Supplementary Information

The oxytocin system mediates neurobiological and behavioral alterations associated with early adversity

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Supplementary Materials and Methods

Animals. 6-8 weeks old C57BL/6J (C57) male and female mice (purchased from Charles River, Italy) were housed in standard conditions (transparent polysulfone cages; 26.7 × 20.7 × 14.0 cm) with water and food available ad libitum. Room temperature (21 ± 1 °C) and a 12:12 h light-dark cycle (lights on at 07.00 AM) were kept constant. Animals were mated at 12 weeks old. The mating protocol consisted of housing 2 females with 1 male in standard cages with water and food ad libitum. After 15 days, pregnant females were isolated in clean cages with bedding materials and inspected twice a day for delivery. Adequate measures were taken to minimize pain or discomfort of mice, and all experiments were carried out in accordance with Italian national laws (DL 116/92 and DL 26/2014) on the use of animals for research based on the European Communities Council Directives (86/609/EEC and 2010/63/UE). Experimental protocols (no. 769/2017, no. 901/2023) were approved by the Italian Ministry of Health. For all the experiments, female mice were used.

Repeated Cross Fostering (RCF). The RCF procedure was conducted as previously reported[1-7]. Litters born on the same day spent the first postnatal day (PND0) with their biological mother; on PND1, litter were randomly assigned to RCF or control conditions (Cont). RCF was performed daily from PND1 to PND4, between 10:00 and 10:30 a.m. each day, by moving the entire litter into the home cage of a different dam, whose pups had just been removed and placed into the cage of a different adoptive mother. On PND4, pups remained with the last adoptive mother until weaning (PND28). The Cont group was picked up daily, from PND1 to PND4, and reintroduced in their home cage within 30s. At weaning (PND28), animals were separated by sex and housed in groups of 4 littermates. To prevent potential estrous cycle group synchronization and avoid litter effects, experimental female groups were sorted by collecting max 2 individuals per litter[2,3,8]. Different cohorts were used for behavior, molecular, and neurochemistry experiments.

Post-natal oxytocin treatment. The post-natal oxytocin treatment was performed during the RCF procedure, from PND1 to PND4. RCF pups were gently lifted and injected with a single subcutaneous injection (2.5 μg/mL) of oxytocin (EDQM - European Pharmacopoeia #O0700000) (RCF-Oxt) dissolved in isotonic saline (20 μL), or with isotonic saline (0.9%; RCF-Sal) for the vehicle control group. This OXT concentration has been selected to prevent homeostatic imbalances associated with sub-chronic treatment in newborns while ensuring it remains within the effective range for OxtR activation[9]. After the injection, pups were rapidly re-introduced in the home- cage of the mother and checked for any possible discomfort.

Maternal Behavior Observation. Maternal Behavior (MO) Observation was conducted as previously described[2,7]. MB observation took place daily, from PND1 to PND7, twice a day (from 12 to 12:30 and from 16 to 16:30), with an instantaneous sampling (2 minutes of sampling/rate) for 16 observation/sampling in each session. The Grooming (G), Nursing (N), Nesting (B), and Other Behaviors (OB) shown by the biological mother (Cont) or the adoptive mother (RCF, RCF-Oxt RCF- Sal) were analyzed. 6 dams x group were used.

Pup Retrieval Test. This assessment was conducted as previously described[10] during the early postpartum period, on day 5 following delivery (PND5), enabling the completion of the RCF procedure. The dam was briefly separated from the litter for less than 3 minutes and placed in a holding standard new cage. Subsequently, the pups were scattered into the opposite corner of the nest site. The mother was then reintroduced to the cage, and the occurrences of specific behaviors were recorded: the latency to the first pup

contact (First Contact, time taken for the initial contact or sniff with the first pup), the latency to the first pup retrieval (First Retrieval, the time taken for the first retrieval), and the latency to retrieve pups back to the nest, normalized on the litter size (mean time). The test concluded after 10 minutes or when the female retrieved all the pups. Dams that didn't retrieve all the pups by 10 minutes were excluded. Pups that their mother did not pick up during the test were returned to the nest site by the observer. 5-6 pups of each group (Cont, RCF, RCF-Oxt, RCF-Sal) belonging to 4-5 different litters were tested.

Ultrasonic vocalizations (USVs). The Ultrasonic Vocalization Calls (USV's) were measured as previously described[5,7]. On test day (PND8), pups were isolated from the mother for 5 minutes. Each pup was then placed individually into a sterilized beaker with home cage bedding material (home-cage) or clean, odorless bedding material (clean) to record vocalizations. UltraSoundGate microphone (CM16, Avisoft Bioacoustics, Berlin, Germany), placed above 1 cm of the beaker, was used for the recording session (5 minutes). The microphone was sensitive to 15-180 kHz frequencies, with a flat response (±6 dB) between 25-140 kHz. The spectrogram was produced at 488 Hz frequency resolution and 0,512 ms temporal resolution after transferring the recorded files in SasLabPro (AvisoftBioacustic) and converted. A threshold-based automatic algorithm was used to detect USVs. To reduce with noise, signal below 20 kHz was deleted. The output was analyzed with Avisoft Bioacoustics software (Berlin, Germany) by a blind trained researcher. No more than 4 pups x litter were tested. 6-8 pups of each group (RCF, Cont and RCF-Oxt, RCF-Sal), were tested.

Conditioned Place Preference (CPP). Conditioned Place Preference (CPP) test was conducted as previously described[2,11]. The apparatus consisted of 2 gray Plexiglas chambers (15 × 15 × 20 cm), a central alley (15 × 5 × 20 cm), and two sliding doors (4 × 20 cm) to connect the alley to the chambers. Two triangular parallelepipeds (5 × 5 × 20 cm of black Plexiglas), arranged in different patterns covering the same surface, were used as conditioned stimuli in each chamber. On day 1 (pretest), mice were free to explore the apparatus for 20 min. During the conditioning phase (following 8 days), mice were confined for 40 min in 1 of the 2 chambers, alternately. One pattern was paired with the drug (cocaine-paired chamber (Paired); 2.5 mg/Kg; i.p.) and the other one with vehicle ((Unpaired); saline 0.9%). Test day (day 10) was conducted as the pretest. Data were collected and analyzed by the automated video tracking system "EthoVision" (Noldus, The Netherlands). Preference scores were assessed by the time spent (seconds) in cocaine- (Paired) and saline- (Unpaired) chambers on the test day[12]. 7-9 mice per group (RCF-Sal, RCF-Oxt) were tested. For the CPP with ZD7288 (ZD; 0.1 µg in NaCl 0.9%), Control group received bilateral injection of ZD (Cont+ZD), or Vehicle (Cont+Veh), for 4 consecutive days. 24 h after the infusion, animals were tested in the CPP test, as described above. 5-8 mice per group were tested.

Stereotaxic surgery and ZD infusion. Animals were deeply anesthetized with Zoletil and Rompun (Zoletil 100 (tiletamine HCl 50 mg/ml + zolazepam HCl 50 mg/ml; from Virbac, Italy); Rompun 20 (xylazine 20 mg/ml; from Bayer S. p.A, Italy); dissolved in saline solution (4.1 mg/ml and 1.6 mg/ml, respectively) prior injection (7.3 ml/kg)) and placed in a stereotaxic apparatus (David Kopf Instruments, CA, USA) with a mouse adapter. Mice were chronically implanted with bilateral 26-gauge guide cannulas, 1 mm from the ventral tegmental area (VTA). The following coordinates were used: AP = 3.2 mm, ML = 0.4 mm, DV = 3.6 mm[13]. After 5-7 days of the post-operative recovery period, control female mice received bilateral injections of ZD7288 (ZD; 0.1 µg in NaCl 0.9%) (Cont+ZD) or vehicle (Saline 0.9%; Cont+Veh) for 4 consecutive days. A total volume of 0.4 µl per side at a continuous infusion rate of 0.15 µl/min, using a micro-infusion pump[4,13-15] was infused. To prevent backflow, 5 minutes after the infusion, the injector cannula was removed, and mice were returned to their home cages to recover for 24 hours.

Three Chamber Test (TCT). The Three Chamber Test (TCT) was conducted in a gray plexiglass rectangular box (60 × 40 × 24 cm), consisting of a central neutral starting chamber connected with two "stimuli chambers" containing one identical clear cylinder (plexiglass, 8cm diameters) each, with multiple

small holes During the habituation session (10 min), mice were placed into the starting chamber and left free to explore the apparatus. During the first test session (10 min), an age- and sex-matched mouse was introduced into one cylinder (pseudo-randomly chosen) as a social stimulus (familiar mouse), and a neutral object was introduced into the other one. In the second test session (10 min), a new age- and sex-matched mouse (novel mouse) was introduced into the cylinder previously containing the neutral object[16]. The familiar and novel mice always belong to different home cages. The position of stimuli within cylinders was alternated to avoid potential confounding effects. The sessions were recorded and analyzed by a video-tracking system (EthoVision) to evaluate the time spent in each chamber. The time spent sniffing each cylinder (i.e. contacts) during the test was manually scored by a blind-trained observer by Boris[17]. To obtain a measure of social preference and social novelty, the percentage of time spent interacting with the subject compared to the object [((time investigating the subject) / (time investigating the subject + object)) ∗ 100] and the novel compared to the familiar mouse [((time investigating the novel mouse) / (time investigating the novel + familiar mouse)) ∗ 100] was calculated[5]. 6-7 mice per group (Cont, RCF, RCF-Sal, RCF-Oxt) were tested.

Open Field (OF). The Open Field test (OF) was performed as previously reported[5]. The apparatus consists of a circular open field with 60 cm of diameter and 20 cm of height. Distance moved (cm) and time spent in the external part of the apparatus (percentage of total time spent in exploration) during the 5 minutes of the test were recorded and analyzed with the fully automated tracking video system "EthoVision" (Noldus, The Netherlands). 7-9 mice per group (RCF-Sal, RCF-Oxt) were tested.

Eleveted Plus Maze (EPM). The Elevated Plus Maze test (EPM) was conducted as previously described[3,5,7]. Mice were individually tested (for 5 min) in an apparatus consisting of two open arms (27 × 5 cm) and two closed arms (27 × 5 × 15 cm) connected by a central platform (starting point), 38.5 cm above the floor. The percentage of time spent in the closed arms [(time in closed)/(time in open + closed)) ∗ 100)]/open + closed x 100) was estimated using the "EthoVision" (Noldus, The Netherlands) fully automated video tracking system. 7-9 mice per group (RCF-Sal, RCF-Oxt) were tested.

Brain and plasma collection. For biochemical experiments of PND5, the pup's brain was collected after decapitation, and olfactory bulbs and cerebellum were removed. Brains collected were divided into the two hemispheres, cutting the medial sagittal line, and stored at -80 °C until the day of the assay. Just one hemisphere for the pup was used for the assay, and the left and right hemispheres were balanced across groups. For biochemical experiments on PND90, the mice's brain was collected after decapitation and immediately frozen at -80 °C until the day of punches collection. For plasma collection, blood samples were collected after decapitation and immediately centrifuged (PND5: 10 minutes, 2000 rcf, 4 °C; PND90: 10 minutes, 1500 rcf, 4 °C). The supernatant was taken and stored at -80 °C until the day of the assay. 5-6 mice per group (Cont, RCF, RCF-Sal, RCF-Oxt; PND5 and PND90) were used for central and peripheral OXT quantification.

Microdissection of brain punches. For biochemical experiments on PND90, brain tissue micro- punches of preFrontal Cortex (pFC), Nucleus Accumbens (NAc), amygdala (Amy), Paraventricular Nucleus of the hypothalamus (PVN), and Ventral tegmental Area (VTA) were obtained from frozen brain slices (coronal sections according to the atlas of Paxinos and Franklin[18] not thicker than 300 μm using stainless-steel tubes of different inner diameters (1.0 mm for pFC, 0.7 mm for NAc, PVN, 0.5 mm for VTA) and stored at -80 °C until the day of the assay[19].

RNA extraction and Quantitative Real Time PCR (qRT-PCR). qPCR was performed for the evaluation of Oxytocin receptor (*OxtR*) mRNA levels in pFC, NAc, PVN, and VTA of Cont and RCF female mice, and in the NAc of RCF-Sal and RCF-Oxt mice. RNA from punches was extracted using microcolumn from Total

RNA purification Plus Kit (Norgen Biotek, Canada). RNA quantity was determined by absorbance at 260 nm using a NanoDrop UV-VIS spectrophotometer. Complementary DNA (cDNA) was obtained using the High-Capacity Reverse Transcription kit (Applied Biosystems, USA). Around 10 ng of cDNA templates were amplified by qPCR using the 7900HT thermal cycler apparatus equipped with SDS software version 2.3 (Applied Biosystems, AB) for data collection. Each data point was obtained from 5 to 6 biological replicates. Ct values were normalized to measures of Tata Binding Protein (TBP, Taqman assay ID Mm00446973_m1) for OxtR (ID Mm01182684_m1) to achieve ΔCt values[19]. OxtR expression was calculated as fold changes using the ΔΔC(t) method. All data were run in triplicate and expressed as means ± SE.

Immunohistochemistry. Animals were perfused transcardially with 50 ml of saline followed by 50 ml of 4% paraformaldehyde in a phosphate buffer (PB; 0.1 M; pH 7.4), under deep anesthesia. Each brain was removed immediately, post-fixed in the same fixative for 24h and, after three washes in PB, transferred to 30% sucrose in PB solution at 4 °C. Brains were cut into four series of 30 µm -thick transverse sections using a freezing microtome and collected in PB. Every fourth section was processed for immunohistochemistry[20]. Before incubation with the primary antibody, free-floating sections were incubated in a blocking solution (5% normal donkey serum, 0.3% Triton X-100, PB) for 2 h at room temperature. Rabbit anti-Oxytocin-neurophysin1 (1:1000; #AB212193; Abcam) was prepared in PB and 0.3% Triton X-100 and incubated for 48 hours at 4 °C. Each incubation step was followed by three 5-minute rinses in PB. Then, sections were incubated with a biotinylated donkey anti-rabbit IgG as secondary antibody (1:200; Jackson Immunoresearch Laboratories, West Grove, PA, USA) followed by the avidin-biotin-peroxidase method (Vectastain, ABC kit, Vector, Burlingame, CA, USA), and 3,3 -diaminobenzidine (Sigma) as a chromogen. Sections were mounted on chrome alum-gelatinized slides and air-dried for at least 24 h, dehydrated, and coverslipped with Entellan (Sigma). The immunoperoxidase material was examined under a light-transmission microscope (Zeiss, Axioskop 2) equipped with a CCD camera (ProgRes C10 plus, Zeiss). Qualitative observations were limited to paraventricular (PVN). Cell counting of oxytocin+ neurons was performed on 10 sections regularly spaced throughout the rostro-caudal extent of the two nuclei by using a 10X objective. Only positive neurons with the nucleus in the focal plane were counted.

Multiplex RNAscope. Mice were transcardially perfused as above and 20 µm coronal sections were mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Pittsburgh, PA). RNAscope was performed by using RNAscope Multiplex Fluorescent Detection Reagents v2 (cat#323110; Advance Cell Diagnostics, Hayward, CA)[21]. The following probes were used: Oxtr-C3 (Mm-Oxtr- C3 cat# 412171-C3; Advance Cell Diagnostics, Hayward, CA) in combination with the Drd1-C2 (Mm-Drd1-C3 cat# 461901-C2; Advance Cell Diagnostics, Hayward, CA) or with Drd2-C1 probe (Mm-Drd2 cat# 406501-C1; Advance Cell Diagnostics, Hayward, CA). Sections were processed for RNAscope by following the company protocol. To label the amplified hybridizations with fluorophores the TSA Vivid Fluorophores 520 (for C2 or C1 probes), 570 (for C3 probe) were used. Images were taken by using Nikon Eclipse Ti1 confocal microscope. Densitometric analysis (optical density, O.D.) of *OxtR* on D1R+ or D2R+ neurons was performed on digital images taken by using the confocal microscope by maintaining confocal settings constant throughout the acquisition of sections from the different experimental groups. After background subtraction, OxtR-associated signals were quantified by manually outlining individual D1R+ or D2R+ neurons at the level of the NAc shell by outlining the dorso-medial and dorso-lateral portion and measuring cell-associated fluorescence intensity with the ImageJ software. Quantification was done on 15 D1R+ or D2R+ neurons per section (n = 4 sections/mouse; N = 4 mice/group).

Enzyme-linked immunosorbent assay (ELISA). For ELISA experiments, total protein content was extracted from all samples (plasma at PND5 and PND90, brain at PND5, punches at PND90) using C18 Spin Columns (C18 Spin Columns, G-Bioscience, 786-931). Brain and punches were previously homogenized in

ice-cold lysis Buffer (1:100 protease inhibitor (Protease Inhibitor Cocktail, P8340 Sigma-Aldrich) in PBS). PND5 brain samples were weighted, and lysis buffer was used at 10ul/mg; for PND90 brain punches, a total amount of 100ul of lysis buffer was used for each punch. After homogenization, samples were centrifuged (14000g, 15 mins, 4°C), then the supernatant was collected and used for the assay. Plasma samples did not undergo any additional treatment before being extracted by C18 columns. Columns were placed in collection tubes, 200ul of activation solution (50% ACN) was added, and then the solution was briefly centrifugated (1500xg for 1 min). The residue was discarded, and the Activation Solution wash was repeated. 200ul of Equilibration Solution (0.5% TFA in 5% ACN) was added, the columns were centrifuged as reported before, and the solution was discarded. The equilibration Solution wash was repeated once. The columns were then transferred to clean collection tubes, and the sample was applied. After a brief centrifugation, the flow-through was recovered and reapplied to ensure complete binding before repeating the centrifugation. Columns were transferred to clean collection tubes, and 200 µL of Wash Solution (0.5% TFA in 5% ACN) was applied before centrifugation. The flow-through was discarded, and the Wash Solution was repeated. The columns were transferred to clean collection tubes, and 20ul of Elution Buffer (70% ACN) was added before centrifugation. The elution step was then repeated, and the final volume of 40ul was collected and stored at -20 °C when not immediately processed for the assay. The ELISA kit was used to analyze OXT content in samples, following the manufacturer's instructions (Oxytocin ELISA kit, Enzo Lifescience, ADI-900- 153A). 5-6 mice per group were used for OXT quantification.

Protein Extract Preparation and Western Blot Analysis. Right and left punches from each brain region were collected for each animal. NAc punches from 2/3 adult mice (PND90) were combined into a single tube and homogenized using ice-cold RIPA buffer (Sigma- Aldrich), supplemented with protease and phosphatase inhibitors (SERVA Electrophoresis GmbH, Germany; 1:10000). To perform total protein extraction, NAc tissues were homogenized in ice-cold RIPA Buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors (SERVA Electrophoresis GmbH, Germany; 1:10000). Upon 30 minutes in ice, the homogenates were centrifuged at 12000 RCF for 15 min at 4°C. The pellets were discarded while the supernatants were stored at -80°C before use[22]. Protein concentrations were determined by Bradford's colorimetric assay (Serva, #P220101, Germany), and protein extract preparations were processed using WB analyses, as previously described[22]. Total proteins were separated, according to their molecular weight, by SDS polyacrylamide gel electrophoresis (30% Acrylamide/Bis solution, 1.5M Tris-HCl, pH 8.8, 20% SDS, TEMED and 10% APS; Sigma-Aldrich), and transferred to a Polyvinylidene Fluoride (PVDF) membrane (GVS, #1212639; North America, Sanford, ME, USA). Subsequently, PVDF membranes were blocked in 3% Bovine Serum Albumin (BSA) (PanReac AppliChem, ITW Reagents) diluted in Tris-Buffered Saline, 0.1% Tween 20 (TBS- T) (Sigma, Milan, Italy) for 1 hour at RT. Then, membranes were overnight incubated at 4°C with Oxytocin-neurophysin1 monoclonal antibody (#ab212193; Abcam), and Tubulin beta polyclonal antibody (#PA5-30380; Invitrogen). Following washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies specific for mouse IgG (Cell Signaling Technology, Danvers, Massachusetts, USA) and rabbit IgG (Cytiva, USA). Blots were developed using the ECL Kit (WesternBright Sirius- femtogram HRP Substrate, Advansta) and chemiluminescence system (iBright 1500, Invitrogen). Blots were quantified by densitometry using ImageJ Fiji software (latest version), and the intensity of each band was normalized to the Tubulin beta signal intensity. The average values were expressed in arbitrary units as a ratio to the control (RCF vs Cont) or the RCF-Sal (RCF-Sal vs RCF-Oxt) mean values.

Dissociation of pFC After animal sacrifice, the pFC was collected and immersed in D-PBS with high glucose buffer at 4°C. According to the manufacturer's protocol, the tissue was immediately dissociated to single-cell suspension by enzymatic degradation and myelin removal using the adult brain dissociation kit and GentleMACS dissociator (Miltenyi Biotec.). The isolated tissues were placed in C-tubes, and a mix containing Enzyme P and Buffer Z was added to the samples. A second mix containing Enzyme A and

Buffer Y is then added, C-tubes tightly closed and attached upside down onto the sleeve of the gentle MACS Octo Dissociator with Heaters (Miltenyi biotec). The appropriate program 37C_ABDK_02 was run for 30 minutes, c-tube was detached and centrifuged to collect the sample, which was then resuspended and filtered to a 70 um MACS SmartStrainer with 10 ml of cold D-PBS with high glucose. After centrifugation at 300g for 10 minutes, the sample was resuspended in the debris removal solution, gently overlayed in cold D- PBS with high glucose, and centrifuged at 3000g for 10 minutes with full acceleration brake. The two top phases were aspirated and discarded, and the sample was resuspended in cold D-PBS with high glucose, centrifuged at 1000g for 10 minutes, and then resuspended in a staining buffer ready for cell count and flow cytometry staining. 6 mice per group (Cont, RCF, RCF-Oxt, RCF-Sal) were used.

Immunophenotyping and RAGE expression by High-dimensional Flow cytometry. Cellular phenotypes were assessed using high-dimensional flow cytometry panels containing markers to identify cell types and to evaluate the expression of RAGE in each cell population. The use of these markers allowed us to exclude all cells of no interest based on physical parameters (side and forward scatter) and to gate on specific cells of interest. For the immunophenotyping of pFC, single suspension cells isolated were stained with different panels of cell surface markers. Upon exclusion of both doublets and debris, single cells were gated. Astrocytes were identified as ACSA-2+ cells and oligodendrocytes as O4+ cells. Negative cells for both ACSA-2 and O4 were further gated to identify CD90.2+ neurons and CD31+ endothelial cells. Negative cells for both CD90-2 and CD31 were further gated to identify resident and infiltrated immune cells as follows: microglial cells as CD45lowCD11b+ cells, myeloid cells as CD45+CD11b+ cells further divided into Ly6G+ neutrophils and Ly6G- macrophages, and CD45+CD11b- non-myeloid cells. Each cell population was evaluated for the expression of RAGE at their cell surface. Samples were acquired on a 13-color Cytoflex (Beckman Coulter) and for each analysis, at least 0.5×10^6 live cells were acquired[23].

Midbrain slice preparation. Horizontal midbrain slices containing the VTA (250 μm; VT1000S, Leica Biosystems) were prepared as previously reported[4,24]. Shortly, following no-return deep anesthesia with halothane and trans-cardiac perfusion with cold (0.5–4 °C), 95%O2–5%CO2 – saturated N-methyl-D-glucamine (NMDG)-based 'slicing' solution, adult C57 females previously exposed to RCF or Control manipulations were decapitated. Brains were rapidly dissected and moved to the vibratome in the same chilled, bubbled (95% O2–5% CO2) 'slicing' solution containing, in mM: 92 NMDG, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl2, and 10 MgSO4 (pH to 7.3–7.4 with 18% hydrochloric acid). After cutting, slices were maintained in 'slicing' solution at approx. 34 °C and added with progressively increasing volumes of the 'sodium-spike solution' (2M NaCl; Ting et al., 2018). For long-term storage, slices were transferred into a Na-based aCSF containing (in mM): 92 NaCl, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na- pyruvate, 2 CaCl2, and 2 MgSO4 (95%O2–5%CO2; pH 7.3–7.4) and let recover for at least 1 hour at room temperature (approx. 24 °C) before starting patch-clamp experiments.

Electrophysiology. For patch-clamp recordings, slices were moved to the recording chamber of an upright microscope (DMLSF; Leica) and continuously perfused (3–4 ml/min) with aCSF solution containing (in mM): 126 NaCl, 24 NaHCO3, 10 glucose, 2.5 KCl, 2.4 CaCl2, 1.2 NaH2PO4 and 1.2 MgCl2, saturated with 95%O2–5%CO2 (pH 7.4; ~290 mOsm). All recordings were performed at near-physiological temperature (32 – 34 °C), with no drugs added to the aCSF and no liquid junction potential correction. Pipettes were pulled from thick-walled capillaries to a final tip resistance of 5– 6 MΩ when filled with an 'intracellular' solution containing (in mM): 125 K-gluconate, 10 KCl, 10 HEPES, 2 MgCl2, 4 ATP-Mg2, 0.3 GTP-Na3, 0.75 EGTA, 0.1 CaCl2, 10 Phosphocreatine-Na2 (pH7.2, ~280 mOsm). Whole-cell patch-clamp recordings were performed from visually identified (40X) VTA DA neurons (MultiClamp 700B and Digidata 1322A; Molecular Devices). Neurons were selected for patching using the following criteria[25] i) location in the

intermediate region of the VTA (iVTA); ii) presence of low-frequency, spontaneous action potentials (APs) firing in cell-attached and whole-cell configuration; iii) presence of *Ih* current. Our criteria were validated by *post-hoc* Tyrosine Hydroxylase (TH) immunostaining on recorded neurons (not shown; [15,25]) . Only neurons meeting these criteria were included in this study. Spontaneous firing was recorded in whole-cell configuration, immediately after membrane rupture (amplifier in-built 8-pole low-pass Bessel filter, *fc* 10 kHz; sampling 50 kHz). The neuron approximate Resting Potential (RP) was also estimated immediately after accessing the cell interior using the amplifier inbuilt voltmeter. Intrinsic properties (input resistance, Ri, membrane time constant, Taum, and membrane capacitance, Cm) were estimated within 2 min after membrane rupture using the in-built Clampex 9 'membrane test' protocol (30 ms-long, + 20 mV step at 20 Hz from VH – 60 mV). In voltage-clamp, the hyperpolarization-activated inward current *Ih* was recorded in whole-cell configuration in response to hyperpolarizing voltage steps (1 sec-long; from – 60 to – 120 mV, 20 mV increment, VH – 60 mV; *fc* 2 kHz; sampling 10 kHz). In current-clamp (*fc* 10 kHz; sampling 50 kHz; membrane potential held at approx. – 60 mV via steady current injection), cell evoked excitability was investigated both by estimating rheobase and by building *f-I* curves. The rheobase (the amplitude of the injected current necessary to induce the first AP) was estimated from the cell response to a series of 5 pA-incremental consecutive steps of depolarizing injection current (I_{inj}; 50 ms-long steps; amplitude range: 0 – 0.2 nA). The '*f*-I' curves, depicting the relationship between the frequency of evoked action potentials (*f*) and the intensity of somatic current injections, were built (I) via presenting the neuron with a family of under- and supra- threshold somatic current injection steps (I_{inj}; 1 sec-long; amplitude range: -0.2 – +0.4 nA; 13 total steps).

Electrophysiology Data Analysis. Analysis of current-clamp and voltage-clamp recordings was performed using Igor. Pro 6.32A (WaveMetrics Inc.) with NeuroMatic 2.8[25]. The amplitude of Ih currents was measured at the current steady-state and after nulling the current baseline at the interception between the offset of the capacitive peak and the onset of the hyperpolarization-activated inward current for each step response[25]. These amplitude values were used to describe the 'I–V curve', the relationship between the voltage command and the elicited current. In current-clamp experiments, APs were detected using a threshold-crossing method.

Statistical Analysis. The normality distribution of data sets was evaluated using the Shapiro-Wilk or Kolmogorov-Smirnov test. All data are presented as mean ± SEM. For behavioral data, statistical analysis was conducted using Super ANOVA or Prism 9 (GraphPad), considering a statistically significant p-value < 0.05. The frequency of maternal behaviors (Grooming, Nursing, Nesting, Other Behaviors) from P1 to P7, as well as First contact, First retrieval, and Mean time of the Pup Retrieval Test, were analyzed by separate Student T-test. Differences in Latency to First Retrieval and Mean time between Cont and RCF groups were analyzed using a non-parametric Mann-Whitney test due to the non-normal distribution of the data. The number of pups USVs emitted during separation from mother was investigated by a separate Student T-test for each experimental group, with Welch's correction when needed. Adult social behavior and cocaine vulnerability were analyzed using Repeated Measures (RM) Two-way ANOVAs with Sidak's *post-hoc* test, and Friedman test with Dunn's *post-hoc* test with data not passing the normality check. Specifically, social preference and social recognition in the TCT were analyzed using RM Two-way ANOVAs (independent factors: treatment, 2 levels: Cont, RCF; RCF-Sal, RCF-Oxt; and stimulus, 2 levels: object, subject (Social preference); familiar, novel (Social recognition)), except for social recognition in Cont vs RCF, that was analyzed by Friedman test. Cocaine preference was evaluated by RM Two-way ANOVAs with Sidak's correction (independent factors: treatment, 2 levels: RCF-Sal, RCF-Oxt or Cont+ZD, Cont+Veh; and pairing, 2 levels: Paired, Unpaired). The moved distance (during OF) and the anxiety-like behavior (percentage of time spent in the external area of OF; time spent in the closed arms of EPM) were analyzed by separate Student T-test. For, ELISA, qRT-PCR, WB, IF IHC, and RNAscope experiments, separate Student T-test (with Welch's correction when needed) were performed, and appropriate non-parametric test (Mann-Whitney

405 *U* test) were performed in case of non-normal distribution of the data. RAGE distribution across cell types in
406 pFC of Cont mice was assessed by One-way ANOVA (independent factor: cell type, 8 levels: non-myeloid
407 leukocytes, endothelial cells, astrocytes, oligodendrocytes, neutrophils, neurons, microglia, macrophages).
408 Differences in the RAGE expression across the different cell types between Cont *vs*. RCF, and RCF-Sal *vs*.
409 RCF-Oxt, were analyzed by separate Student T-test analyses. For electrophysiological data, statistical analysis
410 was run in Prism 9 (GraphPad) using Mann–Whitney *U* test, unpaired Student's *t* -t-tests (with Welch's
411 correction), one-way or two-way repeated measures ANOVA with Bonferroni's *post-hoc* test or Kruskall-
412 Wallis with Dunn's *post-hoc* test, as required. The normality of data sets was evaluated using either the
413 Shapiro–Wilk test or the D'Agostino & Pearson omnibus normality test ($p<0.05$ was considered significant).

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Supplementary Figure Legends

Fig. S1. RCF manipulation impacts the attachment-like behavior in pups but does not alter maternal behavior. (A) Experimental timeline: Maternal behaviors (MB; Grooming, Nursing, Nesting, Other Behaviors) were assessed from PND1 to PND7; Pup Retrieval Test (PRT) and USVs test were performed at PND5 and PND8, respectively. (B) Number of USV calls emitted by Cont and RCF pups when exposed to the home- and clean-cage (Cont group = 6 per each condition; RCF group = 7 (Home cage), 8 (Clean)). Data are presented as mean ± SEM. *P < 0.05. (C-I) Frequency of Grooming (C), Nursing (D), Nesting (E), and Other Behaviors (F) shown by RCF and Cont dams (Cont group = 6; RCF group = 6). (G-I) PRT test in RCF and Cont dams: (G) First contact, (H) First retrieval, (I) Mean time.

Fig. S2. RCF affects RAGE expression in pFC and does not impact OxtR expression in pFC, VTA and PVN. Distribution of RAGE expression across different cell types in the pFC of Cont mice by High-dimensional Flow Cytometry (n = 6). (A-E) RAGE expression in endothelial cells (A), neutrophils (B), microglia (C), neurons (D), and macrophages (E) of Cont and RCF groups. (F-H) OxtR expression was evaluated by qRT-PCR in the pFC (F), VTA (G), and PVN (H) of Cont and RCF mice. Data are presented as mean ± SEM.

Fig. S3. OxtR expression on NAc GABAergic neurons. (A) High-magnification of representative images of *OxtR* RNAscope ISH (red) combined with GAD67-immunofluorencence (green) merged signal in the medium spiny neurons of the NAc shell. Scale bar: 50μm. (B) Histograms showing the OxtR-RNA signal measured as optical density (O.D.) in the dorso-medial and dorso-lateral portions of the NAc shell D2R+ neurons of RCF-Sal and RCF-Oxt (N = 4 animals per group, 4 sections/animal).

Fig. S4. Early pharmacological treatment does not alter maternal behavior and does not impact anxiety-like behavior in adult mice. (A) Experimental timeline: RCF pups were treated with subcutaneous OXT (RCF-Oxt) or Sal (RCF-Sal) from PND1 to PND4; Pup Retrieval Test (PRT) test was performed at PND5, and maternal behavior (MB) was evaluated from PND1 to PND7. Anxiety-like behavior was assessed by the Open Field Test (OF) and Elevated Plus Maze Test (EPM) in adulthood (PND90). (B) Grooming, (C) Nursing, (D) Nesting, and (E) Other Behaviors shown by dams. (F) First contact, (G) First retrieval, (H) Mean time shown by dams in PRT. (I) % time spent in the closed arms of the EPM test by RCF-Sal and RCF-Oxt groups (J) Distanced moved (cm) by RCF-Sal and RCF-Oxt groups in the OF test. (K) % time in periphery shown by RCF- Sal and RCF-Oxt groups in the OF test. Data are presented as mean ± SEM.

Fig. S5. Lack of modulation of VTA DA neurons by early OXT treatment. (A) Experimental timeline: RCF pups were treated with OXT (RCF-Oxt) or saline (RCF-Sal) from PND1 to PND4, and *ex-vivo* path clamp experiments were performed in adulthood to analyze *Ih* current in VTA dopaminergic neurons. (B) Average curves for the relationships between AP frequency and injected current (I_{inj}) intensity depicting lack of differences in evoked excitability of VTA DA neurons (squared brackets indicate analysis range; neurons: 15 RCF-Sal, 21 RCF-Oxt). (C), Plot depicting lack of differences between the same experimental groups for maximum frequency of evoked Aps. (D) I–V relationships for Ih current density (squared brackets indicate analysis range; neurons: 14 RCF-Sal, 18 RCF-Oxt). (E) Pooled data for *Ih* current density at Vm = - 120 mV.

Fig. S6. Effects of pharmacological modulation of the *Ih* current in VTA DA neurons on cocaine-induced CPP. (A) Experimental Timeline: intra-VTA injections of an *Ih* current blocker (ZD7288 (ZD))

were performed in Cont adult mice before the Conditioned Place Preference (CPP) paradigm (cocaine 2.5 mg/kg). (B) Time spent in the cocaine-paired chamber (Paired) and saline- paired chamber (Unpaired) by Cont+ZD and Cont+Veh groups (Cont+ZD group = 7; Cont+Veh group = 6). Data are presented as mean ± SEM.

Table S1.

Experiment	Age	Conditions	Statistic test	Statistics	Figure
Brain mRNA OxtR expression	PND5	Cont vs RCF	Student's T-test	t = 0.07; p = 0.94	1D
pFC mRNA OxtR expression	PND90	Cont vs RCF	Mann Whitney U-test	U = 13; p = 0.48	1G
VTA mRNA OxtR expression	PND90	Cont vs RCF	Student's T-test	t = 0.99; p = 0.345	1H
PVN mRNA OxtR expression	PND90	Cont vs RCF	Student's T-test	t = 0.36; p = 0.69	1I
PVN OXT positive (OXT+) cells	PND90	Cont vs RCF	Student's T-test	t = 0.271; p = 0.79	1K, right panel
RAGE expression (Cont)	PND90		One way ANOVA	F(7,40) = 26.88; p < 0.0001	2B
RAGE expression	PND90	Non-myeloid leukocytes vs. Endothelial cells	Turkey's posthoc	p <0,0001	2B
RAGE expression	PND90	Non-myeloid leukocytes vs. Oligodendrocytes	Turkey's posthoc	p <0,0001	2B
RAGE expression	PND90	Non-myeloid leukocytes vs. Neutrophils	Turkey's posthoc	p <0,0001	2B
RAGE expression	PND90	Non-myeloid leukocytes vs. Astrocytes	Turkey's posthoc	p <0,0001	2B
RAGE expression	PND90	Non-myeloid leukocytes vs. Microglia	Turkey's posthoc	p <0,0001	2B
RAGE expression	PND90	Non-myeloid leukocytes vs. Neurons	Turkey's posthoc	p <0,0001	2B
RAGE expression	PND90	Non-myeloid leukocytes vs. Macrophages	Turkey's posthoc	p <0,0001	2B
RAGE expression	PND90	Endothelial cells vs. Neurons	Turkey's posthoc	p = 0,0292	2B
RAGE expression	PND90	Endothelial cells vs. Macrophages	Turkey's posthoc	p = 0,0109	2B
NAcLat OxtR mRNA	PND90	Cont vs RCF	Student's T-test	t = 1.073; p = 0.324	3J

expression on D1R+ cells					
NAcLat OxtR mRNA expression on D2R+ cells	PND90	Cont vs RCF	Student's T-test	t = 0.7; p = 0.5	3M
USVs (RCF-Sal)	PND8	Home vs clean	Student's T-test	t = 0.691; p = 0.39	4B
NAc OxtR mRNA expression on D2R+ cells	PND90	RCF-Sal vs RCF-Oxt	Student's T-test	t = 1.717; p = 0.137	5G
CPP (RCF-Oxt)	PND90	Paired vs Unpaired	Sidak's posthoc	p = 0.82	6B
Social Preference	PND90	RCF vs Cont, subject vs object	RM 2-way ANOVA	stimulus effect: F (1, 12) = 0,024; p = 0,8802	6C
Social Recognition (RCF)	PND90	familiar vs novel	Dunn's posthoc	p > 0.999	6D
Social Recognition (RCF-Sal)	PND90	familiar vs novel	Sidak's posthoc	p = 0.98	6F
Social Preference	PND90	RCF-Sal vs RCF-Oxt, subject vs object	RM 2-way ANOVA	stimulus effect: F(1,9) = 83.32; p < 0.0001	6E
Social Preference	PND90	RCF-Sal vs RCF-Oxt, subject vs object	RM 2-way ANOVA	stimulus x treatment: F(1,9) = 0.039; p = 0.84	6E
Maternal Behavior (Grooming)		Cont vs RCF	Student's T-test	t = 0.696; p = 0.50	S1B
Maternal Behavior (Nursing)		Cont vs RCF	Student's T-test	t = 0.098; p = 0.923	S1C
Maternal Behavior (Nesting)		Cont vs RCF	Student's T-test	t = 0.259; p = 0.8	S1D
Maternal Behavior (Other Behaviors)		Cont vs RCF	Student's T-test	t = 0.031; p = 0.975	S1E
Pup Retrieval Test (First contact)		Cont vs RCF	Student's T-test	t = 0.69; p = 0.5	S1F
Pup Retrieval Test (First retrieval)		Cont vs RCF	Mann Whitney U-test	U = 13; p = 0.79	S1G

Pup Retrieval Test (Mean time)		Cont vs RCF	Mann Whitney U-test	U = 12; p = 0.66	S1H
USVs (Cont)	PND8	Home vs clean	Student's T-test	t = 4.775; p = 0.0008	S1I
USVs (RCF)	PND8	Home vs clean	Student's T-test	t = 0.354; p = 0.7306	S1I
RAGE expression on Endothelial cells	PND90	Cont vs RCF	Student's T-test	t = 0.67; p = 0.51	S2A
RAGE expression on Neurotrophils	PND90	Cont vs RCF	Student's T-test	t = 0.26; p = 0.79	S2B
RAGE expression on Microglia	PND90	Cont vs RCF	Student's T-test	t = 0.36; p = 0.72	S2C
RAGE expression on Neurons	PND90	Cont vs RCF	Student's T-test	t = 1.45; p = 0.17	S2D
RAGE expression on Macrophages	PND90	Cont vs RCF	Student's T-test	t = 0.95; p = 0.36	S2E
pFC mRNA OxtR expression	PND90	Cont vs RCF	Student's T-test	t = 0.4; p = 0.69	S2F
VTA mRNA OxtR expression	PND90	Cont vs RCF	Student's T-test	t = 0.42; p = 0.68	S2G
PVN mRNA OxtR expression	PND90	Cont vs RCF	Student's T-test	t = 0.59; p = 0.56	S2H
NacMed OxtR mRNA expression on D2R+ cells	PND90	RCF-Sal vs RCF-Oxt	Student's T-test	t = 1.84; p = 0.1154	S3B
NacLat OxtR mRNA expression on D2R+ cells	PND90	RCF-Sal vs RCF-Oxt	Student's T-test	t = 1.232; p = 0.264	S3B
Maternal Behavior (Grooming)		RCF-Sal vs RCF-Oxt	Student's T-test	t = 0.41; p = 0.68	S4B
Maternal Behavior (Nursing)		RCF-Sal vs RCF-Oxt	Student's T-test	t = 0.67; p = 0.51	S4C
Maternal Behavior (Nesting)		RCF-Sal vs RCF-Oxt	Student's T-test	t = 0.08; p = 0.93	S4D
Maternal Behavior (Other Behaviors)		RCF-Sal vs RCF-Oxt	Student's T-test	t = 1.45; p = 0.17	S4E

Pup Retrieval Test (First contact)		RCF-Sal vs RCF-Oxt	Student's T-test	t = 0.29; p = 0.77	S4F
Pup Retrieval Test (First retrieval)		RCF-Sal vs RCF-Oxt	Mann Whitney U-test	U = 9; p = 0.5	S4G
Pup Retrieval Test (Mean time)		RCF-Sal vs RCF-Oxt	Student's T-test	t = 0.89; p = 0.39	S4H
Anxiety behavior (EPM)	PND90	RCF-Sal vs RCF-Oxt	Student's T-test	t = 0.95; p = 0.35	S4I
Anxiety behavior (OF, Distance moved)	PND90	RCF-Sal vs RCF-Oxt	Student's T-test	t = 0.13; p = 0.89	S4J
Anxiety behavior (OF, Time in periphery)	PND90	RCF-Sal vs RCF-Oxt	Student's T-test	t = 0.95; p = 0.35	S4K
CPP (Cont + Veh/ZD)	PND90	Paired vs Unpaired	RM 2-way ANOVA	F(1,11) = 0.431; p = 0.525	S6B

Table S1. The table reports the statistical test and the relative statistical results not reported in the main text, indicating the specific experiment, age of the animals, conditions, and figure reference.

Table S2.

	RP (mV)	Taumemb (ms)	Rin (MΩ)	Cm (pF)	SponAP (Hz)	Rheo (pA)
RCF-Sal	–50±1 (15)	1.4±0.2 (15)	171±21 (15)	66±7 (15)	2.2±0.5 (9)	151±38 (9)
RCF-Oxt	–53±2 (21)	1.9±0.2 (19)	191±20 (20)	62±5 (20)	1.7±0.5 (13)	185±21 (16)
p value	0.2328[1]	0.0654[1]	0.6627[2]	0.6575[1]	0.4379[1]	0.4368[1]

[1] Unpaired *t*-test with Welch's correction

[2] Mann-Whitney test

Table S2. The table summarizes average ± SEM values (cells analyzed in brackets) for membrane approximate 'resting' potential (RP); time constant (Taumemb); input resistance (Rin); capacitance (Cm); spontaneous action potential firing (SponAP, in whole-cell); and rheobase (Rheo). Bottom, relevant *p*-values. Sub- and supra-threshold properties of DA neurons of the intermediate VTA are unaffected by early OXT treatment.

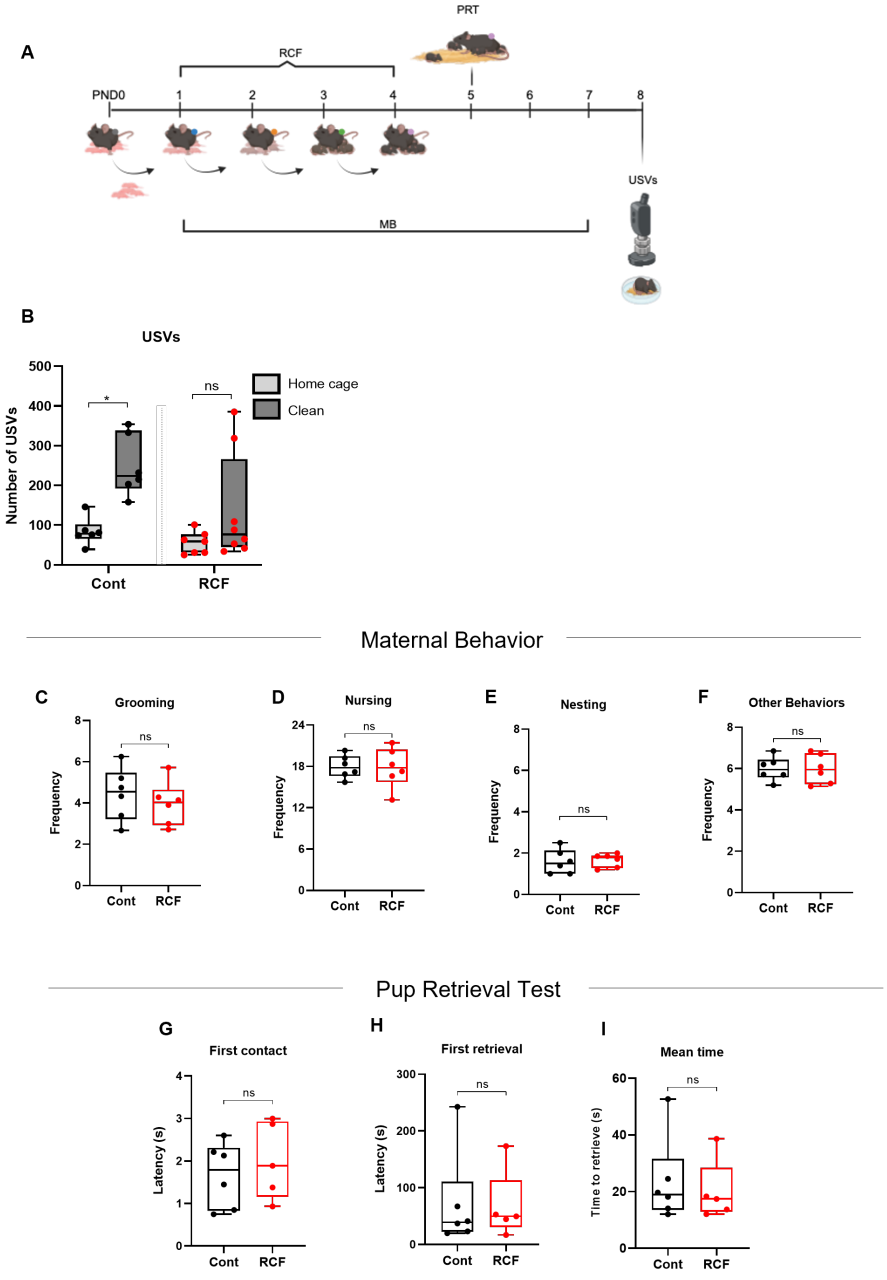

Fig. S1. RCF manipulation impacts the attachment-like behavior in pups but does not alter maternal behavior.

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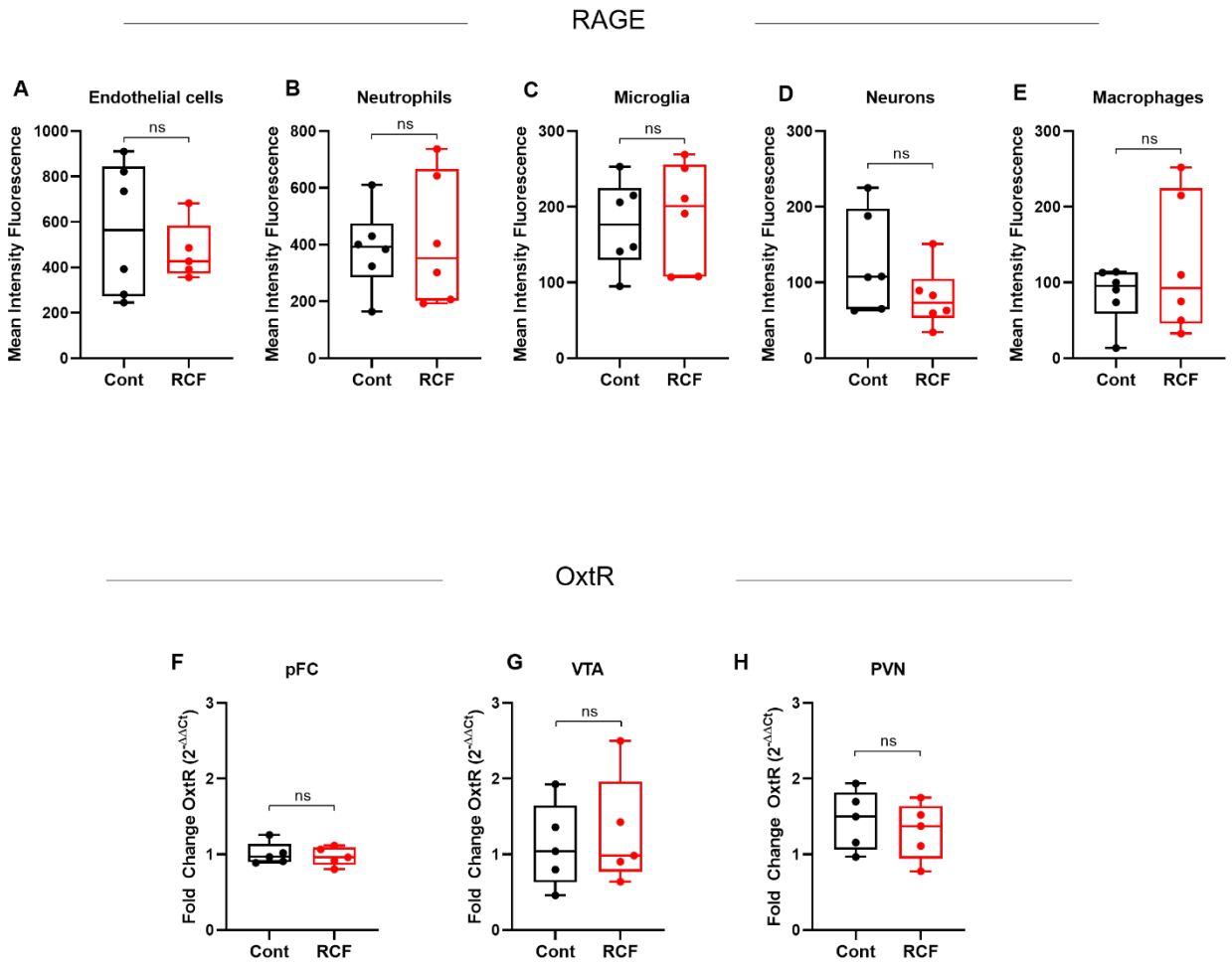
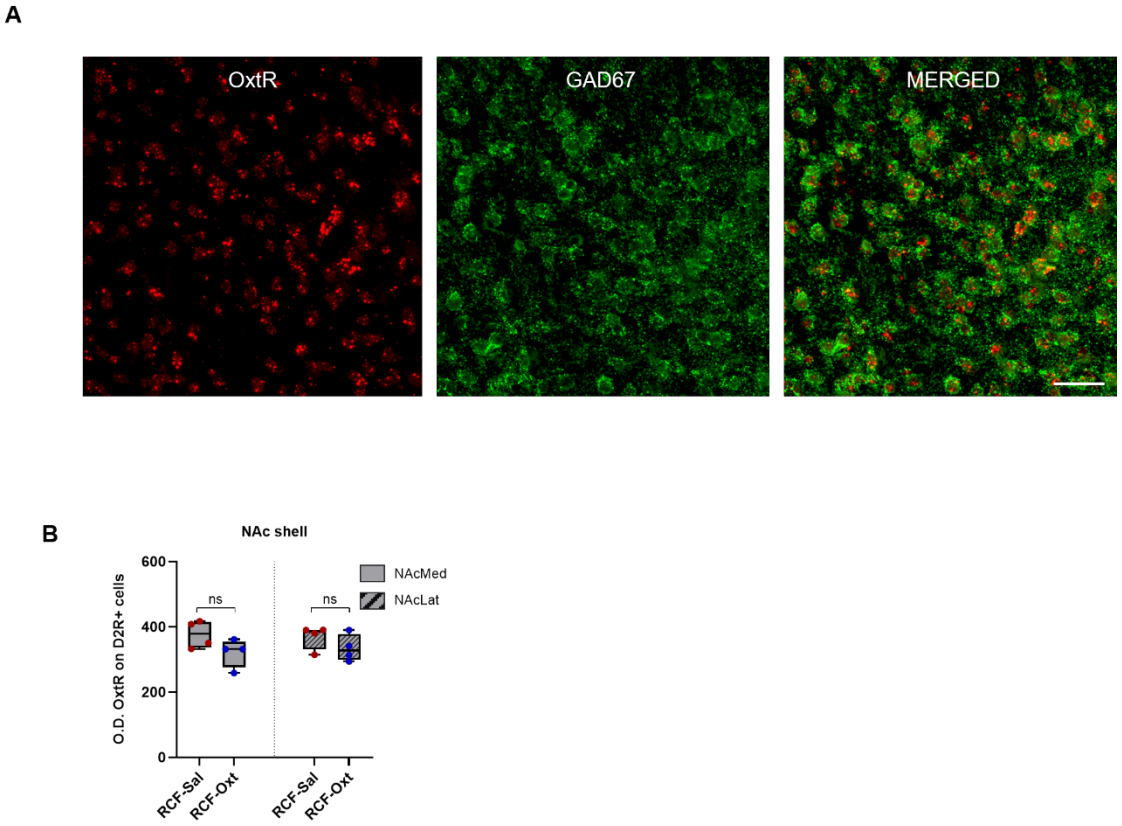


Fig. S2. RCF affects RAGE expression in pFC and does not impact OxtR expression in pFC, VTA and PVN.

565



566
567
568 **Fig. S3. OxtR expression on NAc GABAergic neurons.**

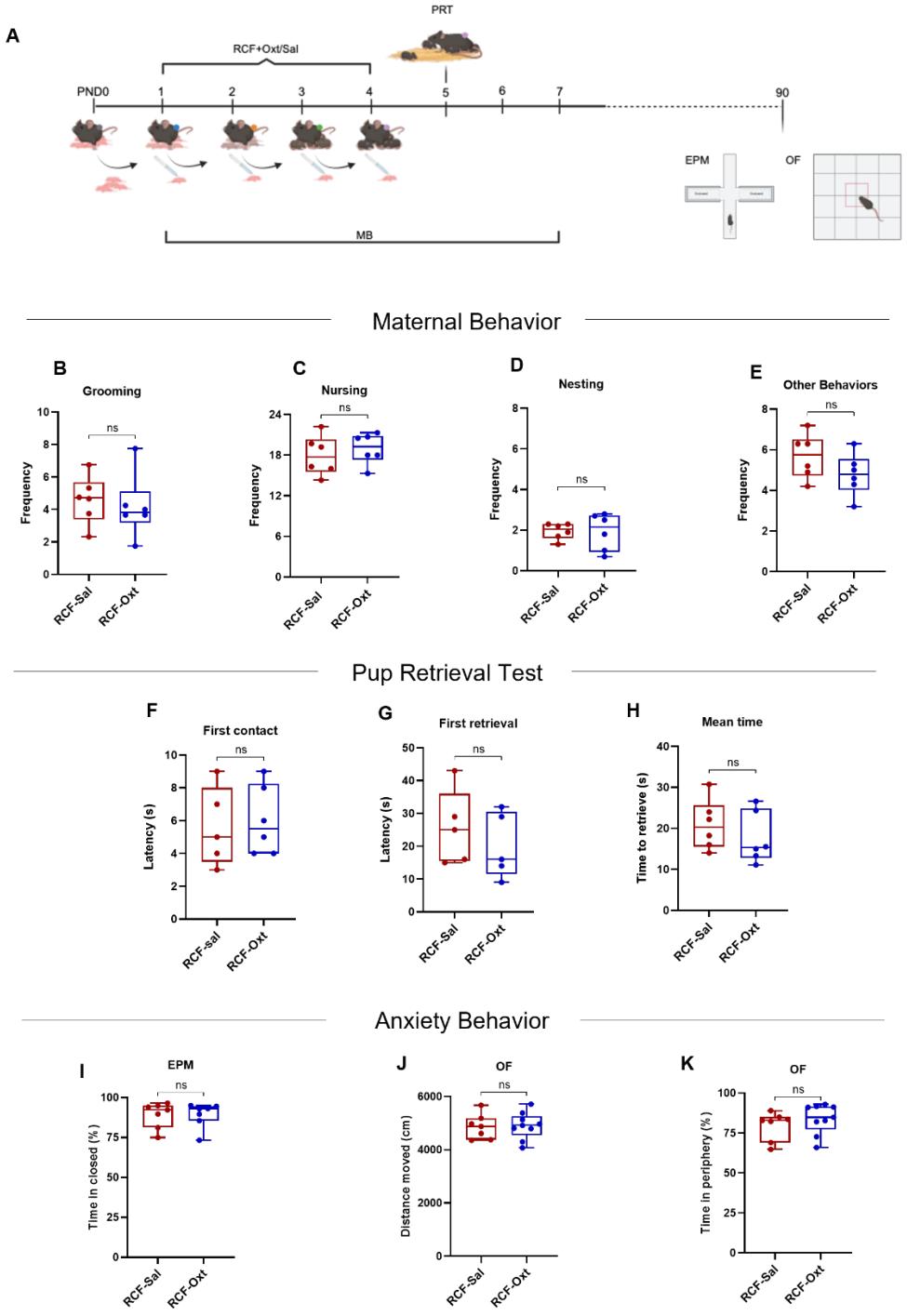


Fig. S4. Early pharmacological treatment does not alter maternal behavior and does not impact anxiety-like behavior in adult mice.

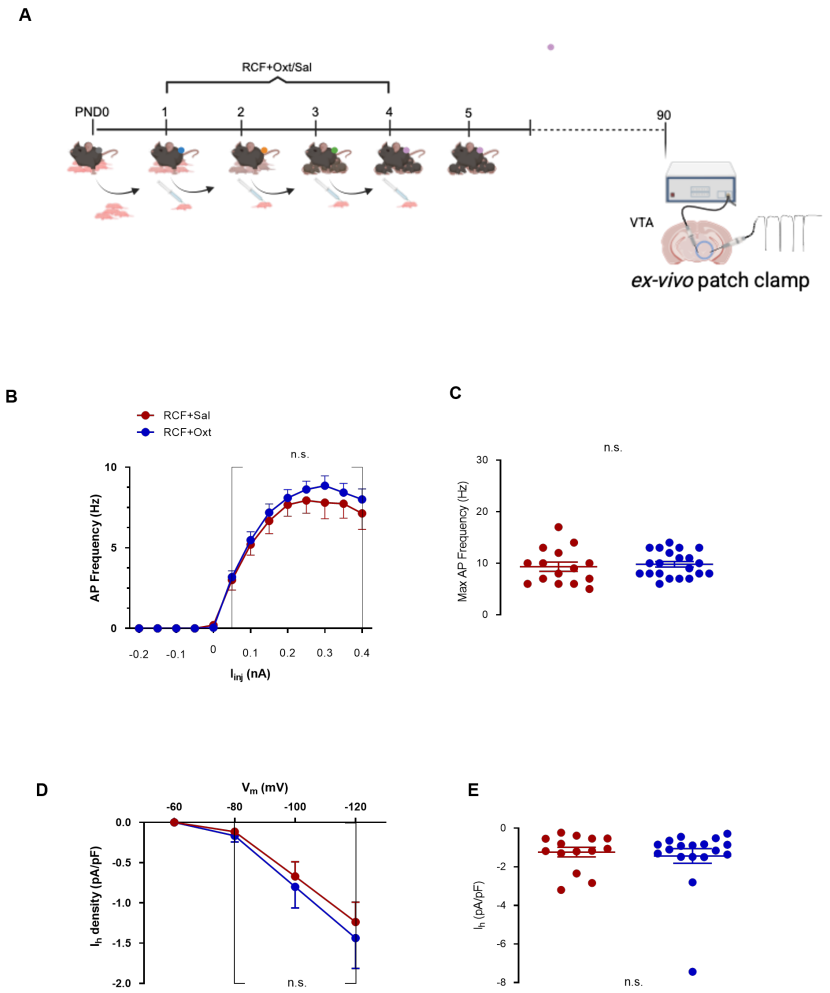


Fig. S5. Lack of modulation of VTA DA neurons by early OXT treatment.

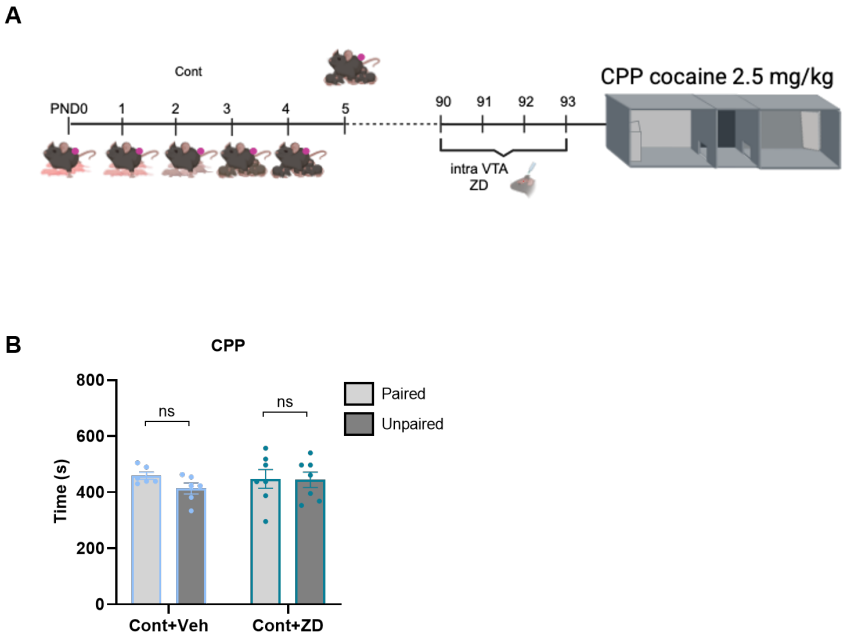


Fig. S6. Effects of pharmacological modulation of the *Ih* current in VTA DA neurons on cocaine-induced C

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