CHARLES UNIVERSITY

Second Faculty of Medicine

Summary of the Dissertation

Molecular mechanism of Cannabinoid receptor 1 regulation by SGIP1

Molekulární mechanismus regulace signalizace kanabinoidního receptoru 1 proteinem SGIP1

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Abstrakt

Src homology 3-domain growth factor receptor-bound 2-like endophilin interacting protein 1 (SGIP1) byl identifikován jako interakční partner kanabinoidního receptoru 1 (CB1R). Jejich protein-proteinová interakce byla potvrzena koimunoprecipitací. SGIP1 brání internalizaci aktivovaného CB1R a moduluje jeho signalizaci v buňkách HEK293. Pomocí elektrofyziologické metody terčíkového zámku jsme prokázali, že SGIP1 ovlivňuje signalizaci CB1R v autaptických hipokampálních neuronech.

Sadou behaviorálních testů jsme zkoumali důsledky delece SGIP1 na chování regulované endokanabinoidním systémem u myší s konstitutivní delecí SGIP1 ($SGIP1^{-/-}$) a myší WT. U myší $SGIP1^{-/-}$ nebylo změněno zkoumání prostředí, pracovní paměť a senzomotorické učení. Myši $SGIP1^{-/-}$ byly méně úzkostlivé a depresivní. U samic $SGIP1^{-/-}$ byla zrychlena extinkce averzivní vzpomínky. Projevy kanabinoidní tetrády byly delecí SGIP1 taktéž ovlivněny. Samci $SGIP1^{-/-}$ vykazovali abnormální příznaky závislosti na THC. Delece SGIP1 také snížila akutní nocicepci a myši $SGIP1^{-/-}$ byly citlivější na antinocicepční účinky agonistů CB1R a morfinu.

Interakce CB1R-SGIP1 vede k významné modifikaci signalizace CB1R. Pozorování *in vivo* dále naznačují, že SGIP1 ovlivňuje projevy chování souvisejícího s CB1R.

Klíčová slova

Autaptické hipokampální neurony, bolest, elektrofyziologie (metoda terčíkového zámku), endokanabinoidní systém, kanabinoidní receptor 1, receptor spojený s G proteinem, SGIP1, tolerance, úzkost

Abstract

Src homology 3-domain growth factor receptor-bound 2-like endophilin interacting protein 1 (SGIP1) has been identified as an interacting partner of cannabinoid receptor 1 (CB1R). Their protein-protein interaction was confirmed by co-immunoprecipitation. SGIP1 hinders the internalization of activated CB1R and modulates its signaling in HEK293 cells. Employing whole-cell patch-clamp electrophysiology, we have shown that SGIP1 affects CB1R signaling in autaptic hippocampal neurons.

Using a battery of behavioral tests in SGIP1 constitutive knock-out ($SGIP1^{-/-}$) and WT mice, we investigated the consequences of SGIP1 deletion on behavior regulated by the endocannabinoid system. In $SGIP1^{-/-}$ mice, exploratory levels, working memory, and sensorimotor gating were unaltered. $SGIP1^{-/-}$ mice showed decreased anxiety-like and depressive-like behaviors. Fear extinction to tone was enhanced in $SGIP1^{-/-}$ females. Several cannabinoid tetrad behaviors were altered in the absence of SGIP1. $SGIP1^{-/-}$ males exhibited abnormal THC withdrawal behaviors. SGIP1 deletion also reduced acute nociception, and $SGIP1^{-/-}$ mice were more sensitive to antinociceptive effects of CB1R agonists and morphine.

CB1R-SGIP1 interaction results in profound modification of CB1R signaling. Furthermore, *in vivo* findings suggest SGIP1 is a novel modulator of CB1R-related behavior.

Keywords

Anxiety, autaptic hippocampal neurons, cannabinoid receptor 1, endocannabinoid system, G protein coupled receptor, pain, SGIP1, tolerance, whole-cell patch-clamp electrophysiology

1. INTRODUCTION

The endocannabinoid system (ECS) is a neuromodulatory system that plays an important role in the development of the central nervous system (CNS) and the body's responses to the external environment [1]. The major ECS receptor is the cannabinoid receptor 1 (CB1R), abundant in the brain [2]. CB1R is found on presynaptic membranes of neurons [3], and upon ligand binding, it inhibits neurotransmitters' release to synaptic clefts [4-6].

CB1R signaling influences behavior such as memory [7], anxiety [8], motor function [9, 10], and pain response [11]. In mice, acute administration of CB1R agonists causes a characteristic combination of four symptoms (called cannabinoid tetrad): catalepsy (decreased mobility), antinociception (decreased sensitivity to pain), hypothermia (decreased body temperature), and impaired motor function [12].

CB1R signaling plays a role in several physiological as well as pathological processes. Therefore, it is important to understand and to be able to modulate the cannabinoid system precisely. Proteins that interact intracellularly with CB1R and affect its signaling are one way to achieve this. This dissertation focuses on the effect of Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1 (SGIP1) on CB1R signaling.

SGIP1 was detected in our laboratory during a search for intracellular interaction partners of CB1R by the yeast two-hybrid system [13]. According to our observation, SGIP1 partially co-localizes with CB1R in neurons [13]. SGIP1 stabilizes and prolongs the association of β arrestin with activated CB1R and hinders its internalization in human embryonic kidney cells (HEK293). A study of SGIP1 in our laboratory revealed its effect on the signaling of activated CB1R. SGIP1 does not affect CB1R signaling via G-proteins. However, ERK1/2 phosphorylation is reduced in the presence of SGIP1 [13]. The mechanism by which SGIP1 fine-tunes the CB1R signaling is interference with activated receptor endocytosis. The possibility that SGIP1 modulates the signaling of other receptors is not ruled out.

This dissertation focuses on the study of the interaction of CB1R and SGIP1 in neurons and its impact on the behavior of mice with a SGIP1 deletion.

2. AIMS AND HYPOTHESES

CB1R signaling is very complex and can be modulated at different levels by interacting partners. An interacting partner's influence on the receptor signaling may be reflected in behavior.

We previously detected the protein-protein interaction of CB1R and SGIP1 and characterized it biochemically and pharmacologically using transfected mammalian cells. This thesis focuses on further characterization of CB1R-SGIP1 interaction and mapping its function in neuronal cultures and *in vivo* using a mouse model.

For this purpose, we used a reverse genetic approach. We prepared a mouse line with SGIP1 gene deletion ($SGIP1^{-/-}$).

This thesis's main aim was to study the phenotype of SGIP1 knock-out ($SGIP1^{-/-}$) mice compared to the WT mice. The behavioral testing was focused on aspects of behavior that are known to be affected by the CB1R signaling.

3. MATERIAL AND METHODS

3.1. Co-immunoprecipitation

The experiment was performed as described previously [14], only minor changes were made in the procedure. The mouse forebrain was homogenized in homogenization buffer with protease inhibitors. Samples were diluted to a total protein concentration of 5 µg/ml. Subsequently, 3 - [(3-cholamidopropyl) dimethylammonio] -1-propanesulfonate hydrate (CHAPS) (total concentration 1%) was added to the samples, and the samples were incubated at 37 ° C for 1 h. The samples were centrifuged for 1 h at 100,000 x g and 4 ° C. The supernatant was diluted 10x with homogenization buffer, which additionally contained 0.1% Triton X-100. Next, 20 µl of agarose beads with immobilized protein A/G and bound anti-CB1R antibody (rabbit antibody produced in our laboratory [13] were added to the sample, and the mixture was incubated for 4 h, at 4 ° C with slow tube rotation. The samples were centrifuged for 1 min at 2000 x g. The beads were washed three times by centrifugation with 0.1% Triton X-100 homogenization buffer. The bead pellet contained a fraction of bound proteins, the supernatant contained the remaining unbound proteins. All samples were dissolved in 50 µl of SDS-PAGE sample buffer, heated to 70 ° C for 10 min. 10 µl of each sample was loaded onto a gel and analyzed by immunoblotting.

3.2. Generation of SGIP1$^{-/-}$ mice

SGIP1$^{-/-}$ mice were generated in cooperation with the Czech Center for Phenogenomics led by doc. Dr. Radislav Sedláček, Ph.D. The embryonic stem cells of C57Bl/NCrl background were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM) [15]. The embryonic stem cells (Sgip1$^{tm1a(EUCOMM)Hmgu}$) carried the SGIP1 gene (GeneBank Accession: NM_001285852) modified by homologous recombination. The FRT sites flanked exon 2 of the SGIP1 gene, and the LoxP sites bordered additional sequences. Using a laser-assisted technique, embryonic stem cells were injected into 8-cell stage embryos to generate chimeric mice. Sgip1$^{tm1a+/-}$ mice were crossed. Selected offspring were bred with Flp-expressing Gt(ROSA)26Sor$^{tm2(CAG-flpo,-EYFP)Ics}$, to delete aberrant sequences, and their offspring were further crossed with a strain expressing Cre recombinase Gt(ROSA)26Sor$^{tm1(ACTB-cre,-EGFP)Ics}$ to excise the Exon 2. Used mouse lines were from the same source [16].

3.3. Autaptic hippocampal neurons cultivation

Neurons were isolated from CA1-CA3 regions of mouse hippocampi (postnatal day 0-2) and plated on a previously prepared feeder layer of astrocytes [17]. Neuronal cultures were kept

in high glucose (20mM) DMEM containing 10% horse serum and used for recording after 8 days in culture. Neurons were used only up to 14 days after isolation.

3.4. Whole-cell patch-clamp electrophysiology

All experiments were performed on isolated autaptic neurons. The cells were kept at room temperature for the whole time of the recording, and they were not used for longer than three hours after removal from culture media. Whole-cell patch-clamp recordings were performed using HEKA Triple Patch Clamp EPC10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) and recording electrode filled with intracellular solution. The extracellular solution was used to fill the chamber. The flow rate of the solution through the chamber was ~3ml/min.

DSE was induced after establishing a 10-20 s 0.5 Hz baseline. For DSE dose-response experiments, depolarization to 0 mV for 50 ms, 100 ms, 300 ms, 500 ms, 1 s, 3 s, 10 s. Values before depolarization were normalized to 1, and the DSE values are presented as fractions of 1.

For 2-AG dose-response experiments, the membrane potential was held at -70 mV, and the excitatory postsynaptic currents (EPSCs) were triggered every 20 s with a 1 ms depolarizing step. After establishing a 5 min baseline without the drug, 2-AG was added to the cells in subsequently higher concentrations (1nM, 10nM, 100nM, 1uM, 5uM), and the EPSC was continuously recorded.

For desensitization experiments neurons were incubated in 100 nM WIN55,212-2 (WIN) in 0.001% DMSO overnight. After the overnight treatment, cells were washed for at least 20 min before they were used to record DSE dose-response (as described above).

3.5. Spontaneous alteration

Assessment of spontaneous alteration (SA) was performed in the Y maze [18]. In this test, the short-term memory of subjects is examined. The mouse is left to freely explore the maze and filmed with a camera from above for 5 minutes. The software calculates how many arm alterations (in percent) the mouse has made in a given time. The equation for calculating the spontaneous alteration was as follows: $\%SA = (TA * 100) / (TE-2)$, where %SA - the percentage of spontaneous alteration, TA - total number of alterations performed by the mouse, TE - total number of maze arm entries.

3.6. Pre-pulse inhibition of the startle response

Sensorimotor gating was monitored by the pre-pulse inhibition (PPI) [19]. Testing took place in soundproof boxes, to which the animals were accustomed 10 minutes before the testing session. Tones of different volumes (70, 77, 82, 85 dB) were presented to the animal either alone or followed by a tone of volume 110 dB, which should frighten the animal. The aversive tone always followed 120 ms after the pre-pulse tone. Each animal was tested six times, and each of these tests consisted of 10 pre-pulse tone pairings with an aversive tone or a non-tone delay. Pre-pulse intensities and their pairing with sound or silent delay were alternated. The response is presented in the graphs as a decrease in the startle response amplitude in the presence of pre-pulse (% PPI).

3.7. Open field test

The open field test (OF) was used to monitor the anxiety of mice and their overall activity [20]. The mouse is placed in an arena, which is virtually divided into center and periphery by software that is connected to the camera recording. The mouse is filmed on video, and the time spent in the middle of the arena and the distance traveled is evaluated by software.

3.8. Elevated plus maze

Elevated plus maze (EPM) assesses mice's anxious behavior and works on a similar principle as OF [21]. The maze consists of four elevated arms, which are crossed into the shape of a plus. Two of the arms are open, and two are protected with walls. Each animal was allowed to explore the maze for 5 minutes. Each animal was filmed with a camera from the top. The record was automatically evaluated by software that calculated the time each mouse spent in the open and closed arms and the center. The total distance that each mouse traveled was also analyzed.

3.9. Tail suspension test

To investigate depression-like behavior, the tail suspension test (TST) was used [22]. In the TST, the tested mouse is secured with adhesive tape to the hook by its tail. The mouse is hung by its tail for 6 minutes and recorded with a camera. From the record, the software calculates the time the mouse spent motionless.

3.10. Fear conditioning

Fear conditioning (FC) is based on the pairing of an electric shock with a context or a conditioned stimulus (cue) such as a specific tone [23]. Each mouse was placed in an experimental box where it was acclimated for 4 minutes. After this time, a conditioned stimulus (tone of 77 dB and 9kHz), which lasted 20 s, was triggered in the box. With the last second of the conditioned stimulus, a weak electric current / unconditional stimulus (0.6 mA for 1 s) was released into the box floor. Contextual fear conditioning was tested 24 hours later. The environment of the test box was the same as during the learning of fear conditioning. Mice were videotaped for 6 min, and their freezing was recorded. After 3 h, the mice were monitored for response to a conditioned stimulus. The mice were placed in a box with a changed pattern on the walls, the box floor was replaced with another material, and also, a pulp with a novel essential oil was placed next to the box. After adaptation to the new environment (2 min), the mice were presented with the conditioned stimulus, and their freezing was recorded for 2 min.

In the extinction experiment, mice were taught fear conditioning in the same manner as in the above-mentioned experiment. The following days, the mice were placed in a box, allowed to become familiar with the environment for 1 min, and after acclimatization, presented with a conditioned stimulus (tone) for 3 min. Their immobility was recorded during these 3 minutes. The experiment was terminated when the extinction trend stopped developing in mice (males - 11 days, females - 5 days).

3.11. Tail immersion test

Nociception was tested by the tail immersion test (TIT). Mice were gently immobilized in a cotton cloth. They were acclimated to this procedure the day before the experiment. During the experiment, 1 cm of the tip of the mouse's tail was immersed in a water bath with a 52 ° C temperature. The time to tail flick was measured. The experiment was repeated 3 times with a 30-minute inter-interval between each measurement.

3.12. Cannabinoid tetrad and withdrawal

Male mice were used for the cannabinoid tetrad that describes the four manifestations of THC intoxication [24]. Their behavior was tested on days 1, 4, and 8 of the experiment, always 1 h after intraperitoneal administration of THC (10 mg/kg/day). The control group of mice was injected with VEH in the same manner. Baseline values were measured on day 1 of the experiment before the first THC administration.

The individual tests were performed in the order in which they are mentioned herein. The catalepsy test was performed by placing the mouse on a 6.35 cm diameter steel ring mounted 16 cm above the base. Mice were monitored for 5 min and the duration of catalepsy, i.e., immobility, was recorded. The results are presented as a percentage of the maximum possible effect (% MPE) according to the equation %MPE = [(immobility after the injection - immobility before injection) / (300 - immobility before injection)] x 100. The TIT was used to determine the nociception (see chapter 3.11). Results are reported as %MPE according to the equation %MPE = [(latency after injection - latency before injection) / (10 - latency before injection)] x 100. Mice body temperature was measured with a rectal thermometer. Results are presented as percent change in body temperature (%ΔBT) according to the equation %ΔBT = [(pre-injection temperature) - (post-injection temperature)] / [pre-injection temperature] x 100. For the rotarod test, mice were trained two days before the experiment. Mice were placed on an accelerating rotating cylinder (4-40 rpm) and the time spent on the cylinder before falling was recorded as latency.

Subjects from the tetrad experiment were used to test the THC withdrawal. On day 9 of the experiment, mice were injected intraperitoneally with THC or VEH. After 30 minutes, the mice received another injection with VEH only, and after another 30 minutes, the mice were injected with 10 mg/kg of rimonabant. Mice were videotaped throughout the experiment, and a blinded observer analyzed behaviors. The incidence of withdrawal behaviors (headshakes, paw shakes, scratching and grooming, and jumping) was manually calculated.

3.13. Testing of antinociception induced by CB1R ligands and morphine

The effect of ligands on nociception in mice was investigated by TIT (see chapter 3.11). First, the baseline latency in TIT was measured, and the test was repeated 1 h after intraperitoneal injection of each dose of the drugs. The individual doses were always injected starting with the lowest and ending with the highest. The cut off of 10 s was used in the experiment with THC and WIN, 15 s was used for the morphine experiment. Data are presented as %MPE = [(latency after injection - latency before injection) / (10 (15 for morphine) - latency before injection)] x 100.

3.14. Order of behavioral tests and statistical analysis of behavioral data

In the case of tests in which both sexes of mice were tested, each sex was tested separately. The order of tests was as follows: OF, SA, EPM, TST, PPI, TIT. New cohorts of mice were used to test FC, extinction of aversive memories, the nociceptive effect of THC, WIN, and morphine.

A new cohort consisted of males only was used for cannabinoid tetrad and THC withdrawal. Finally, a new cohort of males was used to test the nociceptive effect of rimonabant.

The experimental procedures and data analysis were blinded to the experimenter, in cases of video analyses blinded to the observer.

The F test was used to analyze the homogeneity of sample variances in the R program (stats library). No violations of normality or sphericity were detected using the R program (library moments) [25] in our data except the incidence of jumping in THC withdrawal. Here the analysis was done using a general linear model using the Poisson link in the R program (library stats) [26].

Qq plots were used to inspect the normal distribution of residuals and to calculate the correlation coefficient between observed residuals and theoretical residuals, R library olsrr [27]. Log transformation for data that showed an abnormality in the qq plot was used. Bonferroni post hoc test was applied when F in ANOVA achieved $P < 0.05$ only, and there was no significant variance inhomogeneity.

To analyze the ligand dose needed for 50% effect (ED_{50}), the curves were fitted as nonlinear regressions with variable slope (four parameters). The curves were constrained to 0 at the bottom and 100 at the top. The ED_{50} values, the 95% confidence intervals, and Hill slopes were determined from the fit.

T-tests, ANOVA, and nonlinear regression analyses were performed using GraphPad Prism version 8.0.1. for Windows (GraphPad Software, USA). The remaining experiment analysis was performed by the general linear model in the R program (version 4), library stats. $P < 0.05$ was considered significant.

4. RESULTS

The co-localization and interaction of CB1R and SGIP1 *in vitro* were previously verified by microscopic, biochemical, and pharmacological methods [13]. The interaction of CB1R and SGIP1 was confirmed by co-immunoprecipitation (**Fig. 1**). A mouse line with the SGIP1 gene deletion was developed to study the effect of SGIP1 on CB1R signaling further. Modulation of CB1R-SGIP1 signaling was studied by whole-cell patch-clamp electrophysiology in neurons derived from SGIP1$^{-/-}$ and WT mice. Finally, changes in the phenotype of SGIP1$^{-/-}$ mice were monitored.

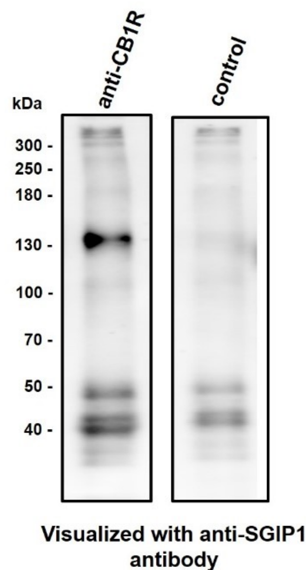

Fig. 1. Co-immunoprecipitation of SGIP1 and CB1R. Cannabinoid receptor 1 (CB1R) was precipitated from the detergent solution-soluble fraction prepared from mouse brain homogenate by anti-CB1R antibody, and anti-metabotropic glutamate receptor 1 antibody was used as a negative control. The precipitated proteins were then electrophoretically separated and visualized by immunoblotting. The anti-SGIP1 antibody was used for visualization, which detected SGIP1 bound to precipitated CB1R (~ 130 kDa).

In neurons, the DSE response to a given depolarization is weaker in the absence of SGIP1. This was particularly evident for longer depolarizations. In WT mice, 2-AG decreased the EPSCs in a concentration-dependent manner as expected. 2-AG was less effective in neurons lacking SGIP1 than in WT neurons, which is reminiscent of the impaired DSE observed in SGIP1$^{-/-}$ neurons. The desensitization was comparable in SGIP1$^{-/-}$ and WT neurons (**Fig. 2**).

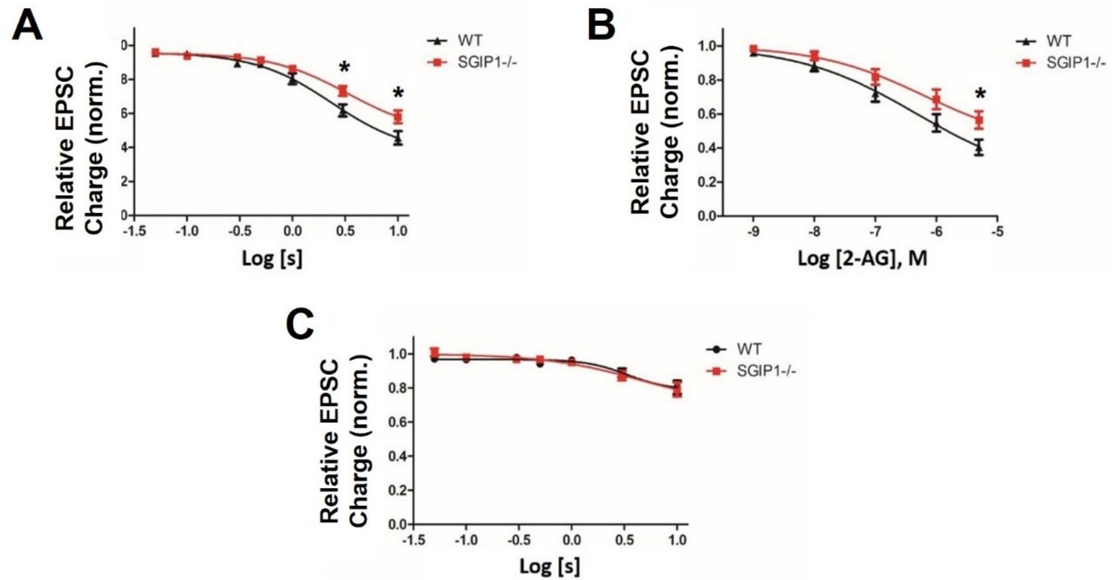

Fig. 2. Depolarization-induced suppression of excitation (DSE) is modulated by SGIP1 protein. Autaptic neurons were depolarized for progressively longer intervals to induce DSE **(A)**. SGIP1$^{-/-}$ neurons are significantly less responsive to depolarization compared to wild-type neurons. (WT: 2.358 s; ED50 SGIP1$^{-/-}$: 3.344 s). (**B**) SGIP1$^{-/-}$ neurons are also less sensitive to 2-AG (ED50 WT: 475.5nM, ED50 SGIP1$^{-/-}$: 639.4nM). Suppression of EPSC charge by increasing concentration of 2-AG was evaluated in autaptic neurons. (**C**) SGIP1$^{-/-}$ neurons desensitize at the same rate and extent as WT neurons. Cells were treated overnight with the CB1R agonist WIN 55,212-2 (100 nM), washed for 20 minutes, and DSE was evaluated. Both SGIP1$^{-/-}$ and WT neurons were almost completely desensitized by treatment with WIN 55,212-2. Baseline response was normalized to 1, and DSE is plotted as fractions of 1. Data are expressed as mean ± SEM. (*n* = 9-24 per group). * $p < 0.05$.

Working memory and exploration in WT and SGIP1$^{-/-}$ mice were assessed in the Y maze. Both groups of mice examined were comparably active during testing, and no change in the number of alterations (arm rotation) was observed when examining SGIP1$^{-/-}$ mice compared to WT mice. Sensorimotor learning was assessed by determination of pre-pulse inhibition of the startle response (PPI). No significant changes in startle response were observed between SGIP1$^{-/-}$ and WT mice (**Fig. 3**).

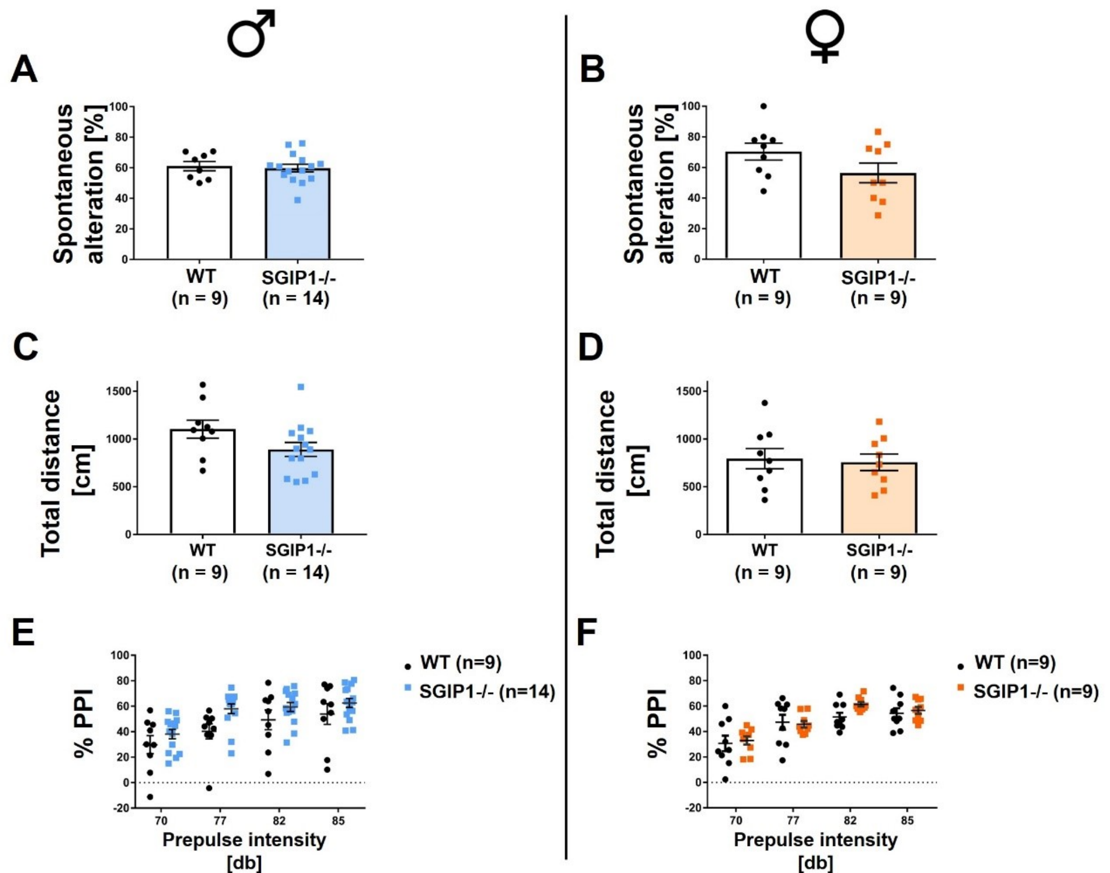

Fig. 3. SGIP1$^{-/-}$ mice have intact short-term memory, environmental exploration, and sensorimotor learning. (**A, B**) No significant changes in spontaneous alterations or (**C, D**) distance traveled in the Ypsilon maze were detected in the compared cohorts of males and females. (**E, F**) Similarly, no significant changes were detected in the SGIP1$^{-/-}$ and WT mice in pre-pulse inhibition of the startle response (PPI). Data are presented as mean ± SEM.

Open field and elevated plus-maze tests were used to test for anxiety-like behavior. The time spent in the center of the OF serves as an indicator of anxiolysis. Less anxious mice spend more time in the middle of an open field. SGIP1$^{-/-}$ males spent significantly more time at the center than the control group while traveling a comparable distance across the arena as male WTs. Comparable distance traveled was also recorded in females, but unlike males, SGIP1$^{-/-}$ females did not spend longer time in the middle of the OF. During the test, other manifestations, e.g., episodes of freezing indicating fear, were observed. There were no significant differences in the number of these episodes in females and SGIP1$^{-/-}$ males compared to controls.

Furthermore, the number of rearings, which are manifestations of active exploration of the environment, was analyzed. SGIP1$^{-/-}$ males had a higher incidence of this behavior than WT males. In females, the frequency was similar in both compared groups (**Fig. 4**).

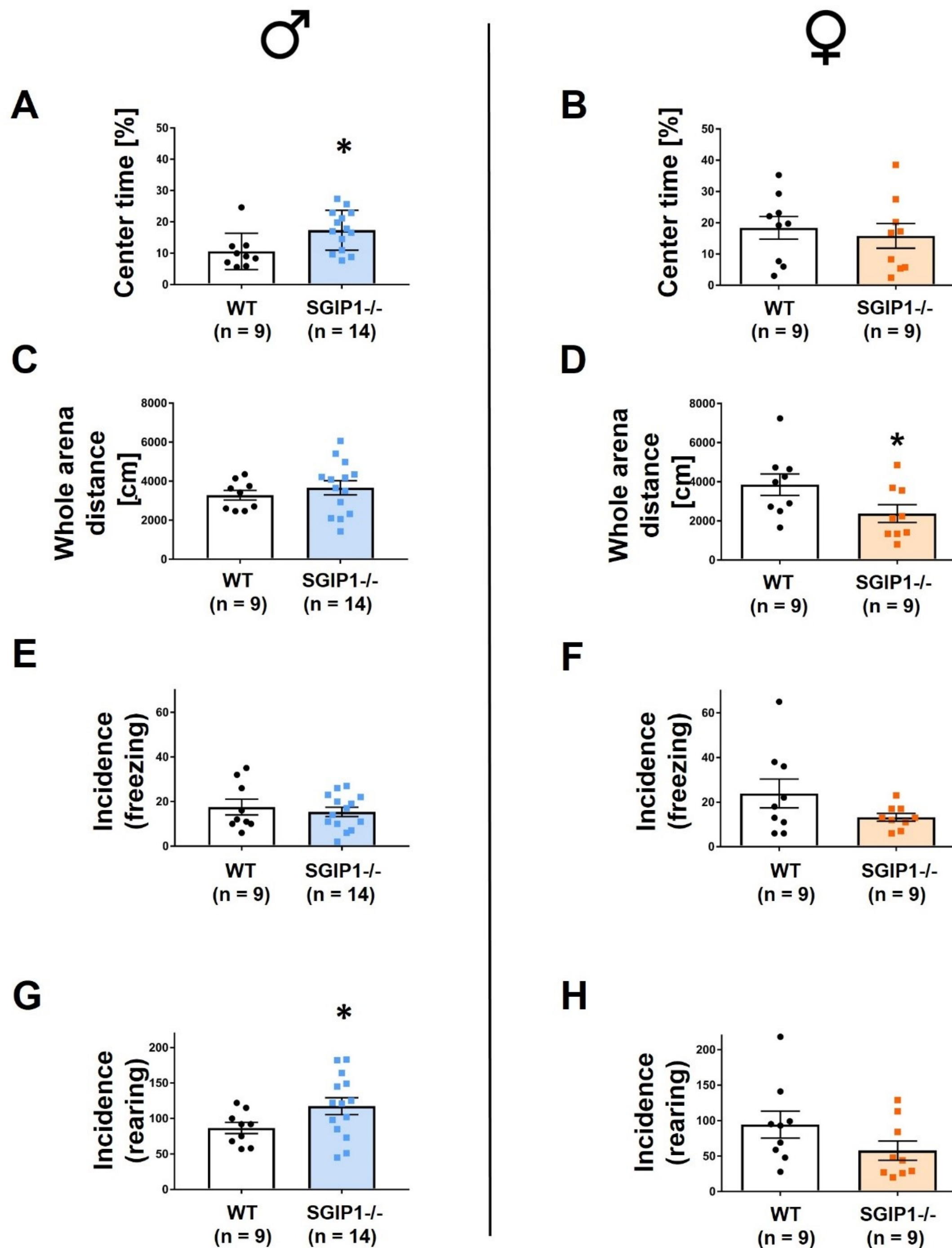

Fig. 4. SGIP1$^{-/-}$ males showed an anxiolytic phenotype in the open field test. (**A**) SGIP1$^{-/-}$ males spent significantly more time in the center of the open field than WT males. (**B**) This difference was not observed in females. (**C**) Both groups of males walked a comparable distance in the arena. (**D**) SGIP1$^{-/-}$ females traveled significantly shorter distances than WT females. (**E-F**) The incidence of freezing was comparable between the studied groups in both males and females. (**G**) The incidence of rearing was higher in SGIP1$^{-/-}$ males than in WT males. (**H**) The difference in rearing incidence was not significant in females. Data are presented as mean ± SEM; * $p < 0.05$.

In the elevated plus-maze both SGIP1$^{-/-}$ males and females spent significantly more time in the open arms and traveled a greater distance than the WT control mice. SGIP1$^{-/-}$ mice were less anxious and generally more active. When comparing the number of open and closed arm visits, the differences between SGIP1$^{-/-}$ and WT mice were not significant. Similarly, no significant difference was observed in the experimental groups of mice in the number of rearings in the maze (**Fig. 5**).

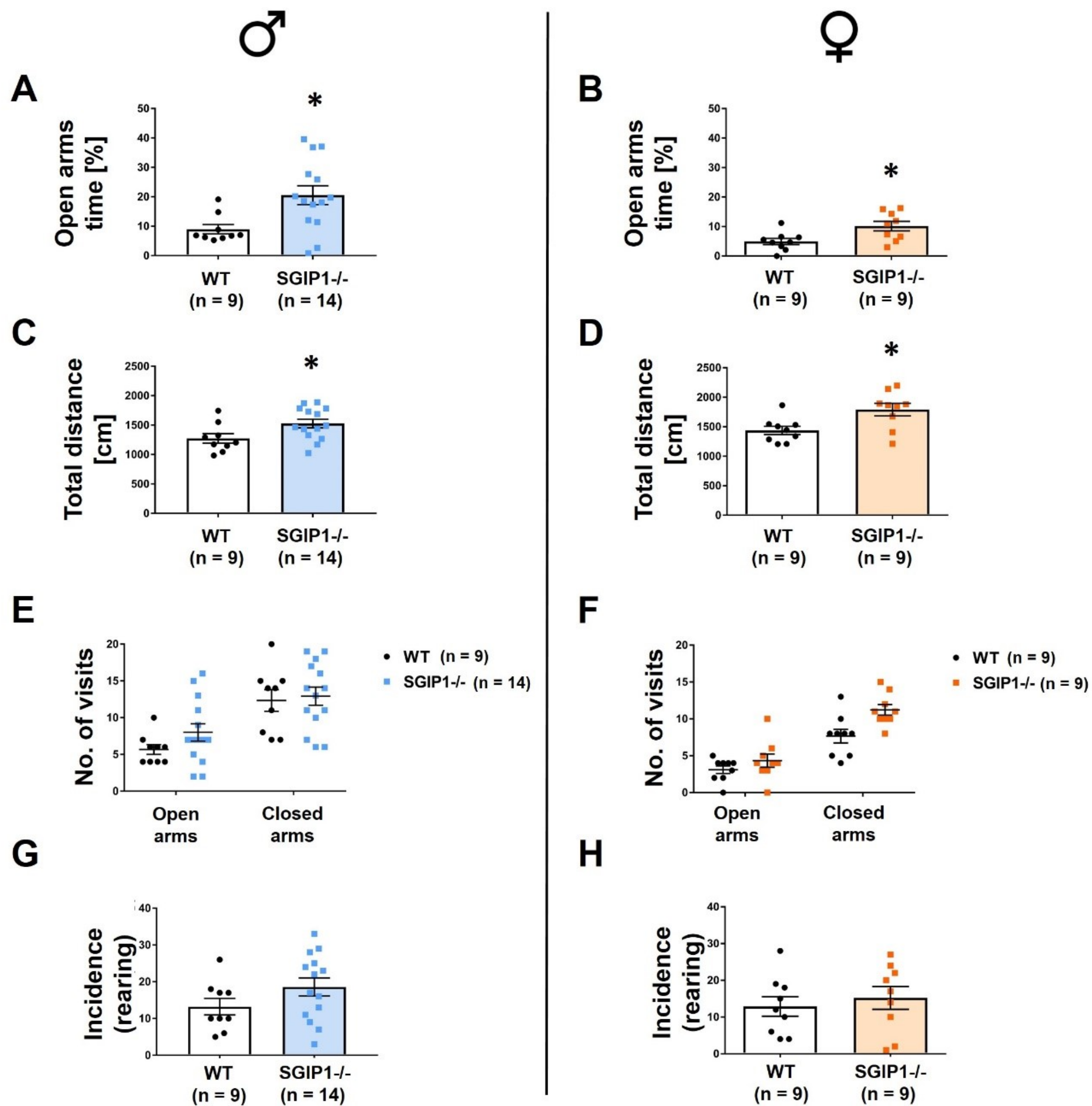

Fig. 5. SGIP1$^{-/-}$ males and females showed an anxiolytic phenotype in the elevated plus maze. (**A-B**) Both SGIP1$^{-/-}$ males and females spent more time in the open arms of the maze than the WT control groups, and also (**C-D**) traveled longer distances in the maze. (**E-F**) No significant changes in the number of open and closed arm visits were observed between the groups. (**G- H**) Also, the incidence of rearings in both SGIP1$^{-/-}$ males and females were comparable to the incidence of rearings in WT mice. Data are presented as mean ± SEM; * $p < 0.05$.

Depressive-like behavior was observed in the tail suspension test. The animals were exposed to a situation from which it was impossible to escape and were expected to try to escape from it nevertheless. Depressive-like animals generally give up their escape efforts earlier. $SGIP1^{-/-}$ mice spent more time active, trying to escape, and demonstrated greater resilience in the unescapable situation (**Fig. 6**).

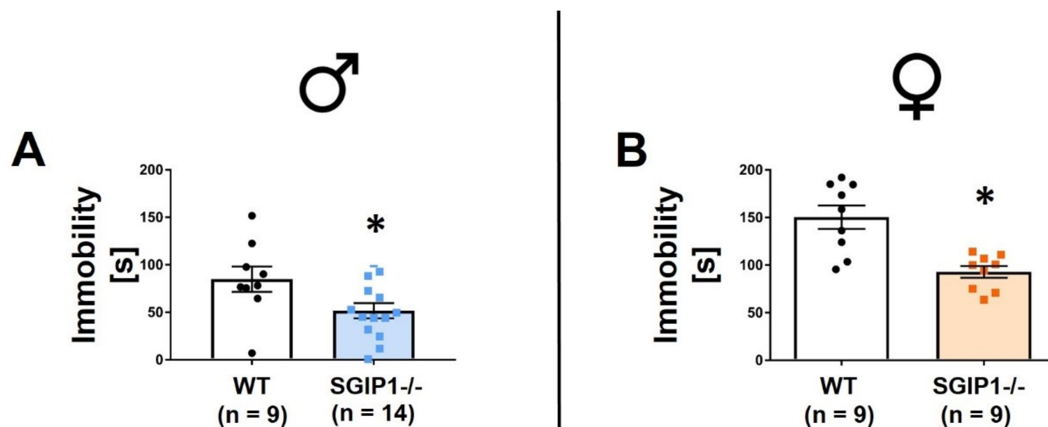

Fig. 6. $SGIP1^{-/-}$ mice cope better with an unescapable situation. The time the mice spent motionless in the tail hinge was significantly lower in both (**A**) male and (**B**) female $SGIP1^{-/-}$ than WT mice. Data are presented as mean ± SEM; * $p < 0.05$.

The fear conditioning of the aversive memory connected with context and a cue was examined. Male and female $SGIP1^{-/-}$ mice spent comparable time freezing as their WT littermates. Extinction of the cued aversive memory occurred at a similar pace for $SGIP1^{-/-}$ and WT male mice. However, in female $SGIP1^{-/-}$ mice, the extinction to tone was facilitated compared to WT female mice (**Fig. 7**).

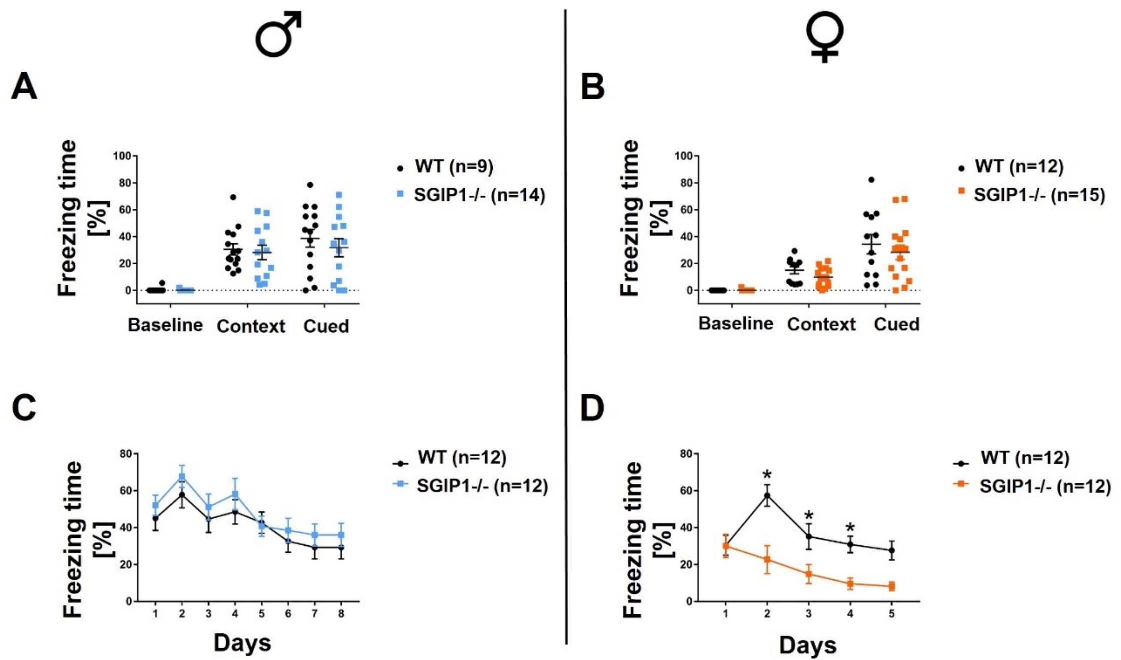

Fig. 7. Fear conditioning was intact in SGIP1$^{-/-}$ mice, SGIP1$^{-/-}$ females showed a faster extinction of aversive memories. Fear conditioning in response to context and cue was comparable in (**A**) males and (**B**) females of SGIP1$^{-/-}$ and WT genotype. (**C**) SGIP1$^{-/-}$ males exerted similar fear extinction as WT males. (**D**) SGIP1$^{-/-}$ females showed accelerated fear extinction. Data are presented as a percentage of the time the mice spent freezing, relative to the total time spent in the test chamber. Data are presented as mean ± SEM; * $p < 0.05$.

The behavior of male SGIP1$^{-/-}$ and WT was compared in a set of tests referred to as the cannabinoid tetrad. These tests evaluate four manifestations of CB1R agonist intoxication - catalepsy, antinociception, hypothermia, and impaired motor skills. The acute response to THC was evaluated as well as the development of tolerance with daily administration of 10 mg/kg THC intraperitoneally for 8 days. Control groups of mice injected with VEH only for the whole time of the experiment were also included in the testing.

Mice were not cataleptic before the first injection of THC. The first injection of THC induced comparable catalepsy in both SGIP1$^{-/-}$ and WT mice. On days 4 and 8 of testing, SGIP1$^{-/-}$ mice were significantly more cataleptic than control WT mice. Control groups that did not receive THC did not develop catalepsy. On the first day of testing, SGIP1$^{-/-}$ mice had an increased latency to tail-flick after the THC administration compared to WT mice. On day one, the hypothermia evoked by acute THC treatment was more profound in SGIP1$^{-/-}$ mice than in WT mice. On days 4 and 8 of testing, this change was no longer observable. In the Rotarod test, the genotype effect between SGIP1$^{-/-}$ and WT mice was not significant before and after the treatments. (**Fig. 8**).

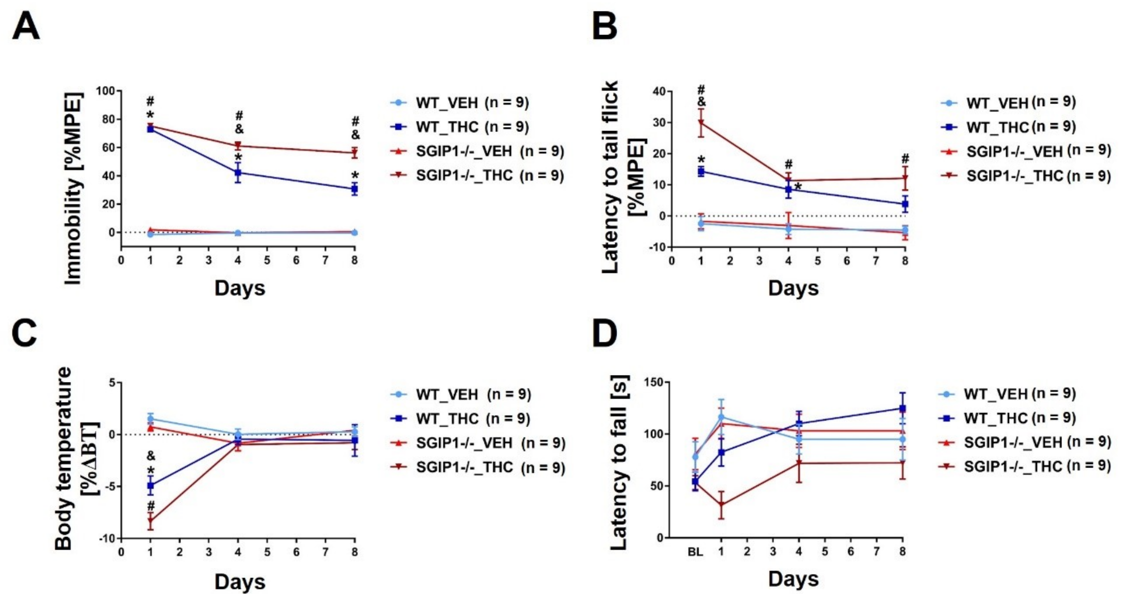

Fig. 8. The cannabinoid tetrad behavior is altered in SGIP1$^{-/-}$ males. The cannabinoid tetrad behavior was observed for 8 days, during which time the tested males received daily intraperitoneal doses of 10 mg/kg Δ^9-tetrahydrocannabinol (THC). Control groups of mice received only vehicle without active substance (VEH). Cannabinoid tetrad was performed 1 h after injection on days 1, 4, and 8 of the experiment. (**A**) In the ring test, THC induced comparable immobility in SGIP1$^{-/-}$ and WT mice. On days 4 and 8, SGIP1 $^{-/-}$ mice were more cataleptic after THC than WT mice. (**B**) In the tail flick test, prolonged tail-flick latencies were detected in SGIP1$^{-/-}$ mice versus WT mice before THC injection. On day 1 of testing, THC injection doubled the latency in SGIP1$^{-/-}$ mice

Symptoms of the THC withdrawal were observed in males that were given 10 mg/kg/day of THC for 9 days. The control group of mice received VEH instead of THC during this time. On the 9th day, the mice were injected with 10 mg/kg THC, followed by VEH injection 30 minutes later, and another 30 minutes later, 10 mg/kg rimonabant was applied. Headshakes, paw shakes, and scratching and grooming were monitored as the withdrawal symptoms. No increased incidence of headshakes or scratching/grooming after the rimonabant injection in THC pretreated WT and SGIP1$^{-/-}$ mice was observed. A higher incidence of paw shakes in THC pretreated WT and SGIP1$^{-/-}$ mice after the rimonabant injection was observed. However, there was no significant difference between the two monitored genotypes. In SGIP1$^{-/-}$ mice, the withdrawal was expressed as intense jumping manifested as straight leaps in the air with a strong charging from all four paws (**Fig. 9**).

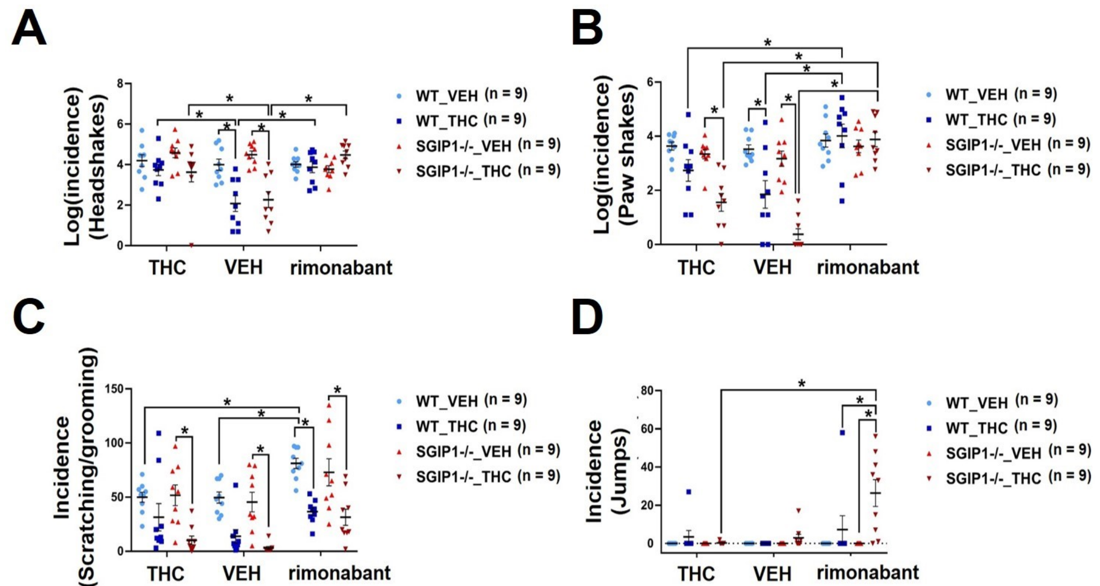

Fig. 9. Jumping as an unusual symptom of Δ[9]-tetrahydrocannabinol (THC) withdrawal in SGIP1$^{-/-}$ mice. After 8 days of daily administration of 10 mg/kg THC or vehicle (VEH), withdrawal symptoms were precipitated with the cannabinoid receptor antagonist 1 rimonabant. On the 9th day of the experiment, mice were injected with THC, then VEH, and finally rimonabant, and their behavior was recorded on video.

The incidence of THC withdrawal signs: headshakes (**A**), paw shakes (**B**), scratching/grooming (**C**) was observed. There were no relevant differences between WT and SGIP1$^{-/-}$ mice in the manifestations of headshakes, paw shakes, and scratching/grooming. However, after rimonabant application, SGIP1$^{-/-}$ mice jumped more frequently (**D**). Data are presented as mean ± SEM; * p <0.05.

THC-induced antinociception was assessed in SGIP1$^{-/-}$ and WT mice. In this experiment, mice were injected with increasing doses of THC (0, 1, 3, 10, 30, 50 mg/kg, intraperitoneally), and after each dose, the latency to tail flick was measured. The latency dose-response curve was shifted to the left in SGIP1$^{-/-}$ males when compared to WTs , but not in females (**Fig. 10**).

The antinociceptive effect of WIN was also observed in the compared groups of mice that received increasing doses of this drug (0, 0.3, 1, 3, 10 mg/kg, intraperitoneally). The antinociceptive effect of WIN is enhanced in both SGIP1$^{-/-}$ males and females (**Fig. 10**).

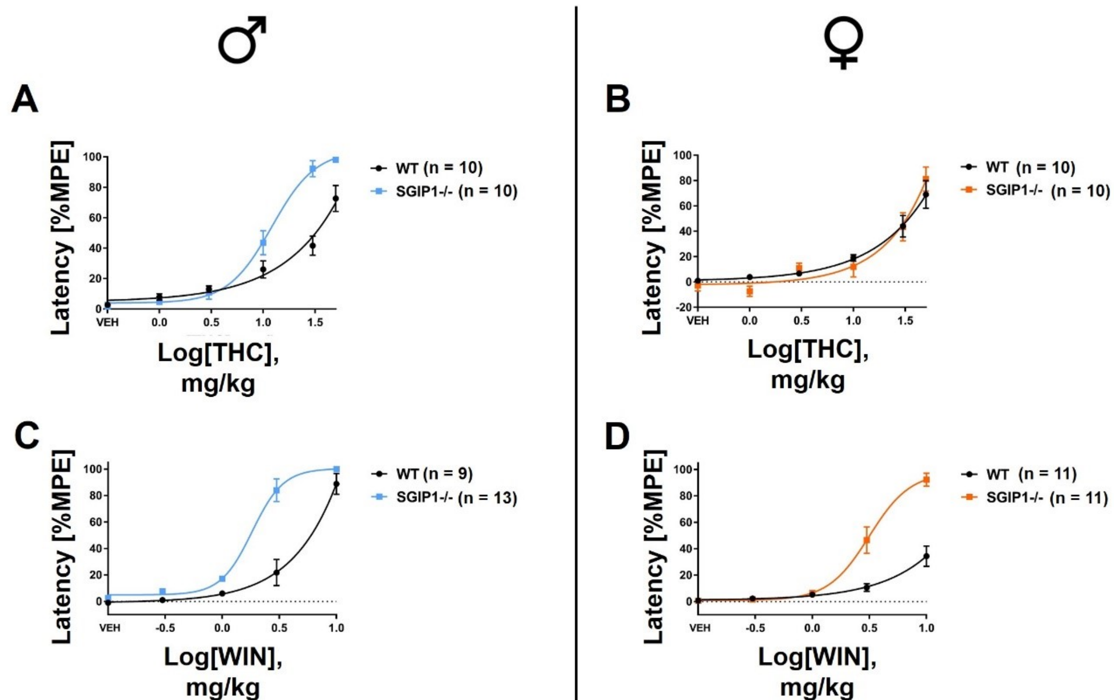

Fig. 10. Effect of cannabinoid receptor 1 (CB1R) agonists on pain perception in mice. Mice were injected with CB1R agonists Δ^9-tetrahydrocannabinol (THC; 0, 1, 3, 10, 30 and 50 mg/kg) or WIN 55, 212-2 (WIN; 0, 0.3, 1, 3, 10 mg/kg) intraperitoneally in gradually increasing doses. VEH on the axis x depicts an administration of a carrier without an active substance. The tail flick latency from the water bath (52 ° C) was measured 1 h after each dose. (**A**) In SGIP1$^{-/-}$ males, the THC dose-response latency curve was shifted to the left compared to the curve measured in WT males, (**C**) the leftward shift in SGIP1$^{-/-}$ males was also observed after WIN administration. (**B**) SGIP1$^{-/-}$ females responded to THC in a comparable way to WT females. (**D**) When WIN was administered, the dose-response latency curve of WIN was shifted to the left from the curve measured in female WTs. Data are presented as mean ± SEM; * p <0.05.

The effect of morphine on acute pain was also studied in our mice. Mice were injected intraperitoneally with morphine in increasing doses (0, 0.3, 1, 3, 10, 30 mg/kg), and their latency to tail flick was monitored. The morphine dose-response curve was shifted to the left in both SGIP1$^{-/-}$ males and females compared to the curves obtained in WTs (**Fig. 11**).

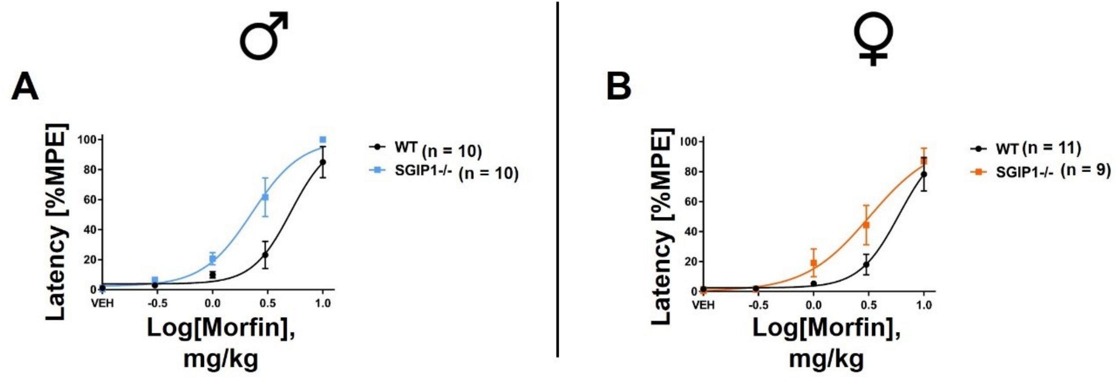

Fig. 11. The reaction to morphine is stronger in SGIP1$^{-/-}$ males than in WT males. Mice were injected with morphine intraperitoneally in gradually increasing doses (0, 0.3, 1, 3, 10 mg/kg). The tail flick latency from the water bath (52 ° C) was measured 1 h after each dose. (**A-B**) In both SGIP1$^{-/-}$ males and females, the morphine dose-response curve was shifted to the left from the WT curve. Data are presented as mean ± SEM; * $p < 0.05$.

5. DISCUSSION

Continuous activation of CB1R leads to its desensitization, internalization [28, 29], and development of tolerance [30]. SGIP1 prevents the internalization of activated CB1R, which causes changes in the CB1R signaling in transfected HEK293 cells [13].

Since CB1R is not normally expressed in HEK293 cells, we wondered if SGIP1 also affects signaling in neurons where CB1R is endogenously expressed. Electrophysiological experiments revealed reduced DSE in SGIP1$^{-/-}$ neurons. After CB1R activation, the absence of SGIP1 can cause very rapid receptor internalization, leading to a reduction in the number of available receptors on the membrane, resulting in reduced DSE.

To determine how SGIP1 affects mouse behavior, we used a reverse genetic approach. We prepared a mouse line with the SGIP1 deletion (SGIP1$^{-/-}$) and compared them with WT mice in behavioral experiments. We focused our testing on behaviors that are documented to be affected by cannabinoid signaling.

The level of exploration of the new environment, mobility, short-term memory and sensorimotor gating were comparable in SGIP1$^{-/-}$ mice and WT mice. However, we noted that deletion of the SGIP1 protein affects anxiety-like behavior, especially in males, depressive-like behavior, and in females also the extinction of aversive memories. Anxiety-like and depressive-like behavior tends to be alleviated with increased ECS activity, and changes in this behavior depend on gender [31, 32]. In a study comparing emotionality, cannabinoids reduced exploration levels and anxiety-like behavior in female rats, but not in males [33].

Mice carrying the S426A, S430A mutations of CB1R that prevent the receptor's desensitization show similar behavior as SGIP1$^{-/-}$ mice. S426A, S430A mice have increased sensitivity to THC and slower development of tolerance, and they jump when the THC withdrawal symptoms are precipitated in them [34]. Mice with a genetic deletion of β arrestin 2 have also increased sensitivity to THC and decreased tolerance to the antinociceptive effects of THC. In contrast, in THC-induced catalepsy, tolerance develops faster in these mice than control mice. These observations point to the importance of knowing the precise mechanisms of receptor signaling modulation. It is clear that changes at different levels of signaling have different effects [35]. The unusual THC withdrawal phenotype indicates the possible involvement of other signaling pathways. Jumping is a common manifestation of morphine withdrawal in rodents [36]. It is possible that the interaction of ECS and the opioid system is responsible for the phenotype observed in SGIP1$^{-/-}$ mice [37, 38].

The very strong antinociceptive effect of WIN in $SGIP1^{-/-}$ mice suggests more than an additive effect of WIN administration and the altered genotype. A study from our laboratory in transfected HEK293 cells showed that in CB1R-SGIP1 co-expressing cells WIN causes more increased β arrestin 2 association and more significant inhibition of CB1R-activated ERK1/2 than 2-AG [13]. Therefore, we believe that in the case of mild CB1R stimulation, the effect of SGIP1 on this receptor is only slight; however, it increases when CB1R is strongly activated.

Co-administration of THC and morphine increases the antinociceptive effects of morphine, even when THC is administered at a dose that has no measurable effect per se [39, 40]. $SGIP1^{-/-}$ mice responded more strongly to administered morphine than WT mice, supporting our hypothesis of a slight increase in endocannabinoid signaling in $SGIP1^{-/-}$ mice and confirms the ECS and opioid system cooperation.

6. CONCLUSIONS

CB1R and SGIP1 partially co-localize in the synaptic parts of neurons. Their protein-protein interaction was demonstrated in this work by immunoprecipitation from mouse brains. We have shown that SGIP1 interferes with the internalization of activated CB1R and modulates its downstream signaling in mammalian cell tissue cultures [13].

The effect of SGIP1 on CB1R signaling is also demonstrated in neurons here. We described the SGIP1 modulation of CB1R signaling in autaptic hippocampal neurons by electrophysiological approach. We used cultured neurons from mice with deleted SGIP1 and compared their signaling properties to WT neurons. The absence of SGIP1 causes a reduction in CB1R-mediated DSE.

Changes in CB1R signaling affect mouse behavior. We investigated the effect of SGIP1 deletion on mouse behavior experimentally by comparing SGIP1$^{-/-}$ mice with WT mice. Cognitive functions, such as short-term memory and sensorimotor learning, were not impaired by SGIP1 deletion. However, SGIP1$^{-/-}$ mice showed decreased anxiety-like behavior and coped better with an unescapable situation, while their fear conditioning remained intact. In SGIP1$^{-/-}$ females, an accelerated extinction of aversive memory occurred compared to WT females. Also, we observed changes in cannabinoid tetrad in SGIP1$^{-/-}$ mice, also stronger withdrawal symptoms were particularly noticeable in these mice. Both males and females SGIP1$^{-/-}$ had a significantly increased pain threshold and were more responsive to cannabinoids and morphine's antinociceptive effects.

Our results suggest that behavior in WT mice is affected by SGIP1 through its action on CB1R. CB1R signaling and its modulation by SGIP1 likely differ in various brain regions and different types of neurons. In the future, it would therefore be appropriate to focus on the study of SGIP1 function in specific parts of the brain and individual types of neurons. Due to the high-level SGIP1 expression in the brain, we cannot rule out that SGIP1 also affects other proteins and receptors. This possibility should also be explored.

This work contributes to the understanding of the SGIP1 function. Our results point out the critical role of SGIP1 in the regulation of CB1R-mediated signaling. Our studies may be of importance in developing drugs that act on CB1R, especially in pain management.

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OVERVIEW OF AUTHOR'S PUBLICATIONS

Publications based on the data shown in the dissertation thesis

Dvorakova, M., Kubik-Zahorodna, A., Straiker, A., Sedlacek, R., Hajkova, A., Mackie, K., Blahos, J., SGIP1 is involved in regulation of emotionality, mood, and nociception and tunes in vivo signaling of Cannabinoid Receptor 1. *British Journal of Pharmacology* 10.1111/bph.15383 (2021) IF 7,730

Hajkova, A., Techlovska, S., **Dvorakova, M.**, Chambers, J.N., Kumpost, J., Hubalkova, P., Prezeau, L., Blahos, J., SGIP1 alters internalization and modulates signaling of activated cannabinoid receptor 1 in a biased manner. *Neuropharmacology* 107, 201-214. (2016) IF 5,273

Other publications

Straiker A., **Dvorakova M.**, Zimmowitch A., Mackie K.. Cannabidiol inhibits endocannabinoid signaling in autaptic hippocampal neurons. *Mol. Pharm.* 94, 743-748. (2018) IF 3,681

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OVERVIEW OF AUTHOR'S PRESENTATIONS

"Distorted endocannabinoid signaling in absence of SGIP1 correlates with altered pain processing and modified responses to THC in knock-out mice" (poster), Gordon Research Conference on Cannabinoid Function, Castelldefels, Spain, July 21-26, 2019

"Distorted endocannabinoid signaling in SGIP1 knock-out mice correlates with altered modulation of anxiety, fear, stress and pain processing" (presentation), Gordon Research Seminar on Cannabinoid Function in the CNS (GRS), Castelldefels, Spain, July 20-21, 2019

"A new piece of the THC and morphine interaction puzzle" (presentation), 2LF Scientific conference, Charles University in Prague, Czech Republic, April 10-11, 2019

"SGIP1 as a New Piece of the THC and Morphine Tolerance Puzzle (The Story of Mellow and Popcorn Mice) " (prezentace), Regular Institutional Seminar, Institute of Molecular Genetics of the ASCR, Prague, Czech Republic, March 20 2019

"Distorted endocannabinoid signaling in SGIP1 knock-out mice correlates with altered modulation of anxiety, fear, stress and pain processing" (poster), 4th Endocannabinoid Pharmacology Meeting, Bern, Switzerland, October 25-26, 2018

"Deletion of SGIP1 Alters Endocannabinoid Signaling and Behavior in Mice" (poster), The 28th Annual International Cannabinoid Research Society Symposium on the Cannabinoids, ICRS Leiden, Netherlands, June 30- July 5, 2018

"SGIP1: New Player in Cannabinoid Signaling", Regular Institutional Seminar, Institute of Molecular Genetics of the ASCR, Prague, Czech Republic, April 1, 2015

"Nový mechanismus regulace signalizace kanabinoidního receptoru CB1" (presentation in Czech language), co-author Alena Hájková, 2LF Scientific conference, Charles University in Prague, Czech Republic, April 9-10 2014