001). *Post hoc* results showed, that SB caused a significant increase after VEH treatment, presenting elevated locomotor activity and this increase occured after AM D5 treatment as well (one-way ANOVA: $F_{3,44}$=6.928, $p$=0.0006; for *post hoc* analysis see Fig. 1, B) showing, that AM D5 treatment did not modulate the effect of SB in this parameter.

In the EPM test, two-way ANOVA analysis showed significant effect of both SB and AM D5 in the CAE parameter ($F_{1,46}$=5.099, $p$=0.0287 and $F_{1,46}$=4.243, $p$=0.0451, respectively). Regarding co-administration of SB and AM D10 in this parameter, significant SB effect and a trend in AM D10 effect were found in two-way ANOVA ($F_{1,44}$=13.33, $p$=0.0007 and $F_{1,44}$=3.124, $p$=0.0841, respectively), and a significant interaction effect as well ($F_{1,44}$=5.624, $p$=0.0222). Thus, two-way ANOVA results presented, that both SB-242084 and AM-251 modulated the locomotor activity in the EPM test. Regarding *post hoc* analysis, AM D5 and AM D10 treated groups showed a significant reduction in the CAE compared to the VEH+VEH treated group ($F_{3, 44}$=6.928, $p$=0.0006, for *post hoc* analysis see Fig. 1, C). This effect of AM-251 on CAE was moderated by SB-242084 pretreatment in the co-treated groups (Fig. 1, C).

## 4.2. Effects of AM-251 and SB-242084 on mainly anxiety-related indices in SI and EPM tests

In the SI test, both SB and AM D5 effects were seen in social interaction time parameter; two-way ANOVA showed a significant SB effect and a tendency in AM D5 effect ($F_{1,54}$=4.161, $p$=0.0463 and $F_{1,54}$=2.817, $p$=0.0990, respectively). This pattern of effects were seen in the number of social interactions as well (two-way ANOVA: $F_{1,54}$=10.12, $p$=0.0024 and

$F_{1,54}$=6.059, $p$=0.0171, respectively). *Post hoc* analysis also showed that SB treatment significantly increased the number of social interactions (one-way ANOVA: $F_{3,54}$=5.777, $p$=0.0017, Fig. 2, B). Considering these measures of social interaction together, we can conclude that SB presented anxiolytic-like effect. AM D5 did not cause significant anxiogenic-like effect on its own in these parameters, but evoked its effect after SB pretreatment (Fig. 2, B). These results presented that both SB-242084 and AM-251 have effect on anxiety-related social behaviors in co-treated group.

Regarding the non-social, but anxiety-related self-grooming behavior, both SB and AM D5 treatment revealed significant effect (two-way ANOVA: $F_{1,53}$=7.745, $p$=0.0074 and $F_{1,53}$=5.559, $p$=0.0221, respectively) in the time spent with self-grooming measure (Fig. 2, C). However, in the number of self-grooming, only AM D5 had significant effect (two-way ANOVA: $F_{1,55}$=10.53, $p$=0.0020), (Fig. 2, D).

In the EPM test, in the SB and AM D5 combination, two-way ANOVA showed a significant effect of SB in absolute indices, like OAT ($F_{1,44}$=15.30, $p$=0.0003) and OAE ($F_{1,46}$=16.61, $p$=0.0002), and in calculated indices, like OAT% ($F_{1,44}$=16.22, $p$=0.0002), OAE% ($F_{1,47}$=6.777, $p$=0.0123). Co-administration of SB with AM D10 also showed a significant effect of SB in two-way ANOVA statistics: OAT ($F_{1,44}$=22.44, $p$<0.0001), OAE ($F_{1,45}$=23.45, $p$<0.0001), OAT% ($F_{1,43}$=19.96, $p$<0.0001), OAE % ($F_{1,47}$=7.645, $p$=0.0081). Significant AM-251 or interaction effects were not observed in the anxiety-related parameters in the EPM test.

Based on one-way ANOVA statistics and *post hoc* analysis, SB significantly increased the OAT% ($F_{5,66}$=6.097, $p$<0.001), the OAE ($F_{5,68}$=6.053, $p$<0.001) as well as the OAE% ($F_{5,68}$=2.684, $p$<0.05) indices showing clear anxiolytic-like effect (for *post hoc* results see Fig. 3). AM D5 and AM D10 caused no difference in the anxiety-related EPM indices compared to the VEH + VEH treated group based on *post hoc* results (Fig. 3.). At the same time, in the co-treated groups, anxiolytic-like effect of SB in some cases was mildly modulated by AM-251 depending on the applied dose (Fig. 3).

## 5. Discussion

Our findings showed, that prior blockade of 5-$HT_{2C}$ receptors was able to prevent the reduction in locomotor and explorative activity caused by $CB_1$ receptor antagonist. Consistent with our results, similar exploration reducing effect of AM-251 have been found in the open

field test [19]. Interestingly, we could not see a consistent effect of AM-251 on anxiety. This is in agreement with results showing that AM-251 had no consistent effects on anxiety in rats [20-23]. Considering the pooled safety results of human RIO (Rimonabant-In-Obesity) studies, rimonabant (an antagonist of $CB_1$ receptors) has also caused anxiety in a relatively low percentage (5.6 %) of patients [1].

Rearing behavior, when animals standing on both hind paws in a vertical upright posture, is definitely considered as locomotor and exploratory activity, but can also be used as an unstable indicator of anxiety, since both increase and decrease of this parameter have been shown to correlate with anxiety [24]. On the other hand, in behavioral studies in rats, increase or decline in locomotor activity have frequently been interpreted as psychomotor agitation and retardation, respectively [25, 26]. Based on this, decline in both rearing behavior and locomotor activity (CAE in the EPM test) as well as the lack of a significant anxiogenic-like effect in our study, suggest that blockade of $CB_1$ receptors produces psychomotor retardation rather than a pronounced anxiety-like effect. Regarding the involvement of the eCB system in locomotor regulation in humans, chronic cannabis smokers showed reduced activation of cortical motor areas in finger sequencing task [27] and a decline in psychomotor function during abstinence [6]. Furthermore, $CB_1$ receptor downregulation has been observed in cortical areas and in the basal ganglia in humans, but also in animals chronically exposed to cannabinoids [7, 8].

Indeed, brain structures that participate in the regulation of movement, like basal ganglia and cortical areas, show high density of $CB_1$ receptors [10]. In Δ9-tetrahydrocannabinol tolerant animals, rimonabant induced c-fos expression and decreased dopamine release in both the nucleus accumbens and the amygdala [28]. This effect is thought to be related to dysphoric consequences of cannabinoid withdrawal, such as psychomotor retardation [8]. Based on these findings, the cause of the decreased locomotor activity by AM-251 in our study might be the decline of $CB_1$ receptor activity in movement regulating brain structures.

Regarding the locomotor effect of selective 5-$HT_{2C}$ receptor antagonist, SB-242084 increased locomotor activity in the SI test in our study. The involvement of 5-$HT_{2C}$ receptors in movement regulation has been suggested by the abundant presence of 5-$HT_{2C}$ receptors in movement regulating brain structures, interestingly in the close proximity of $CB_1$ receptors [9]. This is also supported by data from 5-$HT_{2C}$ receptor null mutant mice showing increased extracellular dopamine levels in the nucleus accumbens [29]. Also, 5-$HT_{2C}$ receptor agonists blocked, whereas antagonists facilitated the cocaine-induced increase in locomotor activity and dopamine signaling in the nucleus accumbens core [30-32]. According to these findings,

increased locomotor activity by SB-242084 in our experiment might have occurred as a result of increased dopamine signaling in the nucleus accumbens region and presumably other regions involved in the regulation of locomotion.

Elevated stress, increased serotonin levels and activation of $G_{q/11}$ protein coupled 5-$HT_{2C}$ receptors can be measured during SI and EPM tests in rodents [33]. In the same tests, 5-$HT_{2C}$ receptor blockade by SB-242084 treatment showed anxiolytic-like effect in our study, in agreement with previous findings [16, 34]. However, the effect of the consecutive administration of 5-$HT_{2C}$ and $CB_1$ receptor antagonists on anxiety-regulation is not clear, because the influence of AM-251 on the SB-242084-induced anxiolytic-like effect seemed to depend on the given behavioral test. AM-251 markedly reduced the effects of SB-242084 in the SI test (number of social interactions), but had minimal or no effect on the OAT and OAE indices in the EPM test. In our study, we applied two different behavioral paradigm: in the SI test, the less avoidable stress is caused by an unfamiliar partner in the SI arena, while in the EPM test, the stress is the effect of the open space and high light that is avoidable in the closed arms. Our finding, that AM-251 modified the anxiolytic-like effect of SB-242084 under less avoidable social stress conditions, have shown the sensitivity of eCB system in terms of the stress controllability. Based on these results, we presume that 5-$HT_{2C}$ and $CB_1$ receptor antagonists might cause an additive pharmacological effect, modifying anxiety-like behavior.

At the same time, several data suggest the interaction between serotonin and $CB_1$ receptors in rodents and humans [3, 35]. Burattini et al. have found that stimulation of 5-$HT_2$ receptors evoked production of 2-arachidonoylglycerol, an endogenous agonist of the $CB_1$ (and $CB_2$ receptors), and activated $CB_1$ receptors in the nucleus accumbens core [36]. Furthermore, in CB1 knockout mice, diminished expression of 5-$HT_{2C}$ receptors has been observed in the nucleus accumbens [37], suggesting their strong interplay in this brain region. A clear interaction between $CB_1$ and 5-$HT_{2C}$ receptors has also been reported in appetite regulation through the modulation of signaling in the nucleus accumbens, demonstrating that SB-242084 pretreatment was able to prevent the hypophagic effect produced by the combination of oleamide (a cannabimimetic drug) and AM-251 [12]. Taken together, blockade of 5-$HT_{2C}$ receptors interferes with the influence of $CB_1$ receptors in locomotor regulation, suggesting that serotonergic and cannabinoid systems are both involved in the regulation of this pathway. This effect is likely pharmacodynamics and not pharmacokinetic, because AM-251 and its structural analog rimonabant are metabolized through microsomal enzimes in vitro [38], but SB-242084 has not influenced the activity of P450 enzimes [39].

## 6. Conclusion

The potential therapeutic use of compounds acting on the eCB system is still an intensively investigated area. Our results point to an interplay between 5-$HT_{2C}$ and $CB_1$ receptors in regulating processes related to the locomotor activity and explorative behavior. Utilizing the advantageous effect of $CB_1$ and 5-$HT_{2C}$ receptor antagonists, their combined application might comprise a promising new direction for the therapeutic application of drugs with $CB_1$ receptor blocking activity.

## 7. Statements

### 7.1. Acknowledgement

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### 7.2. Statement of Ethics

All animal experiments and housing conditions were carried out in accordance with the EU Directive 2010/63/EU and the National Institutes of Health "Principles of Laboratory Animal Care" (NIH Publications No. 85-23, revised 1985), as well as specific national laws (the Hungarian Governmental Regulations on animal studies 40/2013). The experiments were approved by the National Scientific Ethical Committee on Animal Experimentation and permitted by the government (Food Chain Safety and Animal Health Directorate of the Central Agricultural Office, Permit no. 22.1/1375/7/2010).

### 7.3. Disclosure Statement

The authors have no conflicts of interest to declare.

### 7.4. Founding Sources

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## 7.5. Author contributions

EB, DK, PP, SzV and GB designed the experiments. EB, DK, PP and SzV carried out the experiments. EB and DK contributed to data analysis. EB, SzV and GB interpreted the findings and wrote the final version of the manuscript. All authors critically reviewed the content and approved the final version for publication.

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# 9. Figures and figure legends

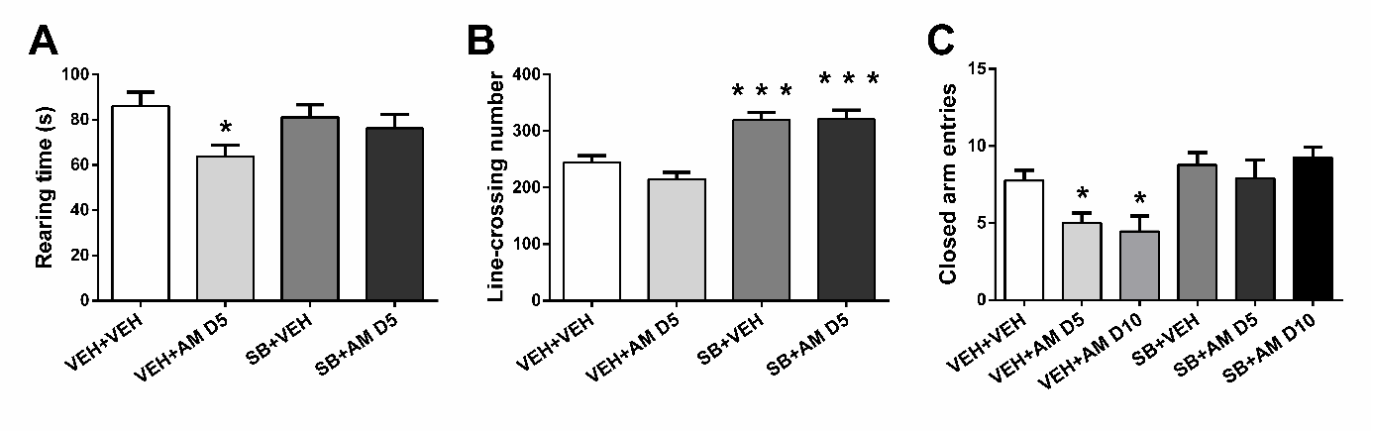



**Fig. 1.** Influence of SB-242084 (SB, 1 mg/kg, ip.), AM-251 (AM D5 and AM D10, 5 and 10 mg/kg, ip.) and their combination on explorative behaviors and locomotor activity in social interaction test (A, B) and elevated plus maze test (C). Graphs show the time spent with rearing (A), the number of line crossings (B) and the number of closed arm entries (C). N=12-16 for all groups. Columns represent mean ± S.E.M. * $p < 0.05$ and *** $p < 0.001$, significant results of Dunnett's *post hoc* test compared to VEH+VEH group.

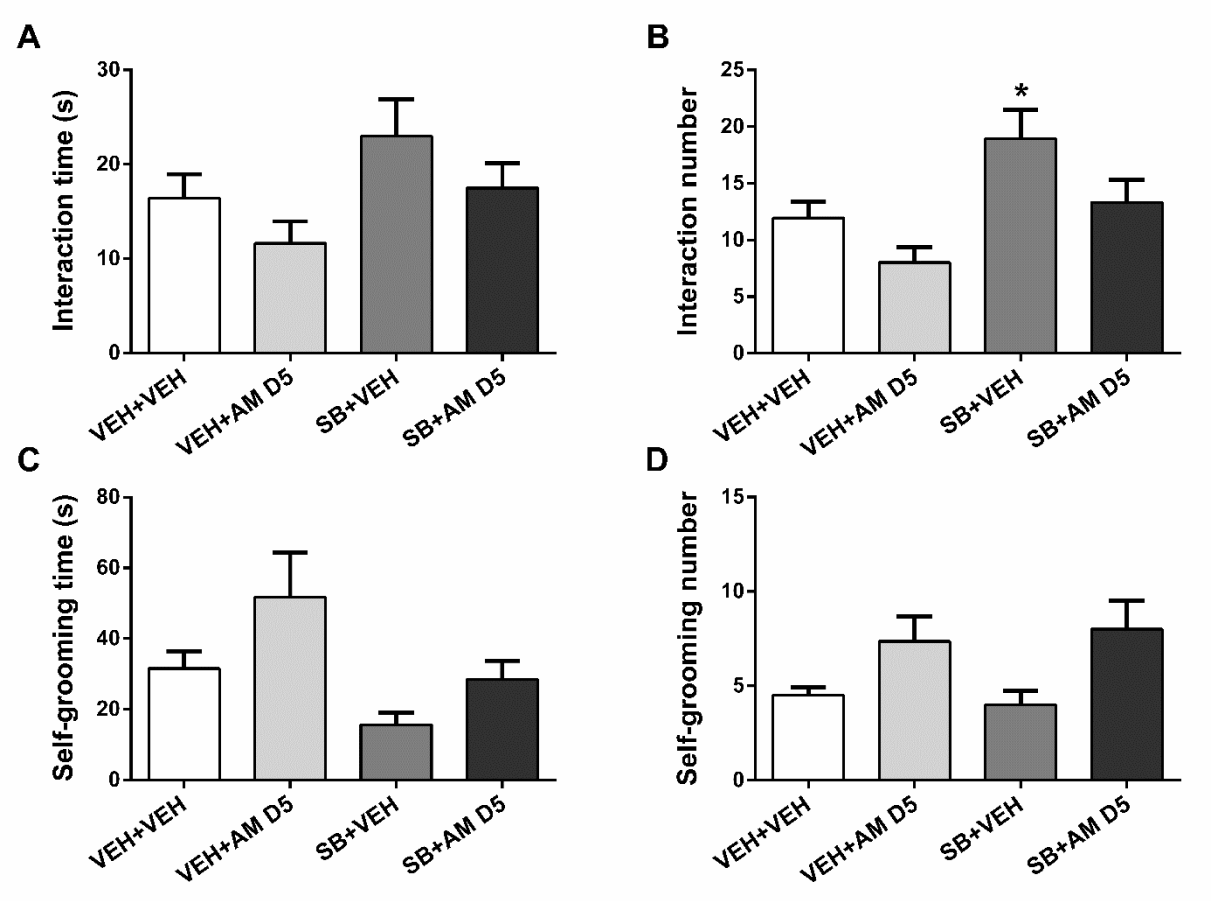
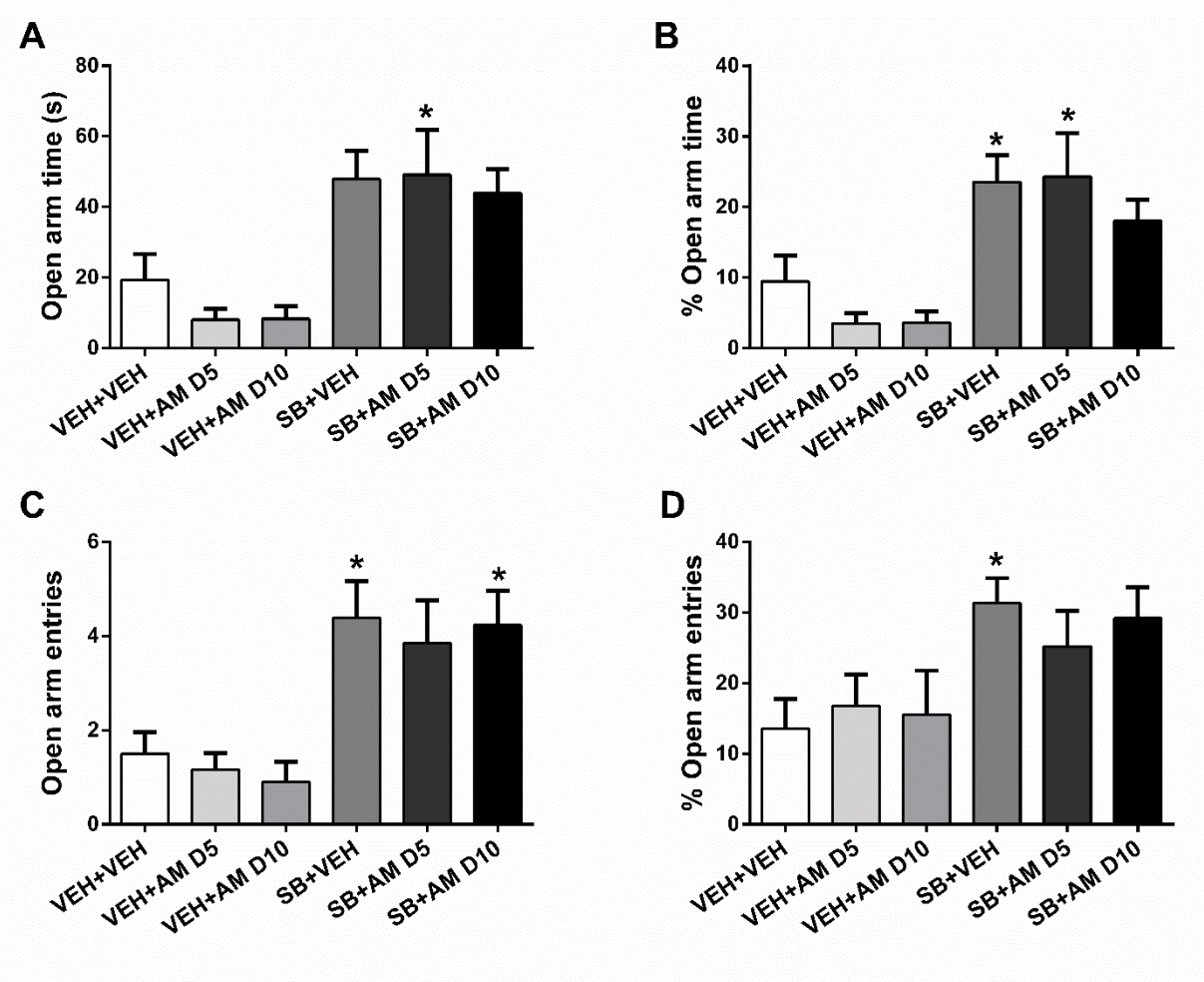



**Fig. 2.** Effects of SB-242084 (1 mg/kg, ip.), AM-251 (5 mg/kg, ip.) and their combination on anxiety-like behaviors in the social interaction test. Graphs show the time (A) and the number (B) of total social interaction, and time (C) and number (D) of self-grooming. N=12-16 for all groups. Columns represent mean ± S.E.M. * $p < 0.05$, significant results of Dunnett's *post hoc* test compared to VEH+VEH group.




403 **Fig. 3.** Influence of SB-242084 (1 mg/kg, ip.), AM-251 (5 or 10 mg/kg, ip.) and their
404 combination on anxiety-related behaviors in elevated plus maze test. Graphs show the
405 absolute time and the percentage of the time spent in open arms (A and B, respectively), the
406 number of open arm entries (C) and the percentage of the number of open arm entries (D).
407 N=10-14 for all groups. Columns represent mean ± S.E.M. * $p < 0.05$, significant results of
408 Dunnett's *post hoc* test compared to VEH+VEH group.