SUPPLEMENTARY INFORMATION

Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression

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Supplementary Methods

2-Deoxy-glucose technique

The $[^{14}\text{C}]$-2-deoxy-glucose procedure (2DG) has been previously described in detail $^{1,2}$. Briefly, animals received an i.p. injection of 165 uCi/kg $[^{14}\text{C}]$-deoxy-D-glucose (specific activity, 53 mCi/mmol; Hartmann Analytic, Germany). Forty-five minutes after the tracer injection, the animals were euthanized by decapitation, and their brains were removed, rapidly frozen in isopentane (-45°C) and stored at -80°C until processing. Coronal sections (20-μm thick) were cut on a cryostat, and one out of six sections was collected on coated slides, rapidly dried on a hot plate at 60°C, and processed for autoradiography along with a calibrated $[^{14}\text{C}]$-microscale on XAR-5 Kodak Biomax MR autoradiography film (Sigma-Aldrich, Switzerland) for 3 days. After developing the films, the slides were counterstained with 0.25% thionin (Sigma-Aldrich, Switzerland), dehydrated through increasing concentrations of ethanol, cleared in xylene and coverslipped with DPX (Sigma, Switzerland) for the histological control.

Image analysis. Digital images from the 2-DG XAR films were obtained with a digital camera and aligned with the corresponding Nissl-stained images to allow for structure identification using MCID Core™ 7.0 software (MCID, UK). $[^{14}\text{C}]$2-deoxy-glucose uptake was measured by densitometric analysis of the XAR films. Briefly, the images were calibrated with $^{13}$C standard curves, the regions of interest were delineated and the optical densities were obtained. The 2-DG uptake was expressed relative to that in the corpus callosum (which did not vary among the experimental groups) to control for differences in film exposure. The analyses of 2DG expression in the different regions of interest were conducted by a researcher who was blind to the experimental condition.

c-Fos immunohistochemistry

The animals were anesthetized with isoflurane and transcardially perfused using a 0.9% saline solution followed by a 4% paraformaldehyde fixative solution in phosphate-buffered saline (PBS, pH = 7.5). After perfusion-fixation, the brains were removed from the skull, post-fixed in the same solution for four hours, and immersed in 30% sucrose solution in PBS at 4°C for cryoprotection until they sank. The brains were
then frozen and stored at -80°C until further processing. Next, 30-μm thick coronal sections were cut on a cryostat and stored at -20°C in cryoprotectant solution (30% ethylene glycol and 20% glycerol in 0.25 mM phosphate buffer, pH 7.3). Every sixth section was processed for free-floating immunohistochemical processing for c-Fos neuron visualization. Briefly, the sections were pre-washed in cold PBS (3 x 10 minutes) and incubated with 3% H2O2 in PBS for 10 minutes to block endogenous peroxidase activity. After washing in PBS, the sections were treated for 1 hour with 0.2% Triton X-100 (Sigma, Switzerland) and 3% non-fat milk in PBS. After washing in PBS, they were incubated overnight at 4°C with polyclonal rabbit antiserum (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution 1:500. PBS containing 0.2% Triton X-100 and 5% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories, West Grove, PA) was used to dilute the primary and secondary antibodies. After washing in PBS, the sections were incubated for 1 h at room temperature (RT) with a biotinylated anti-rabbit IgG (1:200; Southern Biotechnology Assoc., Birmingham, AL, USA). Finally, the sections were incubated in horseradish peroxidase-labeled streptavidin (Southern Biotechnology Assoc.) at a dilution of 1:400 for 1 h at RT. The c-Fos antibody-peroxidase complex was revealed using 0.5 mg/ml diaminobenzidine (DAB, Sigma-Aldrich, Switzerland) and 0.01% H2O2 in PBS. The sections were mounted onto Superfrost slides, dehydrated with an ethanol series, cleared in xylene and coverslipped with DPX (Sigma-Aldrich, Switzerland).

Quantitative analysis of c-Fos immunoreactivity. Grayscale images were taken via a digital camera coupled to a bright-field microscope (Olympus BX51) using a 20X objective. Sample images of the different regions of interest were captured at the same coordinates for each animal using the stereotaxic atlas of Paxinos and Watson (1998) as a reference. Four to ten sample images from each brain region of interest were used for the analyses. To select c-Fos immunopositive nuclei for quantification, PC-based software (Scion Image, Scion Corporation, Frederick, MD, USA) was used, and the nuclei were subsequently identified in the captured images by gray-level thresholding. For each brain area, the threshold for the labeled signal was defined according to the background staining. Size criteria were applied to exclude structures other than c-fos-immunopositive nuclei from the measurements. The number of FLI nuclei per mm² was calculated as the average number obtained from all of the analyzed images.

Effective connectivity analysis

Dynamic brain interactions after the resident-intruder test were modeled through structural equation modeling (SEM) that was applied with maximum likelihood estimation to evaluate the effective network connectivity 3-5 (see Supplementary Methods).

Structural equation modeling (SEM) with maximum likelihood estimation was used to evaluate the effective network connectivity 3-6 produced by the aggressive encounter in the different experimental groups. As a powerful extension of multiple regression analyses, the SEM enables the testing of multiple
variables simultaneously to establish their relative individual predictive ability over one or more dependent variables. The Amos 17.0 program (SPSS, Zurich, Switzerland) was used for the analyses.

By convention, a model is accepted as providing a plausible representation of the underlying variance structure of the data (i.e., a good fit) if several indicators agree. The goodness of fit was evaluated according to the following criteria: a non-significant chi-squared test (χ², i.e., failure to reject the null hypothesis that there was no difference between the predicted and the observed data covariance matrices); a comparative fit index (CFI) ≥0.90 to ≥0.95 (good to very good fit); and a root mean square approximation of variance (RMSEA) that was not significantly greater than 0.05.¹,²

To test whether the activation patterns in the brain regions that showed changes in activity between the control and peripuberty stress groups were related, a path analytic approach was applied. A model was produced comprising the medial orbitofrontal cortex and the medial and central amygdala nuclei. Standardized path coefficients (beta weights) indicated the estimated ability of one variable to predict a dependent variable, ranging in standardized absolute values from zero to one. Each path coefficient was statistically significant (p<0.05). Non-significant paths were removed for parsimony.

The overall difference in fit between the two experimental conditions was evaluated by testing the measurement invariance across the groups. Thus, a multi-group (“stacking”) approach statistically compared the model between the groups (structural weights and intercepts). The multi-group comparison statistics for stacked models specify the statistical significance of χ² differences (Δχ²) when constrained and unconstrained models are compared.

**mRNA quantification (qRT-PCR)**

RNA was isolated using the RNAqueous-Micro kit (Ambion, Applied Biosystems, Rotkreuz, Switzerland). Following ethanol precipitation and spectrophotometric quality verification (NanoDrop, Thermo Fisher Scientific, Wohlen, Switzerland), cDNA was synthesized using the Superscript VILO kit (Invitrogen, Basel, Switzerland) according to the supplier’s recommendations. Quantitative real-time PCR reactions (Applied Biosystems 7900HT cycler) were run in triplicate with the Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was normalized to the internal ribosomal reference genes RPS18 and RPS29, and the analyses were conducted with qBase 1.3.5 using the comparative cycle threshold method, yielding [delta][delta]C_t = [delta]C_t,sample - [delta]C_t,reference. Primers were designed to be complementary to each gene of interest (see Supplementary Table 1 for sequences). The efficiency of all of the primers pairs was confirmed by performing reactions with serially diluted samples. The specificity of all of the primer pairs was confirmed by analyzing the dissociation curve.
Chromatin Immunoprecipitation (ChIP)

The chromatin immunoprecipitation procedures have been previously described in detail. Briefly, frozen prefrontal cortex samples dissected from decapitated rats were minced into approximately 1-mm thick pieces and fixed in 10 ml of 1% formaldehyde for 15 min at room temperature. The fixation was quenched by adding glycine at a final concentration of 0.125 M. The tissue was then rinsed three times with cold PBS containing protease inhibitors and homogenized twice for 10 s in 10 mM Tris, 10 mM NaCl, and 0.2% NP40. The homogenate was centrifuged at 4500 x g for 5 min. The supernatant was removed, and the cell pellet was homogenized twice using nuclear lysis buffer (ChIP kit number 17-295, Upstate Biotechnology). The cell mixture was diluted into 1 ml of nuclear lysis buffer and sheared into 300-700 bp pieces using a Branson 250 digital sonicator. Each sample was sonicated on ice four times for 20 s each at 30% of maximum power.

Two hundred microliters of sonicated lysate were used for each ChIP reaction. One percent of the sample was saved as an input-sample for normalization. The lysate was precleared with 80 μl salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate) and immunoprecipitated overnight at 4°C with 5 μg of antibody directed against acetylated H3 (Upstate, 06-599) or acetylated H4 (Upstate, 06-866). The chromatin-antibody complexes were collected with 60 μl of salmon sperm DNA/protein A-agarose beads for 1 h. The beads were sequentially washed with low salt, high salt, LiCl, and TE (twice) buffers. The chromatin was eluted with 500 μl of 100 mM NaHCO3/1% SDS buffer. The cross-linkages between DNA and histones were reversed in high salt conditions at 65°C overnight. The samples were then treated with Proteinase K for 2.5 h at 55°C, extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 50 μl H2O. The levels of the various modified histones were determined using quantitative real-time PCR (Applied Biosystems 7900HT cycler). The primers were designed to amplify approximately 80-bp amplicons targeted approximately 200 bp upstream of the transcription start site. The primer sequences are shown in Table S1. PCR SYBR green quantitative real-time PCR reactions were run in triplicate with the Power SYBR Green PCR Master Mix (Applied Biosystems) using standard procedures. The real-time polymerase chain reaction (PCR) ChIP data were analyzed using the delta-delta-Ct method in the same manner as the mRNA data, except that the ChIP data were normalized to the respective “input” values.
Supplementary Figures

Figure S1: Fear induction experimental design

Scheme representing the peripuberty stress protocol. (a) Peripubertal stress was induced for 7 days during the developmental period from P28 to P42, which covers the early adolescence period (i.e., P28-P36) and the male puberty period (approximately P41) in rats. Stressors included 2 fear-induction experiences (EP: elevated platform; TMT: exposure to the predator odor TMT), each of which lasted 25 min and were applied in an unpredictable manner. Depending on the day, stressed animals were exposed to either one or two consecutive stress experiences. The cartoon represents the exact schedule of the stressor administration. In addition, on P28, the animals were first exposed to the stressor of an open field (OF) for 5 min, followed by further stressor application. After the end of the stress procedure, the animals were allowed to continue living with their home cage members. Behavioral testing began during adulthood (from P90 onwards; see specific protocols). (b) As a control experiment, the same stress protocol was applied during adulthood. Starting at P90, animals underwent the same stress schedule, and the long-term behavioral effects were evaluated after 2 months.
Figure S2: Experimental design on the different experiments

Schema representing the different experiments carried out to evaluate the behavioral and neurobiological consequences of peripubertal stress. Independent cohorts of animals were used for different porpoises in each experiment, to assess different behavioral aspects of emotionality and social behavior, and pertinent neurobiological end-points after our stress protocol.
**Figure S3: Characterization of the peripubertal stress protocol: Corticosterone response and adaptation**

![Graph showing corticosterone response and adaptation](image)

Corticosterone release after exposure to the elevated platform (25 min) on the first and last days of the **peripubertal stress protocol**. Blood samples were obtained by the tail-nick procedure at basal levels, immediately after the stress exposure (stress) and at 30 and 60 minutes after the end of the stress exposure (rec 30 and rec 60) (n=44). On P28, the animals were exposed for 5 minutes to an open field (OF) prior to the stress exposure, and immediately after the OF exposure, the first blood sample was obtained by the tail-nick procedure (basal). The area under the curve (AUC, right panel) of the plasma levels was calculated using GraphPad Prism (version 4), which computes the AUC using the trapezoid rule. As observed in the corticosterone dynamics (left panel), exposure to the elevated platform induced a robust corticosterone release on the first day of stress exposure that was maintained even 60 min after the termination of the stressor. A significant reduction in the corticosterone levels following the stress protocol was observed in the dynamics of the hormonal response (left panel) and in the AUC (right panel) at the end of the peripuberty stress protocol. Paired sample test \[t(1,43)=16.88, \text{*** } p<0.001\].
Peripuberty stress model induced a delay in the onset of puberty. Puberty onset was determined in control (n=46) and peripubertally stressed animals (n=44) by checking the onset of preputial separation, which is considered to be an external sign of sexual development in male rats. (a) Cumulative percentage of animals from each experimental group that showed preputial separation on each of the observation days. (b) Cumulative survival graph for the frequency of animals from each group that were not on puberty over time. Survival statistical analysis for each experimental group was performed by means of Kaplan-Meier test. LogRank (Mantel-Cox) test showed significant differences between the survival curves of control and peripubertally stressed animals (Chi-Square=7.194, dg=1, P=0.007).
Figure S5: Corticosterone and testosterone responses after the resident-intruder test

Plasma corticosterone and testosterone hormone levels. Peripubertally stressed animals displayed higher corticosterone/testosterone ratios after the resident-intruder test, as shown in Fig. 1h. The absolute values for each time point (a, c) and the area under the curve (b, c) are shown for corticosterone (upper panels) and testosterone (lower panels).
Increased anxiety-like behaviors

**a**

![Graph a](image)

**b**

![Graph b](image)

**c**

![Graph c](image)

Increased depression-like behaviors

**d**

![Graph d](image)

**e**

![Graph e](image)

**f**

![Graph f](image)

Adult peripubertally stressed animals displayed increased anxiety- and depression-like behaviors, as indicated by their spending significantly less time (a) and moving less (b) in the open, unprotected arms of the elevated plus maze [t(1, 42.8) = 3.91, p<0.001; t(1, 44.4) = 3.58, p=0.001, respectively], with higher latencies to explore the open arms (c) [t(1, 35.3) = -2.12, p<0.05] and without differences in locomotor activity, as indicated by the total distance traveled in the maze (d) and the frequency of entries into the closed arms (e). No differences in anxiety-like behaviors were observed when the stress protocol was applied during adulthood (f). Peripubertally stressed animals displayed depression-like behaviors as shown by the observed passive coping styles in an inescapable situation (g) [increased floating time in the first exposure to the forced swimming test [t(1,15) = -2.40, p<0.05], and anhedonia (h) [decreased overnight 1% sucrose consumption t(1,10) = 2.65, P<0.05], which was not observed in the adult stress protocol (i). *** p<0.001; * p<0.05
Effective connectivity analyses were applied to the 2-deoxy-glucose data to evaluate whether the network changes in the stress group seen in the resident-intruder aggression challenge (Fig. 2f) were also detectable under basal conditions. The application of the same model to the 2DG data revealed no basal group differences (see also Table S3), supporting the idea that the effective network connectivity difference described for the resident-intruder aggression challenge emerged within that particular context.
**Figure S8: MAOA and 5HTT expression in the amygdala**

**Gene expression of MAOA and 5HTT in the amygdala.** Quantitative RT-PCR revealed no significant differences between the control and peripubertally stressed groups in the expression of either (a) MAOA or (b) 5HTT mRNA in the amygdala at basal levels or after a 3-hour social challenge (resident-intruder test).
Figure S9: Peripubertal stress does not induce changes in histone acetylation of the 5HTT gene in the PFC.

Chromatin immunoprecipitation showing the relative enrichment of H3 and H4 acetylation of the 5HTT gene in the prefrontal cortex (PFC). Peripubertal stress did not modify the (a) acetylation levels of histone 3 or (b) the histone 4 acetylation of the 5HTT gene in the PFC either under basal conditions or after the 3-h resident-intruder test. (Note that extremely low levels of acetylation were detected for the 5HTT gene.)
Table S1: Primer sequences

| mRNA primers          |            |
|-----------------------|------------|
| MAOA Forward (F)      | TGACCCAGTATGGGAGGGTGAT |
| MAOA Reverse (R)      | TCTGTGCGCTGCAAGTAAATCC |
| 5HTT F                | AAACGGGTGCAATTTCCCATATG |
| 5HTT R                | GCCGTAACCAATGCCCCGTATTT |
| RPS18 F               | TCAATGCAACCCACGACAAA |
| RPS18 R               | TCACGTCTCTGCTGTCAAGG |
| RPS29 F               | GCCCGTCTGCTTCAACC |
| RPS29 R               | GCACATGTTCAGCCCGTATTT |
| ChIP primers          |            |
| MAOA F                | AACGTCGCGTGAGCGAGCTCT |
| MAOA R                | GGTAGCCCCCTCTGGAATG |
| 5HTT F                | CGTGACCTGGGCAACA |
| 5HTT R                | CGGCCATTCTGAGCATATG |
| RPS18 F               | TCAATGCAACCCACGACAAA |
| RPS18 R               | TCACGTCTCTGCTGTCAAG |
| EEF1a F               | CTCCGGCGCGACTATGC |
| EEF1a R               | CCTCGCTTCAAACACTTCAAGG |
Table S2: Effective connectivity analysis related to the neural engagement in the resident-intruder test.

The strength and significance of the paths are presented; other anatomically plausible but non-significant paths are not shown. The level of aggression was related to the influence of the medial orbital cortex on the medial amygdala, which engaged the central amygdala. Only the latter was directly predictive of aggressive behaviors.

| Path          | est.   | P     |
|---------------|--------|-------|
| MO $\rightarrow$ MeA | -0.472 | 0.037 |
| MeA $\rightarrow$ CeA  | 0.716  | <0.001|
| CeA $\rightarrow$ Total aggressive (RESIDENT) | 0.524  | 0.016 |

Abbreviations: MO, medial orbital cortex; MeA and CeA, medial and central amygdala nuclei, respectively; est., standardized estimate; P, probability. N = 8 and 9 for the control and stress groups, respectively.
Table S3: Separate group effective connectivity analyses. The strength and significance of the paths are presented; other anatomically plausible but non-significant paths are not shown. A direct statistical comparison of the groups revealed that, as a result of peripubertal stress, differential network activation occurred upon experiencing the presence of an intruder (c-Fos imaging). No such differential network dynamics were observed under basal brain activation (2DG imaging).

| Path      | c-Fos (resident-intruder test) | 2DG (basal) |
|-----------|--------------------------------|-------------|
|           | Group comparison               | Group       | Group comparison |
|           | ALL | Control | PPS | ALL | Control | PPS | ALL | Control | PPS | ALL | Control | PPS | ALL | Control | PPS | ALL | Control | PPS | ALL | Control | PPS |
| MO → MeA  |                 |             |     |       |                 |             |     |       |                 |             |     |       |                 |             |     |       |                 |             |     |       |                 |             |
| MeA → CeA | .72 | <.001   | .99 | <.001 | 12.4 | 3 | .006 | .93 | <.001 | .89 | <.001 | .93 | <.001 | 3.9 | 3 | .27 |

Abbreviations: MO, medial orbital cortex; MeA and CeA, medial and central amygdala nuclei, respectively; est., standardized estimate; df, degrees of freedom; χ², chi-squared; 2DG, 2-deoxyglucose; N = 8, 9 for the control and stress groups, respectively.

ALL: includes all animals in both groups.
Supplementary references

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