

Anxiety, Stress and Fear Response in Mice with Reduced Endocannabinoid Levels

Supplemental Information

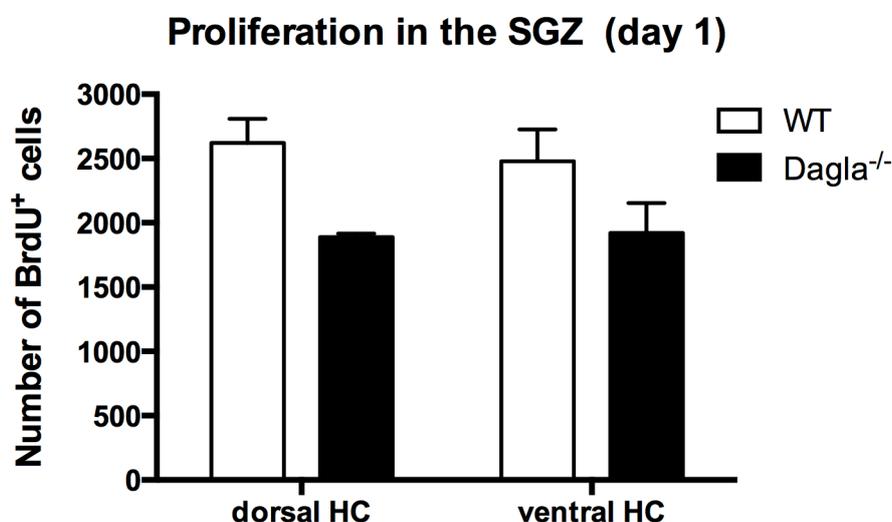


Figure S1. No region-specific difference in adult hippocampal neurogenesis of Dagla^{-/-} mice. Reduced adult neurogenesis did not significantly differ between the dorsal and ventral part of the hippocampus in Dagla^{-/-} or WT mice (two-way ANOVA, Bonferroni's post-hoc test, values represent mean \pm SEM: $n = 3$ animals/group, $p = ns$).

Supplemental Methods and Materials

Measurement of Neuronal Proliferation

To label dividing cells, 5-bromo-2'-deoxyuridine (BrdU, 50 mg/kg; Sigma Aldrich) was administered four times with 2 h intervals. Mice were sacrificed with CO₂, 24 h and 21 days later, respectively, and transcardially perfused with 4% PFA in PBS. Brains were cryoprotected with 20% sucrose for 24 h and frozen in dry ice cooled isopentane. The brains were then embedded in Tissue Tek and serial coronal free-floating cryosections (40 μ m) were cut through the rostrocaudal axis of the

hippocampus from bregma -0.94 to -3.34 mm. Every sixth slide was stained with anti-BrdU rat IgG monoclonal antibody (1:500; Abcam) and Alexa Fluor 594-conjugated secondary antibody (goat anti-rat IgG, 1:500; Life Technologies). The sections were counterstained with a mouse monoclonal antibody against NeuN (Alexa Fluor 488 conjugated, 1:250; Life Technologies). Fluorescence images were obtained with a Zeiss Axiovert 200 M fluorescent microscope (Carl Zeiss Microimaging) with 20x objective lens. BrdU-positive cells were counted in the subgranular zone of the hippocampus. The number of positive cells was then multiplied by the factor of 6, because every sixth section was used for the analysis.

Analysis of Quantitative RT-PCR Data

Total RNA from brain tissue was extracted using Trizol® Reagent (Life Technologies). Purity and RNA concentration was evaluated by optical density measurements at 260 and 280 nm. cDNAs were synthesized using SuperScript® II Reverse Transcriptase and Oligo(dT)₁₂₋₁₈ primers (Life Technologies). Differences in mRNA expression were determined in triplicate by custom TaqMan® Gene Expression Assays (Applied Biosystems, Darmstadt, Germany; *Dagla*: Mm00813830_m1; *Daglb*: Mm00523381_m1; CB1 receptor (*Cnr1*): Mm00432621_s1; monoacylglycerol lipase (*Mgl1*): Mm00449275_m1; fatty acid amide hydrolase (*FAAH*): Mm00515684_m1); Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): Mm99999915_g1 was used as a housekeeping control. Each 10 µl reaction consisted of 1x TaqMan® universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany), 4 µl cDNA and 1x Custom TaqMan® Gene Expression Assay. Samples were processed in a LightCycler® 480 (Roche, Germany) with the following cycling parameters: 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min.

Analysis was performed using the LightCycler® 480SW Software version 1.5.1 (Roche, Germany) and analyzed with the $2^{-\Delta\Delta C_T}$ method (1).

Animal Experiments

All the behavioral experiments were performed at least two times with different cohorts of animals. In all cases, we first performed a sucrose preference test, followed by an open field, social preference, light-dark test and forced swim test, or alternatively open field, zero-maze and forced swim tests. The tests were performed with weekly intervals. Another cohort of animals was housed in a room with an inverse light-dark cycle and tested for home cage activity. The fear extinction, freezing-response (Fig. 8F) and hot plate tests were also performed with independent animal cohorts.

Home Cage Activity Measurement

Home cage activity was recorded using an infrared system (Mouse-E-Motion, Infra-e-motion, Henstedt-Ulzburg, Germany). Mice were housed in single-cages and habituated to the home-cage and to the reversed light/dark cycle (light phase for 12 hours, light on at 9:00 pm) for at least seven days. Movements were sampled and averaged over 1 h.

Fear Conditioning Paradigm

A sound-protected box equipped with a foot shock device, loud speakers and a vibration-sensitive platform to detect movements was utilized (TSE-Systems GmbH, Bad Homburg, Germany). For conditioning, mice were placed in a small grid chamber and after 3 minutes a tone (80 dB, 9 kHz) was presented for 20 s, which co-

terminated with a 2 s electric foot shock of 0.7 mA. Furthermore one group of animals was only presented with the sound cue (without the foot shock) to analyze the response to the auditory cue alone. To evaluate the conditioned freezing response, mice were placed into a glass cylinder (diameter 8 cm) in the same box and after 3 min habituation, the same tone was presented. Vibrations caused by the movements of the test animal were recorded during conditioning and on days 1, 2, 3 and 6 after conditioning (extinction trial 1, 2, 3, 6). Animals that were conditioned only with the sound-cue were analyzed for freezing behavior on day 1 after conditioning. Periods of immobility exceeding 2 s were considered as freezing behavior.

Forced Swim Test

Mice were placed individually into a glass cylinder (height 28 cm, diameter 20 cm) containing water (height 14 cm, 24-25°C). Immobility time was recorded during the last 4 min of the 6 min testing period. For the pharmacological treatment, mice were either treated with 10 mg/kg amitriptyline i.p. 30 min prior to the test, or with 20 mg/kg JZL184 or 0.5 mg/kg URB597 2 h before the forced swim test. The control animals were treated with 0.9% saline i.p. and either tested 30 min or 2 h after injection.

Sucrose Preference Test

Mice were housed individually and could freely select from two bottles containing 1% sucrose solution or water. Consumption was measured for 48 h and the position of the bottles was changed after 24 h. Sucrose preference (%) was calculated as sucrose solution consumed divided by the total amount of solution consumed.

Social Preference Test

Mice were first habituated to a transparent open-field box (44 cm x 44 cm) for 5 min on three consecutive days. The floor was covered with bedding material and the arena contained two metal grid cages in opposing corners. On the testing day an unfamiliar mouse of the same gender and age was placed in one of the metal cages. The test animals were introduced and their location recorded for 10 min using the “EthoVision XT” software (Noldus, Wageningen, Netherlands). Calculated was the time spent investigating the partner mouse compared to the empty cage.

Open-Field Test

The animals were tested in a sound-isolated, dimly illuminated room (20 lux) in an open-field box (44 cm x 44 cm). Mice were allowed to explore the box freely and their behavior was recorded for 30 min.

Light/Dark Box Test

Animals were tested in a sound-isolated room in an open-field box (44 cm x 44 cm) divided into a light (2/3 of area) and a dark (1/3 of the area) compartment. The light compartment was brightly illuminated (800 lux). Each animal was placed in the dark compartment and allowed to explore the arena freely for 10 min, while their behavior was recorded.

Zero-Maze Test

Anxiety-related behavior was tested in the zero-maze (height 40 cm, internal diameter 46 cm, width 5.6 cm) in a sound-isolated room. The maze was divided into four equal quadrants, with non-transparent walls enclosing the two opposite

quadrants. The animals were placed in the open area of the zero-maze and their movements were recorded for 5 min with 600-700 lux illumination. Time spent in the different areas and distance travelled was analyzed using “EthoVision XT” software (Noldus, Wageningen, Netherlands).

Pup Retrieval Test

Maternal care was assessed by the pup retrieval test. In this test, the entire litter was removed and after 5 minutes 3 pups (day 2-7 after birth) were returned to the home cage away from the nest at an opposite end of the cage. The entire test was recorded on video. We then evaluated the latency (in seconds) to sniff a pup and retrieve it to the nest. If a female had not retrieved all pups within 5 min the test was terminated, resulting in a latency of 300 s. In addition, the pups were checked for the presence of a milk spot as an indicator for nursing behavior.

Chemicals

URB597, JZL184, amitriptyline hydrochloride and Tween20 were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). JZL184, URB597 and amitriptyline hydrochloride were dissolved in Tween20 and then diluted in 0.9% saline (final concentration of Tween20 < 0.1%). Vehicle control animals were treated with Tween20 (final concentration < 0.1%) diluted in 0.9% saline.

Supplemental Reference

1. Livak K.J., Schmittgen T.D. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25(4):402-8.