Social but not metabolic stress in adolescence alters offspring social behavior and oocyte *Crhr1*/miR-34c expression: a four-generation study

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Supplementary Methods and Results

Experimental Procedures

Blood collection

Bloodserum corticosterone was assessed 24h after the 7-day stress ended; blood BDNF and bloodserum Oxytocin levels were assessed on postnatal day 60 (PD60) in behaviorally-naïve animals. To produce serum, 2 mL of blood were extracted from each animal. Each tube was incubated at room temperature for 40-120 minutes, and then centrifuged for 20 minutes at 22°C and 3,000 rpm. Serum tubes were stored at -80°C freezer until further analysis.

Hormone Treatment and Sample Collection

Hormone treatment was administered to induce ovulation in a subset of rats (n=8 per group). Ovulation was induced using a two-step hormonal regimen. Initially, rats (PD63) received an

intraperitoneal (i.p.) injection of 40 IU of pregnant mare serum gonadotropin (PMSG, St. Louis, MO, United States) to stimulate follicular development. Forty-eight hours later, rats were administered 40 IU of human chorionic gonadotropin (Sigma-Aldrich) to induce ovulation (PD65). On PD66, mature oocytes were collected from oviducts. Oocyte samples were flash-frozen and stored at -80°C for subsequent mRNA and miRNA expression analysis. Brain and blood samples were collected from the same rats for gene expression and hormone/protein analysis.

RNA Extraction and RT-PCR for mRNA Expression

For oocytes, RNA was extracted using the miRNeasy Micro Kit (Qiagen, GmbH, Hilden, Germany), with a slight modification: 10 µL of glycogen was added after homogenization with TRIzol to enhance RNA recovery.

Brain tissue samples were prepared as previously described[1,2]. Briefly, samples were homogenized in 300 µL of TRIzol and suspended in total 0.5 mL, with 7 µL of glycogen added. After adding, 200 µL of chloroform, phase separation was done by centrifugation at 14,000 rpm for 15 minutes at 4°C. The aqueous phase was collected, mixed with 250 µL of isopropanol, and incubated overnight at −20°C. RNA was then pelleted by centrifugation at 14,000 rpm for 17 minutes at 4°C.

The RNA pellet was washed twice: first with 500 µL of cold 100% ethanol, then with 500 µL of cold 75% ethanol, each wash followed by centrifugation at 7,500 rpm for 5 minutes. The pellet was then air-dried and resuspended in 25 µL of RNase-free water. RNA concentration and purity were assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The 260:280 nm absorbance ratio was used to evaluate RNA quality, and samples were excluded if the ratio was outside the range of 1.7–2.0 or if RNA concentration was below 40 ng/µL.

DNA digestion was carried out using 1 µL of DNase (Fermentas), incubated at 37°C for 30 minutes, followed by heat deactivation at 65°C for 10 minutes. Reverse transcription was performed using the qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, USA), according to the manufacturer's protocol.

For quantitative real-time PCR (qRT-PCR), cDNA was amplified in a 10 µL reaction containing 0.6 µL of 10 µM primers, 5 µL of PerfeCTA SYBR Green (Quanta Biosciences, Gaithersburg, USA), 2 µL of cDNA (diluted 1:9 with H2O), and 2.4 µL of ddH2O. The RT-PCR was performed using a Step One RT-PCR machine (Applied Biosystems, Carlsbad, CA) under the following conditions: 95°C for 20 seconds (holding stage), followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Primers for *Crhr1* were designed using Primer3 software[3] and *Oxtr,* and *Bdnf* primers were synthesized by Agentek (Tel Aviv, Israel). Primer suitability was confirmed through standard curve analysis, melting curve analysis, and linearity at threshold (see Supplementary **Table S1** for sequences).

mRNA expression levels of *Crhr1*, *Oxtr*, and *Bdnf* were analyzed. Fold-change values were calculated using the ΔΔCt method relative to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) or 18s (for oocytes) as previously described[2]. dCt values for housekeeping genes were consistent across groups (<0.5 Ct difference, all p's > 0.1).

miRNA Expression Analysis for Oocytes

miRNA expression analysis was conducted as previously described[2]. Briefly, RT reactions were performed using the qScript microRNA cDNA Synthesis Kit. The expression of miR-34a-5p and miR-34c-5p was assessed using SYBR Green qRT-PCR amplification, with 5 ng of total RNA in a 20 µL reaction volume. Specific primers (0.4 µL each, Quanta Biosciences, Gaithersburg, USA) were used according to the manufacturer's instructions. RT reactions were conducted using a Step One real-time PCR system (Applied Biosystems). Fold-change values were calculated using the ΔΔCt method, normalized to the housekeeping gene RNU6.

Maternal Care

Maternal behavior of F0 dams and their interaction with F1 pups was monitored using instantaneous sampling, as previously described[4]. Daily maternal observations were conducted from PD0-14, with each session lasting 20 minutes, carried out between 8:00 a.m. and 12:00 p.m.

Cages were placed on a table in the breeding room, and no disturbance or direct contact with the animals occurred during the recording periods. All video recordings were made while the dam and litter were in their home cage. The video recordings were randomly assigned to two independent scorers, blind to the experimental conditions, who recorded each dam's behavior every minute, resulting in 20 observations per session.

Observers manually identified specific behaviors. Observer reliability was evaluated by having the two observers rate several overlapping videos, chosen randomly. Maternal behaviors were divided into three main categories: Dam Self-Care behaviors, Pup-Care behaviors, and Aggressive behaviors. Self-Care behaviors included independent movement, where the dam moved around the cage, reared, or sniffed the walls; self-grooming, involving wiping, licking, combing, or scratching any part of her body; resting away from pups, where the dam lay alone out of the nest without contact with pups; along with eating and drinking behaviors.

Pup-Care behaviors involved moving the pups, where the dam carried a pup to or from the nest; resting by the pups, where the dam interacted with the pups without nursing them; licking and grooming (LG) any part of the pup's body; tidying the nest, where the dam manipulated bedding material to maintain the nest; arched-back nursing (ABN), where the dam nursed the pups with an arched back and splayed hind limbs; blanket nursing, where the dam nursed the pups by lying over them without arching her back or extending her legs; and passive nursing, where the dam lay on her back or side while the pups nursed.

Aggressive behaviors were recorded, including rough handling and aggressive grooming of the pups, transporting pups by a limb, stepping or jumping on pups, throwing or dragging them across the cage, or dropping them during transport. Additionally, pushing-away behavior was noted, where the dam actively moved away from an approaching pup or pushed it away. The frequencies of these behaviors were compared across experimental and control groups, and the averages for each behavior were calculated for each dam across the observation period, providing a detailed analysis of maternal care patterns during the first 14 postnatal days.

It should be noted that behavioral categories were not mutually exclusive, as certain behaviors, such as LG, could occur while the mother was nursing the pups. Arched-back nursing, blanket

nursing, and passive nursing were grouped together under the Nursing variable. The average of the two observers' assessments for each video was calculated, and daily observations of each dam were averaged.

Forced Swim Test analysis in DeepLabCut (DLC)

DeepLabCut (DLC) 2.2.1 was used to track rodent behavior during Forced Swim Test (FST) sessions across different generations, based on previous work[5,6]. Due to differences in video quality (e.g., resolution, lighting) between generations, two separate pre-trained models were fine-tuned: one for the F0 generation and another for generations F1-F3. Each model was fine-tuned using 500,000 iterations with default settings (multistep: 0.005, 0.02, 0.002, 0.001).

Custom Python scripts were developed and used for each generation to enhance data analysis. The calibration script optimized the "sense" threshold to classify 'moving' vs. 'not moving' based on distance between frames, maximizing correlation with manual assessments. The analyzer script applied this threshold to analyze DLC data and output movement metrics. The data generated by DLC was then processed using these custom scripts, available in the supplementary data on GitHub: https://github.com/IlyaDobrovinsky/DLC_FST_Analysis.

Supplementary Results

Figure S1

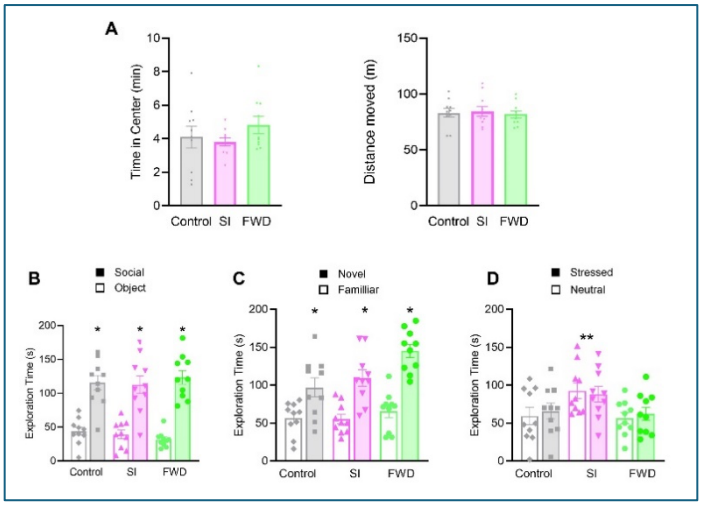

Fig S1. SI or FWD stress in adolescence have no effect on adult behavior in Open Field (OF), social preference, social recognition or Emotional State Preference (ESP). (**A**) SI and FWD-exposed female rats showed Control-like behavior in total locomotor activity (m) and time in the center (**B**) All 3 groups showed social preference, i.e., increased

exploration of the social stimulus vs. the object. (**C**) All 3 groups showed social recognition, i.e., increased exploration of the novel vs. familiar social stimulus (**D**) In ESP, SI-exposed rats exhibited increased exploration compared to the FWD and Control groups. Data presented as means and standard errors relative to Control. *p<0.05. **p<0.01.

Figure S2

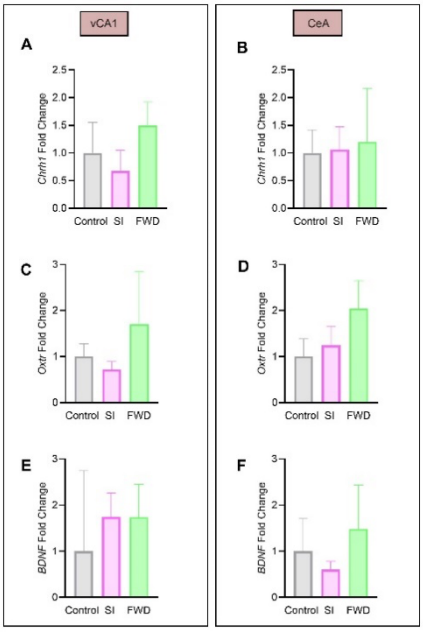

Fig S2. *Crhr1*, *Oxtr* and *Bdnf* expression in vCA1 and CeA of F0-exposed rats. No changes in *Crhr1* (**A, B**), *Oxtr* (**C, D**) or *Bdnf* (**E, F**) mRNA expression were observed in either ventral CA1 of hippocampus (vCA1) or Central Amygdala (CeA), respectively.

Figure S3

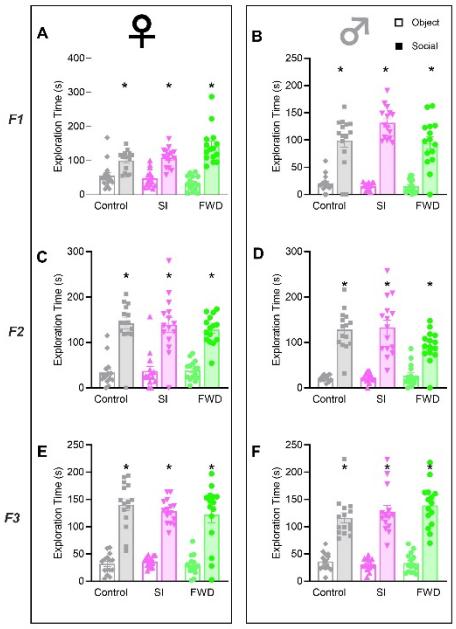

Fig S3. Social preference remains intact in F1-F3 offspring of FWD- and SI-exposed rats. (**A-B).** Female (left) and male (right) F1 offspring of SI- and FWD-exposed females showed intact social preference, i.e., increased exploration of the social stimulus vs. the object (**C-D**) Female (left) and male (right) F2 offspring of SI- and FWD-exposed females showed intact social preference. (**E-F**) Female (left) and male (right) F3 offspring of SI- and FWD-exposed females showed intact social preference. Data presented as means and standard errors. *p<0.05 post-hoc Social vs. Object.

Figure S4

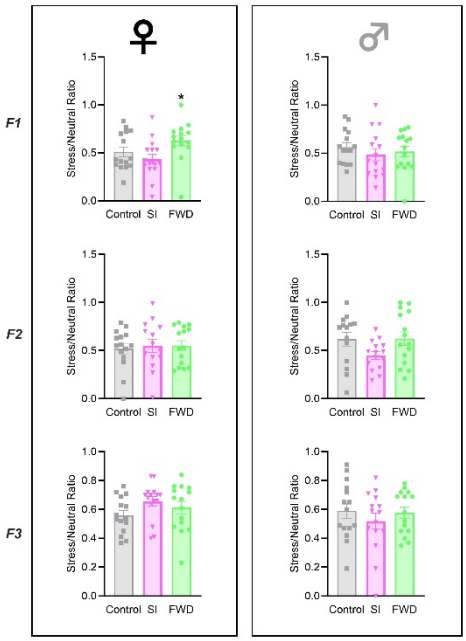

Fig S4. The exploration ratio of the stress-exposed vs. neutral social stimulus in ESP in F1-F3 offspring. The Control group exhibited no preference for the stressed over the neutral stimulus in females (left) or males in any of the generations. Female F1 offspring showed a slight preference for the emotional stimulus (Top, left).

Figure S5

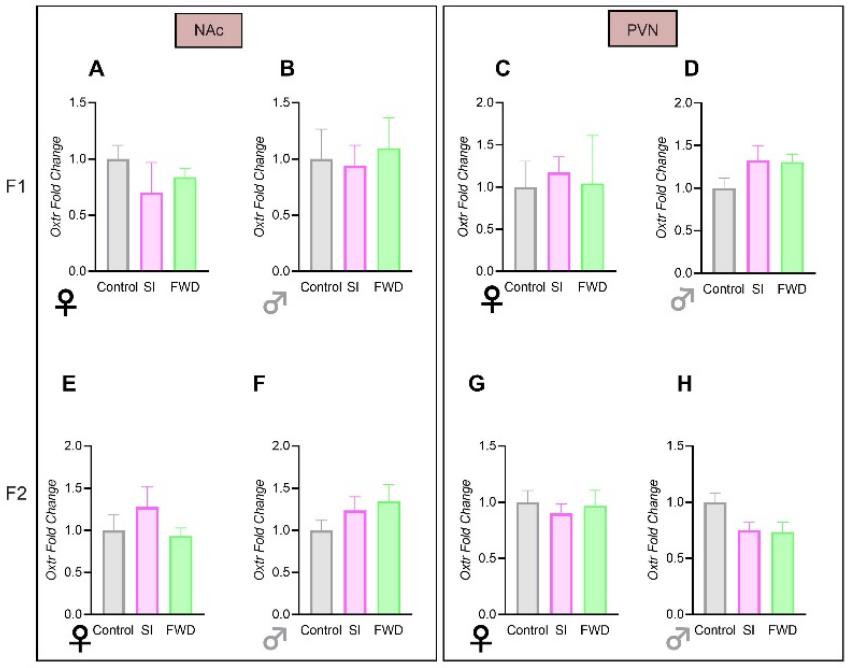

Fig S5. *Oxtr* mRNA expression in Nucleus Accumbens (NAc) and Paraventricular nucleus of the hypothalamus (PVN) of offspring. In F1, no changes were found in *Oxtr* expression in NAc of (**A**) female and (**B**) male offspring, and in the PVN of (**C**) female and (**D**) male offspring. In F2, no changes were found in *Oxtr* expression in NAc of (**E**) female and (**F**) male offspring, and in PVN of (**G**) female and (**H**) male offspring.

Supplementary Tables

Table S1*: Complementary Statistics of Behavioral Tests Not Significant. male and female were analyzed separately when sex effect or sex interaction were significant.*

Behavioral Test		***F0***	***F1***	***F2***	***F3***
Open Field (OF)	**Distance Moved (m)**	F= 0.132, *p*=n.s., η^2= 0.10	F=3.02, *p*=n.s., η^2= 0.073	F=0.392, *p*=n.s., η^2= 0.009	F=0.326, *p*=n.s., η^2= 0.008
	Time in Center (min)	F=1.119, *p*= n.s., η^2=0.077	F=0.863, *p*=n.s., η^2= 0.02	F=2.02, *p*=n.s., η^2= 0.046	F=0.579, *p*=n.s., η^2= 0.014
Social Preference	**Group * factor Interaction**	F=0.103. p=n.s., η^2=0.008	Females F=1.177, *p*=n.s., η^2= 0.01 Males F=2.967, *p*=n.s., η^2= 0.162	Females F=0.142, *p*=n.s., η^2= 0.04 Males F=2.114, *p*=n.s., η^2= 0.091	Females F=0.78, p=n.s., η^2= 0.036 Males F=1.066, p=n.s., η^2= 0.049
Elevated Plus Maze (EPM)	**Open/closed Arms Frequency Ratio**	Significant Changes (see Manuscript)	F=0.547, *p*=n.s., η^2= 0.013	F=1.547, *p*=n.s., η^2= 0.036	F=0.535, *p*=n.s., η^2= 0.015
Forced Swim Test (FST)	**Immobility Ratio* factor Interaction**	Significant Changes (see Manuscript)	F=0.027, *p*=n.s., η^2= 0.001	F=0.647, *p*=n.s., η^2= 0.015	F=0.511, *p*=n.s., η^2= 0.013

Table S2: *F1-F3 neoatets weight in birth (gram)*

	F1		***F2***		***F3***
	Mean, SE		***Mean, SE***		***Mean, SE***
Control	5.928	0.053	6.373	0.043	6.165, 0.057
SI	6.152	0.0348	6.441	0.049	6.835, 0.116
FWD	6.139	0.0494	6.342	0.049	6.337, 0.446

Table S3: *Averaged behavior frequencies in all 4 categories examined during Maternal Care analysis: Mom Self-Care, Pups Care, Aggressiveness, and Nursing per specific animal.*

Animal	Group	Mom Self-Care	Pups Care	Aggressiveness	Nursing
1.2	SI	134	203	2	141
1.3	SI	108	215	12	159
2.2	SI	75	289	19	202
2.3	SI	98	223	20	159
2.4	SI	63	267	21	215
4.4	SI	96	212	8	129
5.1	SI	111	204	7	152

7.3	Control	160	173	4	104
8.1	Control	122	199	4	154
8.2	Control	117	213	1	148
10.3	Control	127	205	20	132
11.1	Control	87	253	22	177
11.3	Control	108	213	19	166
11.4	Control	79	243	27	176
3.1	FWD	129	168	5	136
3.4	FWD	64	249	19	197
6.1	FWD	120	208	21	154
9.1	FWD	218	109	14	72
9.2	FWD	139	176	4	109
12.1	FWD	83	232	10	171
12.3	FWD	68	262	22	217

Table S4: *Primers used in the real-time PCR*

Gene	***Forward Primer***	***Reverse Primer***
S18	5′GTGACCACGGGTGACG3′	5′TTTTCGTCACTACCTCCCCG3′
HPRT	5′CGCCAGCTTCCTCCTCAG3′	5′ATAACCTGGTTCATCATCACTAATCAC3′
Crhr1	5'ACGAAGAGAAGAAGAGCAAAGTACA3′	5'AGATGCAGTGACCCAGGTAGTTG3′
Oxtr	5' AATGCGCCCAAGGAAGCT3'	5'GCACGAGTTCGTGGAAGA3'
BDNF	5'AGGCTGCGCCCATGAAAGAAG3'	5'GTCCGCACAGGTGGGTAG3'

References

1 Zaidan, H., Leshem, M. & Gaisler-Salomon, I. Prereproductive stress to female rats alters corticotropin releasing factor type 1 expression in ova and behavior and brain corticotropin releasing factor type 1 expression in offspring. *Biol Psychiatry* **74**, 680-687 (2013). https://doi.org/10.1016/j.biopsych.2013.04.014

2 Zaidan, H., Galiani, D. & Gaisler-Salomon, I. Pre-reproductive stress in adolescent female rats alters oocyte microRNA expression and offspring phenotypes: pharmacological interventions and putative mechanisms. *Transl Psychiatry* **11**, 113 (2021). https://doi.org/10.1038/s41398-021-01220-1

3 Rozen, S. & Skaletsky, H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365-386 (2000). https://doi.org/10.1385/1-59259-192-2:365

4 Zaidan, H., Wnuk, A., Aderka, I. M., Kajta, M. & Gaisler-Salomon, I. Pre-reproductive stress in adolescent female rats alters maternal care and DNA methylation patterns across generations. *Stress* **26**, 2201325 (2023). https://doi.org/10.1080/10253890.2023.2201325

5 Ritter, A., Habusha, S., Givon, L., Edut, S. & Klavir, O. Prefrontal control of superior colliculus modulates innate escape behavior following adversity. *Nat Commun* **15**, 2158 (2024). https://doi.org/10.1038/s41467-024-46460-z

6 Sturman, O. *et al.* Deep learning-based behavioral analysis reaches human accuracy and is capable of outperforming commercial solutions. *Neuropsychopharmacology* **45**, 1942-1952 (2020). https://doi.org/10.1038/s41386-020-0776-y