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Supplementary Information for

What happens to the infant during maltreatment? Stress targets hippocampus, but stress with other targets amygdala and social behavior

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Supplementary Text

Online Materials

Subjects. Male and female Long-Evans rats born and bred in our colony (originally from Harlan Laboratories) were used as subjects. They were housed in polypropylene cages (34 × 29 × 17 cm) with wood shavings, in a 20 ± 1°C environment on a 12 h light/dark cycle. The day of birth was considered PN0 and litters were culled to 12 pups (6 males, 6 females) on PN1. Food and water were available *ad libitum*. Unless otherwise noted, only one male and/or one female were used from each litter per experimental group. Experimental groups have an approximately equal representation of males and females. All procedures were approved by the Institutional Animal Care and Use Committee of Nathan Kline Institute and New York University, in accordance with guidelines from the National Institutes of Health.

Maltreatment Scarcity-Adversity Model rearing. All pups experienced typical rearing until PN8 and again after PN12. At PN8, half of the litters were randomly assigned to the maltreatment rearing, where cage bedding was reduced to a 1.2 cm layer nesting/bedding material from PN8-12. These ages were selected based on previous research from our laboratory and others that suggested experiencing adversity during this age range produces enduring neurobehavioral effects across development (1-4). Furthermore, previous research had shown that young infant rats had corticosterone increases from adversity at this age and that corticosterone was highly correlated with pup neurobehavioral function (1, 5, 6).

This limited bedding environment decreased mothers' ability to construct a nest, which resulted in frequent nest building, spending more time away from the nest and pups, rough handling, and stepping on pups (see Fig. 1a,d). This procedure does not alter pup body weight at any age, including PN13 ($t_{(8)}$ = 0.100, p = 0.923) but increases corticosterone levels in response to a mother-pup social behavior test ($t_{(6)}$=6.950, p<0.05)(2, 4, 7). Control litters continued to have typical rearing with 5–7 cm layer nesting/bedding material throughout development. All cages were cleaned at least twice a week throughout development, including during the reduced bedding procedure. Maternal and pup behaviors were scored daily during the experimental manipulations for all litters.

Corticosterone increase and decrease paired with mother. Daily from PN8-12, one male and one female from each litter received either an injection of corticosterone (3 mg/kg, i.p.; Sigma) or saline (0.9%) to increase pups' corticosterone levels (continuously for 5 days). The injection protocol did not produce vocalization in the pup and was completed within 10s. After the injection, pups were divided in the following treatments: 1) awake mother: these pups lived with their biological mother and were simply removed from the litter, injected with corticosterone or saline and placed back in the homecage, 2) anesthetized mother: these pups were removed from their biological mother, injected, kept in a warm incubator to maintain thermoneutral body temperature for 30 minutes, placed with an anesthetized mother (urethane to prevent milk letdown) for 90 minutes and then returned to their biological mother and, 3) tube nonsocial: these pups were removed from their biological mother, injected, kept in a warm incubator for 30 minutes, placed with a polyethylene tube for 90 minutes and then returned to their biological mother.

We used a two-pronged approach to testing by using a naturalistic observation of pups within the nest with the mother, combined with an out of nest test with an anesthetized mother to eliminate the impact of maternal behavior, which can obscure pup behavioral competence and deficits. Importantly, pups do not discriminate between their own mother or an unrelated same age postpartum mother provided they eat the same diet (maternal odor is diet-dependent) (8, 9). Thus, pups removed from the nest for experiments were returned to their home cage with an undisturbed mother with typical maternal care.

To block corticosterone changes associated with maltreatment, PN8-12 pups experienced 5 days with 1 hour daily experience with low bedding. Each day, 1 male and 1 female from each litter received an i.p. injection of metyrapone HCL (50 mg/kg, Sigma) or an equal volume of 0.9% saline and were returned to the homecage. 90 minutes after injection, half

of the nursing dams' bedding was reduced from 4000 mL to 100 mL (low bedding), for 1 hour and then control bedding levels returned. Timing of this procedure limited corticosterone reduction effects to within the hour-long window of abuse.

Corticosterone radioimmunoassay. Sixty minutes after the final treatment, pups were decapitated and trunk blood collected and centrifuged at 14,000 rpm for 6 minutes. Serum was stored at -70°C until radioimmunoassay was performed. Duplicate serum samples were analyzed for corticosterone using the Rat Corticosterone Coat-a-Count kit (Diagnostic Products Corporation; sensitivity 5 ng/mL).

Mother-pup social behavior test. At PN13, all pups received a 30 minute social behavior test with an anesthetized mother (milk letdown blocked) placed on her side to give pups access to nipples. Pups were individually placed with the mother and permitted to freely interact with the mother. Tests were recorded and scored blind to experimental condition. The Ethogram included time nipple attached and time behind the mother's back. We used an anesthetized mother to eliminate the impact of maternal behavior, which can obscure pup behavioral competence and deficits. Since pup behavior towards the mother is guided by maternal odor and the maternal odor is learned and diet-dependent, the biological mother or a mother of similar postpartum age was used as the anesthetized mother for treatment and testing. Indeed, pups will orient towards a dam and nipple attach if her diet is the same as the diet fed to the biological mother (8-10).

c-Fos immunohistochemistry. Sixty minutes after the 30 minute mother-pup social behavior brains were removed and processed using a standard c-Fos staining. Briefly, brains were sliced into 20 µm coronal sections and mounted on slides. After 1 hour of postfix in 4% paraformaldehyde/0.1 mol/L phosphate buffer (PB) (pH 7.2) each slide was rinsed in PB (pH 7.2) and dried by cool airstream. To eliminate peroxidase activity, sections were incubated in 0.1 mol/L phosphate buffer saline (PBS) (pH 7.2) containing 3% hydrogen peroxide (H_2O_2) and 10% methanol for 5 minutes. Following PBS rinses and 15 minutes incubation in .2% Triton X-100 (Sigma, St. Louis, Missouri), slides were incubated in 3% Bovine Serum Albumin (Sigma) for 1 hour. After additional PBS rinses, slides were treated overnight at 4°C with the primary antibody (c-Fos, sc-52, Santa Cruz Biotechnology, Santa Cruz, California) diluted 1:500 in PBS. Afterward, they were rinsed in PBS, incubated in the secondary biotinylated antibody (goat anti-rabbit, Vector Laboratories, Burlingame, California) for 2 hours at room temperature, and then incubated for 90 minutes in avidin-biotin-peroxidase (ABC) complex solution. Slides were then treated with PB containing 0.1% 3,3'-diaminobenzidine and H_2O_2 and dehydrated in alcohol and Histoclear (National Diagnostics, Atlanta, Georgia) followed by coverslipping for microscope examination. A microscope equipped with a drawing tube (Olympus Optical Co., Tokyo, Japan; 10 X objectives) was used to count c-Fos-positive cells. Brains were outlined delineating the amygdala (BLA, CoA, MeA, and CeA) and hippocampus (CA1, CA3 and DG). All c-Fos cells were counted without prior knowledge of treatment and were distinguished from background based on density of staining, shape and size of the cell. The mean number of c-Fos positive cells per brain area was determined by averaging the bilateral cell count from all sections (three sections per brain).

Amygdala cannula implantation and muscimol infusions. At PN12, pups were anesthetized by isoflurane inhalation and cannulae implanted bilaterally into the amygdaloid complex targeting the BLA (caudal: -0.90mm; lateral ±4.50mm from bregma), lowered 6.00mm and fixed to the skull. Within 30 minutes, pups were returned to the mother. At PN13, the cannulae were used to bilaterally deliver vehicle or muscimol via a Harvard syringe and pups were placed in the social behavior test described above. After testing, brains were removed, frozen, sectioned (20µm) in a -20°C cryostat, and cresyl violet stained for identification of the cannula placement in relation to amygdala using an atlas (11). Eight rats were eliminated because their cannula placements were off-target.

Anatomy*:* Volume measurements*.* To obtain a volume measurement for the amygdala and hippocampus, separate cohorts of pups received abusive or control rearing (Experiment 1) or corticosterone/saline treatments (Experiment 2) from PN8-12 and were perfused with ice cold 4%

PFA at PN13 and brains stored until sectioning (1:12, 40 um) on a vibratome. Sections were stained with DAPI and area measurements taken using StereoInvestigator software by manually tracing the basolateral and central amygdala and hippocampus on all slices containing those areas as defined by Paxinos and Watson (11). The volume of each amygdala nuclei and hippocampus were calculated using Cavalieri's principle using the formula for a truncated cone (12).

Neurogenesis. To quantify endogenous markers of immature neurons, separate cohorts were perfused with cold 4% PFA, cryoprotected and sectioned on a freezing microtome. Sections were processed and incubated overnight with primary antibody against doublecortin (DCX, 1:100, SC Biotechnologies sc-8066) and DAPI (ThermoFisher USA, cat. No. 62248) using established protocols (13-15). Brains were sectioned in a 1:12 series and 3 sections per brain with highest amygdala DCX counts were included in group comparisons (16). Following counterstaining with secondary antibodies, DCX-expressing cells were counted in the region of interest and multiplied by 24 to obtain a stereological estimate. A density measure was obtained by dividing total counts over the total cross sectional volume (see Volume).

Electrophysiology. Separate cohorts of animals were exposed to either, maltreatment or control rearing (Experiment 1) or corticosterone/saline treatments (Experiment 2) beginning at PN8. At PN13, wireless telemetry was employed to measure LFP oscillations in the amygdala during untethered social behavior with the mother to ensure minimal interference with other ongoing behaviors. Importantly, our LFP analyses allow an assessment of the temporal course of activity during free behavior in pups.

For electrode implanting, the PN12 pups were anesthetized and kept unconscious with an isoflurane anesthesia system (E-Z Systems, Palmer, PA) during surgery. Pups were placed in a stereotaxic apparatus under aseptic conditions. The scalp was reflected and skull dried. A hole was drilled for the recording electrode using coordinates to target the left amygdala (region, coordinates (~0.9mm posterior to bregma, ~4.5mm laterally over the left hemisphere, ~6.0mm ventral to the surface of the brain) and a hole drilled for the reference electrode over the right hemisphere. A Teflon-coated 0.18 mm diameter stainless steel electrode was lowered to the desired depth and dental cement was placed over the hole to hold the electrode in place. The electrode was connected to a telemetry pack (ETA-F10 telemetric device, DSI) inserted into the pup subcutaneously over the animal's back. Topical lidocaine hydrochloride jelly (2% Akorn) was applied to the wound and closed with sutures. A small amount of glue (Vetbond) was then placed over each suture. Anti-nail biter liquid was also applied to the area around the sutures to prevent the mother from over-grooming the wound. Prior to waking, each pup was injected with 1cc of 0.9% saline. Upon waking, pups were placed in an incubator for 30 minutes to 1 hour until observed to be fully recovered and mobile, then returned to the mother and continuously observed for several hours to ensure that the mother allowed the pup to nurse. Histological confirmation of electrode placement after the experiment permitted us to exclude two animals with off-target locations from analysis.

LFP Recordings. Spontaneous LFP recordings were obtained 24 hours after electrode implantation during a social behavior test with an anesthetized mother. Briefly, for a typical recording session, the pup was placed in a small arena in a sound-attenuated recording booth and amygdala LFP activity was recorded continuously throughout a 10 minute habituation. Immediately following this, a mother (anesthetized with urethane to block milk letdown) was placed in the arena and the pup was allowed to interact with the mother for 20 additional minutes. Behavioral activity of the pup was indicated on the neural trace continuously for offline analysis. Movement artifacts were analyzed offline using established protocols. Neural signals were amplified, filtered (0.5 to 300 Hz) digitized at 2 kHz with Spike2 software (CED, Inc) and analyzed offline. Recordings were all from the left amygdala. Previous work has suggested occasional lateral asymmetry in rodent amygdala functions (17) and this will be assessed in future work. After the recordings, the brains were removed and sectioned to verify electrode placement.

LFP data analysis. Fast Fourier Transform (FFT) power analyses were performed on the raw LFP data in intervals taken from sections of each day's neural trace that correlate with a specific behavioral state (alone versus with mom) to quantify LFP oscillatory power in 2.9 Hz frequency bins from 0–100 Hz (Hanning). Power in the theta (5–15 Hz), beta (15-35 Hz) and gamma (35-80 Hz) frequency bands was calculated for each specified window. The change in LFP oscillatory power as a function of the mother's presence was calculated as ratio of LFP power during maternal presence versus alone. One minute in each state (alone versus with mom) was used to calculate ratios. Repeated measures ANOVA's were run to test for main effects of maternal presence duration on LFP power, followed by *post hoc* analyses to examine differences between specific LFP frequencies (ANOVAs or *t*-tests to compare specific frequency bins).

Statistical analysis. Depending on the number of groups to be analyzed, behavioral and neural data were analyzed with Student's *t*-tests, two-way analysis of variance (ANOVA) or repeated measures ANOVA. When significant, ANOVAs were followed by Newman–Keuls *post hoc* tests. Further analyses utilized planned comparisons to test the *a priori* hypothesis that: 1) maltreatment or corticosterone injection will alter behavioral or neural outcomes compared to controls, and 2) blocking corticosterone will prevent the behavioral effects of high corticosterone. Data used for figures are expressed as mean (±SEM) and in all cases differences were considered significant when $p \leq 0.05$.

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