Chronic Social Instability Induces Anxiety and Defective Social Interactions Across Generations

Supplemental Information

Supplemental Methods and Materials

Animals

CD1 females and males were obtained from Charles River (Wilmington, Massachusetts). All mice were maintained in a temperature- and humidity-controlled animal facility on a 12 hour light-dark cycle with food and water ad libitum. All procedures were carried out in accordance with the Institutional Animal Care and Use Committee guidelines of Tufts University.

Chronic Social Instability

The chronic social stress procedure was performed as previously established (1). In this model, female and male mice were exposed separately to a highly unstable and unpredictable social environment during the adolescent and young adult period, starting at postnatal day 27, and finishing at day 76. To achieve that, the group composition in each cage was changed twice per week for seven weeks, and each time, four mice from different cages were placed together in a clean cage. The rotation schedule was randomized to minimize the likelihood of a repeated encounter with the same mice throughout the experiment (1). In contrast, control mice remained housed with the same cage mates in groups of four. At the end of the treatment, stressed and control mice were separated and housed in pairs (with cage mates from the last change in the case of stressed animals), not individually to avoid further stress by isolation. Two months after treatment, stressed and control females and males were used for evaluation of the long-term behavioral effects of chronic social instability.

Breeding

For evaluation of the transgenerational effects of chronic social instability, breeding pairs were formed two weeks after the end of the stress exposure. Stressed mice were selected for breeding according to their increased anxiety levels, measured five days after the last cage composition change, using the elevated plus maze and the open field tests. In our experiment, out of 10 individuals submitted to chronic social instability, 1 or 2 were resistant to the effects of this stress treatment, and not used for breeding.

To produce the first generation, stressed females and males were mated with other stressed or control mice. In total, 28 different breeding pairs were formed as followed: 11 breeding pairs composed of both stressed females and males, 5 breeding pairs composed of stressed females and control males, 5 breeding pairs composed of stressed males and control females, and 7 breeding pairs composed of both control mice. Unless otherwise stated, males were kept in with females during pregnancy, birth of pups, and weaning. To produce the second generation, F1 females and males from both stressed parents were mated with other F1 stressed or control mice. In total, 24 different breeding pairs were formed as followed: 5 breeding pairs composed of both F1 parents from stressed mice, 6 breeding pairs composed of F1 females from stressed mice and F1 control males, 6 breeding pairs composed of F1 males from stressed mice and F1 control females, and 7 breeding pairs composed of both F1 control mice. To produce the third generation, F2 males from F1 males from stressed parents and F1 control females were mated with F2 control females. In total, 8 breeding pairs were formed, in addition to 5 breeding pairs composed of both F2 control mice. F1, F2, and F3 pups were weaned at postnatal day 21, and behaviorally tested when they were 2 months old.

For the cross-fostering experiments, F1 pups born to control or both stressed parents, were raised by both stressed or control parents, respectively, and then tested when they were 2

months old. To discern the influence of the male's presence during female pregnancy on F2 offspring behavior, F1 males from stressed mice were removed from the breeding cages after 2 weeks of mating, and F2 offspring were tested as previously described. In the case of the third generation, F2 males were removed from the breeding cages 2 weeks after mating.

Behavioral Analysis

F0, F1, F2 and F3 females and males from control and stressed groups were tested in a series of behavioral assays in the following order: elevated plus maze, open field, and direct social interaction with a juvenile. These behavioral tests were done 3 days apart of each other, and were used to measure anxiety-related phenotypes. F0, F1, F2 and F3 mice were also used first for the sociability and preference for social novelty tests, designed to measure social tendencies, and then for the forced swimming test, designed to measure depression-like behaviors. All behavioral tests were carried out during the light phase of the cycle, and mice were acclimated to the testing room 1 h prior to any behavioral procedure. To minimize litter effects, all behavioral measurements from animals belonging to the same litter were averaged and considered as an individual value. At least two animals from the same litter were used in the behavioral assays.

Elevated Plus Maze Test

Mice were placed in the center of a plus-shaped maze elevated 40 cm from the floor, composed of two open and two closed arms, each 35.5 cm long and 5 cm wide (Campden Instruments Ltd, Lafayette, IN). General mouse activity was analyzed for 5 min, and percent of time spent in the open arms was recorded using the Motor Monitor software (Campden Instruments Ltd, Lafayette, IN).

Open Field Test

Mice were placed in a 16 x 16 cm open arena connected to an activity frame (Campden Instruments Ltd, Lafayette, IN) and allowed to move under even illumination for 5 min. General locomotor activity was analyzed and total distance was recorded using the Motor Monitor software. The adaptation of locomotor activity over time, measured by distance traveled in the first minute minus distance traveled in the fifth minute, was used as a measure of anxiety, where high adaptation was a sign of low anxiety (2).

Direct Social Interaction with a Juvenile Test

Direct social interaction was assessed in a clean cage, similar to the home cage in which the animals were regularly housed, with bedding on the floor, and covered by a plastic lid to avoid direct light exposure. Each experimental mouse was placed into this cage and left to habituate for 15 min. Then, an unfamiliar same-sex juvenile (26-28 days old) was introduced and left there for 3 min. Testing sessions were recorded and videos were analyzed by an observer (blind to the treatments) taking only the experimental animal into account. During the 3 min test, time spent performing the following affiliative and social behaviors was measured: sniffing the juvenile, grooming it, following it, crawling over or under it, doing passive physical contact with it, and walking side by side. To assess social memory, two days after this first encounter, experimental animals were exposed again to the same juvenile, and affiliative and social behaviors were measured for 3 min.

Sociability and Preference for Social Novelty Tests

These two social assays were done in a three-chambered box, previously described (3). The box consisted of a central chamber connected on each side to another compartment (40 x 20 x 22 cm, each) through a small rectangular opening. Each side contained a round wired cage. Initially, each experimental mouse was placed in the middle chamber and allowed to explore the

entire apparatus for 10 min, in the presence of the two empty wired cages. For the sociability test, after this habituation period, an unfamiliar adult same-sex mouse (stranger 1) was placed in one of the side chambers, enclosed in a cage, while the opposite side contained an empty cage. The placement of the stranger 1 was counterbalanced between animals. The experimental mouse was allowed to explore the entire box during 10 min. This session was recorded and time spent in each side of the social box, and time spent sniffing and interacting with each cage was measured. For the preference for social novelty test, a second unfamiliar same-sex mouse (stranger 2) was placed in the previously empty side, also enclosed in a cage. The experimental mouse was allowed to explore the box for an additional period of 10 min, where it had to choose between the first, already-investigated unfamiliar mouse (stranger 1), and the novel unfamiliar mouse (stranger 2). As described above, this session was recorded and time spent in each side of the social box, and time spent sniffing and interacting with each cage was measured.

Although time spent in each side of the social box was the parameter used to evaluate sociability tendencies in the mice directly submitted to social instability, interaction time with the social targets, calculated as time spent sniffing and interacting with the enclosed strangers was also used for the mice from first, second, and third generations. Given that these two parameters offered us similar results, we decided to show only the interaction time with the enclosed strangers for the F1, F2, and F3 mice.

Forced Swimming Test

Mice were placed in a 10-cm clear plastic cylinder (diameter 8.5 cm) containing 6 cm of water at 25 ± 1°C. All sessions were recorded, and latency to immobility and total immobility were manually scored during the 6-min swimming test by a blind observer.

Corticosterone Measures

F1, F2, and F3 offspring from control and stressed mice, not submitted to any behavioral procedure, were decapitated at 2 months of age, and trunk blood was collected individually in 1.5 ml EDTA-coated microcentrifuge tubes (Fischer Scientific). All blood samples were maintained on ice, and later centrifuged for 15 min at 6000 rpm at 4˚C. Plasma was separated and stored at -80˚C until analysis. Corticosterone levels in plasma were measured using a commercially available immunoassay kit (Corticosterone EIA Kit, Enzo Life Sciences, Farmingdale, NY), according to the manufacturer's instructions. The intra-assay variability ranged from 6.6% to 8.0%, inter-assay variability ranged from 7.8% to 13.1%, and mean assay sensitivity was 26.99 pg/mL. All blood samples were collected during the lights-on period between 1:00 pm and 2:00 pm.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction (PCR)

A group of stressed and control females and males, not used for behavioral analysis or breeding, was decapitated 2 months after the end of the social instability. F1, F2, and F3 offspring, not submitted to behavioral tests, were also decapitated at the age of 2 months. Brains were rapidly extracted, chilled in ice-cold phosphate buffered saline 1X, and transferred to a cold surface to perform hippocampal dissections as previously described (4). Briefly, the hippocampus was first separated as an intact structure after peeling back the overlying cortex. Then, areas CA1 and CA3 were isolated from one another. Hippocampal areas from stressed and control mice, as well as offspring, were pooled according to the treatment (n = 3-4 animals in each pool), and total RNA extraction was done using an RNA isolation kit (QIAGEN) following the manufacturer's instructions. RNA concentration was determined using the NanoDrop-1000 Spectrophotometer (NanoDrop Technologies).

Complementary DNA (cDNA) was obtained from total RNA (250 ng) samples, using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Real-time PCR was performed using an iCycler iQ Real-time PCR Detection System (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad). Briefly, 2 µl of cDNA were combined with 0.5 µM of each primer, 1× iQ SYBR Green Supermix, and PCR-grade water to a volume of 25 µl. The following forward and reverse primers were used: *Rcan1*, forward, 5′- GAC TGG AGC TTC ATC GAC TGC -3′; *Rcan1*, reverse, 5′- CCC AGG AAC TCG GTC TTG T -3′; *Rcan2*, forward, 5′- CAA CCT TTG CTG TCC AAA CCA CCT -3′; *Rcan2*, reverse, 5′- GCA TGG GCA TTG GAT GAA TGG GAA -3′; *Rps29*, forward, 5′- GTC TGA TCC GCA AAT ACG GG -3′; *Rps29*, reverse, 5′- AGC CTA TGT CCT TCG CGT ACT -3′. All primers were synthesized by Integrated DNA technologies (IDT-DNA). The cycling conditions for all primers were as follows: 95°C for 15 min to activate the HotStarTaq polymerase, followed by 40 cycles consisting of three steps, 45 s at 95°C (denaturation), 30 s at 58.5°C (annealing), and 30 s at 72°C (extension). The PCR program was completed by a melting temperature analysis consisting of 1 min at 95°C (denaturation), 2 min at 55°C (annealing), and then 101 steps lasting 8 s each, through which temperature ranged from 55 to 95°C. Amplification plots were used to calculate the threshold cycle (Ct) for both target (*Rcan1* and *Rcan2*) and reference (*Rps29*) genes. All reactions were done in triplicates, and the Ct was used for quantification. The $2^{-\Delta\Delta Ct}$ method for relative gene expression analysis was used for normalization and quantification of the expression ratios between each *Rcan* gene and *Rps29*.

Statistical Analysis

All statistical analyses were performed with Prism 4 software (GraphPad Software). For all the behavioral and endocrine measures, statistical significance was assumed at $p < 0.05$. Data

from female mice were analyzed separately from male mice. Results from the elevated plus maze, open field, direct social interaction with a juvenile, forced swimming test, and the corticosterone measures were analyzed using Student's *t* tests when comparing just control and stressed mice in each generation. To compare the results of the sociability and preference for social novelty tests, two-way analysis of variance (ANOVA) and Bonferroni's posttesting were applied. To analyze the behavioral data (elevated plus maze, open field, and direct social interaction with a juvenile test) and corticosterone measures of F1 and F2 mice from single-stressed parents, one-way ANOVA and Newman–Keuls posttests were used. To analyze the mRNA expression data from the real-time PCR experiments between control and stressed animals, including their offspring, one-way ANOVA and Newman–Keuls posttests were also used.

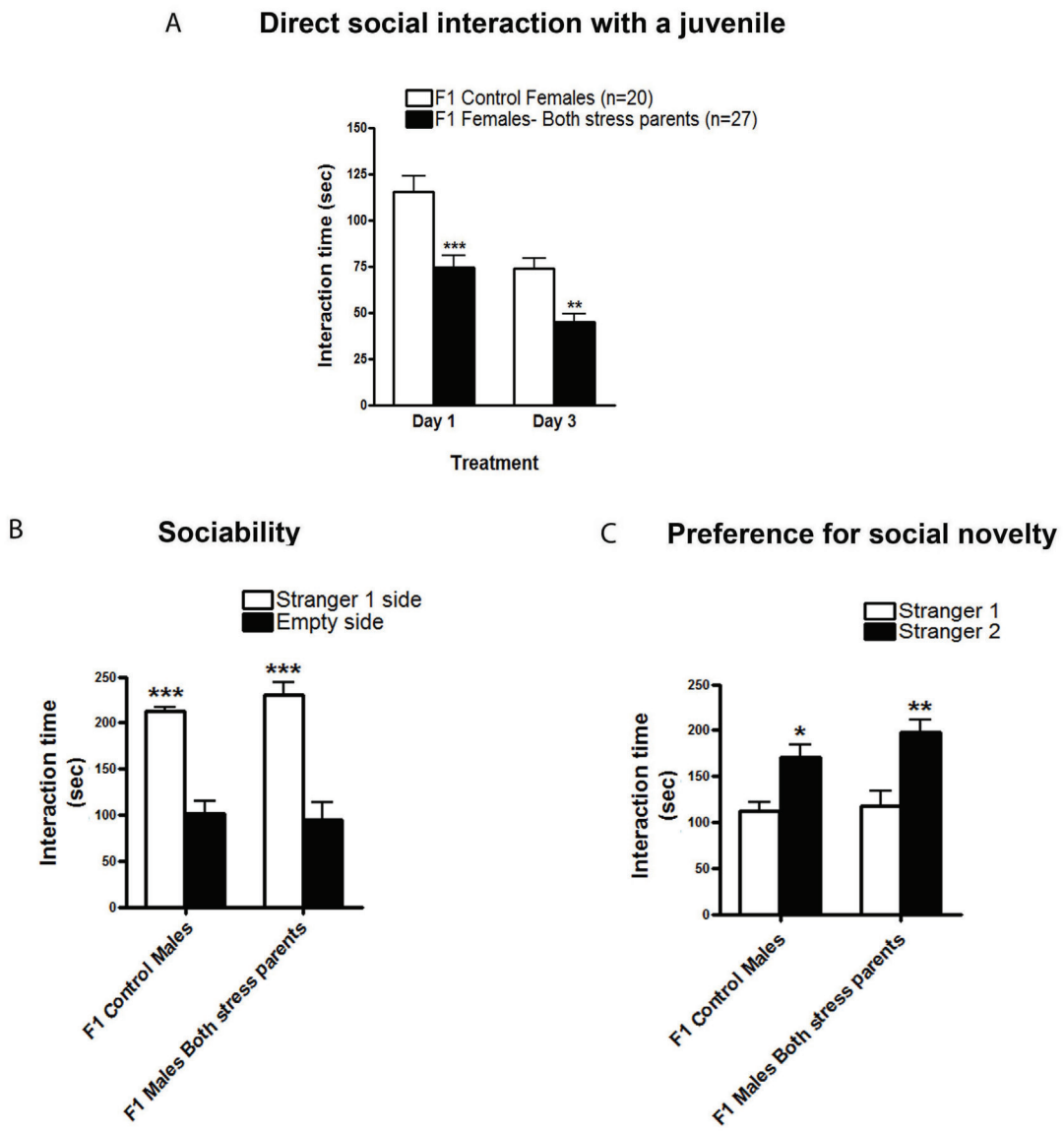

Figure S1. Transmission of defective social interactions to F1 female but not male offspring is not related to social memory deficits. **(A)** In the direct social interaction test with a juvenile, F1 females from both stressed parents spent significantly less time [$F(1,46) = 30.61$, $p < 0.001$] interacting with and exploring the juvenile in comparison with F1 control females on days 1($p < 0.001$) and 3 ($p < 0.01$) of the test. On day 3, both F1 control and stress females reduced significantly the interaction time with the juvenile in comparison with day 1 [$F(1,46) = 31.73$, $p < 0.001$]. **(B)** In the sociability test, both F1 control males ($n = 7$) and F1 males from stressed parents ($n = 6$) showed a significant preference [$F(1,16) = 59.87$, $p < 0.001$] for spending more time interacting with the stranger 1 in comparison with the empty cage ($p < 0.001$). **(C)** In the preference for social novelty test, both F1 control males ($n = 7$) and F1 males from stressed

parents ($n = 6$) exhibited a significant preference [$F(1,16) = 23.06$, $p < 0.001$] for spending more time interacting with the stranger 2 in comparison with the stranger 1 ($p < 0.05$ for F1 control males, and $p < 0.01$ for F1 males from both stress parents). Error bars indicate SEM. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

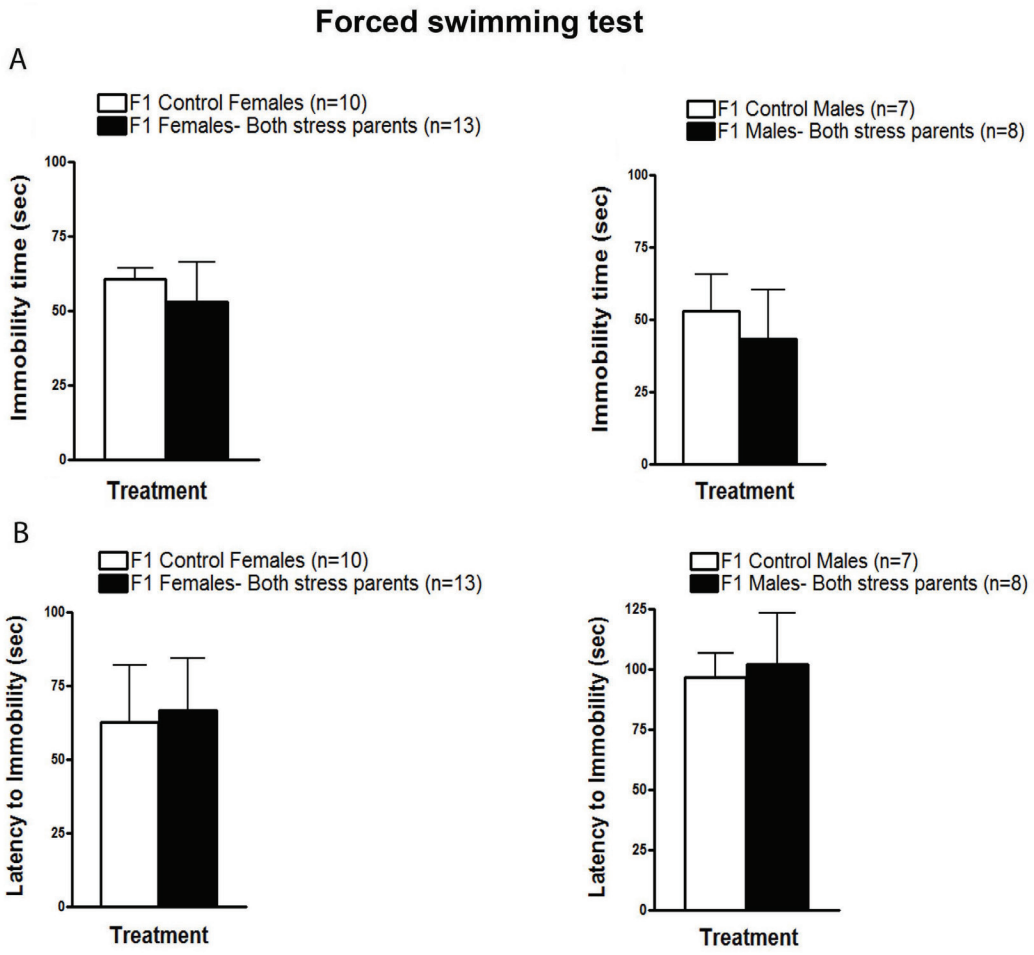

Figure S2. Evaluation of a depressive-like phenotype in F1 offspring from both stressed mice. In the forced swimming test, no significant differences were found in total immobility time [$t(4) = 0.3890$, $p > 0.05$] or latency to immobility [$t(4) = 0.1530$, $p > 0.05$] between F1 control females and F1 females from stressed parents (**A**). Similarly, F1 males from stressed parents did not show significant differences in total immobility time [$t(4) = 0.4559$, $p > 0.05$] or latency to immobility [$t(4) = 0.2523$, $p > 0.05$] in comparison with F1 control males (**B**). Error bars indicate SEM.

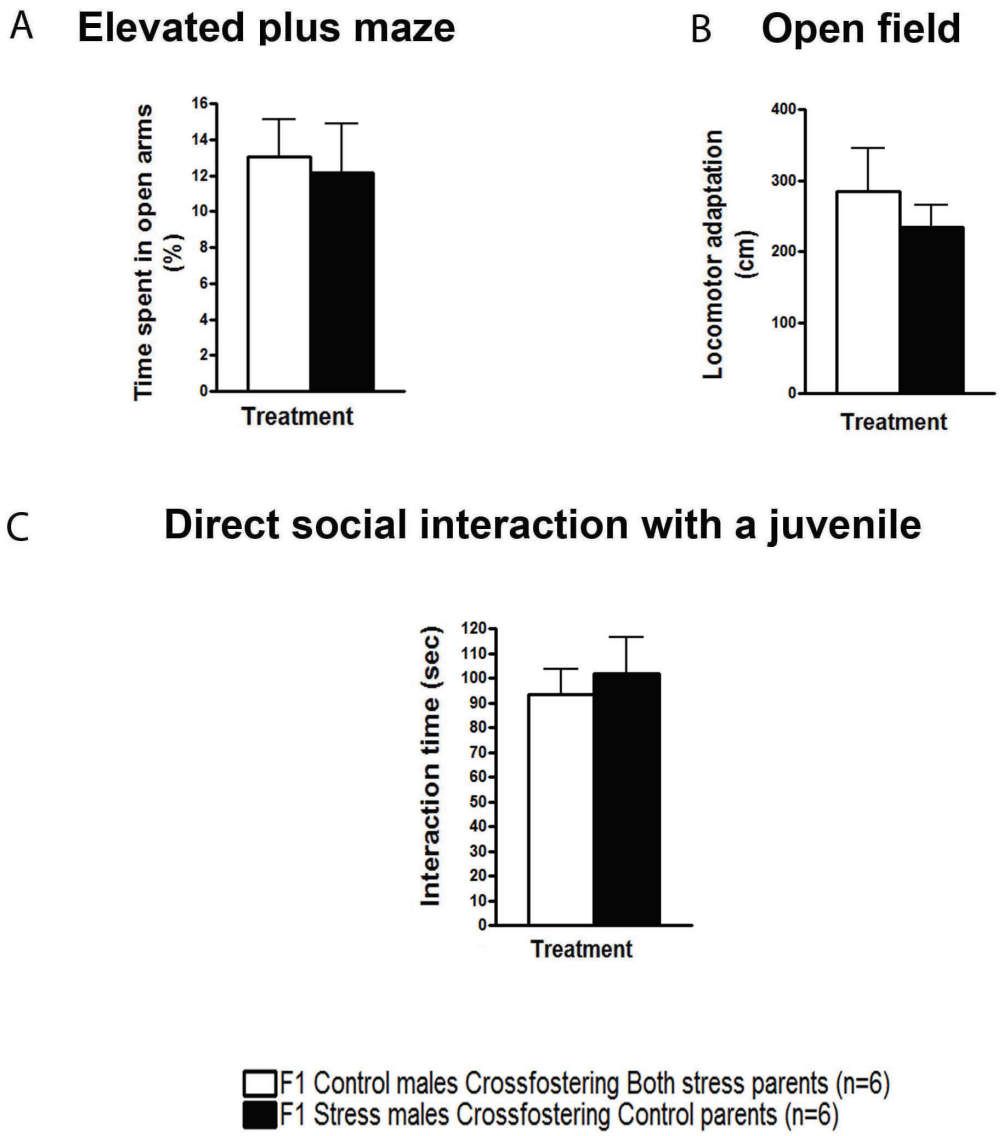

Figure S3. Anxiety-related and social behaviors of F1 male offspring are not affected by postnatal behaviors of stressed parents. Cross-fostering experiments were performed and both stressed parents were replaced at birth of their offspring with control foster parents, and vice versa. The male offspring of these crossings were tested when they were two months old. **(A)** In the elevated plus maze, no significant differences in time spent in the open arms were detected between F1 males born to stressed parents but raised by control parents (F1 Stress males Crossfostering Both control parents) and F1 males born to control parents but raised by stressed parents (F1 Control males Crossfostering Both stress parents) [$t(5) = 0.2367$, $p > 0.05$]. **(B)** In the open field, F1 males born to stressed parents but raised by control parents displayed similar

levels of locomotor adaptation to the new environment as F1 males born to control parents but raised by stressed parents [$t(5) = 0.7369$, $p > 0.05$]. **(C)** In the direct social interaction test with a juvenile, no significant differences in time spent interacting with the juvenile were found between F1 males born to stressed parents but raised by control parents and F1 males born to control parents but raised by stressed parents [$t(5) = 0.4549$, $p > 0.05$]. Error bars indicate SEM.

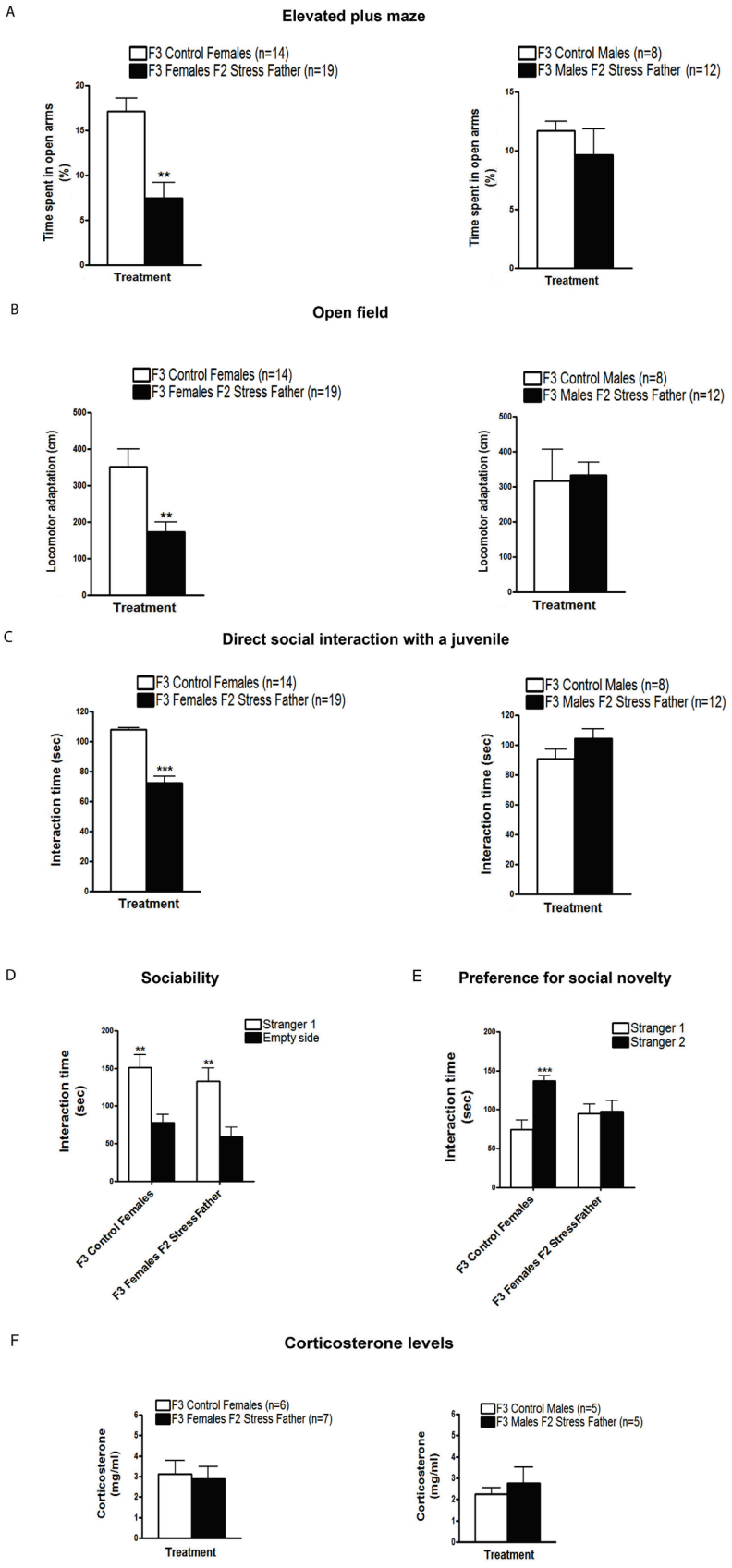
A
Elevated plus maze
F3 Control Females (n=14)
F3 Females F2 Stress Father (n=19)
Time spent in open arms (%)
Treatment
F3 Control Males (n=8)
F3 Males F2 Stress Father (n=12)
B
Open field
Locomotor adaptation (cm)
C
Direct social interaction with a juvenile
Interaction time (sec)
D
Sociability
Stranger 1
Empty side
F3 Control Females
F3 Females F2 Stress Father
E
Preference for social novelty
Stranger 1
Stranger 2
F
Corticosterone levels
Corticosterone (mg/ml)
F3 Control Females (n=6)
F3 Females F2 Stress Father (n=7)
F3 Control Males (n=5)
F3 Males F2 Stress Father (n=5)

Figure S4. Transmission of enhanced anxiety and defective social interactions to F3 female offspring. F2 males from F1 males from both stressed parents and F1 control females were mated with F2 control females, and their offspring were tested when they were two months old. F3 offspring from control mice were also generated. **(A)** In the elevated plus maze, F3 females from F2 males from the stressed parental lineage spent significantly less time in the open arms in comparison with F3 females from control mice [$t(12) = 4.041$, $p < 0.01$] (Left panel). F3 males from F2 males from the stressed parental lineage did not show significant differences in comparison with F3 control males in terms of time spent in the open arms [$t(5) = 0.5526$, $p > 0.05$] (Right panel). **(B)** In the open field, F3 females from F2 males from the stressed parental lineage showed a significant reduction in locomotor adaptation in comparison with F3 control females [$t(12) = 3.388$, $p < 0.01$] (Left panel). F3 males from F2 males from the stressed parental lineage showed similar levels of locomotor adaptation as F3 control males [$t(5) = 0.2124$, $p > 0.05$] (Right panel). **(C)** In the direct social interaction test with a juvenile, F3 females from F2 males from the stressed parental lineage spent significantly less time interacting with and investigating the juvenile in contrast to F3 control females [$t(12) = 5.042$, $p < 0.001$] (Left panel). F3 males from F2 males from the stressed parental lineage displayed similar interaction levels with the juvenile as F3 control males [$t(5) = 1.278$, $p > 0.05$] (Right panel). **(D)** In the sociability test, both F3 control females ($n = 7$) and F3 females from F2 males from the stressed parental lineage ($n = 6$) spent significantly more time sniffing and interacting with the cage containing the stranger 1 than with the empty cage [$F(1,20) = 24.63$, $p < 0.01$]. **(E)** In the preference for social novelty test, F3 control females ($n = 7$) spent significantly more time interacting with and sniffing the cage containing the stranger 2 in comparison to the cage containing the stranger 1 [$F(1,20) = 9.177$, $p < 0.001$]. In contrast, F3 females from F2 males from the stressed parental lineage ($n = 6$) did not show any difference in time spent interacting with both enclosed strangers ($p > 0.05$). **(F)** Basal corticosterone levels in plasma were similar between F3 females from F2 males from the stressed parental lineage and F3 control females [$t(10) = 0.2452$, $p > 0.05$] (Left panel). Likewise, no differences in basal corticosterone levels were found between F3 males from F2 males from the stressed parental lineage and F3 control males [$t(8) = 0.6882$, $p > 0.05$] (Right panel). Error bars indicate SEM. (** $p < 0.01$, *** $p < 0.001$).

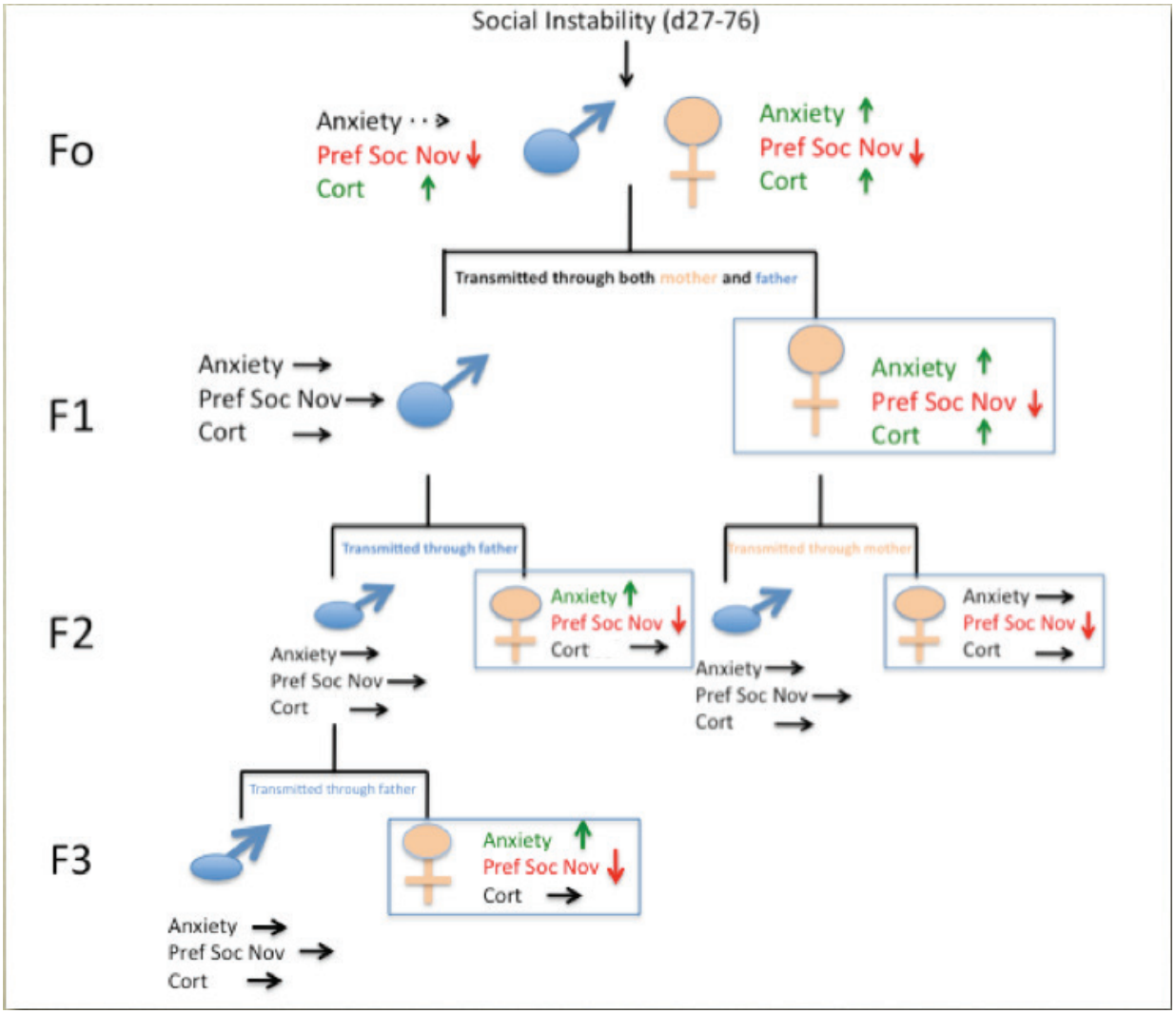

Figure S5. Differences in the transgenerational transmission patterns of anxiety and preference for social novelty observed in female offspring after chronic social instability through the male and female lineages. Social instability was introduced from postnatal day 27-76 on both male and female CD1 mice. F0 mice were tested for corticosterone (Cort) levels 3 weeks later and in behavioral assays for anxiety (elevated plus maze, open field and direct social interaction) and preference for social novelty (Pref Soc Nov) 2 months after stress exposure. Green lettering and up arrow signify elevated level, red lettering and down arrow signify decreased level, and black lettering and level arrow mean no change. F0 males showed normal elevated plus maze and open field performance but decreased direct social interaction, so a dotted arrow signifies a mixed result.

Another set of stressed mice was mated with each other or control mates. F1 offspring were raised in a control environment and tested for cited behaviors and cortisone levels at 2 months of age. The results were the same when two previously stressed mice were mated, when stressed males were mated with control females or stressed females were mated with control males. However, different inheritance patterns were observed in the F2 generation, when male or female stressed mice were mated with control partners as indicated. Finally, F2 males were mated with wild-type mice to generate F3 offspring. Differences in the phenotypes of F2 and F3 females generated from either F1 males or F1 females are highlighted by enclosure in box.

Supplemental References

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