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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 **1. Abstract**

2 The endocannabinoid (eCB) and serotonin (5-HT) systems have key roles in the regulation of
3 several physiological functions like motor activity and food intake but also in the
4 development of psychiatric disorders. Here we tested the hypothesis, whether blockade of
5 serotonin 2C (5-HT_{2C}) receptors prevents the reduced locomotor activity and other behavioral
6 effects caused by a cannabinoid 1 (CB₁) receptor antagonist. As a pretreatment, we
7 administered SB-242084 (1 mg/kg, ip.), a 5-HT_{2C} receptor antagonist or vehicle (VEH)
8 followed by the treatment with AM-251 (5 or 10 mg/kg, ip.), a CB₁ receptor antagonist or
9 VEH. The effects of the two drugs alone or in co-administration were investigated in social
10 interaction (SI) and elevated plus maze (EPM) tests in male Wistar rats. Our results show that
11 AM-251 decreased the time spent with rearing in the SI test and decreased locomotor activity
12 in EPM test. In contrast, SB-242084 produced increased locomotor activity in SI test and
13 evoked anxiolytic-like effect in both SI and EPM tests. When applied the drugs in
14 combination, these behavioral effects of AM-251 were moderated by SB-242084. Based on
15 these findings we conclude that certain unwanted behavioral effects of CB₁ receptor
16 antagonists could be prevented by pretreatment with 5-HT_{2C} receptor antagonists.

17 2. Introduction

18 The potential therapeutic modulation of the endocannabinoid (eCB) system and the role of
19 cannabinoid 1 (CB₁) receptors in the regulation of various physiological functions have been
20 extensively investigated in the past decade. The most promising CB₁ receptor antagonist
21 drugs had been developed for the therapy of obesity and metabolic syndrome, however, these
22 drugs have been suspended due to their psychiatric side effects, such as depressive-like
23 symptoms, psychomotor retardation and anxiety [1]. Exploration of the eCB system is still in
24 the focus of medical research, so, understanding the mechanism of the side effects caused by
25 CB₁ receptor antagonists is sufficient to work out interventions to prevent them, which may
26 open a way for new therapeutic application of these drugs. Regarding the development of
27 psychiatric side effects of CB₁ receptor antagonists, animal studies have shown that activation
28 or blockade of CB₁ receptors modulate the excitability of serotonergic neurons in dorsal raphe
29 nucleus and influences the serotonin release [2]. Furthermore, a growing body of human
30 studies suggests that serotonergic neurons appreciably contribute to the development of
31 psychiatric side effects induced by CB₁ receptor antagonists [3]. At the same time, the key
32 role of different 5-HT₂ receptor subtypes has been demonstrated in the regulation of neuronal
33 excitability, sleep-wake cycle and also in the control of anxiety and locomotor activity [4, 5].
34 The link between eCB system and locomotor regulation is shown by the fact that
35 psychomotor performance of chronic cannabis smokers during abstinence is decreased [6],
36 presumably as a result of the down regulation of CB₁ receptors and eCB dysfunction in
37 cortical areas and in basal ganglia [7, 8].

38 In terms of the interplay between the serotonergic and cannabinoid systems, it is important to
39 emphasize, that the G_{q/11} protein coupled 5-HT_{2C} receptors and the G_i/G_o-linked CB₁ receptors
40 are co-distributed in high density in brain regions related to mood and locomotor regulation
41 together [9, 10]. The interaction between CB₁ and 5-HT_{2C} receptors has been demonstrated in
42 the regulation of appetite too. Namely, administration of the CB₁ receptor antagonist AM-251,
43 either by microinjection into the nucleus accumbens or intraperitoneally, has been shown to
44 produce hypophagia which effect was preventable with the 5-HT_{2C} receptor antagonist SB-
45 242084 [11, 12]. However, interaction between these receptors has also been postulated in the
46 control of other physiological processes, like movement and mood regulation [13, 14].

47 Based on the above mentioned evidences, here we test the hypothesis if blockade of 5-HT_{2C}
48 receptors is able to prevent the reduced locomotor activity and anxiety-like effect caused by
49 CB₁ receptor antagonist, similarly to their interaction on food intake. For that, we injected the

50 highly selective 5-HT_{2C} receptor antagonist, SB 242084, as a pretreatment, before the
51 application of AM-251 and tested the effect of the drugs using behavioral tests. We applied
52 social interaction (SI) and elevated plus maze (EPM) tests, which apply psychological (social
53 or environmental) stress factors, have remarkable locomotor component and were used most
54 frequently to investigate the behavioral effects of AM-251 and SB-242084 [15].
55

56 **3. Materials and methods**

57 3.1. Animal maintenance

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

68 3.2. Drugs

69 SB-242084 (SB) [6-chloro-5-methyl-1-[2-(2-methylpyrid-3-yloxy)-pyrid-5-yl carbamoyl]
70 indoline] and AM-251 (AM) [N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4- dichlorophenyl)-4-
71 methyl-1H-pyrazole-3-carboxamide] were purchased from Tocris Cookson™ (Bristol, UK).
72 Both compounds were dissolved in vehicle (VEH) consisted of 70% PBS (phosphate buffered
73 saline, pH=7.4), 20% dimethylsulfoxide and 10% Tween 80. Animals were randomly
74 assigned to the treatment groups. The applied doses of the drugs, namely 1 mg/kg for SB-
75 242084 and 5 or 10 mg/kg for AM-251 (AM D5 and AM D10, respectively) were chosen
76 based on our previous experiments or publications demonstrating significant behavioral
77 effects [16, 17]. All injections were performed intraperitoneally (ip.) in 1 ml/kg volume.

78 3.3. SI test

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

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

90 3.4. EPM test

91 The test was performed in the housing room of the rats, under artificial laboratory
92 illumination (200 lx at maze level). The EPM apparatus (metal, painted black, arm length, 50
93 cm; arm width, 15 cm; central platform, 15×15 cm; closed arm walls height, 40 cm) was
94 elevated 50 cm above the floor. The test began by placing a single rat on the central platform
95 facing an open arm. The first 5 min of free exploration were recorded with a camcorder. At
96 the end of the test, the maze was cleaned thoroughly and dried. All rats were treated by one of
97 the following treatments: VEH+VEH, VEH+AM D5, VEH+AM D10, SB+VEH, SB+AM D5
98 or SB+AM D10. The second injection was given 10 min after the first one. The test was
99 begun 30 min following the second treatment. Percentage of time spent in open arms (OAT
100 %) and open/total (open plus closed) arm entries ratio (OAE %) were calculated and used as
101 measures of anxiety. Closed arm entries (CAE) were considered as indicators of general
102 locomotor activity.

103 3.5. Data analysis

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

112 4. Results

113 4.1. Effects of AM-251 and SB-242084 on explorative behavior in the SI, and locomotor 114 activity in the SI and EPM tests

115 In the SI test, we found a significant AM D5 effect in the rearing time and number (two-way
116 ANOVA: $F_{1,56}=5.436$, $p=0.0233$ and $F_{1,56}=6.418$, $p=0.0141$, respectively). *Post hoc* result
117 also showed, that AM D5 significantly reduced the rearing time (one-way ANOVA:
118 $F_{3,56}=2.665$, $p=0.0565$), presenting decline in explorative behavior. This effect was
119 compensated, but not thoroughly blocked by SB pretreatment (for *post hoc* results see Fig. 1,
120 A).

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

126 In the EPM test, two-way ANOVA analysis showed significant effect of both SB and AM D5
127 in the CAE parameter ($F_{1,46}=5.099$, $p=0.0287$ and $F_{1,46}=4.243$, $p=0.0451$, respectively).
128 Regarding co-administration of SB and AM D10 in this parameter, significant SB effect and a
129 trend in AM D10 effect were found in two-way ANOVA ($F_{1,44}=13.33$, $p=0.0007$ and
130 $F_{1,44}=3.124$, $p=0.0841$, respectively), and a significant interaction effect as well ($F_{1,44}=5.624$,
131 $p=0.0222$). Thus, two-way ANOVA results presented, that both SB-242084 and AM-251
132 modulated the locomotor activity in the EPM test. Regarding *post hoc* analysis, AM D5 and
133 AM D10 treated groups showed a significant reduction in the CAE compared to the
134 VEH+VEH treated group ($F_{3,44}=6.928$, $p=0.0006$, for *post hoc* analysis see Fig. 1, C). This
135 effect of AM-251 on CAE was moderated by SB-242084 pretreatment in the co-treated
136 groups (Fig. 1, C).

137

138 4.2. Effects of AM-251 and SB-242084 on mainly anxiety-related indices in SI and EPM 139 tests

140 In the SI test, both SB and AM D5 effects were seen in social interaction time parameter; two-
141 way ANOVA showed a significant SB effect and a tendency in AM D5 effect ($F_{1,54}=4.161$,
142 $p=0.0463$ and $F_{1,54}=2.817$, $p=0.0990$, respectively). This pattern of effects were seen in the
143 number of social interactions as well (two-way ANOVA: $F_{1,54}=10.12$, $p=0.0024$ and

144 $F_{1,54}=6.059$, $p=0.0171$, respectively). *Post hoc* analysis also showed that SB treatment
145 significantly increased the number of social interactions (one-way ANOVA: $F_{3,54}=5.777$,
146 $p=0.0017$, Fig. 2, B). Considering these measures of social interaction together, we can
147 conclude that SB presented anxiolytic-like effect. AM D5 did not cause significant
148 anxiogenic-like effect on its own in these parameters, but evoked its effect after SB
149 pretreatment (Fig. 2, B). These results presented that both SB-242084 and AM-251 have
150 effect on anxiety-related social behaviors in co-treated group.

151 Regarding the non-social, but anxiety-related self-grooming behavior, both SB and AM D5
152 treatment revealed significant effect (two-way ANOVA: $F_{1,53}=7.745$, $p=0.0074$ and
153 $F_{1,53}=5.559$, $p=0.0221$, respectively) in the time spent with self-grooming measure (Fig. 2, C).
154 However, in the number of self-grooming, only AM D5 had significant effect (two-way
155 ANOVA: $F_{1,55}=10.53$, $p=0.0020$), (Fig. 2, D).

156 In the EPM test, in the SB and AM D5 combination, two-way ANOVA showed a significant
157 effect of SB in absolute indices, like OAT ($F_{1,44}=15.30$, $p=0.0003$) and OAE ($F_{1,46}=16.61$,
158 $p=0.0002$), and in calculated indices, like OAT% ($F_{1,44}=16.22$, $p=0.0002$), OAE%
159 ($F_{1,47}=6.777$, $p=0.0123$). Co-administration of SB with AM D10 also showed a significant
160 effect of SB in two-way ANOVA statistics: OAT ($F_{1,44}=22.44$, $p<0.0001$), OAE ($F_{1,45}=23.45$,
161 $p<0.0001$), OAT% ($F_{1,43}=19.96$, $p<0.0001$), OAE % ($F_{1,47}=7.645$, $p=0.0081$). Significant
162 AM-251 or interaction effects were not observed in the anxiety-related parameters in the EPM
163 test.

164 Based on one-way ANOVA statistics and *post hoc* analysis, SB significantly increased the
165 OAT% ($F_{5,66}=6.097$, $p<0.001$), the OAE ($F_{5,68}=6.053$, $p<0.001$) as well as the OAE%
166 ($F_{5,68}=2.684$, $p<0.05$) indices showing clear anxiolytic-like effect (for *post hoc* results see Fig.
167 3). AM D5 and AM D10 caused no difference in the anxiety-related EPM indices compared
168 to the VEH + VEH treated group based on *post hoc* results (Fig. 3.). At the same time, in the
169 co-treated groups, anxiolytic-like effect of SB in some cases was mildly modulated by AM-
170 251 depending on the applied dose (Fig. 3).

171

172 **5. Discussion**

173 Our findings showed, that prior blockade of 5-HT_{2C} receptors was able to prevent the
174 reduction in locomotor and explorative activity caused by CB₁ receptor antagonist. Consistent
175 with our results, similar exploration reducing effect of AM-251 have been found in the open

176 field test [19]. Interestingly, we could not see a consistent effect of AM-251 on anxiety. This
177 is in agreement with results showing that AM-251 had no consistent effects on anxiety in rats
178 [20-23]. Considering the pooled safety results of human RIO (Rimonabant-In-Obesity)
179 studies, rimonabant (an antagonist of CB₁ receptors) has also caused anxiety in a relatively
180 low percentage (5.6 %) of patients [1].

181 Rearing behavior, when animals standing on both hind paws in a vertical upright posture, is
182 definitely considered as locomotor and exploratory activity, but can also be used as an
183 unstable indicator of anxiety, since both increase and decrease of this parameter have been
184 shown to correlate with anxiety [24]. On the other hand, in behavioral studies in rats, increase
185 or decline in locomotor activity have frequently been interpreted as psychomotor agitation
186 and retardation, respectively [25, 26]. Based on this, decline in both rearing behavior and
187 locomotor activity (CAE in the EPM test) as well as the lack of a significant anxiogenic-like
188 effect in our study, suggest that blockade of CB₁ receptors produces psychomotor retardation
189 rather than a pronounced anxiety-like effect. Regarding the involvement of the eCB system in
190 locomotor regulation in humans, chronic cannabis smokers showed reduced activation of
191 cortical motor areas in finger sequencing task [27] and a decline in psychomotor function
192 during abstinence [6]. Furthermore, CB₁ receptor downregulation has been observed in
193 cortical areas and in the basal ganglia in humans, but also in animals chronically exposed to
194 cannabinoids [7, 8].

195 Indeed, brain structures that participate in the regulation of movement, like basal ganglia and
196 cortical areas, show high density of CB₁ receptors [10]. In Δ^9 -tetrahydrocannabinol tolerant
197 animals, rimonabant induced c-fos expression and decreased dopamine release in both the
198 nucleus accumbens and the amygdala [28]. This effect is thought to be related to dysphoric
199 consequences of cannabinoid withdrawal, such as psychomotor retardation [8]. Based on
200 these findings, the cause of the decreased locomotor activity by AM-251 in our study might
201 be the decline of CB₁ receptor activity in movement regulating brain structures.

202 Regarding the locomotor effect of selective 5-HT_{2C} receptor antagonist, SB-242084 increased
203 locomotor activity in the SI test in our study. The involvement of 5-HT_{2C} receptors in
204 movement regulation has been suggested by the abundant presence of 5-HT_{2C} receptors in
205 movement regulating brain structures, interestingly in the close proximity of CB₁ receptors
206 [9]. This is also supported by data from 5-HT_{2C} receptor null mutant mice showing increased
207 extracellular dopamine levels in the nucleus accumbens [29]. Also, 5-HT_{2C} receptor agonists
208 blocked, whereas antagonists facilitated the cocaine-induced increase in locomotor activity
209 and dopamine signaling in the nucleus accumbens core [30-32]. According to these findings,

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

213 Elevated stress, increased serotonin levels and activation of G_{q/11} protein coupled 5-HT_{2C}
214 receptors can be measured during SI and EPM tests in rodents [33]. In the same tests, 5-HT_{2C}
215 receptor blockade by SB-242084 treatment showed anxiolytic-like effect in our study, in
216 agreement with previous findings [16, 34]. However, the effect of the consecutive
217 administration of 5-HT_{2C} and CB₁ receptor antagonists on anxiety-regulation is not clear,
218 because the influence of AM-251 on the SB-242084-induced anxiolytic-like effect seemed to
219 depend on the given behavioral test. AM-251 markedly reduced the effects of SB-242084 in
220 the SI test (number of social interactions), but had minimal or no effect on the OAT and OAE
221 indices in the EPM test. In our study, we applied two different behavioral paradigm: in the SI
222 test, the less avoidable stress is caused by an unfamiliar partner in the SI arena, while in the
223 EPM test, the stress is the effect of the open space and high light that is avoidable in the
224 closed arms. Our finding, that AM-251 modified the anxiolytic-like effect of SB-242084
225 under less avoidable social stress conditions, have shown the sensitivity of eCB system in
226 terms of the stress controllability. Based on these results, we presume that 5-HT_{2C} and CB₁
227 receptor antagonists might cause an additive pharmacological effect, modifying anxiety-like
228 behavior.

229 At the same time, several data suggest the interaction between serotonin and CB₁ receptors in
230 rodents and humans [3, 35]. Burattini et al. have found that stimulation of 5-HT₂ receptors
231 evoked production of 2-arachidonoylglycerol, an endogenous agonist of the CB₁ (and CB₂
232 receptors), and activated CB₁ receptors in the nucleus accumbens core [36]. Furthermore, in
233 CB1 knockout mice, diminished expression of 5-HT_{2C} receptors has been observed in the
234 nucleus accumbens [37], suggesting their strong interplay in this brain region. A clear
235 interaction between CB₁ and 5-HT_{2C} receptors has also been reported in appetite regulation
236 through the modulation of signaling in the nucleus accumbens, demonstrating that SB-242084
237 pretreatment was able to prevent the hypophagic effect produced by the combination of
238 oleamide (a cannabimimetic drug) and AM-251 [12]. Taken together, blockade of 5-HT_{2C}
239 receptors interferes with the influence of CB₁ receptors in locomotor regulation, suggesting
240 that serotonergic and cannabinoid systems are both involved in the regulation of this pathway.
241 This effect is likely pharmacodynamics and not pharmacokinetic, because AM-251 and its
242 structural analog rimonabant are metabolized through microsomal enzymes in vitro [38], but
243 SB-242084 has not influenced the activity of P450 enzymes [39].

244

245 **6. Conclusion**

246 The potential therapeutic use of compounds acting on the eCB system is still an intensively
247 investigated area. Our results point to an interplay between 5-HT_{2C} and CB₁ receptors in
248 regulating processes related to the locomotor activity and explorative behavior. Utilizing the
249 advantageous effect of CB₁ and 5-HT_{2C} receptor antagonists, their combined application
250 might comprise a promising new direction for the therapeutic application of drugs with CB₁
251 receptor blocking activity.

252

253 **7. Statements**

254 7.1. Acknowledgement

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260 7.2. Statement of Ethics

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

268 7.3. Disclosure Statement

269 The authors have no conflicts of interest to declare.

270 7.4. Founding Sources

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275

276 7.5. Author contributions

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

281

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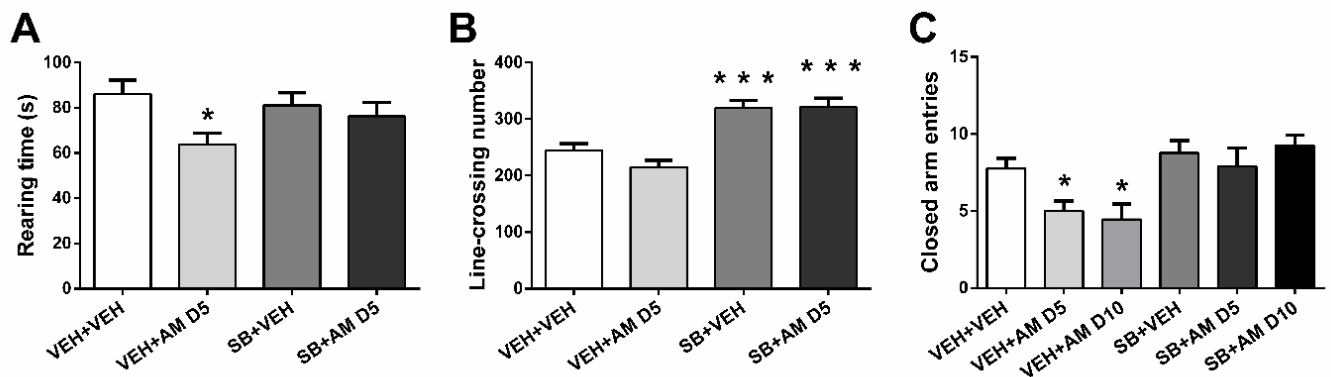
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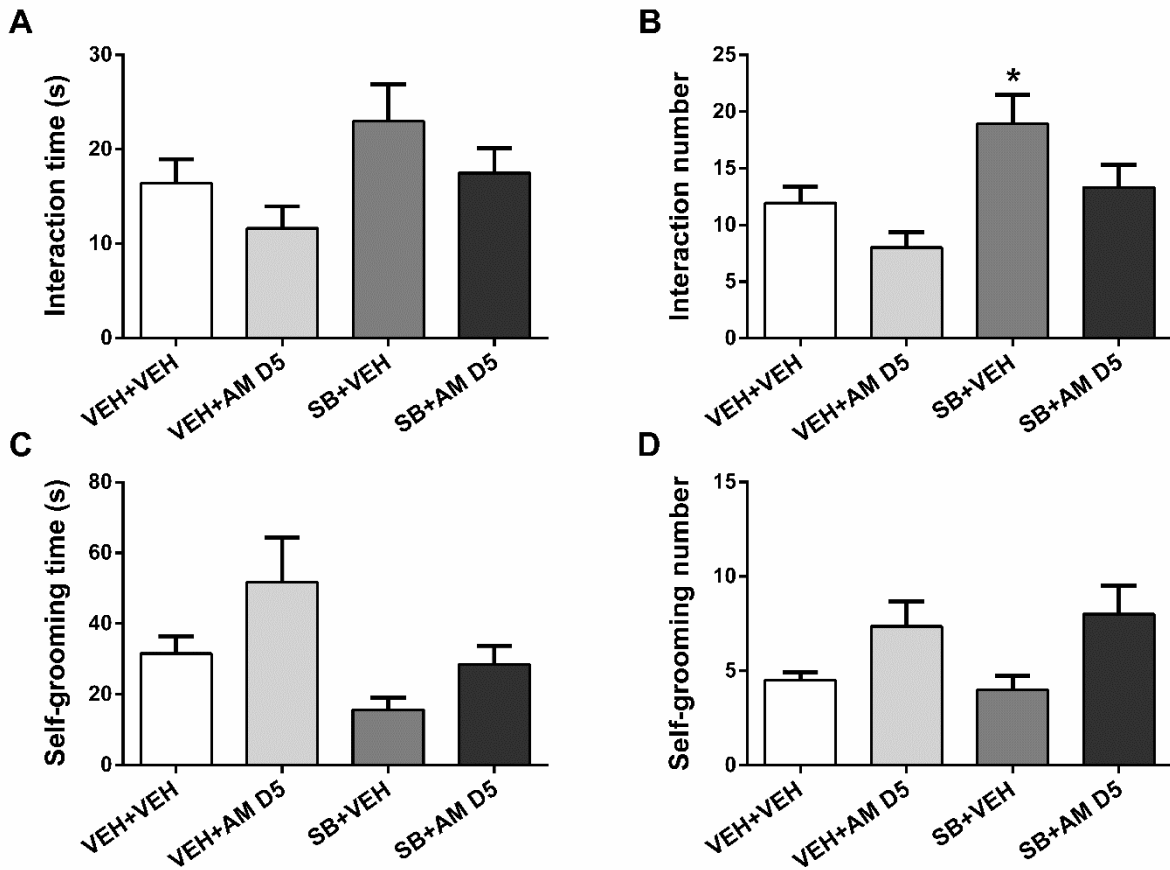
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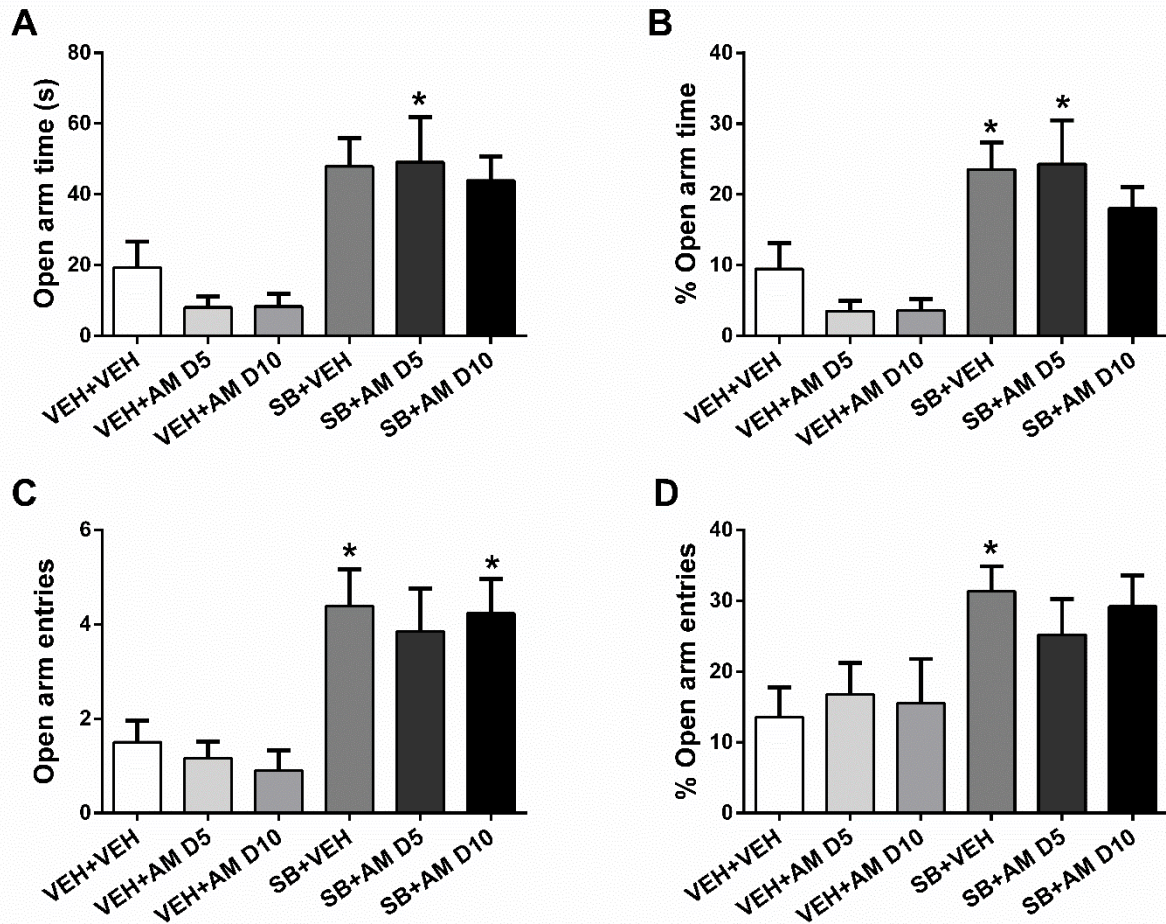
391 **9. Figures and figure legends**



392 **Fig. 1.** Influence of SB-242084 (SB, 1 mg/kg, ip.), AM-251 (AM D5 and AM D10, 5 and 10
393 mg/kg, ip.) and their combination on explorative behaviors and locomotor activity in social
394 interaction test (A, B) and elevated plus maze test (C). Graphs show the time spent with
395 rearing (A), the number of line crossings (B) and the number of closed arm entries (C). N=12-
396 16 for all groups. Columns represent mean \pm S.E.M. * $p < 0.05$ and *** $p < 0.001$, significant
397 results of Dunnett's *post hoc* test compared to VEH+VEH group.



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