Animals

Female Sprague-Dawley rats (250g – 280g; Charles River Laboratories, Sulzfeld, Germany) were mated overnight. After giving birth, mothers were housed with their pups for one day (LD1) and either separated (LD1+19) or continued to be housed with their offspring until LD20 (NMS20).

Pharmacological treatment

In Experiment 1, animals were sacrificed without any pharmacological manipulation. Immediately following, brains were rapidly extracted, fresh-frozen on dry ice, and stored at –80 °C for further analysis.

In Experiment 2, all animals were postpartum rat dams that had undergone pup loss. These mothers were randomly assigned to one of two experimental groups to ensure unbiased distribution. On the day of behavioral testing (day 2), animals received bilateral injection of either vehicle (Ringer; pH adjusted to 7.4) or the selective CRFR2 antagonist Astressin 2B (Tocris Bioscience, Cat. No. 2391). Injections were administered 10 minutes prior to the start of the modified forced swim test, allowing sufficient time for the compound to exert central effects. The Astressin 2B dosage was 4 µg in 0.5 µL per side.

In Experiment 3, a separate cohort of postpartum mothers who had lost their pups was used, and animals were randomly allocated into three treatment groups. As in Experiment 2, injections were performed 10 minutes before behavioral testing (day 2). The groups received either oxytocin (OT; 0.01 µg in 0.5 µL vehicle per side), oxytocin receptor antagonist (OTR-A; 0.1 µg in 0.5 µL vehicle per side), or vehicle (0.5 µL Ringer's solution adjusted to pH 7.4 per side). All injections were administered bilaterally into the VMH using the following stereotaxic coordinates relative to bregma: anteroposterior (AP): –2.5 mm; mediolateral (ML): ±0.7 mm; dorsoventral (DV): –8.4 mm, based on the Paxinos and Watson rat brain atlas.

In Experiment 4, animals were sacrificed without any pharmacological manipulation and transcardially perfused, first with phosphate-buffered saline (PBS) to remove blood, followed by 4% paraformaldehyde (PFA) for fixation. Brains were then extracted, post-fixed, and prepared for further analysis.

Measurement of mRNA with qPCR

The analysis was performed following an established protocol. Briefly, flash frozen brains were stored at -80°C, cryosectioned with 250 µm thickness, and punched with different puncher sizes. RNA isolation was conducted using 1 ml of peqGold Trifast (VWR LIFE SCIENCE, Darmstadt, Germany). After 5 min incubation at RT, 200 µl of chloroform was added. Supernatants were collected and centrifuging for 15 min at 17,000 rpm. To reach 45% of the final volume, 500 µl of isopropanol was added to each sample. RNA concentraion was measured using the NanoDrop, RNA was transcribed into cDNA. Then, Samples were measured in the PCR Cycler (Quantstudio^{TM5}, Applied Biosystems, Thermofischer Scientific, Darmstadt, Germany).

Modified Forced Swim Test (mFST)

Passive stress-coping behavior was assessed using the modified forced swim test, conducted over two consecutive days between 9:00 a.m. and 12:00 p.m. to minimize variability due to circadian influences.

On day 1 (pre-test session), each rat was individually placed in a transparent cylindrical tank (50 cm in height, 30 cm in diameter) filled with tap water maintained at 23 ± 1 °C. The depth of the water was sufficient to prevent the animal from touching the bottom with its tail or hind limbs, ensuring it had to swim or float. Rats remained in the tank for 10 minutes to allow habituation to the testing conditions.

On day 2 (test session), rats were again placed in the same tank under identical conditions for a 10-minute session, which was used to assess their stress-coping behaviors. All test sessions were video recorded, and behavioral scoring was performed using JWatcher software (available at https://www.jwatcher.ucla.edu). Scoring was conducted by an experimenter blind to the treatment to prevent observer bias. Behavioral parameters analyzed included immobility (a measure of passive stress coping), swimming, and struggling behaviors.

After each session, animals were gently dried with a towel and returned to their home cages. The water in the tank was changed between animals.

Verification of cannula placement

After the final behavioral test, rats were sacrificed with CO_2, and blue dye was injected via the infusion system through the guide cannula. Only animals with correct, histologically verified infusion sites were included in the statistical analyses.

Confocal Microscopy:

For the evaluation of different activation states of microglia, confocal images were acquired on Zeiss LSM 880 (Carl Zeiss Microscopy GmbH, Jena, Germany). Adjusting digital images and image capture were performed using ZEN (ZEN 2.3 SP1, Carl Zeiss Microscopy GmbH, Jena, Germany) in conjunction with the Zeiss system. Images were captured using 40´ magnification and 1´ digital zoom. Imaging settings were adjusted for optimal acquisition quality and were kept constant throughout the acquisition of all stacks for the experiment. Pictures with a resolution of 512 x 512 pixels were acquired with a 0.5 µm z-step size, resulting in a Z-stack with a thickness of 30 µm. Image acquisition included both hemispheres and was pooled in the subsequent analysis.

Image analysis:

Sholl Analysis

The microglia complexity was measured by sholl analysis. Confocal images were imported to Fiji for ImageJ (ver. 1.52p, (Schindelin et al., 2012; Schneider et al., 2012)), the channels were split and a maximum intensity z projection was created for the channel containing IBA1 staining. The image LUT color was converted to gray scale. Following, the image color was inverted. Subsequently, the brightness and contrast were adjusted by setting the minimum and maximum limits of the histogram to the borders of the intensity curve. Following, D was determined by using the function “Set scale” and using the value of the correspondence between pixels and µm. Per region, three microglial cells were chosen randomly, resulting in a total of 6 analyzed cells. For the creation of concentric circles, the cell center of a microglia cell was picked by hovering with the mouse over the center and noting its position with the help of the x and y coordinates. The plugin Concentric Circles was executed, and the x and y center values were set by multiplying the respective x and y values by D. The inner center

value was set to 10 µm. The outer radius value was calculated by 2´D+(N´S´D), whereas N corresponds to the number of concentric circles additionally to the inner circle and S corresponds to the spacing value between two circles in µm. In the case of this study, 4 µm of between-circle distance was selected. The circle value was then set to N+1 resulting in a total of 9 circles and the number of intersections of the processes with the circles was noted.

Morphology

Microglia activation states were determined using Fiji for ImageJ (ver. 1.52p, (Schindelin et al., 2012; Schneider, Rasband, & Eliceiri, 2012)). Image processing was kept uniform throughout the analysis. To prepare the images for the morphological analysis, pseudo-colors were assigned to the blue, green, and red channels, and the channels were merged. Following, a maximum intensity projection was applied on the merged image as well as on the green channel separately. To facilitate easier visual detection of microglia soma, gray scale attribute filtering was applied on the green channel with the following settings: operation=Opening, attribute=Area, minimum=25, connectivity=8. Using the “Polygon selections” tool, ROIs were created at the border of the soma of each microglial cell. Microglia whose soma areas extended past the borders on the x, y, or z axis were excluded from further analyses since no precise measurement of microglial soma area, shape, and counting of primary processes and endpoints could be ensured. Microglia processes and end points were counted with the aid of the counter function of the multi-point tool. The total numbers of primary processes and endpoints were noted. For the evaluation of the microglial reactivity states, all four parameters (soma area, soma roundness, number of primary processes, and number of endpoints were measured and analyzed. In the case of soma with an area of more than 35 µm2, a roundness of >0.7, and >5 primary processes, end points were not counted since the microglia could already be characterized as belonging to the ramified state

Percentage Area

First, image channels were separated, and the green and blue channels were individually stacked using *Image → Stacks → Z Project* with “Max Intensity” projection,

while excluding poor-quality slices. The green channel was then converted to 8-bit (*Image* → *Type* → *8-bit*) and used for all subsequent analyses. Regions of interest (ROIs) were defined manually using the polygon selection tool and stored in the ROI Manager. Thresholding was applied (*Image* → *Adjust* → *Threshold*) to ensure microglia were represented as white against a dark background, with adjustments made to clearly visualize microglial branches while excluding background signal. Measurement parameters were set under *Analyze* → *Measurements* to include area, minimum/maximum gray values, integrated density, area fraction, and mean gray value, restricted to the defined threshold. Finally, ROIs were quantified by selecting each region and applying *Analyze* → *Measure* (or using the ROI Manager).