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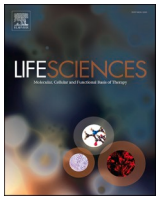

Evidence for functional interaction between the CB1 and the mGlu7 receptors mediated signaling in modulation of anxiety behavior and cognition.

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ABSTRACT

Anxiety is a severe social problem. It is a disease entity that occurs alone or accompanies other diseases such as depression, phobia, or post-traumatic stress disorder. Our earlier studies demonstrated that blockage of arachidonic acid (AA) pathway via inhibition of cyclooxygenase-2 (COX-2) enzyme can modulate mGluRs-induced anxiety-like behavior. Here, we hypothesized that modulation of 2-arachidoglycerol (2-AG), a component of the AA pathway, concomitantly with modulation of mGluR7 signaling, should be adequate to trigger a similar response from the test organism. Since 2-AG is an endogenous agonist for CB1 receptors, we used a CB1/GPR55/μ-opioid receptor antagonist (AM251) alone and in combination with mGluR7 allosteric agonist (AMN082). Stress-induced hyperthermia (SIH) test was performed as a behavioral readout. AM251 has a dual mode on AMN082-mediated effects in SIH in CD-1 mice. Furthermore, the CB1 receptor ligand influenced adaptation to stress in repeated SIH procedures and learning possibilities of mice in the Barnes maze. We also found changes in mGluR7 protein expression levels in the prefrontal cortex (PFC) after mice were exposed to AM251, which showed the potential to attenuate the AMN082-induced decline in mGluR7 levels. The changes induced by AM251 on AMN082-mediated behavioral and biochemical effects were confirmed in electrophysiological experiments in which AM251 abolished AMN082-mediated LTP escalation in PFC. The mGluR7 overexpressed cell line was used to exclude the direct involvement of mGluR7 in AM251 activity. All the above results and the co-localization of CB1 and mGlu7 receptors detected in specific brain regions strongly suggest the specific interaction between CB1 and mGlu7 receptors and their signaling.

1. Introduction

Incomprehensible emotions in the interior, such as unjustified anxiety, are devastating for the body. Generalized anxiety disorder exists as a separate disease entity, or it may accompany other mental disorders, such as depression, often exacerbating its symptoms. Anxiety and depression often use the same neurotransmitter pathways. However, the underlying causes of either disease are not completely known. Recent studies indicate a significant role of the fatty acid pathway in shaping emotions [1–4]. Parallel to discovering the role of the metabolism of a fatty acid pathway on the psychical possibilities of the brain, the role of metabotropic glutamate receptors (mGluRs) in these processes has been well documented. The mGluRs belong to class III G-protein coupled receptors (GPCRs), which possess seven transmembrane domains and exert influence on intracellular signaling cascades via G proteins [5–7]. There are eight different mGluR subtypes divided into three groups based on their sequence homology, pharmacological profile, and signal transduction mechanism [6]: group I (mGluR1 and mGluR5), group II

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(mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7 and mGluR8).

The anxiolytic-like potential of mGluRs group III ligands, especially ligands of mGluR7, is now well documented [8,9,5]. AMN082, mGluR7 allosteric agonist, has been shown to produce anxiolytic-like effects in a wide range of tests, including SIH [10]. Studies indicate that both receptors belong to GPCRs, and their interaction affects the release of neurotransmitters, including glutamate. For example, mGluR7, acting presynaptically, inhibits excessive glutamate release, which may be modulated by CB1 activity. It has also been shown that group III mGluR agonists, including mGluR7, may have neuroprotective effects in models of neurodegenerative disease and oxidative stress [11,12]. At the same time, the involvement of cannabinoid type 1 (CB1) receptors in modulation of anxiety behavior was demonstrated [13]. Therefore, the search for common mechanisms of action on these two paths seems logical.

Our earlier studies showed that blockade of arachidonic acid (AA) transformation via inhibition of cyclooxygenase-2 (COX-2) possesses therapeutic effects in rodents [3,4]. The blockade of AA metabolism via inhibition of the COX-2 enzyme was shown to modulate the cognitive abilities of rodents but not anxiety [3,4]. Moreover, the interaction of COX-2 with mGluRs ligands on cognition and depressive-like behavior was found [3,4]. Further, inhibition of COX-2 was enough to modulate mGluRs action during tolerance development in the anxiety test, but not anxiety itself [3]. Because AA simultaneously is a component of 2-arachidoyl glycerol (2-AG), we decided to check if modulation of the 2-AG pathway may be involved in modeling anxiety behavior. Since 2-AG is an endogenous agonist for CB1 receptors [13], we used AM251, a CB1/GPR55/μ-opioid receptor antagonist in combination with mGluRs group III ligands: AMN082 (an allosteric agonist of mGluR7) and LSP4-2022 (an orthosteric agonist of mGluR4) [14,15] on anxiety-like behavior in mice. The engagement of mGluR7 and mGluR4 ligands in mental processes is well-known [8,9,16,17].

Our investigation found that AM251 did not act as an anxiolytic by itself in SIH. However, it intensified the anxiolytic-like effect of the mGluR7 ligand, suggesting the interaction of these two paths in the modulation of anxiety behavior. Interestingly, our results showed that the AM251 impaired the adaptation of animals to stressful situations, and mGluR7 engagement was postulated to be involved in that effect. Therefore, the following experiments checked the cognitive possibilities of mice after the treatment and changes in mGluR7 protein level in brain structures such as PFC, HC, and Amy. Electrophysiological recordings and colocalization of CB1 and mGlu7 receptors were performed to investigate the interaction between these two receptors and their signaling as a possible mechanism for the observed behavioral changes.

2. Materials and methods

2.1. Animals and housing

Male CD-1 mice (30-40 g, 242 mice) were used for testing. Animals were group-housed with the following conditions: 12/12 h light/dark cycle (6:00–18:00 and 18:00–6:00, respectively) and temperature 21 ± 1 °C. Free access to feed and water was ensured. Experimental procedures were conducted between 9:00 and 17:00 (the light period). Guidelines issued by the National Institutes of Health Animal Care and Use Committee were followed concerning all tests performed. The Ethics Committee Institute of Pharmacology, Polish Academy of Sciences in Kraków (0006) verified and approved the experimental design (Approval no.: 1206 and 309/2022).

2.2. Drug treatment

AM251: 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide, (0.3 and 3 mg/kg, Abcam Biochemicals, UK), AMN082: *N*,*N*′-Bis(diphenylmethyl)-1,2-ethanediamine dihydrochloride (6 mg/kg, Tocris Cookson ltd., Bristol, UK), and LSP4-2022 (2*S*)-2-amino-4-[[[4-(carboxymethoxy)phenyl]-hydroxymethyl]-hydroxyphosphoryl]butanoic acid (2 mg/kg), were dissolved according to the manufacturer's instructions, and were administered to animals through intraperitoneal route (*i.p.*) along with respective vehicle, 60 min before the tests, LSP4-2022 was synthesized in the laboratory of F.A. following a previously described procedure [18].

2.3. Modified stress induced hyperthermia (SIH) test in singly housed mice

The modified stress-induced hyperthermia (SIH) test was adopted from Van der Heyden et al. [19]. The SIH test is a commonly used test for assessing generalized anxiety in mice. For the SIH procedure, mice were single-housed for 24 h before the experiment. During the test day, body temperature was measured in each mouse at $T = 0$ min (T1) and T = +15 min (T2). Mice were placed into a new cage immediately after T1 measurement. The difference in temperature T2-T1 is considered a SIH. Rectal temperature was measured using an Ellab thermometer with an exact temperature of 0.1 °C. Details of the procedure are described in Stachowicz [3]. Furthermore, the mouse weight was measured every day before injections of tested compounds.

2.4. The modified Barnes maze (MBM) test

The modified Barnes maze (MBM) test is applied to evaluate the memory of mice, with particular emphasis on spatial memory [20,4]. The Barnes maze consists of a gray circular platform (ϕ122 cm) mounted above the floor (at a height of 113 cm) on a leg with 40 holes and an escape box (17.8 × 5.1 × 10.2 × 7 cm). Stachowicz [3] provided particulars of the test. The MBM test includes three stages: training session, retention interval, and test session. The mice were placed individually on a platform and allowed 5-min exploration during training and test sessions. Each animal was tested twice during one session. The following variables were monitored throughout the experiment: latency to find an escape hole, the number of errors, strategy, working memory, and wiping of memory traces. After each trial, the odor was removed from the platform using a 20 % ethanol solution. All stages of the experiment were videotaped.

2.5. Tissues collection

Decapitation was performed one hour after the last drug injection. Animal brains were removed at once. The hippocampus (HC), prefrontal cortex (PFC), and amygdala (Amy) were obtained following the coordinates included in the Mouse Brain Atlas [21]. PFC was obtained by cutting the anterior part of the forebrain at Bregma 2.20 mm. Olfactory bulbs and the anterior striatum were cut off. Therefore, the tissue taken for analysis contained most of the prefrontal cortex. The amygdala has been extruded through the cortex from the right and left sides of the cerebrum. Subsequently, the brain was cut into two hemispheres in the sagittal line. Then, the whole HC was taken out from each hemisphere. Before biochemical analysis, the tissues were frozen on dry ice and stored at −80 °C.

2.6. Western blot analysis

Protein levels in the PFC, HC, and Amy were determined using the Western blot method. The tissues were homogenized in 2 % SDS, denaturated (10 min at 95 °C), and centrifugated (5 min at 9000 xg; 4 °C). The BCA method (Thermo Fisher Scientific; Rockfold, IL, USA) was used to establish the total protein concentration in the resulting supernatants. Then the samples containing 20 μg of total protein were prepared and loaded on gels. The following steps involved SDS-PAGE fractionation and transferring the proteins from the gel to nitrocellulose membrane (Bio-Rad; Frankfurt, Germany). 1 % blocking solution (BM Chemiluminescence Western Blotting Kit; Mouse/Rabbit; Roche;

Basel, Switzerland) was used to prevent non-specific binding (30 min at 4 °C). Next, the membranes underwent overnight incubation at 4 °C with primary antibodies: rabbit polyclonal anti-mGluR7 (diluted 1:1000; Abcam, Cambridge, UK, ab2723). The successive stage involved washing membranes in Tris-buffered saline with 1 % Tween 20 (TBS-T) and incubating for 30 min at room temperature with a goat HRP-conjugated anti-rabbit/mouse IgG (diluted 1:25,000; Roche; Basel, Switzerland). TBS-T was used for subsequent washing. The luminescent signal was detected employing an enhanced chemiluminescence reaction (BM Chemiluminescence Western Blotting Kit; Roche; Basel, Switzerland) and analyzed using Fuji-Las 1000 with Fuji Image Gauge v.4.0 software. β-actin was monitored on each membrane to verify the amount of total proteins in each sample. To this end, a mouse monoclonal anti-β-actin antibody (diluted 1:7000; Sigma-Aldrich; Darmstadt, Germany) was used. Final results for each sample are expressed as the ratio of the optical density of a particular protein to the optical density of β-actin.

2.7. Electrophysiological studies – slice preparation

Brain slices (400-μm-thick) were prepared one hour after the last drug injection. Mice were anesthetized with isoflurane (0.2 ml, Aerrane, Baxter), and decapitated. Brains were removed into an ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl (130), KCl (5), CaCl2 (2.5), MgSO4 (1.3), KH2PO4 (1.25), NaHCO3 (26), and D-glucose (10), bubbled with a mixture of 95 % O2 and 5 % CO2. Frontal cortical (FC) 400 μm thick, were cut in the coronal plane using a vibrating microtome (VT 1000 Leica). Slices were stored at 32.0 ± 0.5 °C at least 1 h.

2.8. Extracellular recording and LTP induction

Individual slices were placed in the recording chamber of an interface type which was superfused (2.5 ml/min) with a modified ACSF containing (in mM) NaCl (132), KCl (2), CaCl2 (2.5), MgSO4 (1.3), KH2PO4 (1.25), NaHCO3 (26), and D-glucose (10), bubbled with 95 % O2 and 5 % CO2 (temperature 32.0 ± 0.5 °C). Cortical field potentials (FPs) were evoked by stimulation (0.033 Hz, duration 200 μs) using a constant-current stimulus isolation unit (WPI) and a bipolar Pt—Ir electrode (CBARC85, FHC) placed in layer V. FPs were recorded using ACSF-filled glass micropipettes (~1 MΩ) placed in layer II/III. The responses were amplified (EXT 10–2F amplifier, NPI), filtered (1 Hz–1 kHz), A/D converted (10-kHz sampling rate, Axon Instruments), and stored on PC using the Micro1401 interface, and Signal 4 software (CED). A stimulus–response (input–output) curve was made for each slice. To obtain the curve, stimulation intensity was gradually increased stepwise (16 steps; 0–100 μA). One response was recorded at each stimulation intensity. Then, stimulation intensity was adjusted to evoke responses of 30 % of the maximum amplitude. Cortical LTP was induced by theta burst stimulation (TBS). TBS consisted of 10 trains of stimuli at 5 Hz, repeated 5 times every 15 s. Each train was composed of five pulses at 100 Hz. During TBS, pulse duration was increased to 0.3 ms. Amplitude was measured in FC.

2.9. mGlu7 receptor expressing cells

T-REx 293 cell line (HEK293 cell line containing an inducible expression system, Invitrogen) with stable and inducible expression of the human mGlu7 receptor (NM_000844.2) was established as previously described [22]. The cell line was maintained in DMEM supplemented with 10 % tetracycline free FBS, 2 mM Glutamax I (Lonza, Basel, Switzerland), 100 μg/ml Hygromycin B and 10 μg/ml blasticidin (Invitrogen). Expression of the mGlu7 receptor was induced by the incubation of cells with 0.75 μg/ml tetracycline for 24 h. The expression of mGlu7 receptor was validated by RT-PCR and Western blotting as previously described [22].

2.10. Forskolin-induced cAMP accumulation assay

The detection of cAMP in T-Rex 293 cells expressing mGlu7 receptor was performed with the use of a homogeneous time-resolved fluorescence (HTRF) cAMP dynamic 2 assay, according to the manual's instruction from Cisbio (Codolet, France) with minor modifications [22]. Cells were grown in standard conditions to 60 % confluency. 24 h before the experiment mGlu7 receptor expression was induced by adding tetracycline to the cell medium. 16 h before the experiment, growth media was replaced with serum-free DMEM. Directly before the experiment, cells were scraped and centrifuged. A cell pellet was suspended in Hanks-HEPES buffer (130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 0.9 mM NaH2PO4, 20 mM HEPES, and 3.25 mM glucose, pH 7.4). Then, 50 μl of cell suspension was pipetted to a 96-well plate containing 3 μM forskolin and appropriate concentrations of tested compounds. Cells were incubated together with forskolin and compounds for 5 min at 37 °C. Stimulation step was stopped by adding 10 μl of the reaction mixture to 5 μl of cAMP-d2 conjugate suspended in lysis buffer using an automated pipetting system (Tecan Evo 200; Tecan, Mannedorf, Switzerland). Next, 5 μl of anti-cAMP cryptate conjugate was added. After 1 h of incubation at room temperature, the fluorescence at 620 nm and 665 nm was read (Tecan Infinite M1000). The results were calculated as the 665-nm/620-nm ratio multiplied by 10^4. The specific signal is inversely proportional to the concentration of cAMP in the sample.

2.11. CB1 and mGlu7 receptors co-localization

Frozen mouse brains were cut into 14 μm thick slices. The fixation process was performed in ice-cold methanol for 10 min. After washing twice in PBS for 5 min, the antigen retrieval process was carried out in PBS with 0.2 % hydrogen peroxide, 0.2 % Triton X-100 and 20 % methanol for 25 min at room temperature. After washing twice in PBS for 5 min, an antibody solution (PBS with 0.3 % Triton X-100, 0.05 % Tween-20 and 2 % normal goat serum – NGS) containing primary antibodies: CB1 (1:200, #ACR-001-GP, Alomone Labs) and mGluR7 (1:400, #AGC-017, Alomone Labs) were incubated for 1 h at room temperature and then overnight at 4 °C. The slices were then incubated twice with 2 % NGS for 5 min and then incubated with antibody solution (PBS with 0.3 % Triton X-100, 0.05 % Tween-20 and 2 % NGS) containing secondary antibodies: anti-guinea pig Alexa 555 (1:400, #A21435, Invitrogen) and anti-rabbit Alexa 488 (1:400, #A21206, Life technologies) for 1 h at room temperature and then overnight at 4 °C. The slices were then incubated twice with 2 % NGS and stained with 4,6-diamidino-2-phenylindole (DAPI). Slides were covered with VECTASHIELD Antifade Mounting Medium (Vector Laboratories). Images were acquired using an Axio Imager A2 fluorescence microscope (Carl Zeiss, Germany). The colocalization was measured using ImageJ and the JACoP plugin. The degree of co-localization is determined by the value of the Pearson's Coefficient (r). R equal 1 means that there is complete colocalization, while R equal 0 means no colocalization.

2.12. Statistical analysis

The results are presented as the means ± S.E.M. A one-way or two-way (followed by the Tukey's test) analysis of variance (ANOVA), when appropriate, were used to analyze data. GraphPad Prism software 8.0 (San Diego, CA, USA) was used for calculation. $P < 0.05$ was considered significant. Fig. 2. - calculated by ANOVA with repeated measures using Statistica 14.00.15 for Windows. MBM data were analyzed using three-way mixed-design ANOVA with two between-subject factors (AM251 and AMN082 treatment) and day as a repeated measure, using Statistica 14.00.15 for Windows. Histogram of frequency distribution was adopted for analysis of strategy in MBM.

3. Results

3.1. The effect of AM251 on SIH in CD-1 mice

Dose dependence study of AM251 effects (0.3 mg/kg and 3 mg/kg) on T1 (Fig. 1A) and SIH (Fig. 1B) showed no effect of single injections on T1 [F (2, 27)=0.0685; $P = 0.934$] or SIH [F (2, 27)=0.585; $P = 0.564$]. AMN082 is known as potent compound in SIH [10,12] in the dose dependent manner presented in our previous studies [10]. However, AMN082 is not able to change T1 after single injection [10]. Here, single injection of AMN082 (6 mg/kg) or AM251 (0.3 mg/kg) had no effect on T1 in CD-1 mice (Fig. 1C). However, co-treatment of these two compounds resulted in significant decrease of T1 [F(3,34) = 4.297; $P = 0.011$] (Fig. 1C). The above results and our previous data may suggest that the GABA-ergic component takes part in the action of both compounds [10,17,9].

In the second part of experiment SIH was detected (Fig. 1D). Significant decrease in SIH after single injection of AMN082 (6 mg/kg) and

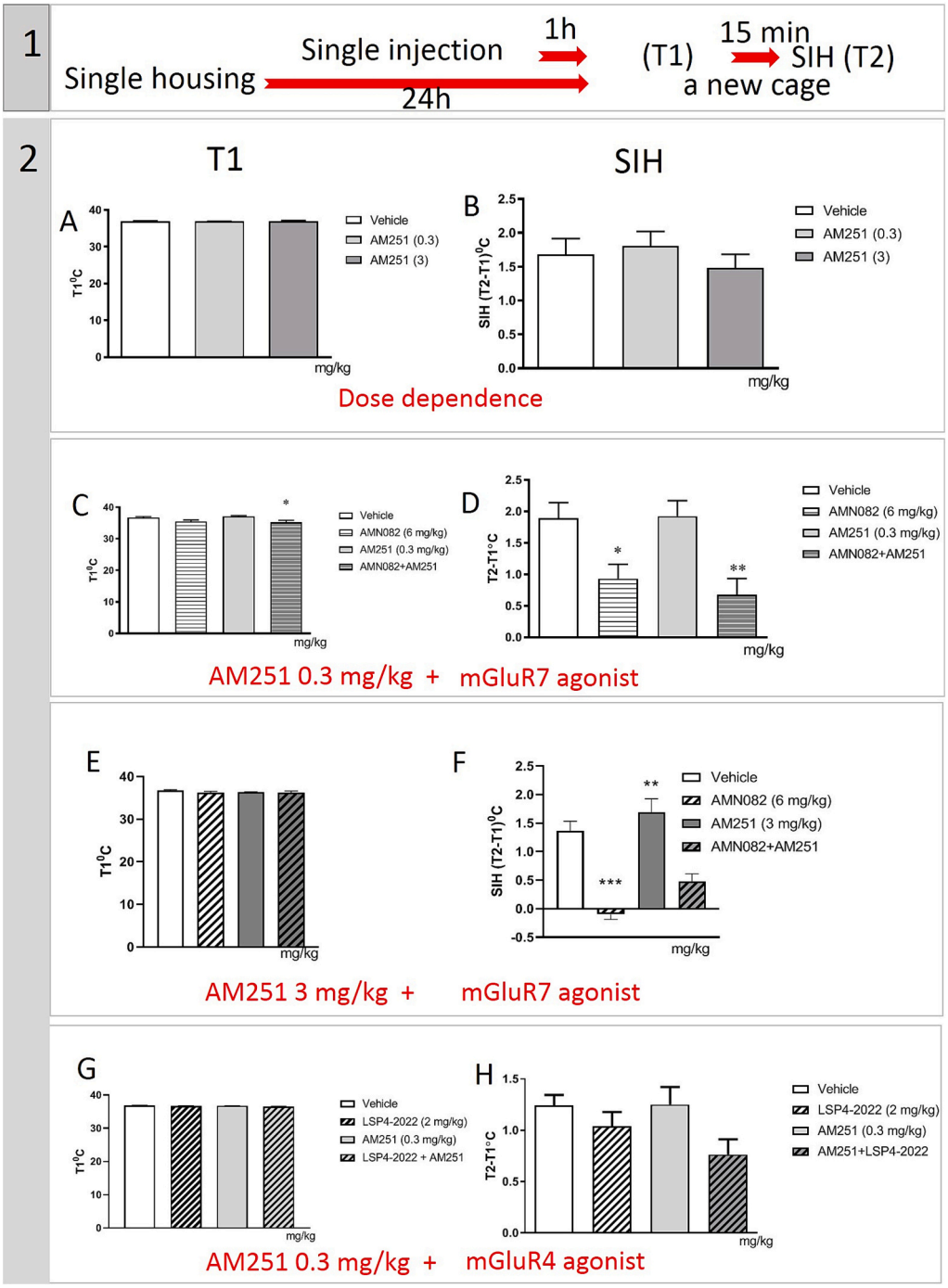


Fig. 1. The effect of AM251 on SIH in CD-1 mice. (A-B) Dose dependence study of single injection of AM251 (0.3 - 3 mg/kg) on SIH in CD-1 mice. (C—D) The effect of co-treatment of AM251 (0.3 mg/kg) with AMN082 (6 mg/kg) on T1 and SIH in CD-1 mice. (*E*-F) The effect of co-treatment of AM251 (3 mg/kg) with AMN082 (6 mg/kg) on T1 and SIH in CD-1 mice. (G-H) The effect of co-treatment of AM251 (0.3 mg/kg) with LSP4-2022 (2 mg/kg) on T1 and SIH in CD-1 mice. CD-1 mice were injected *i.p.* 60 min before the test. Values are expressed as the means ± S.E.M., a one-way ANOVA (Tukey test), $n = 8$–10. (Left panel) shows T1, and (Right panel) shows SIH.

no effect of AM251 (0.3 mg/kg) in SIH was found. One-way ANOVA showed a significant effect of co-treatment with AMN082 and AM251 in SIH [one-way ANOVA: F(3,34) = 6.774; $P = 0.001$] (Fig. 1D). However, two-way ANOVA did not found interaction [F(1,34) = 0.333; $P = 0.568$].

Further, we demonstrate that **AM251 weakens the anxiolytic-like properties of AMN082 when given at a higher dose of 3 mg/kg**. Treatment of mice with AMN082 (6 mg/kg), AM251 (3 mg/kg) or their combination had no effect on T1 in CD-1 mice [F (3, 30)=0.612; $P = 0.613$], (Fig. 1E). At the same time, significant treatment effects were found in SIH (Fig. 1F). A two-way ANOVA detected a significant effect of AMN082 (6 mg / kg), [F (1, 30) = 68.14; $P < 0.0001$], a significant effect of AM251 (3 mg / kg), [F (1, 30) = 7.77; $P = 0.009$], and no interaction [F (1, 30) = 0.6094; $P = 0.441$]. These results may suggests that the anxiolytic-like effect of AMN082 is weakened by the CB1 receptor ligand (Fig. 1F).

AMN082 has been shown to activate also mGluR4 if a higher dose is used [23]. Therefore, our responsibility was to check if our effects can be partially driven also by the mGluR4. For this purpose a novel, selective mGluR4 agonist, LSP4-2022 was chosen for the study [14]. The dose of LSP4-2022 used here, 2 mg/kg, was selected based on our earlier experiments (data not shown). Neither single injection of AM251 (0.3 mg/kg), LSP4-2022 (2 mg/kg), or their combination had effect on T1 in CD-1 mice [One-way ANOVA: F(3,34) = 1.262; $P = 0.303$] (Fig. 1G). Similarly no effect of the above treatment was found in SIH [One-way ANOVA: F (3,34) = 2.338; $P = 0.091$] (Fig. 1H). Two-way ANOVA showed no significant effect of AM251 [F(1,34) = 0.866; $P = 0.359$], significant effect of LSP4-2022 [F(1,34) = 5.720; $P = 0.023$], and no interaction [F (1,34) = 1.000; $P = 0.324$]. It seems that these two compounds, CB1 receptors antagonist and mGluR4 agonist, act via separately mediated mechanisms, with no interaction during anxiogenic situations in mice.

3.2. Repeated administration of AM251 blocks mice's adaptation to stress induced by a repeated procedure of SIH

There is growing evidence for the involvement of CB1 receptors in fear conditioning, especially in the reconsolidation of fear memory in the basolateral amygdala [24,25]. Thus, we were interested in checking the effects of AM251 on adaptation to the repeated procedure of SIH and learning possibilities of mice. To this end, the SIH experiments were repeated three times over four weeks.

Vehicle-injected mice adapted to the procedure overtime, with almost 45 % decrease in SIH. However, mice treated with AM251 did not adapt to the procedure, and this effect was dose-dependent (Fig. 2). Repeated measure ANOVA revealed not significant effect of the treatment [F (1, 24)=0.996; $P = 0.383$] and a significant effect of the day [F (3,72) = 2.998; $P = 0.036$].

3.3. The effects of co-treatment of AM251 with AMN082 on learning possibilities of CD-1 mice in MBM

The observed changes in adaptation of animals to stress in the SIH test after AM251 (3 mg/kg) administration (see Fig. 2) could be related with memory disturbance. Therefore, we decided to test the potential effects of both compounds on memory parameters in mice. To this end, we have made a series of observations using the Barnes maze.

3.3.1. Effect of simultaneous treatment with AM251 and AMN082 on the latency to find an escape hole, the number of mistakes made and the strategy of CD-1 mice on the MBM

A three-way mixed design ANOVA demonstrated a non-significant effect of AM251 (3 mg/kg) treatment [F (1, 24)=0.124; $P = 0.728$], AMN082 (6 mg/kg) treatment [F (1, 24)=0.774; $P = 0.387$], or their interaction [F (1, 24)=0.487; $P = 0.388$] on latency to find the escape box in the MBM test (Fig. 3A). There was a significant effect of the day of the test [F(3,72) = 18.109; $P = 0.000$], demonstrating that all groups made significant progress during the test. Furthermore, there was a significant effect of the day vs. AMN082-treated group [F(3,72) = 7.898, $P = 0.0001$], although post-hoc analysis showed no significant difference between groups [Newman-Keuls test, ns]. A three-way mixed design ANOVA showed a non-significant effect of AM251 treatment [F (1, 24)=0.002; $P = 0.963$], AMN082 treatment [F (1, 24)=0.298; $P = 0.589$], or their interaction [F (1, 24)=1.427; $P = 0.243$], on the number of errors made in the MBM test (Fig. 3B). The study revealed a significant effect of the day of the test [F(2,48) = 4.521; $P = 0.015$], demonstrating that all mice made significant progress during the test.

An interesting finding of this study is that a single injection of AMN082 (6 mg/kg) was able to completely block the use of the spatial strategy in CD-1 mice (Fig. 3D). This effect was observed even on the second day after treatment. However, on the third day after treatment, the group of mice previously treated with AMN082 used the most spatial strategy (about 36 %), suggesting some functional changes after the treatment. While AM251 (3 mg/kg) had no effect on the spatial strategy of the mice, the compound was able to release the spatial strategy block induced by AMN082 (Fig. 3D), suggesting interactions between these two pathways.

3.3.2. There was no effect of simultaneous treatment with AM251 and AMN082 on the memory flexibility, working memory, and wiping memory traces of CD-1 mice on the MBM

Three parameters were tested to determine the quality of the memory, namely: working memory (Fig. 4A), wiping memory traces (Fig. 4B), and memory flexibility (Fig. 4C). There was no effect of the administration of the tested substances on the above parameters using 2-way ANOVA. Regarding Fig. 4A, subtle differences between AM251 (3 mg/kg) and AMN082 + AM251 were visible in the context of working memory, but not statistically significant. A two-way ANOVA found no

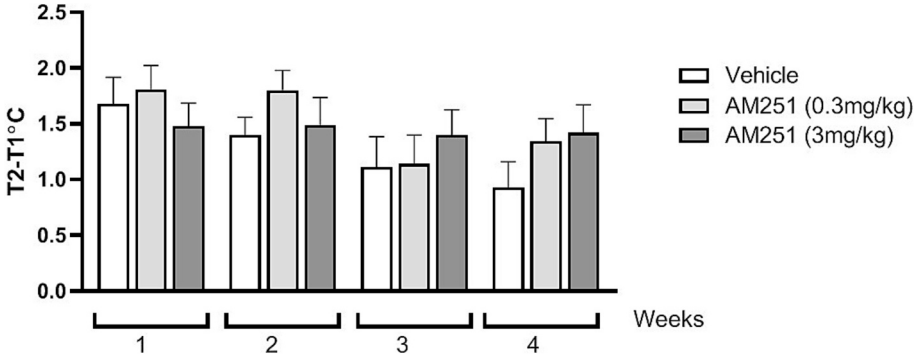


Fig. 2. The effect of repeated treatment with AM251 (0.3 and 3 mg/kg) on SIH in CD-1 mice. Compound was injected *i.p.* once a week 60 min before the test. Values are expressed as the means ± S.E.M., a repeated measure ANOVA, $n = 8–10$.

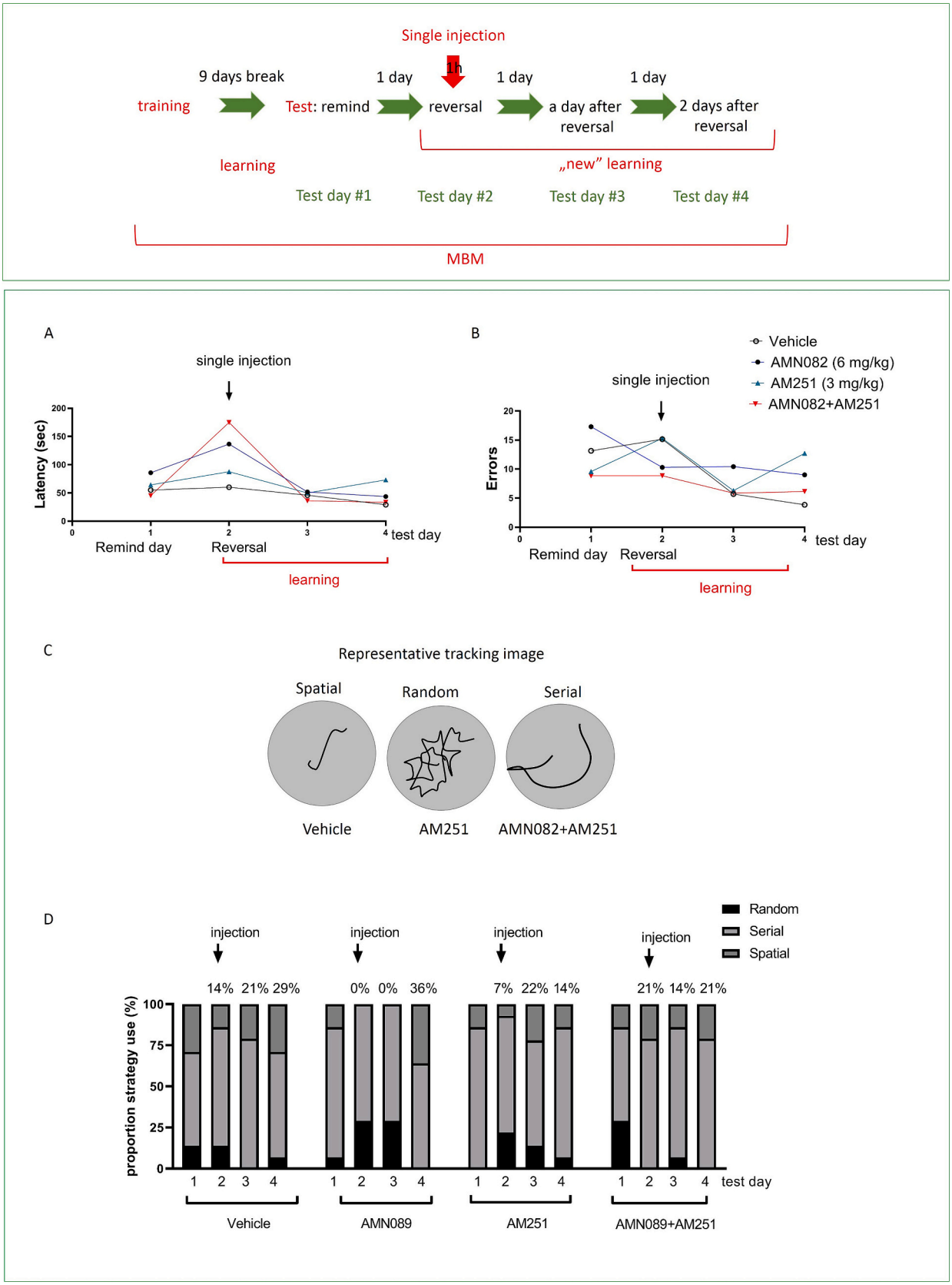


Fig. 3. The first part of the panel presents schematic diagram of injections and experimental schedule. The second part of the panel presents the effect of co-treatment with AMN082 (6 mg/kg) and AM251 (3 mg/kg) on behavior of CD-1 mice in the MBM. Latency to find escape box is shown in (A), the number of errors is shown in (B), representative tracking image (C), strategy of mice in (D). Compounds were injected *i.p.* 1 h before the test on the second day of the experiment, during "new learning" (after remind day) - formulation used for the purposes of the manuscript. Values are expressed as the means ± S.E.M., three-way mixed design ANOVA, (n = 7).

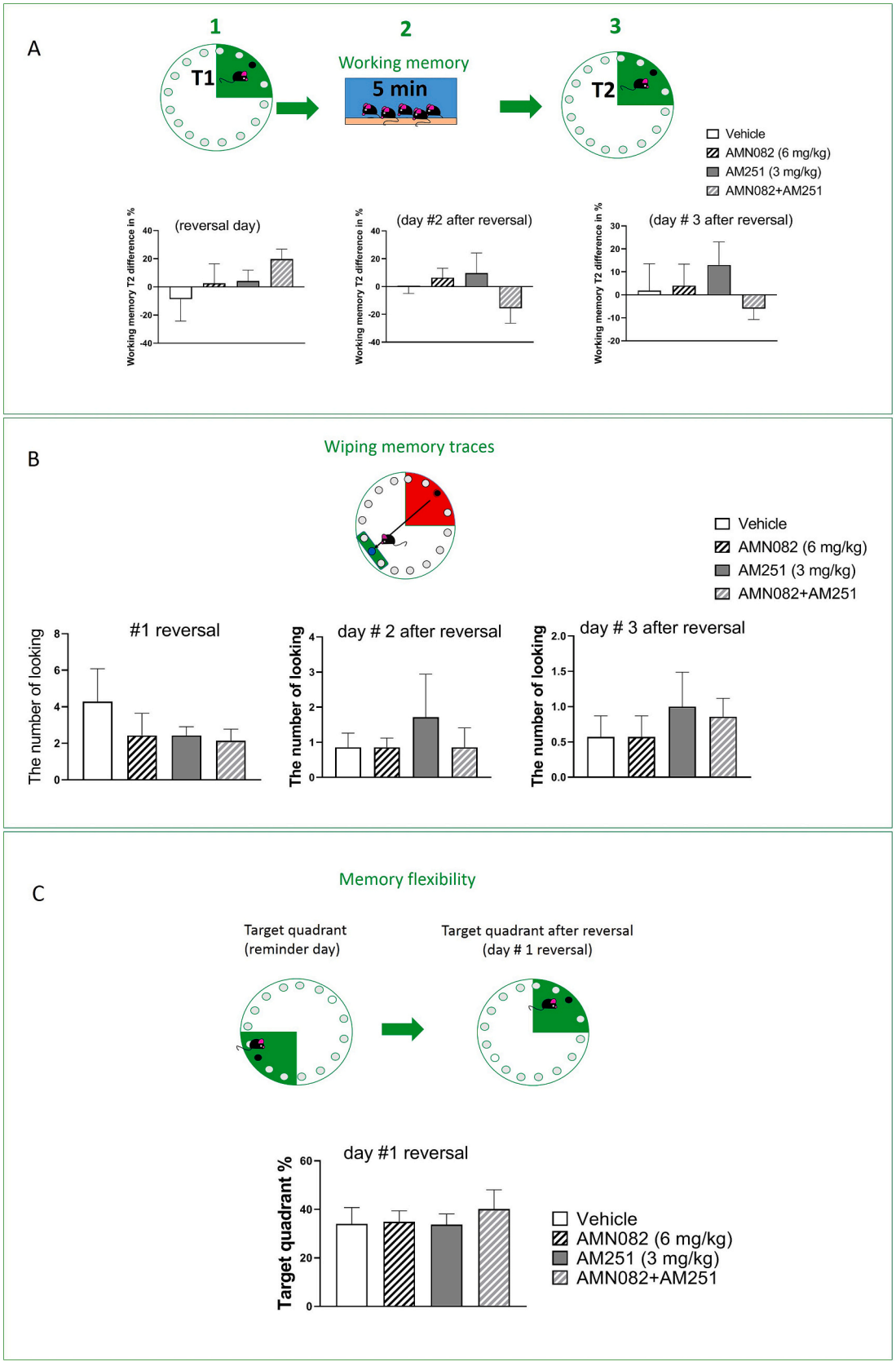


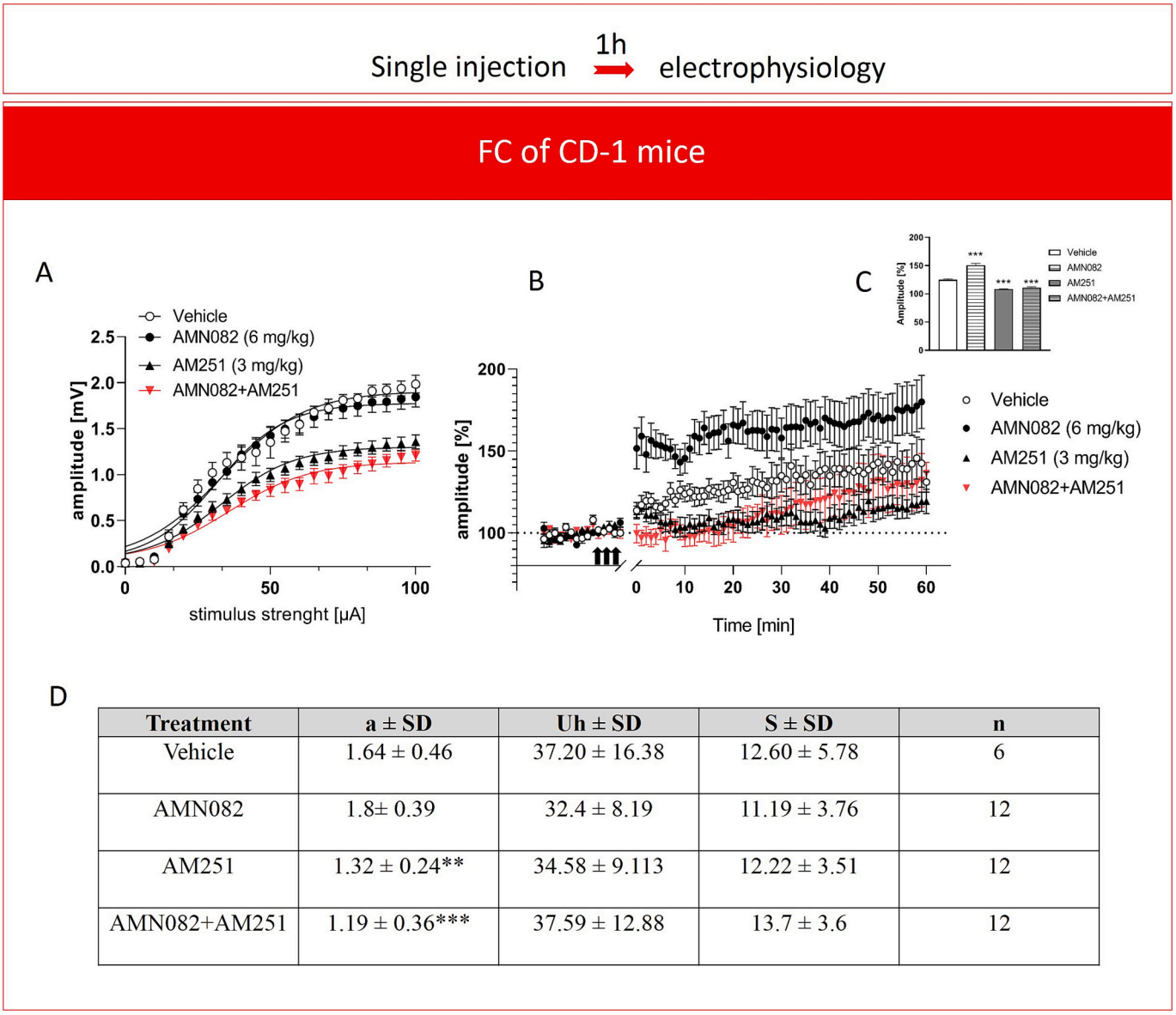
Fig. 4. The effect of co-treatment with AMN082 (6 mg/kg) and AM251 (3 mg/kg) on behavior of CD-1 mice in the MBM. Working memory is shown in (A), wiping memory traces is shown in (B), memory flexibility of mice in (C). Compounds were injected *i.p.* 1 h before the test on the second day of the experiment, during "new learning" (after remind day) - Formulation used for the purposes of the manuscript. Values are expressed as the means ± S.E.M., two-way ANOVA, ($n = 7$).

significant effect of AMN082 (6 mg/kg), [F (1, 24)=1.33; $P = 0.261$], no significant effect of AM251 (3 mg/kg), [F (1, 24)=1.66; $P = 0.210$], and no interaction [F (1, 24)=0.0338; $P = 0.856$] on working memory during first day of reversal ("new learning"). Similar lack of effects were found during second and third day of the test. A two-way ANOVA found no effect of AMN082 (6 mg/kg), [F (1, 24)=0.911; $P = 0.349$], no effect of AM251 (3 mg/kg), [F (1, 24)=0.372; $P = 0.548$], and no interaction [F (1, 24)=2.56; $P = 0.123$] during second day after reversal on working memory in mice. Furthermore, there was no effect of AMN082 on this memory parameter during third day of the test [F (1, 24)=0.818; $P = 0.375$], no effect of AM251 [F (1, 24)=0.00376; $P = 0.952$], and no interaction [F (1, 24)=1.29; $P = 0.268$], (Fig. 4A).

Regarding parameter of wiping memory traces (Fig. 4B) there was no effect of AMN082 [F (1, 24)=0.862; $P = 0.362$], no effect of AM251 [F (1, 24)= 0.862; P = 0.362], and no interaction [F (1, 24)=0.463; $P = 0.503$] during the first reversal day. Similarly there was no effect of AMN082 [F (1, 24)=0.359; $P = 0.555$], no effect of AM251 [F (1, 24)= 0.359; P = 0.555], and no interaction [F (1, 24)=0.359; P = 0.555]. During third day of the test there was no effect of AMN082 [F (1, 24) = 0,04227; P = 0,8388], no effect of AM251 [F (1, 24)=1.056; $P = 0.314$], and no interaction [F (1, 24)=0.0423; $P = 0.839$] on wiping memory traces in CD-1 mice (Fig. 4B).

As it states to memory flexibility of mice (Fig. 4C) the parameter may be assessed during reversal day. However during reversal day, there was no effect of AMN082 [F (1, 24)=0.358; P = 0.555], no effect of AM251 [F (1, 24)=0.168; $P = 0.686$], and no interaction [F (1, 24)=0.209; $P = 0.652$] on memory flexibility of mice (Fig. 4C).

The above results suggest that both AMN082 and AM251 or their



Treatment	a ± SD	Uh ± SD	S ± SD	n
Vehicle	1.64 ± 0.46	37.20 ± 16.38	12.60 ± 5.78	6
AMN082	1.8± 0.39	32.4 ± 8.19	11.19 ± 3.76	12
AM251	1.32 ± 0.24**	34.58 ± 9.113	12.22 ± 3.51	12
AMN082+AM251	1.19 ± 0.36***	37.59 ± 12.88	13.7 ± 3.6	12

Fig. 5. The effect of AMN082 (6 mg/kg), AM251 (3 mg/kg), and co-administration of both compounds on the relationship between stimulus intensity, and amplitude of field potentials (FPs) (A), and LTP (B) in FC of C57Bl/6 J mice. Compounds were injected *i.p.* 60 min before the test. Graphs represent plots of the mean FP amplitude (±S.E.M.). FPs recorded in slices prepared from control (open circles) rats receiving AMN082 (black circles), AM251 (black triangles), and AMN082 + AM251 (red triangles). (C) insert - shows superposition of averaged FPs recorded in representative experiments. Arrow denotes the time of the beginning of TBS. C – mean amplitude of FPs recorded between 45 and 60 min after TBS in control slices, and slices prepared from experimental animals. (D) shows parameters characterizing stimulus–response curves of field potentials in FC of CD-1 mice after treatment, calculated using the Boltzmann fits. Data are presented as means ± S.E.M. Stimulus intensity evoking FP of approximation 0.1 mV in amplitude; Uh – half maximum stimulation; S – factor proportional to slope of the curve; n – number of slices. $^{**}P < 0.01$, $^{***}P < 0.001$ vs vehicle group.

combination did not interfere with actively changing memory parameters after a single injection.

3.4. The effects of treatment with AM251 on mGluR7 level in brains of CD-1 mice (HC, PFC, Amy) detected in Western blot – supplemental data

PFC: A not significant decrease in mGluR7 protein levels was detected in the PFC of CD-1 mice after injection of AMN082 (6 mg/kg), [F (1, 28)=2.824; $P = 0.104$], (Suppl. 1A). Similarly, when mice were treated with AM251 (3 mg/kg), [F (1, 28)=1.212; $P = 0.280$], (Suppl. 1A) and significant while treated with both compounds simultaneously [F(1, 28)=6.67; $P = 0.015$], (Suppl. 1A).

HC: A two-way ANOVA (followed by Tukey) showed a non-significant effect of single injection with AMN082, AM251 or their combination on mGluR7 protein level in HC of CD-1 mice [F (1, 28)= 0.218; $P = 0.644$; F (1, 28)=3.80; $P = 0.061$; F (1, 28)=2.42; $P = 0.131$, respectively], (Suppl. 1B).

Amy: A two-way ANOVA (followed by Tukey) showed a non-significant effect of single injection with AMN082, AM251 or their combination on mGluR7 protein level in Amy of CD-1 mice [F (1, 23)= 0.154; $P = 0.699$; F (1, 23)=1.23; $P = 0.278$; F (1, 23)=0.00712; $P =$ 0.934, respectively], (Suppl. 1C).

3.5. The effect of co-administration of AM251 with AMN082 on field potentials and LTP in FC of CD-1 mice

Analyses of field potentials (FPs) recorded in slices obtained from CD-1 mice, receiving injections of AM251 or AMN082 + AM251 revealed a significant decrease in the relationship between stimulus intensity, and FP amplitude (input–output curve) compared with the slices obtained from control animals (Fig. 5A). Parameters characterizing input–output curves of FPs, calculated using the Boltzmann fits, are summarized in Fig. 5D. In slices prepared from control animals, receiving vehicle injections, the mean amplitude of FPs, measured between 45 and 60 min after TBS delivery, was 140 % of baseline. As illustrated in Fig. 5B, in slices prepared from animals treated with AMN082, long-term potentiation (LTP) was significantly stronger, 172 %. AM251 and AMN082 + AM251 treatment weakness LTP phenomena, and the measured amplitude of FPs were about 115 % and 129 % of baseline, respectively. Statistically significant interaction between AMN082 and AM251 was found by two-way ANOVA: [F1,31 = 37.30; $p < 0.0001$], (Fig. 5C).

3.6. Activity of AM251 in mGlu7 receptor expressing cells (in vitro studies)

mGlu7 receptors are primarily linked to Gαi/o proteins and negatively coupled to adenylyl cyclase to inhibit the production of the second messenger, cAMP. Thus, a forskolin-induced cAMP accumulation assay has been used to study the activity of the mGlu7 receptor expressed in T-Rex 293 cells after incubation with tested compounds. As expected, significant changes in cAMP accumulation in mGlu7 receptor-expressing cells have been detected following the addition of endogenous receptor ligand L-Glu (Fig. 6B). The potency of L-Glu is 3.5 mM (EC50 = 3.5 mM) which is consistent with the literature and confirms the functionality of the mGlu7 receptor in our in vitro cell model. In contrast, AM251 didn't show any changes in the forskolin-induced cAMP accumulation assay in the concentration range from 0.1 nM to 100 μM (Fig. 6A). This result demonstrates that AM251 does not activate mGlu7 receptor mediated signaling in T-Rex 293 cells (Fig. 6A). Furthermore, no significant difference between the potency of L-Glu and the potency of L-Glu in the presence of 10 μM AM251 (EC50 = 4.2 mM) has been detected (Fig. 6B). This result suggests that AM251 does not affect (enhance or inhibit) the activity of mGlu7 receptor mediated signaling induced by the increasing concentration of L-Glu in in vitro cell model (Fig. 6B).

3.7. The effects of treatment with AM251 on EAAT3 level in brains of CD-1 mice (HC, PFC, Amy) detected in Western blot – supplemental data

PFC: A two-way ANOVA (followed by Tukey) showed a non-significant effect of single injection with AMN082 (6 mg/kg), [F (1, 28)=3.495; $P = 0.136$], a non-significant effect of AM251 (3 mg/kg) [F (1, 28)=3.66; $P = 0.066$], and a non-significant interaction [F (1, 28)= 0.299; $P = 0.589$], (Suppl. 2A).

HC: A two-way ANOVA (followed by Tukey) showed a non-significant effect of single injection with AMN082, AM251 or their combination on mGluR7 protein level in HC of CD-1 mice [F (1, 28)= 0.157; $P = 0.695$; F (1, 28)=1.61; $P = 0.215$; F (1, 28)=0.0034; $P =$ 0.954, respectively], (Suppl. 2B).

Amy: A two-way ANOVA (followed by Tukey) showed a non-significant effect of single injection with AMN082, AM251 or their combination on mGluR7 protein level in Amy of CD-1 mice [F (1, 28)= 0.0827; $P = 0.776$; F (1, 28)=3.17; $P = 0.086$; F (1, 28)=2.134; $P =$ 0.155, respectively], (Suppl. 2C).

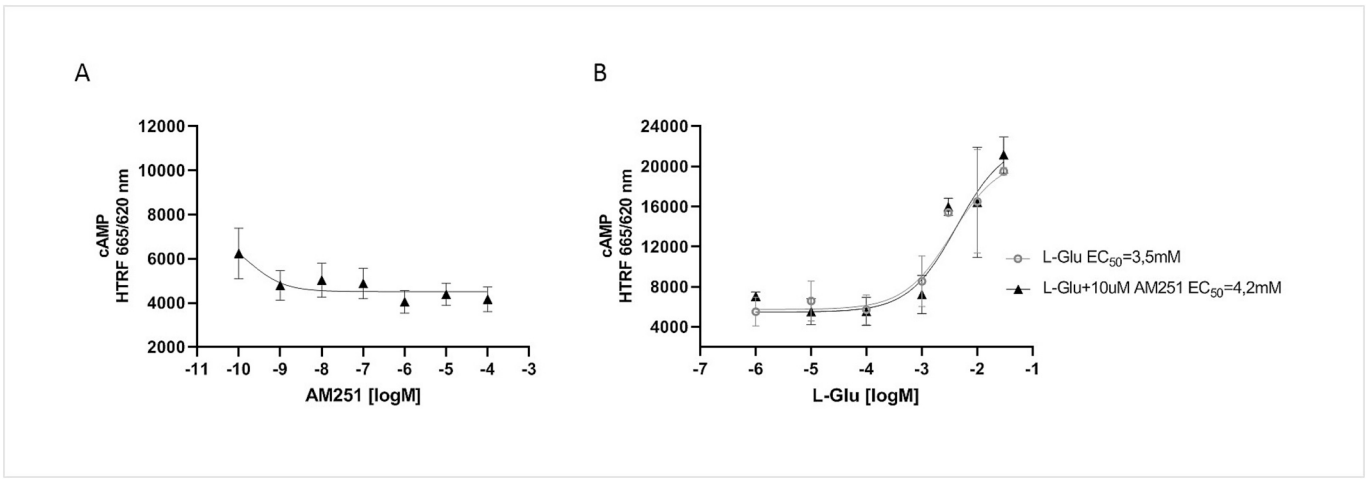


Fig. 6. AM251 does not affect the cAMP level in mGlu7 expressing cells. (A) The level of cAMP in mGlu7 expressing T-Rex 293 cells after incubation with increasing concentration of AM251 (0.1 nM - 100 μM). (B) cAMP level in mGlu7 expressing cells after incubation with increasing concentration of L-Glu (1 μM – 30 mM) alone and in the presence of 10 μM AM251. Graphs represent means ± SEM from three independent experiments run in duplicates.

3.8. The correlation coefficient calculations for CB1 and mGluR7 in PFC, Amy and HC

PFC: Acute administration of AMN082 induced a significant increase in the colocalization of mGluR7 and CB1 receptors in the Prefrontal cortex, [F (3, 16) = 4.062; $P = 0.0253$]. While, neither the administration of AM251 nor the co-administration of ligands for both receptors influenced the colocalization of the receptors under study (Fig. 7A-B).

Amy: No significant changes in CB1 and mGluR7 colocalization were observed in the amygdala (Fig. 7C-D).

HC: A one-way ANOVA (followed by Tukey) showed a significant effect of single injection with AM251 [F (3, 13)= 4.877; $P = 0.0174$] in the CA1 filed of hippocampus on the mGluR7 and CB1 colocalization (Fig. 7E-F).

3.9. Supplemental data of mice body weight

Repeated weekly administration of AM251 at doses of 0.3 mg/kg or 3 mg/kg showed no effect on the body weight of CD-1 mice (Suppl. 3).

4. Discussion

Here, we hypothesized that anxiety-like behavior in mice is modulated by influencing the 2-AG pathway via inhibition of the CB1 receptor using its antagonist (AM251). Moreover, based on our and other previous studies, CB1 receptor blockade should influence glutamatergic signaling mainly through mGluR7. The basis for our hypothesis was studies of the Vanderbilt University group, showing the effect of CB1 receptor modulation on anxiety [25]. Our earlier results documented that inhibition of COX-2 modulates activation of mGluR7, a presynaptically localized representant of group III of mGluRs, and intensely engaged in anxiety [10,11,26].

We found that single injections of AM251 were not enough to influence the anxiety-like behavior of mice in SIH. SIH is a susceptible procedure in which the mouse, separated from the group for 24 h and removed to a new environment after the first temperature measurement during the test day, reacts with a substantial temperature increase a few minutes later [19]. The mouse reaction is similar to the human anxiety reaction [19]. The anxiolytic-like action of the compound is manifested by the failure of the temperature rise [19]. The above-cited reaction was not observed after AM251 treatment. Results were surprising, while CB1 receptor ligands were previously documented as anxiolytics or anxiogenics depending on the dose used [13], but not as neutral compounds. It was proposed, however, that inconsistent behavioral effects of CB1 receptor ligands may be explained by different responsiveness of GABAergic or Glutamatergic neurons [13]. Therefore, we decided to check the effects of AM251 injections on anxiolytic-like effects mediated by mGluR7 agonist, AMN082 with documented anxiolytic-like potential. Our results demonstrated that a single injection of AM251 intensified significantly the anxiolytic-like potential of AMN082 in SIH. Moreover, the intensification of the observed anxiolytic-like effects after co-treatment with AM251 and AMN082 may be connected with GABA-

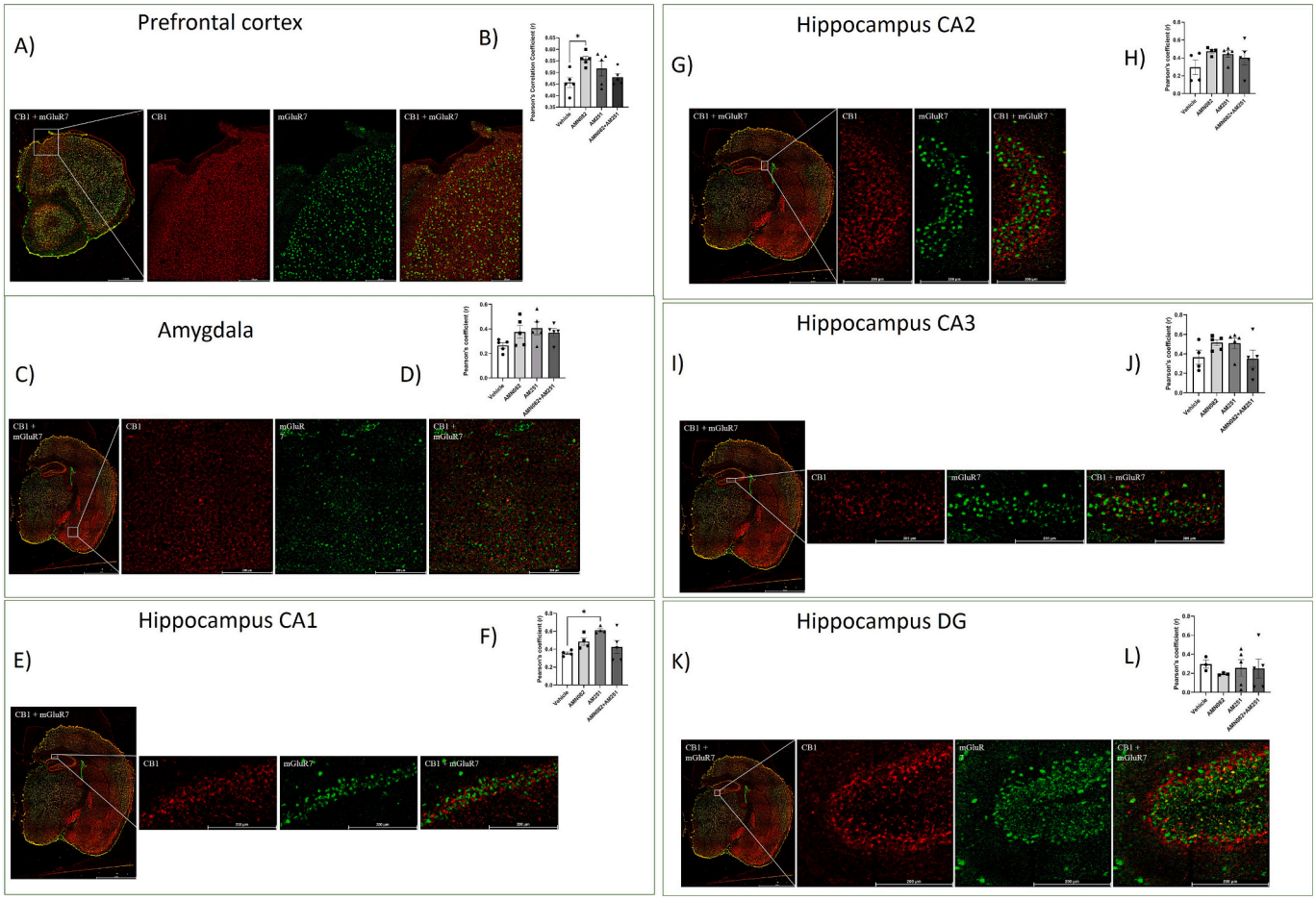


Fig. 7. The figure presents representative images with highlighted brain regions where correlation coefficient calculations for CB1 and mGlu7 receptors were performed: prefrontal cortex (respectively A,B); amygdala (respectively C,D); hippocampal area CA1 (E,F); hippocampal area CA2 (G,H); hippocampal area CA3 (I,J); and the hippocampal dentate gyrus (DG) (*K,L*). Results are presented as Pearson correlation coefficient (mean +/− S.E.M); $n = 3$–5 slices per group in three technical replicates. * indicates $p < 0.05$ vs control group.

ergic transmission, while a decrease in T1 was found [10]. This effect was not observed when compounds were injected separately. Such speculation is also based on the fact that CB1 and mGlu7 receptors are localized presynaptically and on GABAergic terminals [13,27]. Strengthening our hypothesis is provided by previous studies in which an ineffective dose of diazepam and fatty acid amide hydrolase (FAAH) inhibitor became effective in open arms and elevated plus maze [28]. What is more, co-treatment of AM251 with an adequate dose of diazepam blocked its effectiveness in open arms [28], while agonist of CB1 receptors (WIN55212–2) intensified its action [28].

Because AMN082 may also act via mGluR4 [23], we performed similar experiments with the use of a selective ligand of mGluR4, LSP4-2022 to confirm the involvement of mGluR7 in the observed behavioral effects. We found that modulation of 2-AG did not influence the mGluR4 action when ligands of both pathways were co-administered. The opposite behavioral effects between mGluR7 and mGluR4 are unsurprising and were documented in rodents [17]. Opposite results obtained for mGluR4 and mGluR7 ligands are postulated to be connected with their localization in a synapse and other effects exerted on Glu or GABAergic transmission [17,23]. However, no impact of co-administration of AM251 and LSP4-2022 confirms that the anxiolytic-like effects we observed after AM251 + AMN082 treatment, were strictly connected with the interaction between the two receptors, CB1 and mGlu7, and their specific signaling.

The described results raised the suspicion that the 2-AG pathway may be involved in the anxiety connected with cognitive abilities. However, in the case of pure emotional response observed in SIH, it may act only via regulation of the glutamatergic system. AM251 did not induce any effects in SIH by itself, but it influenced the effects of AMN082. We repeated experiments three times over four weeks to incorporate a cognitive component into the SIH procedure. It is well known that repetition of a stressful situation should make the body adapt to the stress. Indeed, vehicle-injected mice showed a gradually decreasing response to stress in SIH. However, the AM251-injected mice in the same situation did not adapt to the stressful situation. Moreover, the observed effects were dose-dependent, and complete blockage of adaptation to stress in SIH was observed at a higher (3 mg/kg) dose of AM251. Two scenarios can explain the observed phenomenon. Firstly, the anxiogenic-like action as an effect of subsequent injections, and secondly, the forgetfulness of re-entering similar stressful situations. We tend to lean towards the second hypothesis based on published data concerning CB1 receptor-mediated behavioral actions. Ratano et al. [24] discovered that AM251 impairs the reconsolidation of Pavlovian fear memory in the rat basolateral amygdala. Furthermore, the effect of amnesia was evident only if AM251 was given in conjunction with memory reactivation [24]. This scenario fits our experimental setup, where mice were injected with AM251 after 24 h separation into single cages, thus resembling stressful situations. We performed the Barnes maze test to check the effects of the CB1 receptor ligand on memory parameters. Our studies have shown that AM251 does not interfere with spatial memory or learning in mice given a single dose of 3 mg/kg. However, it improved the spatial orientation of mice treated with AMN082 in a 6 mg/kg dose. This result suggests that hippocampal neurotransmission may be involved in the stress adaptation observed in SIH after AM251 and AMN082 + AM251 treatment.

Considering the CB1R/mGluR7 interaction, it has been documented that mGluR7 is a practical component of the acquisition and extinction of conditioned responses [29]. Given this, we cannot rule out changes in amygdala-hippocampus-cerebral cortex transmission. Thus, we performed Western blot experiments to determine which brain structure is most susceptible to changes in mGluR7 protein levels (PFC, HC, or Amy). We found decreased levels of mGluR7 in PFC in the group of mice treated with AMN082. This effect was attenuated in mice treated with AMN082 + AM251 simultaneously, suggesting brain region-specific interaction between the two pathways. The changes induced by AM251 on AMN082-mediated behavioral and biochemical effects were confirmed in electrophysiological experiments in which AM251 abolished AMN082-mediated LTP escalation in PFC, which suggests functional interaction between these two signaling pathways.

Further in vitro experiments were performed to exclude the direct activation of mGluR7-mediated intracellular signaling by AM251. For this purpose, we incubated AM251 with cells overexpression of the mGluR7 alone or in the presence of L-Glu. No changes in the activation or modulation of mGluR7-mediated signaling in the cell model were observed. These results show that the observed effects of AM251 on mGluR7-mediated behavioral, biochemical, and electrophysiological outputs are not driven by the binding of AM251 to mGluR7.

To decipher the mechanism responsible for the observed changes, we performed immunohistochemical studies that allowed us to visualize CB1 receptors, mGlu7 receptors, and their colocalization in the specific mouse brain regions after the treatment. Interestingly, changes in colocalization between CB1 and mGlu7 receptors were detected in the PFC suggesting that at least partially the observed behavioral effects of tested compounds can be driven by physical interaction between these two receptors. Changes in co-localization of both receptors have been detected also in CA1 of the hippocampus. In Western blot analysis, we found no differences in the whole structure. On the other hand, in staining, we were able to detect changes in the level of mGluR7 depending on the region (in one region there was an increase, in another, there was a decrease, so overall, we could come out with no change in the whole structure in Western blot). Our study is consistent, and the behavioral results confirm the involvement of HC in the interaction in question.

In addition, we can rule out the involvement of other pathway components, such as COX-2, as our unpublished studies using ELISA showed that AMN082 did not affect COX-2 levels in PFC, HC, or Amy after a single administration. However, these studies were performed on a different strain of mice, i.e., C57Bl/6 J, so we treat them only as additional information.

We cannot exclude the involvement of other receptors in the observed effects, such as mGluR5. The mGluR5/CB1 interaction has been documented previously [30–32]. For example, Qin et al. [32] showed that impairment of a cascade involving mGluR5, among others, impairs eCB signaling in the amygdala, contributing to the development of anxiety. This hypothesis is confirmed by our earlier results demonstrating the involvement of mGluR5/COX-2 pathways in spatial memory changes in mice [3], and here, AM251 improved the spatial strategy of mice treated with AMN082.

4.1. Conclusions

We demonstrated specific interactions between CB1 and mGlu7 receptors mediated signaling in anxiolytic-like response in the SIH. The CB1 receptor blockage with AM251 intensified the anxiolytic-like effects of AMN082 when given at a lower dose of 0.3 mg/kg and attenuated it when given at a higher dose of 3 mg/kg. Furthermore, we confirmed the involvement of HC memory mechanisms in anxiety acquisition using the Barnes maze, which we detected by repeating the SIH protocol. Significantly, the observed behavioral changes correlated with biochemical and electrophysiological results. The reduction in LTP and mGluR7 levels after CB1 receptor ligand treatment and region-specific changes in colocalized expression of mGlu7 and CB1 receptors in PFC and CA1 of mice indicate the existence of a functional relationship between the mGlu7 and CB1 receptor-mediated pathways.

In summary, the presented research results indicate the discovery of a new mechanism for regulating Glu neurotransmission homeostasis in the mouse brain via the interaction of mGlu7 and CB1 receptor pathways, which impacts emotions, especially anxiety. Continued research is needed to determine the mechanism of the interaction we discovered deeply.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2024.123313.

CRediT authorship contribution statement

Barbara Chruścicka-Smaga: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Magdalena Sowa-Kućma:** Methodology, Investigation, Formal analysis, Data curation. **Patrycja Pańczyszyn-Trzewik:** Methodology, Investigation, Formal analysis, Data curation. **Bartosz Bobula:** Methodology, Investigation, Formal analysis, Data curation. **Agata Korlatowicz:** Methodology, Investigation, Formal analysis, Data curation. **Katarzyna Latocha:** Methodology, Investigation, Formal analysis, Data curation. **Paulina Pabian:** Methodology, Investigation, Formal analysis, Data curation. **Ewelina Czechowska:** Methodology, Investigation, Formal analysis, Data curation. **Tomasz Lenda:** Methodology, Investigation, Formal analysis, Data curation. **Agata Faron-Górecka:** Methodology, Investigation, Formal analysis, Data curation. **Katarzyna Stachowicz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors state no conflict of interest.

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