Supporting Information

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SI Methods and Materials

Maternal Behavior Measurements. Maternal behavior in dams was observed from P5–P14, over 1 h (eight observations per hour), five times a day (0900, 1100, 1400, 1630, and 1900 hours). The total number of observations for one mother was 40 per day. The following parameters of maternal behavior were estimated: mother carrying, mother licking pup, and mother nursing pups (51, 52). Nursing posture was rated as either an arched-back posture, when the mother was arched over pups with legs extended, a blanket posture in which the mother lays over the pups with no leg extension, or a passive posture in which the mother is lying on her back or side while the pups nurse. The number of occurrences of each behavior was assessed every observation time.

Locomotion Test. Rats were placed in a 72-cm × 72-cm open field for a 10-min period. Total locomotor activity was scored as the number of line crossings (forelimbs crossing a line) and rearings.

Food Dominance Test. The food dominance test was used to test social dominance, as previously described in detail (53). The dominance testing apparatus was a modified V-maze. The two side arms were 20.0 cm long, 11.8 cm wide, and 20.8 cm high. There were guillotine doors placed 8 cm from the food cup end of the arms. The center arm was 8 cm long, and had a 1.5-cm diameter, 1-cm high, round food cup on a 2-cm raised platform that was 10 cm long and 3 cm wide. The juvenile rats were deprived of food for 2 d before the start of the experiment. All rats were first trained to run to the food cup following the lifting of the door. Each rat was placed on one side of the maze for 15 s. The door was raised and the rat was allowed to explore the apparatus until a pellet from the food cup was eaten. Rats were then given an additional 2 min before being placed in the other side, where the process was repeated. This training was given to each rat on 3 consecutive days. The side on which the rat started was alternated each day. The rats were given dominance tests on days 4 (test day 1) and 5 (test day 2). Dominance tests lasted 120 s on each side, with this time being divided between the two rats.

Agonistic Behavior Test. The agonistic behavior test was also used to test social dominance, as previously described in detail (8). Briefly, juvenile rats housed together for a while do not exhibit extensive aggressive behavior toward each other. However, right after they are switched to a new cage, a dirty cage previously inhabited by other rats, they tend to engage in more agonistic activities, presumably due to the need to claim territory in the new environment. We videotaped the rats after their cage was inhabited by other rats, they tend to engage in more agonistic activities.

Preparation of PSD Fractions. PSD fractions were prepared as described previously (54). Dounce homogenate was prepared from the mPFC and centrifuged at 1,000 × g for 10 min to remove nuclei and debris (P1). The supernatant was spun at 12,000 × g for 20 min to obtain a P2 fraction. P1 and P2 fractions were resuspended and centrifuged twice to remove contaminants. The P2 fraction was then resuspended in buffer containing 0.5% Triton X-100 and rotated for 15 min. This fraction was then centrifuged at 12,000 × g for 20 min to yield soluble and insoluble fractions, and the insoluble fraction (PSD enriched fraction) was then solubilized into 2% (wt/vol) LDS. All fractionation steps were performed at 4 °C in the presence of 0.3 M sucrose and 4 mM Hepes, containing complete protease inhibitor mixture (Roche Applied Science).

Golgi Staining. Rats were anesthetized with an isoflurane–oxygen mixture and the brain hemispheres were removed. The impregnation procedure was carried out using FD Rapid GolgiStain kit (FD NeuroTechnologies) according to the manufacturer’s protocol; tissue was then sectioned at 200-μm thickness and mounted on gelatin-coated slides. The sections were then stained, dehydrated, and coverslipped using Permunt (Fisher).

Image Analyses. The dendritic spine density of pyramidal neurons in the mPFC was analyzed by counting the tertiary basal dendrites. Spines were morphologically characterized and classified as filopodia, stubby, or mushroom-shaped spines (55). Spines were classified as filopodia if the spine’s length was greater than its uniform diameter, as stubby if the diameter was similar to the length, and as mushroom if the diameter of the head was much greater than the diameter of the neck. Spine density was normalized as the number of spines per 30 μm of dendrite length.

Antibodies. Antibodies to phospho-cofilin (ab12866; Abcam), cofilin (ab42824; Abcam), phospho-LIMK (07–850; Millipore), LIMK (3842; Cell Signaling Technology), GluA1 (04-855; Millipore), GAPDH (14C10; Cell Signaling Technology), CaMKII (ab22609; Abcam), GABA (H4830; Sigma), and GFP (598; MBL and 012-20461; Wako) were used.

Immunohistochemistry and Counting. Lenti-S3A-IRES-Venus virus was injected into the rat mPFC unilaterally. After 1 wk of expression, the rats were perfused and the brain was removed. Serial brain sections (30 μm) were incubated with mouse anti-CaMKII antibody (1:1,000) and rabbit anti-GFP antibody (1:1,000), or rabbit anti-GABA antibody (1:1,000) and mouse anti-GFP antibody (1:1,000) overnight at 4 °C. After extensive washing with TBS, the preparation was overlaid with secondary antibody solution for 1 h at room temperature. Sections were assessed for the number of infected cells, CaMKII or GABA-positive cells, and double-labeled cells. The numbers were counted in three sections using the Zeiss LSM Image Browser.
Fig. S1. Social isolation does not affect maternal behavior. Average number of observed occurrences for maternal behaviors [(A) carry, (B) lick, (C) blanket nurse, (D) arched-back nurse, (E) passive nurse, and (F) off] obtained with dams with control litters (nonisolated) or dams with litters exposed to 6-h isolation (isolated) \( (n = 6 \text{ mother rats) beginning at 1000 hours. Observation sessions began at 0900 (nonisolated and isolated), 1100 (nonisolated), 1400 (nonisolated), 1630 (nonisolated and isolated), and 1900 hours (nonisolated and isolated). The maximum possible score for any dam at each relevant observation time was 8 (eight samples per observation) before/isoration periods/after isolation: carry \( [F(1, 58) = 1.055/F(1, 34) = 1.000/F(1, 58) = 0.144], \text{blanket nurse} \( [F(1, 58) = 0.012/F(1, 34) = 0.153/F(1, 58) = 0.028], \text{arched-back nurse} \( [F(1, 58) = 0.000/F(1, 34) = 0.000/F(1, 58) = 0.000], \text{passive nurse} \( [F(1, 34) = 0.012/F(1, 34) = 0.039/F(1, 34) = 0.485], \text{off} \( [F(1, 58) = 0.000/F(1, 34) = 0.032/F(1, 34) = 0.158]. \text{Error bars represent SEM. The statistical analyses for the 1100 and 1400 hours observations during isolation periods were not included. n.s., not statistically significant (repeated measures ANOVA).}
Fig. S2. Social isolation does not affect body weight and locomotion. Body weight, number of rearings, and number of line crossings in the open field. There was no significant difference among animals with (A) nonisolated and isolated (n = 9 rats nonisolated and 10 rats isolated), (B) nonisolated with vehicle and isolated with RU486 (n = 7 rats nonisolated with vehicle and 7 rats isolated with RU486), or (C) nonisolated with vector and isolated with S3A (n = 8 rats isolated with S3A and 6 rats isolated with vector). Error bars represent SEM. n.s., not statistically significant (unpaired Student’s t test).

Fig. S3. Social isolation does not affect locomotion in the tube. There was no significant difference among animals in nonisolated versus isolated groups in the latency of movement in the tube during eight training trials on each of two successive days (n = 25 rats nonisolated and 25 rats isolated) [repeated measures ANOVA F(1, 733) = 0.874]. Error bars represent SEM.
Fig. S4. Additional dominance tests between socially isolated and nonisolated rats. (A) Food dominance test. Isolation increased rats’ dominance in a food-competition situation relative to nonisolation (time of occupied food) (four matches). (B) Agonistic behavior test. Rats that underwent neonatal isolation exhibited an increased number of offensive behaviors relative to rats that were not isolated (n = 8 rats nonisolated and 8 rats isolated). *P < 0.05 (unpaired Student’s t test). Error bars represent SEM.

Fig. S5. Social isolation does not affect electrophysiological properties ([A] membrane capacitance, [B] membrane resistance and [C] resting membrane potential). There was no significant difference between nonisolated and isolated rats (n = 8 neurons nonisolated and 8 neurons isolated). Error bars represent SEM. n.s., not statistically significant (unpaired Student’s t test).

Fig. S6. (A) (Top) Representative Western blots of PSD fractions obtained from the mPFC of socially isolated and nonisolated rats. (Bottom) Graph depicting the average ratio of GluA1 to GAPDH (n = 6 rats nonisolated and 6 rats isolated). (B) (Left) Representative mIPSC traces obtained from layer 2/3 pyramidal neurons in the mPFC of socially isolated or nonisolated juvenile rats. (Right) Graphs depicting the average mIPSC amplitude and frequency (n = 10 neurons nonisolated and 6 neurons isolated). (C) (Left) Representative NMDA-mEPSC traces obtained from layer 2/3 pyramidal neurons in the mPFC of socially isolated or nonisolated juvenile rats. (Right) Graphs depicting the average NMDA-mEPSC amplitude and frequency (n = 7 neurons nonisolated and 6 neurons isolated). *P < 0.05 (unpaired Student’s t test). Error bars represent SEM. n.s., not statistically significant.
Fig. S7. (A) FRAP analysis of spines of GFP-actin- and tdTomato-expressing neurons in the mPFC. Although the fluorescence levels of tdTomato are completely recovered, the fluorescence recovery of GFP-actin is partial, and there will be an unrecoverable fraction (n = 20 spines). (B) Social isolation does not affect spine size. Representative images showing tdTomato fluorescence on spines in the mPFC of socially isolated or nonisolated rats. There was no significant difference in the tdTomato spine/dendrite ratio between nonisolated and isolated rats (n = 33 spines nonisolated and 39 spines isolated). Representative images shown in the figures were unsharp mask-filtered. Arrowheads indicate dendritic spines. Error bars represent SEM. n.s., not statistically significant (unpaired Student’s t test). (Scale bars: 1 μm.)

Fig. S8. (A) (Left) Representative photomicrographs of basal dendrites of mPFC neurons. Arrowheads indicate mushroom-shaped spines; arrows indicate stubby spines. (Scale bars: 3 μm.) (Right) High-magnification images of dendritic spine enclosed with rectangles. Representative images shown in the figures were unsharp mask-filtered. (Scale bars: 1 μm.) (B) The number of each type of dendritic spine from the mPFC of socially isolated and nonisolated rats. Social isolation significantly increased the number of mushroom-shaped spines and decreased the number of stubby spines on mPFC pyramidal neuron (nonisolated, n = 30 dendrites; isolated, n = 32 dendrites). *P < 0.05 (unpaired Student’s t test). Error bars represent SEM. n.s., not statistically significant.
Constitutive-active ADF/cofilin is moderately overexpressed in electroporated animals. (A) The level of endogenous cofilin is similar between RFP-S3A transfected (n = 35 neurons) and untransfected (n = 35 neurons) neurons. Arrowheads indicate S3A transfected neurons. (Scale bar: 20 μm.) (B) Representative images showing GFP fluorescence on spines in the mPFC of socially isolated or nonisolated rats. Arrowheads indicate mushroom-shaped spines; arrows indicate stubby spines. (Scale bars: 2 μm.) (Right) High-magnification images of dendritic spine enclosed with rectangles. Representative images shown in the figures were Gaussian- and unsharp mask-filtered. (Scale bars: 1 μm.) (C) There was no significant difference of spine shapes (n = 25 dendrites, vector; n = 26 dendrites, S3A). (D) There was no significant difference in the GFP spine/dendrite ratio between nonisolated and isolated rats (n = 61 spines, vector; n = 61 spines, S3A). (E and F) Sholl analysis of basal dendrites in the mPFC layer 2/3 pyramidal cell based on distance from the cell body. (E) Mean length of basal dendrite branches of PrL regions (n = 10 neurons, vector; n = 11 neurons, S3A). (F) Mean number of intersections of basal dendrite branches with consecutive 5-μm-spaced concentric spheres of PrL regions (n = 10 neurons, vector; n = 11 neurons, S3A) [repeated measures ANOVA F(1, 73) = 0.506]. Error bars represent SEM. n.s., not statistically significant (A, C, D, and E, unpaired Student's t test; F, repeated measures ANOVA).
Fig. S10. Social isolation impaired increase of mEPSC amplitude by TEA cLTP induction. Sample traces of mEPSC from mPFC layer 2/3 neurons of nonisolated and isolated rats before and after 10-min exposure to 25 mM TEA without TTX and picrotoxin. The whole-cell patch-clamp recordings were performed on the same neurons before and after 10-min TEA treatment. mEPSC amplitude was increased in the neurons from nonisolated rats but not isolated rats (n = 7 neurons nonisolated and 8 neurons isolated). *P < 0.05 (unpaired Student’s t test). Error bars represent SEM. n.s., not statistically significant.

Fig. S11. Lentivirus preferentially infected pyramidal neurons. (A and B) Immunohistological staining for CaMKII-positive pyramidal neurons (A) and GABA-positive neurons (B) of layer 2/3 of the mPFC after Lenti-S3A-IRES-Venus injection. (C) Quantification of infection rates in A and B (n = 4 slices, CaMKII and 5 slices, GABA). Arrowheads indicate CaMKII-positive pyramidal neurons expressing S3A; arrows indicate GABA-positive pyramidal neurons expressing S3A. *P < 0.05 (unpaired Student’s t test, C). Error bars represent SEM. (Scale bars: 20 μm.)