Materials and Methods

Animals

Pregnant female (GD4) C57BL/6J mice aged 8- 10 weeks were ordered from Jackson Laboratory (Bar Harbor, ME). Animals received from Jackson were singly housed and habituated to our facility for 7 days before experimental manipulation. Mice were maintained in a temperature- and humidity-controlled room on a 12h:12h dark light cycle (10:30am-10:30 pm dark phase) with access to food and water ad libitum. Pups remained with the mother until the end of the experiment. Experimental animals were randomly allocated to experimental groups using an online random number generator. All experiments were performed in accordance with the ARRIVE guidelines and NIH guidelines, and were approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee (IACUC; protocols 20180110, 20180111, 00001386). Dams were weighed every other day during the experimental period. No pups were experimentally culled in this study. Offspring were weaned from the experimental dams at postnatal day 21 (PND 21) and separated by sex. Offspring were randomly assigned to adolescent or adult groups and subjected to further behavioral testing as described (Fig. 1A).

Prenatal Corticosterone Administration

Corticosterone (CORT; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 5% ethanol in sesame oil (Sigma-Aldrich, St. Louis, MO, USA) and administered at a dose of 20 mg/kg subcutaneously to the nape of the neck. Vehicle mice were injected with sesame oil at the same volume. CORT dose was chosen based on our prior study with

maternal caregiving (1). Mice were weighed and injections started on gestation day 12 (GD12) and ended on gestational day 18 (GD18) for a total of 7 daily injections (Fig. 1A). All injections were done within 2 hours of the start of the dark phase.

Cross-fostering

For cross-fostering experiments, dams were treated with corticosterone or vehicle as described above. Within 24 hours of birth, pups were swapped between a CORT and vehicle dam with equivalent litter sizes.

Serum Corticosterone Measurement

Trunk blood samples were taken at the time of sacrifice after brief anesthetization with inhaled isoflurane and serum was isolated from whole blood by centrifugation. A high-sensitivity corticosterone (CORT) enzyme immunoassay (EIA) was used and analyzed according to manufacturer's instructions (Immunodiagnostic Systems Ltd, Fountain Hills, AZ, USA). Briefly, the percent binding (B/Bo%) of each calibrator, control, and sample was calculated by dividing the mean absorbance over the mean absorbance for '0' calibrator and multiplied by 100. A calibration curve was used to plot B/Bo% on the ordinate against the concentration of corticosterone. A 4PL curve fit was applied.

Behavior Assays

Experiments were conducted during the dark phase under dim red light. Tests were recorded by Fly Capture cameras (Point Grey, Richmond, BC, Canada), and behaviors were scored by an observer blind to experimental conditions using Ethovision XT 13 or 18 (Noldus Information Technology, Leesburg, VA, USA).

Appetitive Maternal Behavior

Appetitive maternal behavior tests were conducted in the mouse's home cage as previously described on postnatal day 10 (PND 10; Fig. 1A) (1-5). Mice were habituated to the testing room for 1-2 hours and then the lid of their cage was replaced with a clear Plexiglas lid with holes and were habituated for an additional 10 minutes. Two C57BL/6J pups 1-3 days old from a non-experimental C57BL/6J dam were presented in the cage in the opposite corner to the undisturbed home cage nest containing all the dam's own pups. Test sessions started at pup approach (female first touches the pup with its snout) and lasted for 10 minutes. The following behaviors were quantified: latency to retrieve, pup investigation (sniffing, close contact with snout), grooming (handling with forepaws and licking), nest building, time spent in the nest, crouching, rearing, digging, and self-grooming.

Behavioral sequences analysis

Behavioral sequences were quantified using several complexity metrics: Transition Entropy was computed as the Shannon entropy of transition probabilities between behaviors in ethogram sequences, reflecting the unpredictability of behavioral shifts; Normalized Transition Entropy divided this by the logarithm of unique behavior counts for sequence-length invariance; Switching Rate was calculated as the number of behavioral transitions per unit time, capturing dynamism; and LZC Normalized employed Lempel-Ziv complexity (LZC) to measure sequence compressibility normalized by sequence length to enable cross-comparison. Linear mixed-effects models (LMM) were fitted using statsmodels.mixedlm (6) with the formula Metric ~ C(Group)+C(Phase)+C(Group):C(Phase), where Group and Phase are

categorical, incorporating random intercepts for mouse ID to account for repeated measures. Post-hoc simple effects analyses included Wilcoxon signed-rank tests for paired phase comparisons within groups (with rank-biserial correlation as effect size) and independent tests (t-test or Mann-Whitney U, based on normality via Shapiro-Wilcoxon) for group effects per phase, supplemented by permutation tests (10,000 iterations) and bootstrapped 95% confidence intervals (10,000 resamples) for robustness in small samples.

Transition Probability Analysis

Transition probability matrices were constructed for each mouse by tallying behavioral shifts in ethogram sequences, normalized by row sums to yield probabilities, and aggregated per group. Stationary distributions were derived via eigenvalue decomposition of smoothed matrices (epsilon=1e-9 added for stability), representing long-term behavior centrality. Group differences in node centrality were assessed using bootstrapped contrasts (1,000 resamples with replacement), computing p-values from the proportion of resamples exceeding observed differences. Edge (transition) probabilities were compared via Mann-Whitney U tests on per-mouse values, with two-tailed alternatives. Significant differences ($p<0.05$) informed network visualizations using NetworkX (7), where nodes were sized and colored by centrality shifts (red for increases, blue for decreases in the experimental group), and edges styled by probability differences (solid/thick for significant, dashed/thin for trends). A comprehensive summary table compiled means, differences, p-values, and significance for all nodes and edges across comparisons.

Open field

Mice were assessed for activity in a 45cm x 45 cm open field at 40 lux for 5 min as previously described (8). The center was considered 25 cm x 25 cm, and the borders were 10 cm around the perimeter of the box. Time and frequency in the center and border, as well as distance moved and velocity, were calculated using Ethovision XT13.

Odor preference test

Odor preference was assessed in the open-field arena, adapted from protocol established in Zou et al 2015 (9). Newborn pups from a non-experimental dam were swabbed with a Q-tip around their mouths and anogenital regions to transfer odors onto the swabs. A swab from a male pup and a swab from a female pup were placed in opposite quadrants of the arena. Dams were introduced to the arena and allowed to explore for 5 minutes. Fresh swabs were used for each test. Snout contact with the swabs and movement in the arena was quantified in Ethovision XT13 using head position tracking.

Elevated plus maze

The elevated plus maze (EPM) was conducted as previously described (10). Briefly, our EPM consisted of a plus-shaped custom-made apparatus with two 30 cm open arms without walls and two 30 cm enclosed arms with walls 16cm high. The apparatus stands at a height of 42 cm from the ground. The mice were placed at the junction of the open and closed arms and allowed to explore the maze for 5 minutes. The maze was cleaned with ethanol between trials. Movement and time spent in the zones of the apparatus was quantified in Ethovision XT13.

Novel object recognition test

Novel object recognition training and testing was conducted in the open field arena as previously described (11). On the training day, mice were presented with two identical objects in the open field arena and allowed to explore them for 5 minutes. The following day, mice were presented with one familiar object and one novel object and allowed to explore for 5 minutes. Movement in the arena and time spent in the contact zone of each object was quantified in Ethovision XT13.

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) was performed as recommended by ACD Bio (Newark, CA, USA) using V2 RNAscope reagents. Brains were embedded in OCT (Tissue-Tek) and frozen with dry ice. Sections of 25 µm were prepared on a cryostat. Protease 3 was used for pretreatment. Ucn3 (Cat No. 464861), MR (Nr3c2; Cat No. 456331), GR (Nr3c1; Cat No. 475261), and CRFR1 (Cat No. 418011) probes were used as per the manufacturer's instructions. Slides were mounted using Prolong Gold with DAPI. Three to eight brains per group were analyzed as in our previous histology experiments (2-5). A Zeiss Axioscan was used for imaging at 10-20X magnification. The Zeiss AxioScan resolution for our experiments was 0.650 μm^2 per pixel with an effective NA of 0.45 using the 10X objective or 0.325 μm^2 with an effective NA of 0.8 using the 20X objective.

Image Analysis

Images were exported from Zen Blue software after linear spectral unmixing. For the maternal brains, all Ucn3+ cells were counted and quantified for colocalization with

MR and GR. Cells were manually counted in a region of interest (ROI; 1.3mm × 0.75mm) around the 3rd ventricle at approximately Bregma AP -0.94 by an observer blinded to condition using FIJI Cell Counter. Two ROIs per mouse were quantified and averaged. For the offspring brains, mean fluorescence intensity (background subtracted) was measured in the pyramidal cell layers of dentate gyrus (DG), CA1, and CA3 for each channel in FIJI (ImageJ, NIH) from a 16-bit non-compressed manually thresholded image. The hippocampal ROI was acquired at approximately Bregma AP -1.94 (ROI; 1.8mm × 2.8mm). Two ROIs per mouse were quantified and averaged.

Data Analysis and Statistics

Data were analyzed in Graphpad Prism 10 with the investigator blinded to the experimental condition of the mice. Sample sizes were selected based on previous experiments (1, 2, 4, 5, 8). We first determined if the data were normally distributed using a Shapiro-Wilk test. If data were normally distributed, we used an unpaired two-tailed t-test to compare two groups. For non-normally distributed data, we used a two-tailed Mann-Whitney U test. For repeated measures data, we used a Two-Way Repeated Measures ANOVA or Mixed Effects Repeated Measures test followed by Tukey or Fisher's post-hoc correction. For colocalization experiments, we used Fisher's exact test to compare the total number of Fos/marker double positive cells to the total number of Fos-/marker+ cell populations across all mice and expressed the data as proportional percentage in bars. Outliers were identified by Grubb's outlier test or ROUT. P values reported as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. All data are expressed as mean ± SEM.

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