Reducing Behavioral Inhibition to Novelty via Systematic Neonatal Novelty Exposure: The Influence of Maternal Hypothalamic-Pituitary-Adrenal Regulation

Supplemental Information

Supplemental Methods

All experimental procedures were in accordance with the Institutional Animal Care and Use Committee at the University of New Mexico.

Neonatal Novelty Exposure (1-3)

On post-natal day (PND) 0, approximately one-half of each litter was pseudo-randomly assigned to the Novel and the other half to the Home conditions (split-litter design), with body size counter-balanced between the two groups by visual inspection. Apparent body size rather than precisely measured body weight was used to minimize experimenter manipulation that was not pertinent to key experimental manipulations. In comparison to the default random assignment procedure, by taking apparent body size into consideration, additional information was used to counter-balance the distribution of body size across the Novel and Home groups, thus contributing to an improved experimental design. Group membership was marked by tattooing the left and right hind paws of the Novel and Home pups using two different digit combinations: (1) left first and right fifth, and (2) right first and left fifth. The precise patterns of marking were counterbalanced between the Novel and Home groups across litters.

During PND1–21 between 1000 and 1500 h, the neonatal novelty exposure procedure (Figure 1A and B) was carried out in the animal housing room and involved half of each litter spending 3 min away from the familiarity of their home cage (Novel group) and their matched
control siblings remaining in the home cage (Home group). Specifically, the dam was first removed from the home cage. The Novel and Home pups were then identified by examining toe markings. Once identified, Novel rats were placed in a new cage (30 cm × 19 cm × 13 cm) lined with fresh bedding for their 3-min exposure and subsequently returned to their home cage in which the Home rats remained. During this transfer, each Novel pup was yoked to a Home pup that received a matching amount of experimenter contact at approximately the same time as the yoked Novel pup. The dam was returned to the litter after both the Novel and Home pups were reunited in the home cage. The amount of touching by the experimenter and the duration of maternal separation during this novelty exposure procedure was matched between the Novel and Home rats, thus ensuring that any difference in outcome measures between the two groups was attributable to neither dam separation nor experimenter touch. It should be noted that the total duration of separation between dams and all of her pups was less than 15 min, which does not constitute the kind of prolonged maternal separation (>3 h) known to result in deficits in offspring emotional, cognitive, and neuroendocrine function (for review see 4).

This procedure is in contrast to the commonly used neonatal handling procedure (5-7) that utilizes a between-litter design in which the Non-Handled litters remained entirely undisturbed and the Handled litters experienced a combination of at least four manipulations: 1) “handling” of experimental animals by the experimenter; 2) separating the neonates from their mother; 3) increasing the mother’s stress by separating her from her pups; and 4) exposing the neonates to an unfamiliar environment, i.e. novelty. Therefore, by using a split-litter or within-litter design, the novelty exposure component is isolated from the other three confounding factors, including maternal stress and associated maternal behavioral differences, inherent to the neonatal handling procedure (1-3).
Disinhibition to Novelty

When exposed to a novel environment, animals typically show at least a brief period of behavioral inhibition, expressed as freezing or displaying little movement, followed by a period of increased exploration, which we refer to here as disinhibition. This rapid change in the initial behavioral response to novelty was examined using a unique open field procedure consisting of multiple shortly spaced 20-s long exposures (8, 9). In contrast to the typically used open field testing parameters which include several minutes (up to 30 min) of continuous exposure, this procedure allows efficient assessment of the rat’s initial response to a novel environment.

Specifically, on PND24, animals were exposed to a novel open field (60 cm × 60 cm × 20 cm) during eight 20-s trials. An experimenter who was blind to the treatment condition tested animals in groups of eight. To maximize the initial fear of novelty, individual pups were placed in the center of the open field. At the beginning of each trial, rats were briefly covered by a cardboard box similar to the size of their body. The trial began immediately after the box was lifted and the rat was allowed to ambulate freely. The inter-trial interval was approximately 5 min. To minimize interference with the rat’s ongoing behavior, the experimenter remained still and in the same location during all trials. All trials were videotaped by a camera mounted directly above the open field. Activity level was defined as the number of 12 cm × 12 cm squares traversed. To quantify the rapid initial change in behavioral inhibition upon entering the open field, we used a disinhibition score, defined as the difference in open field activity between Trial 2 and Trial 1 (8, 9). We believe that this measure is fundamentally different from the measure of activity when a rat is exposed to a clearly life-threatening situation (such as predator odor) (10, 11), because while it seems adaptive for a rat to show consistent, thus non-habituating fear of
predator odor over time, it is maladaptive to show non-habituating fear to a once novel, but non-threatening, physical stimulus or social conspecific.

**Maternal Physiological Measures**

Shortly after weaning, maternal basal corticosterone (CORT$_B$) and post-swim circulating CORT (CORT$_S$) levels were measured from blood samples collected on PND26 and PND27. Blood samples for CORT$_B$ and CORT$_E$ were collected from the dams by tail nicking five and six days after weaning respectively. These specific delays from weaning were selected based on a balance among the following constraints: that they should not be 1) too distant temporally from the pups’ weaning to fail capturing the pups’ pre-weaning maternal environment; 2) too close to weaning as the disturbances associated with weaning may potentially affect the maternal CORT levels measured; and 3) measured during nursing to avoid further stressing the dams due to blood sampling.

Blood samples were collected between 1300 and 1700 hours. Because we are interested in maternal individual differences in these CORT measures, it is critical that we minimize variance in the measures, particularly variance possibly associated with circadian rhythm which can vary at least 10 fold from the trough (~ 10 ng/ml) to peak (> 100 ng/ml) during a 24 h cycle (12). Therefore, for each type of the two CORT measures, we assessed all dams over 2 consecutive days (Day 1: CORT$_B$; Day 2: CORT$_S$) within a relatively narrow time window of 4 hours and during the middle period of the light cycle when the variation is minimal. We further checked for time-of-day effects within this narrow time window across different dams and found no significant correlation between the CORT concentration levels and time of day (all $p$s > .20).
We matched the times of day for collection of CORT\textsubscript{B} and CORT\textsubscript{S} to insure that the basal and evoked measures were obtained at the same time of day for a given rat.

CORT\textsubscript{B} collection was completed in a blood collection room and took no more than 3 min from the time the dam was removed from the housing room. For CORT\textsubscript{B} samples, dams were transported from the housing room directly to the blood collection room, and for CORT\textsubscript{S} samples, dams were transported from the swim test room to the blood collection room 5 min after the onset of the 1-min swim test (water temperature of $\sim 21^\circ$C). An evoked CORT response, CORT\textsubscript{E} was defined as the difference between CORT\textsubscript{S} and CORT\textsubscript{B} normalized by CORT\textsubscript{B} (CORT\textsubscript{E} = (CORT\textsubscript{S} - CORT\textsubscript{B})/ CORT\textsubscript{B} × 100). It should be noted that we were unable to obtain a sufficient amount of serum from some dams at all time points. Hence, the \textit{n}s for the three evoked CORT measures (\textit{n}s = 13, 14, and 15) were smaller than the \textit{n} for the basal CORT measure (\textit{n} = 19). This is likely due to increased constriction of blood flow in the tail after exposure to the cold water of the swim stressor.

A high CORT\textsubscript{E} here is conceptually and operationally different from the high cortisol measures used in some studies of children in which samples were collected during school hours and the corresponding cortisol levels do not reflect responses evoked by explicit and discrete events (13). This measure also differs from a “high CORT” level observed a couple of hours after prolonged restraint (\textgeq 20 min) (14) which reflects the rate of return to baseline instead of the rising rate of CORT output. The CORT\textsubscript{E} measure here further distinguishes itself from the more typical difference-based “stress reactivity” measure (i.e. post-stress level minus baseline level) in that the CORT\textsubscript{E} here takes into consideration that the same amount of CORT increase from the baseline is likely to have differential impact on neuronal function depending upon the baseline value. For example, an increase of 100 ng/mL from a baseline of 20 ng/mL to 120
ng/mL would mean a post-stress CORT concentration that is a five-fold increase from baseline, therefore having a far greater impact on the neural system than the same 100 ng/mL increase from a baseline of 200 ng/mL to 300 ng/mL, a mere 50% increase. We believe that such a difference in the dynamics of maternal stress regulation is potentially an important part of maternal signaling to her developing infants.

All samples were processed in a single assay in duplicate. Each sample, containing ~200 μL of blood, was centrifuged, and the plasma was extracted and then stored at −20°C until radioimmunoassay (RIA) was performed. Plasma CORT concentration was measured using the Coat-a-Count CORT Kit (Diagnostic Products, Los Angeles CA). The lower limit of detection was 10.4 ng/mL and the intra-assay coefficient of variation was 13%. The descriptive statistics for CORT_B and CORT_E are 1) CORT_B: n = 19, 39.4 ± 7.8 ng/mL; 2) CORT_E5: n = 13, 223.5 ± 19.3 ng/mL; 3) CORT_E15: n = 14, 420.6 ± 17.2 ng/mL; 4) CORT_E30: n = 15, 463.0 ± 14.1 ng/mL.

Statistical Analysis

Prior to analysis, raw data was examined for violation of normality and equal variance assumptions. If detected for the individual pup measures, outliers were removed prior to computing litter averages. Specifically, one Home and 2 Novel rats were statistical outliers resulting in the removal of 2 litters from the current data set. If detected for maternal CORT measures, rank transformation was used to avoid the loss of an entire litter. Along with reports of F- and p-values, effect size (Cohen’s f) is also reported and indicated as large (f ≥ 0.4), medium (0.4 > f ≥ 0.25), or small (0.25 > f ≥ 0.1) effects (15).
Supplemental References


