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Long-term offspring loss in lactating rats: Neurobiological and emotional consequences in a novel animal model

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The maternal bond is a vital social connection that supports the survival and well-being of both the caregiver and offspring. Disruption of this bond, particularly following offspring loss, can result in profound trauma with long-lasting consequences. While considerable research has focused on the impact of maternal separation on offspring development, the biological effects of offspring loss on the mother remain largely unexplored. In this study, we examined the long-term effects of offspring loss on neuroplasticity, the oxytocin (OXT) and corticotropin-releasing factor (CRF) systems, and stress-coping behaviors in Sprague-Dawley rat mothers. We compared mothers that experienced one day of motherhood followed by 19 days of offspring loss, mothers that were with their pups throughout the 20 days, and naive virgin rats.
Our results reveal that mothers who lost their pups expressed increased oxytocin receptor binding and decreased dendritic spine density in limbic brain regions, with no changes in estrogen receptor α or calbindin cells. Additionally, separated mothers showed increased passive stress-coping behaviors in the forced swim test and elevated plasma corticosterone levels. Remarkably, the passive stress-coping behavior was rescued by central CRF receptor blockade but not by oxytocin treatment, suggesting that the CRF system plays a key role in the grieving process following offspring loss, and highlights the suitability of the rat model for studying maternal grief. This research provides insights into the complex neurobiology of grief, and suggests potential directions for future studies.

Introduction

The maternal-infant bond is widely recognized as one of the strongest social bonds in mammals[1]. In humans, disruption of this bond, such as following offspring loss, triggers grief-like reactions in bereaved mothers, encompassing a range of emotional, cognitive, and behavioral responses, including depression and altered stress response. These dysregulations can lead to prolonged grief disorder (PGD)[2,3,4]. However, the neurobiological consequences of offspring loss on the mother's brain, particularly in terms of neuroplasticity, oxytocin (OXT), and corticotropin-releasing factor (CRF) systems, remain poorly understood. Extensive research has explored the maternal bond in rats, showing that reduced maternal care negatively impacts the offspring development and acts as emotional trauma (for review see: [5]). On the other hand, significative behavioral consequences have also been found in the mothers experiencing a repeated offspring separation[6, 7,8]. While previous research mainly utilized paradigms of repeated maternal separation in rodents (for review see: [9]), these models do not fully replicate the experience of complete offspring loss, a more accurate model for human maternal grief. A limited number of studies have specifically addressed the impact of permanent offspring loss on rat mothers[10,11] (for review see [12]). In our previous work, we explored the effects of offspring loss after birth on maternal brain function and stress-coping behavior during the first postpartum week, revealing heightened passive stress-coping responses, altered neuronal activity, and decreased oxytocin receptor (OXT-R) binding in the central amygdala (CeA)[13]. Building on this, we hypothesized that prolonged offspring loss over 19 days in rats could trigger neurobiological changes paralleling aspects of grief in humans[12], with particular emphasis on the CRF and OXT systems. Social bond disruptions in rodents have been shown to alter neurotransmitter systems, neuroplasticity, and behavior[14,15]. As an example, in the monogamous and biparental prairie vole (*Microtus ochrogaster*) partner-loss increases CRF signaling thereby leading to reduced OXT signaling in the nucleus accumbens (NAcc) as well as changes in microglia activity and morphology in a brain region- and sex-specific manner[16-17,18].
The CRF system, involved in stress response and depression, has been studied in both humans and rodents, with dysregulated CRF activity linked to mood disorders[19-24]. The neuropeptidergic ligands CRF and Urocortin 1-3 as well as their receptors CRF-R1 and -R2 are expressed both peripherally and centrally, and they are involved in physiological, autonomic, and behavioral responses to stress[20,25]. Depressed patients often have increased CRF levels in the cerebrospinal fluid and elevated CRF receptor binding in specific brain regions[26,27], suggesting an upregulated CRF system activity[28,29]. CRF overexpression in rats has been associated with depressive symptoms, altered HPA axis function, and behavioral changes[30], while chronic stress can lead to CRF system sensitization[31].
OXT, another key neuropeptide in social bonding, plays a crucial role in the formation of the maternal bond[32-33,34], among other social bonds[35]. OXT-R are distributed throughout the brain, and OXT signaling is involved in modulating stress responses and facilitates social behavior in both humans and rodents[36]. OXT is thought to have antidepressant effects, potentially by reducing the HPA axis reactivity[37,38-39], making it an attractive candidate for understanding grief-

related disorders such as depression[36,35] including postpartum depression[40].

Brain neuroplasticity, or the brain's ability to reorganize itself in response to experiences, is another important factor that may contribute to depressive and grief symptoms. Studies have suggested that changes in dendritic spine density, a key marker of neuroplasticity, may be implicated in depression[41], though the precise mechanisms remain unclear[42].

Estrogen receptor α (ESR1) has been identified as a crucial regulator of neuroplasticity and emotional processing, influencing dendritic spine formation and OXT-R expression[43-44,45]. Particularly, high density of ESR1 positive cells are found in calbindin immunoreactive (ir+) cells of the ventromedial hypothalamus (VMH)[46] and calbindin ir+ cells are decreased in post-mortem depressed patients' occipital cortex[47]. Given these insights, we hypothesized that early loss of offspring would lead to enduring changes in the maternal emotionality, neuroendocrine systems and neuroplasticity. To test this hypothesis, we studied lactating female Sprague-Dawley rats that experienced 1-day motherhood followed by 19 days of offspring loss. We assessed the impact of this experience on their emotionality, HPA axis reactivity, OXT-R binding, dendritic spine density, and ESR1 and calbindin ir+ cells expression in key limbic and maternal brain regions. This study offers valuable insights into the potential use of lactating rats as an animal model for studying the complex phenomenon of grief, with potential therapeutic implications for central CRF receptor modulation.

Materials and Methods

Animals

Sprague-Dawley rats were obtained from Charles River Laboratories (Sulzfeld, Germany) and kept under standard laboratory conditions (12:12h light/dark cycle, lights on at 7 a.m., room temperature 22 ± 1°C, relative humidity 55 ± 5°C) with access to standard rat chow (ssniff-Spezialdiäten GmbH, Soest, Germany) and water ad libitum. After 7 days of habituation, females were mated with sexually experienced male Sprague-Dawley rats in standard laboratory cages (Eurostandard Type IV, 60 cm x 40 cm x 20 cm) for 10 days. From pregnancy day 18 on, pregnant females were single housed for undisturbed delivery in observational cages (plexiglass, 38 cm x 22 cm x 35 cm); all rats delivered within 3-4 days after being single housed. The studies were conducted in accordance with the ARRIVE guidelines, the European regulations of animal experimentation (European Directive 2010/63/EU), and were approved by the local government of Unterfranken (Bavaria, Germany). According to the 3- Rs principles, all efforts were made to minimize the number of animals and their distress or suffering.

Study design

In this study, we focus on two primary groups: control lactating rats that were non-maternally separated (NMS20) and separated lactating rats (LD1+19) that were separated from their offspring on lactation day 1 (LD1) for a total duration of 19 days (**Fig. 1**). The 19-day period was chosen as it precedes weaning (LD21), a critical timepoint when the mother-offspring bond remains strong in rats[48]. We first analyzed the effects of prolonged offspring loss on OXT-R

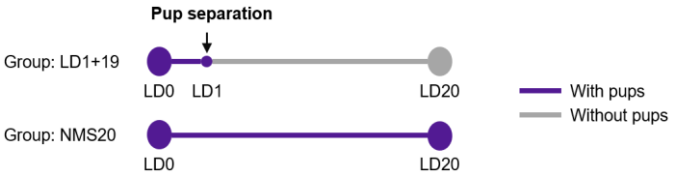

Figure 1: Schematic timelines summarizing the maternal separation paradigm. Abbreviations: LD, lactation day; NMS, non-maternal separation.

binding expression and ESR1-ir+ cells in brain regions involved in maternal behavior. This initial step allowed us to characterize neuroendocrine adaptations specifically within the maternal brain in response to offspring loss and to identify target brain regions for further analyses. Subsequently, we introduced an additional group of Virgin rats to examine dendritic spine density and behavioral changes, providing insight into experience-dependent plasticity in maternal circuits.

Experimental groups

Rats were randomly assigned to the following experimental groups: Virgin (control group), non-maternally separated for 20 days (NMS20; control group), mothers separated on lactation day 1 for 19 days (LD1+19). Each litter was adjusted to 8 pups of mixed sexes on LD0 (delivery day). In the LD1+19 group, all pups were removed on LD1 at 10.00 a.m. Afterwards, LD1+19 mothers were left undisturbed in their home cage. For comparison of the single housing duration, Virgin rats were kept single-housed for the same amount of time.

Experimental design

Experiment 1: Rats were treated as above and sacrificed on LD20 without further manipulations (**Fig. 1**). Brains were taken for analysis of OXT-R binding (NMS20 = 6; LD1+19 = 6), for immunofluorescence analysis (NMS20 = 7; LD1+19 = 5), and for Golgi staining (Virgin = 5; NMS20 = 4; LD1+19 = 4).

Experiment 2: Rats from all three groups (Virgin = 9; NMS20 = 15; LD1+19 = 15) were tested for locomotor and anxiety-like behavior in the light dark box (LDB) on LD 19 and in the open field (OF) on LD 20, after which they were sacrificed.

Experiment 3: Rats from all three groups (Virgin = 8; NMS20 = 13; LD1+19 = 14) were tested for stress-coping behavior in the classic forced swim test (FST) on LD20. Within 5 min afterwards, they were sacrificed, and trunk blood was collected for plasma CORT analysis.

Experiment 4 and 5: Rats were treated as above. On LD15, they underwent stereotaxic implantation of an intracerebroventricular (icv) guide cannula for acute substance infusion. On LD19, rats were subjected to the pre-test of the modified FST (mFST). On LD20, all rats were tested in the mFST after infusion of vehicle (VEH) or either the CRF-R1/2 antagonist D-Phe (Experiment 4: NMS20 VEH = 10; LD1+19 VEH = 8; LD1+19 D-Phe = 9) or synthetic OXT (Experiment 5: NMS20 VEH = 8; LD1+19 VEH = 8; LD1+19 OXT = 7). Afterwards, all rats were sacrificed.

Brain analyses

OXT-R autoradiography. Rats from NMS20 and LD1+19 were anesthetized and underwent cardiac perfusion with ice-cold 1x PBS, were decapitated, brains were flash-frozen in n-methylbutane, and stored at -20 °C until cutting into

coronal sections of 16 µm using a cryostat (CM3050S, Leica Microsystem GmbH). For each brain region of interest, i.e., bed nucleus of the stria terminalis (BNST), nucleus accumbens shell (NAcc shell), medial preoptic area (MPOA), accessory olfactory bulbs (AOB), central amygdala (CeA), agranular insular cortex (AIP), ventromedial hypothalamus (VMH), lateral septum (LS), prelimbic cortex (PL), six slices per rat were collected on SUPERFROST microscope slides and stored at -20 °C until further processing. The OXT-R autoradiography was performed following an established protocol[49]. Briefly, the ornithin-vasotocin analogue [125I]-OVTA [d(CH2)5[Tyr(Me)2,Thr4,Orn8,[125I]Tyr9-NH2]; Perkin Elmer, USA) was used as a tracer. First, the slides were thawed and allowed to dry thoroughly at room temperature. The tissue was shortly fixed via 0.1 % PFA, washed 2 x in Tris (50 mM, pH 7.4), covered with the tracer solution (50 mM tracer, 10 mM MgCl2, 0.1 % BSA) for 60 min, washed 3 x in Tris / MgCl2 buffer for 7 min, each, followed by 30 min spinning in Tris / MgCl2. Finally, slides were dipped into water and air dried before being exposed to Biomax MR films (Kodak, Cedex, France) for 15 days. The films were scanned using the EPSON Perfection V800 Scanner (Epson GmbH, Munich, Germany), and the optical density of each region of interest was analyzed using ImageJ[50] by subtracting the background activity as previously described[49,51]. The analyses were performed simultaneously for 6 slices per rat and per region.

Immunofluorescence staining. Rats from NMS20 and LD1+19 were anesthetized and perfused with ice-cold 1x PBS and subsequently with 4 % paraformaldehyde (PFA) dissolved in 1x PBS. Brains were removed, fixed overnight in 4 % PFA, and subsequently incubated in 30 % sucrose in 1x PBS until the brain sank. After fixation and cryoprotection in sucrose, brains were flash-frozen in n-methylbutane, and stored at -20 °C until cutting into coronal sections of 16 µm using a cryostat (CM3050S, Leica Microsystem GmbH, Nussloch, Germany). Six consecutive slices containing the VMH region were collected per rat on SUPERFROST microscope slides and stored at -20 °C until further processing. Slides were washed in 1x PBS, permeabilized with 0.3 % Triton, blocked with 5 % normal goat serum (Vector Laboratories, Newark, USA), and incubated overnight at 4 °C with primary antibodies (Merck Millipore, rabbit anti ER alpha 1 : 2000; Merck, mouse anti-NeuN 1 : 1000; Synaptic Systems, chicken anti-calbindin 1 : 2000). The next day, slides went through 1 h incubation with secondary antibodies (Alexa Fluor 488-conjugated anti-rabbit IgG 1 : 800, Alexa Fluor 594-conjugated anti-mouse IgG 1 : 800 and Alexa Fluor 649-conjugated anti-chicken IgG 1 : 800) at room temperature. All slides were finally mounted (ROTI®Mount FluorCare DAPI, Carl Roth, Karlsruhe, Germany).

Golgi staining for spine density visualization. The spine density was assessed in Virgin, NMS20 and LD1+19 to investigate neuronal plasticity. Firstly, the Rapid GolgiStainTM Kit (FD NeuroTechnologies, Columbia, USA) was used and applied according to the manufacturer's protocol. Rats were sacrificed with CO2 and brains were collected. Brains were cut into 100 µm slices using a cryostat (CM3050S, Leica Microsystem GmbH) at -28° and placed on gelatin-coated microscope slides (FD NeuroTechnologies, Columbia, USA). After complete drying, Golgi staining was performed, and sections were stored at room temperature in the dark before acquiring images using a brightfield microscope (Leica Thunder DM6 B, Camera Leica DFC9000 GT). Secondly, ImageJ[50] was used to count the spines of secondary dendrites in the PL, AIP, BLA and VMH. Secondary dendrites from different neurons (between 4 and 12, depending on the brain region) were chosen from 10 X overview preview images followed by acquiring 40 X focus images for analysis. The ROI of each dendrite was selected, brightness and sharpening function were adjusted. Spines were counted on a 25 - 30 µm2 section of the dendrite (each dendrite belonging to a different neuron) and the absolute spine density was calculated by using the following formula:

(n = number of spines; µm = dendritic length): $\frac{n-1}{\mu m}$ [52].

Behavioral experiments

Light dark box (LDB). Rats of experiment 2 were tested in the LDB to measure anxiety-like behavior[53]. The apparatus consisted of a light (40 cm x 50 cm, 180 lux) and a dark compartment (40 cm x 30 cm, 0 lux) connected via an opening (7.5 cm x 7.5 cm). At the beginning of the test, rats were placed in the center of the light box, and the behavior was recorded for 10 min for later analysis by an experimenter blind to the treatment with EthoVision XT (Noldus, Wageningen, the Netherlands). The following behaviors were scored: (a) percentage of time in the light box; (b) latency to re-enter the light compartment; (c) locomotion. Before each test, the arena was cleaned with tap water and dried thoroughly.

Open-field (OF). Rats of esperiment 2 were tested in the OF to measure locomotor- and anxiety-like behavior[54]. The apparatus consisted of an empty rectangular arena (80 x 80 cm). Rats were placed in one corner of the apparatus and left to freely explore the arena for 10 min. The behavior was recorded for later analysis by an experimenter blind to the treatment with EthoVision XT (Noldus, Wageningen, the Netherlands). The following behaviors were scored: (a) locomotion; (b) velocity; (c) center entries. Before each test, the arena was cleaned with tap water and dried thoroughly.

Classic forced swim test (FST). Passive stress-coping behavior was tested in the classic FST[55]. Between 9.00 a.m. and 12.00 p.m., rats of experiment 3 were placed for 10 min in a cylindrical tank (50 cm high, 30 cm diameter) filled with tap water (23 ± 1 °C) to a depth that rats could not touch the bottom with their hind paws or tail. Trials were recorded for later analysis using the software JWatcher (https://www.jwatcher.ucla.edu) by an experimenter blind to the treatment. The total time spent on floating, i.e., passive stress-coping behavior, indicative of depressive-like behavior[55], was analyzed.

Modified forced swim test (mFST). The mFST was performed as it is best for testing antidepressant drugs[55] on two consecutive days between 9.00 a.m. and 12.00 p.m. On LD19, rats underwent a pre-test session for 15 min. On LD20, rats were tested in a 10 min FST following pharmacological manipulations. All other test conditions and behavioral analyses were as described above.

Stereotaxic surgery

On LD15, rats of experiments 4 and 5 were implanted with a stainless steel guide cannula (21-G, length 12 mm), above the right ventricle (1.0 mm posterior, 1.6 mm lateral to

bregma, 1.8 mm ventral)[56,57] under isoflurane anesthesia as described before[57]. The guide cannula was closed using a 25-G stainless steel stylet of the same length as the guide cannula. All rats received a subcutaneous injection of analgesics (0.05 mg/kg Buprenorphine, Bayer Vital GmbH, Leverkusen, Germany) pre-surgery to avoid post-surgical pain.

Drug administration

On LD20, rats received one acute icv infusion of either VEH (5 µL sterile Ringer's solution; pH adjusted to 7.4; Braun, Melsungen, Germany), the CRF-R1/2 antagonist D-Phe [(D-Phe12, Nle21-38, a-Me-Leu37)-CRF (12–41); human/rat; 10 µg / 5 µL]; Bachem, Bubendorf, Switzerland] or synthetic OXT (1 µg / 5 µL; Tocris, Nordenstadt, Germany), 10 min prior to the mFST on the second day of testing. Doses were chosen based on previous studies[57]. For the acute icv infusion, a 25-G stainless steel infusion cannula (length 14 mm) was connected to a 10 µL micro syringe via PE-50 tubing (50 cm) and lowered into the guide cannula, where it was kept in place by a piece of silicon tubing during slow substance infusion for approximately 30s[39].

Verification of cannula placement

After the final behavioral test, rats were sacrificed with CO2 and blue dye was injected via the infusion system into the guide cannula. Verification of the icv location of the infusion site was observed by dye spread throughout the ventricular system. Only animals with correct, histologically verified infusion sites were included in the statistical analyses.

ELISA for plasma CORT

Within 5 min after the classic FST, rats were sacrificed and approximately 1 ml trunk blood was collected in EDTA-coated tubes on ice (Sarstedt, Numbrecht, Germany), centrifuged at 4 °C (10,000 rpm/12298 rcf, 10 min), aliquoted and stored at −20 °C until the assay was performed using a commercially available ELISA kit for corticosterone (CORT) (Tecan IBL International GmbH, Hamburg, Germany) following the manufacturer's protocol.

Statistical analysis

All statistical analyses were performed with GraphPad PRISM 9 (GraphPad Software, San Diego, USA). Normality and homoscedasticity were verified (Shapiro-Wilk or Kolmogorov–Smirnov test and Brown-Forsythe test, respectively) and analysis of outliers was run via the ROUT method.

OXT-R binding, ESR1 and calbindin ir+ cells analyses were performed via a two-sample Student's t-test. Dendritic spine density, plasma CORT concentration, anxiety-like, locomotor and stress-coping behaviors were analyzed via one-way ANOVA followed by Sidak post hoc multiple comparisons. Data are presented as mean ± SEM; $p \leq .05$ was considered significant.

Results

Increased OXT-R binding in the VMH and PL after offspring loss

To assess whether the offspring loss experience could affect OXT-R binding in limbic and maternal network regions, we performed a receptor autoradiography (**Fig. 2a**) comparing non-maternally separated mothers (NMS20) and separated mothers (LD1+19). In detail, LD1+19 dams had significantly higher levels of OXT-R binding in the VMH (M = 0.257, SD = 0.083; **Fig. 2b, c**) and the PL (M = 0.070, SD = 0.005; **Fig. 2b, d**) than the NMS20 [VMH: M = 0.147, SD = 0.077, t (10) = 2.366, p < .039; PL: M = 0.066, SD = 0.002, t (10) = 2.203, *p* < .052]. No significant differences were found in any other region analyzed [MPOA (*t* (10) = 0.215, *p* < .834); BNST (*t* (10) = 0.828, *p* < .427); CeA (*t* (10) = 0.061, *p* < .952); OB (*t* (10) = 0.595, *p* < .565); NAcc shell (*t* (10) = 1.440, *p* < .180); AIP (*t* (10) = 1.212, *p* < .254); LS (*t* (10) = 0.613, *p* < .554)].

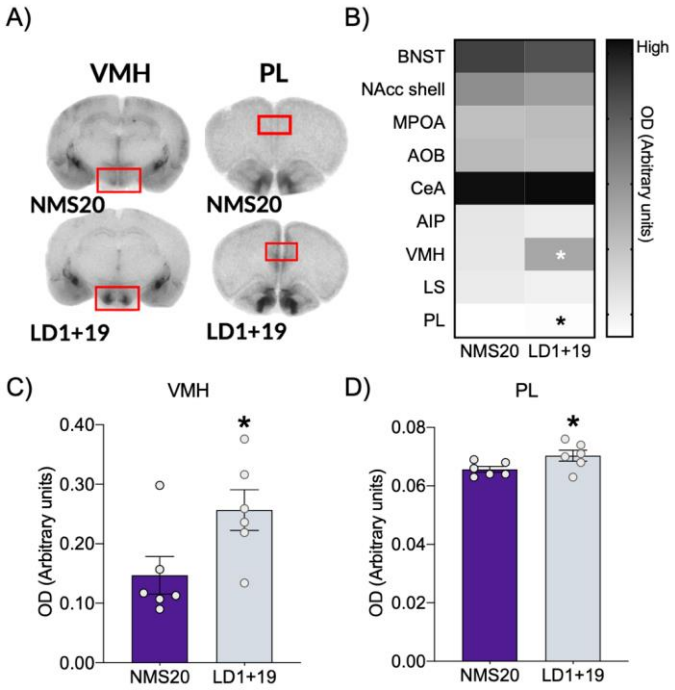

Figure 2: OXT-R binding in brain regions of the limbic system and maternal network. (A) Representative coronal brain sections of the VMH and PL showing differences in OXT-R binding. **(B)** Overview heatmap of gray density (arbitrary units) for OXT-R binding in the analyzed brain areas (* p ≤ .05 versus NMS20). Optical density (arbitrary units) for OXT-R binding in the **(C)** VMH and **(D)** PL. Abbreviations: AIP, agranular insular cortex; AOB, accessory olfactory nuclei; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; LS, lateral septum; MPOA, medial preoptic area; NAcc, nucleus accumbens; PL, prelimbic cortex; VMH, ventral medial hypothalamus. Student's t Test. Data are expressed as mean ±SEM. * p ≤ .05 versus NMS20.

ESR1 and calbindin ir+ cells in the VMH and PL did not differ between groups

To find a possible link between the observed changes in OXT-R binding and the expression of ESR1 in both the VMH and PL, we analyzed the expression of ESR1 and calbindin ir+ cells of NMS20 and LD1+19 rats. There was no significant difference in the percentage of ESR1-ir+ cells (t (10) = 0.456, p < .658; **Fig. 3a, b**) or of calbindin ir+ cells (t (10) = 0.706, p < .496; **Fig. 3a, c**) between the groups in the VMH and not detectable ESR1-ir+ cells in the PL (data not shown).

Reduced secondary dendritic spine density in the VMH after offspring loss

To determine the effects of offspring loss on neuroplasticity, we analyzed the secondary dendritic spine density in the VMH, PL, BLA and AIP of NMS20 and LD1+19

and Virgin group. The statistical analysis revealed a significant difference in secondary dendritic spine density between the groups in the VMH, only (one-way ANOVA: F (2, 10) = 8.734; p < .006; **Fig. 4**), and post hoc multiple comparisons showed significantly lower levels in LD1+19 dams compared to Virgin (p < .024) and NMS20 (p < .009). No significant differences were found in the PL [Virgin: 0.64 ± 0.07; NMS20: 0.53 ± 0.04; LD1+19: 0.62 ± 0.04; one-way ANOVA: F (2,15) = 0.590, p < .356], the BLA [Virgin: 0.61 ± 0.03; NMS20: 0.64 ± 0.009; LD1+19: 0.61 ± 0.02; one-way ANOVA: F (2,16) = 0.158, p < .855], and the AIP [Virgin: 0.62 ± 0.05; NMS20: 0.69 ± 0.04; LD1+19: 0.68 ± 0.04; one-way ANOVA: F (2,15) = 0.599, p < .562] .

Anxiety-like behavior was not affected by offspring loss

To evaluate if offspring loss could alter anxiety-like behavior, Virgin, NMS20 and LD1+19 rats were tested in the LDB on LD19. Groups differed in the latency to re-enter the light box as a measure of anxiety-like behavior [Virgin: 213.5 ± 77.5 sec; NMS20: 48.6 ± 15.9 sec; LD1+19: 121.9 ± 31.9 sec; Kruskal-Wallis test, p < .045] with the Virgin group tending to have a higher latency compared to NMS20 (Dunn's test, p < .058). No significant differences were found in the time spent in the light box [Virgin: 34.0 ± 6.3 sec; NMS20: 36.6 ± 3.6 sec; LD1+19: 42.0 ± 5.1 sec; one-way ANOVA: F (2,36) = 0.676, p < .515] or in locomotion [Virgin: 3778 ± 326 cm; NMS20: 3733 ± 194 cm; LD1+19: 3851 ± 312 cm; one-way ANOVA: F (2,36) = 0.054, p < .948]. LD1+19 were not different from the other groups in any measured parameter.

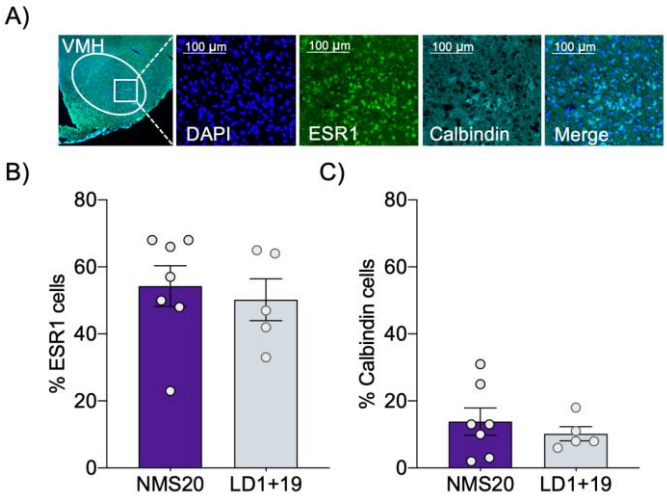

Figure 3: ESR1 and calbindin ir+ cells in the VMH. (A) Representative fluorescent images showing DAPI (blue), ESR1 cells (green), calbindin (cyan) and merged channels in the VMH. Percentage of **(B)** ESR1 and **(C)** calbindin ir+ cells in the VMH. One-way ANOVA. Data are expressed as mean ± SEM.

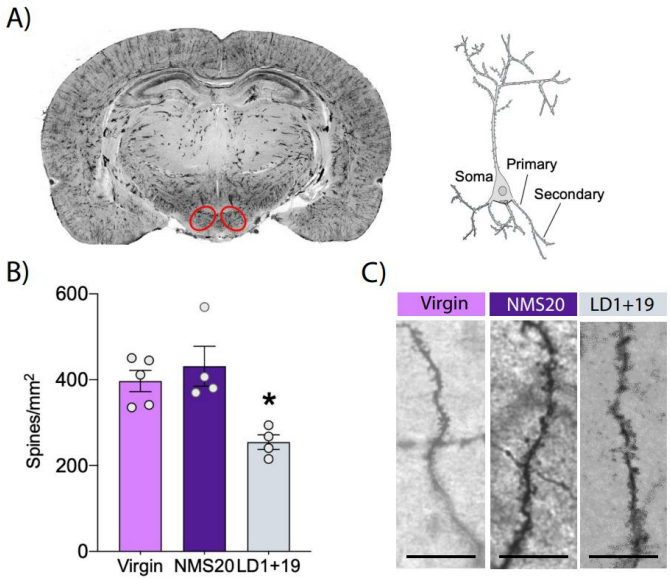

Figure 4: Secondary dendritic spine density in the VMH. (A) Representative coronal whole brain section with Golgi staining. **(B)** Absolute spine density / mm2 across experimental groups. **(C)** Schematic illustration of the neuronal dendritic arbor. One-way ANOVA. Data are expressed as mean ± SEM. * p < .05 versus all other groups. Scale bar 10 µm.

Locomotor activity was not affected by offspring loss

The same animals were tested in the OF for locomotor activity on LD20. The groups significantly differed in the distance travelled (one-way ANOVA: F (2,36) = 3.773, p < .032; **Fig. 5a**) and in the velocity (one-way ANOVA; [F (2,36) = 3.788], p < .032; **Fig. 5b**). In detail, Sidak post hoc test revealed increased locomotion (p < .029) and velocity (p < .028) in Virgin compared to NMS20. No significant differences were found in the number of entries in the center of the arena [Virgin: 21.4 ± 2.7; NMS20: 16.3 ± 1.5; LD1+19: 20.5 ± 2.0; one-way ANOVA: F (2,36) = 1.858, p < .170]. LD1+19 were not different from the other groups in any measured parameter.

Increased passive stress-coping behavior and virgin-like stress response after offspring loss

A different cohort of Virgin, NMS20 and LD1+19 rats were tested for passive stress-coping behavior in the FST on LD20. Groups significantly differed in the time spent floating during the 10-min test (one-way ANOVA: F (2,32) = 6.233, p < .005; **Fig. 5c**), and Sidak post hoc multiple comparisons revealed that LD1+19 spent more time floating compared to Virgin (p < .019) and NMS20 (p < .014). The rats' plasma was collected for CORT measurement 15 min after the start of the FST, i.e., 5 min after termination. The statistical analysis showed that plasma CORT levels significantly differed between the groups (one-way ANOVA: F (2,28) = 13.50, p < .0001; **Fig. 5d**), and Sidak post hoc multiple comparisons revealed lower plasma CORT concentration in NMS20 compared to Virgin (p < .002) and LD1+19 (p < .0001). Interestingly, plasma CORT concentration in LD1+19 was not different from Virgin (p > .05 vs Virgin group), suggesting that the separation experience reverted the lactation-induced changes in the stress axis response.

Central CRF-R1/2 blockade normalized passive-stress coping behavior in the mFST after offspring loss

Following a pre-test on LD19, NMS20 and LD1+19 rats were tested in the mFST after central pharmacological manipulation. Dams received a single acute icv infusion of either VEH (NMS20, LD1+19) or CRF-R1/2 antagonist D-Phe (LD1+19) 10 min prior to the mFST. The statistical analysis revealed a significant difference between the groups (one-way ANOVA: $F_{(2,23)} = 5.828$, $p < .009$; **Fig. 6a**). In detail, LD1+19 VEH spent more time floating compared to NMS20 VEH (Sidak post hoc multiple comparisons, $p < 0.047$), thereby confirming our previous results in the classical FST. Most importantly, the percentage time spent on floating was significantly lower in LD1+19 D-Phe compared to LD1+19 VEH ($p < .014$), even reaching control levels ($p > .05$ vs NMS20 VEH).

Central OXT infusion did not alter passive-stress coping behavior in the mFST

A different cohort of rats were tested in the mFST after receiving a single acute icv infusion of either VEH (NMS20, LD1+19) or OXT (LD1+19) 10 min prior to the test. The groups differed significantly in the percentage time spent on floating (one-way ANOVA: $F_{(2,20)} = 5.273$, $p < .042$; **Fig. 6b**); we confirmed again our previous results demonstrating increased percentage time spent on floating in the LD1+19 VEH versus NMS20 VEH (Sidak post hoc multiple comparisons: $p < .046$), but LD1+19 OXT rats were not different from LD1+19 VEH ($p < .187$).

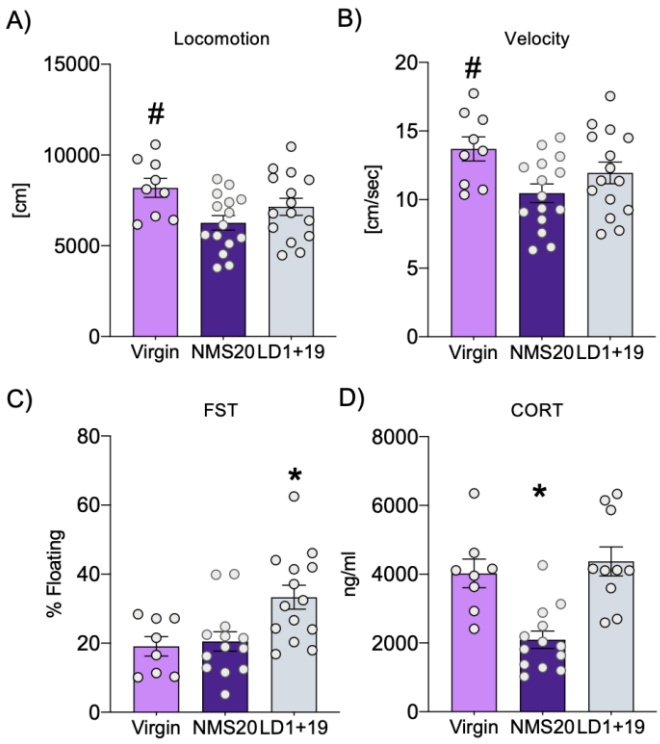

Figure 5: Locomotor behavior in the OF, passive stress-coping behavior in the classic FST, and plasma CORT. (A) Total distance traveled in cm and **(B)** mean velocity over the 10 min OF test. **(C)** Percentage of floating during the 10-min classic FST. **(D)** Plasma CORT concentration immediately after termination of the FST. One-way ANOVA. Data are expressed as mean ± SEM. # $p < .05$ versus NMS20; * $p < .05$ versus all other groups.

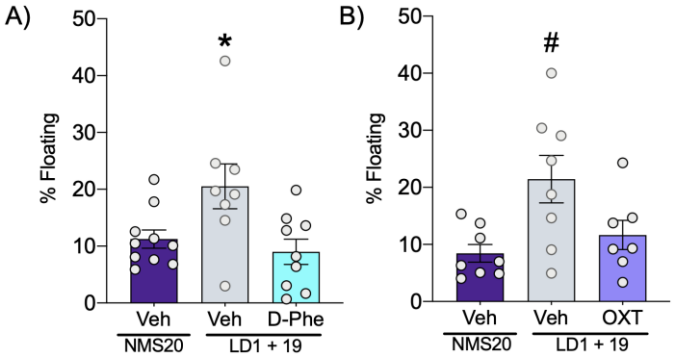

Figure 6: Passive stress-coping behavior in the modified FST following central pharmacological manipulation of the brain CRF and OXT systems. Percentage of floating during the 10 min modified FST following icv infusion of **(A)** VEH or the CRF-R1/2 antagonist D-Phe, and **(B)** of VEH or synthetic OXT. One-way ANOVA. Data are expressed as mean ± SEM. * $p < .05$ versus all other groups; # $p < .05$ versus NMS20.

Discussion

Losing a child is one of the most devastating experiencing in life. When grieving over such loss, parents experience a negative impact in their physical and mental well-being [58,59] with a greater risk of developing an invaliding PGD[60,4]. Adequate animal models are required to identify and comprehend the neurobiology of offspring loss to help develop effective treatments. In the present study, we used rat dams as a novel animal model with the potential to reconstitute the parallels to grieving mothers. Specifically, following 19-days of offspring loss we observed increased OXT-R binding and reduced dendritic spine density in the VMH, and increased OXT-R binding in the PL. Furthermore, our findings show that, compared to control mothers and virgins, the separated mothers' stress response and passive stress-coping behavior were increased. Importantly, the impaired phenotype was rescued by central CRF-R1/2 antagonism, but not by OXT-R agonism, providing significant insight for potential therapeutical approaches.

Increased OXT-R binding in the VMH and PL after offspring loss

To prepare the female for the new challenges as a mother, dynamic changes occur peripartum with respect to the brain OXT system[61,62]. Maternal attachment is modulated by OXT-R distribution[63]. Therefore, we examined whether the long-term offspring loss mirrored changes in OXT-R. Indeed, OXT-R binding was significantly higher in the VMH and PL in LD1+19 dams (**Fig. 2**), a finding we did not expect as we hypothesized that offspring loss would result in less OXT signaling.

The VMH is involved in regulating various social behaviors, including maternal behavior[64] and female aggression[65]. Moreover, VMH neurons are essential in controlling defensive behaviors like maternal defense[66]. Due to the high concentration of OXT-R positive cells in the VMH[67], especially in females[68], it is plausible that OXT signaling in the VMH could modulate maternal behaviors[68]. However, an involvement of OXT within the VMH in maternal behavior has not been studied in detail, yet. The PL has been associated to cognitive and emotional functions[69,70]. Interestingly, OXT-R blockade in the PL in post-partum Sprague-Dawley rats impairs maternal behavior[71]. This

highlights the role of the PL in the neural circuitry of OXT-mediated maternal bonding. Together, the data for PL and VMH suggests that the increased OXT-R binding of LD1+19 mothers reflect a compensatory mechanism triggered by forced maternal bond disruption.

Interestingly, we recently observed a trend towards increased OXT-R binding in the PL but a significant increase in the MPOA after one week of repeated 3-h maternal separation[6]. In that line, other studies also found changes in OXT-R binding associated with altered maternal behaviors, further emphasizing the crucial role of OXT-R in regulating maternal behaviors[72,73]. Prolonged separation from offspring results in reduced interaction with the pups and fewer occurrences of milk ejection reflexes[74], potentially leading to dampened OXT signaling in the brain. Indeed, prior research has shown a decrease in c-Fos expression in OXT-positive (OXT+) neurons of the supraoptic nucleus and a decrease in the number of OXT immunoreactive (OXT ir+) neurons in the paraventricular nucleus (PVN) of separated mothers[75,7]. Therefore, we speculate that the sudden offspring loss may induce alterations in OXT signaling in the brain, and increasing OXT-R in certain brain areas might be a compensatory mechanism for the less available OXT. While no changes were detected in the other regions analyzed, we cannot exclude that relevant changes may occur in those regions at the level of OXT release or mRNA expression, which is not captured by the receptor autoradiography. Future research could specifically analyze those parameters in certain brain areas to provide a better picture of their biodynamic changes.

ESR1 were not involved in increased OXT-R binding in the VMH after offspring loss

The relationship between the observed increase of the OXT-R binding and the distress experienced from offspring loss is not yet fully understood, and it remains unclear whether these changes could be attributed to endocrine factors. Estrogen, a hormone primarily associated with female reproductive function, can trigger the expression and function of OXT-R via ESR1, potentially influencing social and reproductive behaviors[44]. Furthermore, estrogen concentrations are linked to calbindin[76], which is highly expressed in the VMH[77,78]. Therefore, we examined the percentage of ESR1 and calbindin ir+ cells in the VMH and PL to see if these proteins underly the changes in local OXT-R binding after offspring loss (**Fig. 3**). As we did not observe any differences between the groups, the increase in OXT-R binding is unlikely to be caused by this specific estrogen receptor changes. However, at this point we cannot exclude that other neurohormonal systems, like the CRF system (as discussed below), might have contributed to the observed changes in OXT-R binding.

Decreased spine density in the VMH after offspring loss

In rats, the maternal brain network undergoes neuronal morphological changes in the dendritic spine density during pregnancy and the postpartum period[79]. Both human studies and rat animal models of postpartum depression demonstrate reduced dendritic spine density and neuronal changes in the hippocampus[80], prefrontal cortex[81], amygdala and insular cortex[82,83]. Due to the substantial body of research indicating a connection between alterations in spine density and OXT-R[84,85], we analyzed secondary dendritic spine density, which revealed a notable decrease in the VMH, only (**Fig. 4**). In response to chronic stress and social isolation, dendritic spine changes have been found in the BLA, PL and AIP[86,87-88]. More importantly, numerous studies have consistently highlighted the link between reduced spine density and depression (for review see: [89]). Similarly, also in humans a low spine density correlates with depressive symptoms[90]. Therefore, we speculate that the VMH, renowned for its involvement in aggression, stress response, and pain attacks, in both humans and rats[91-93], likely plays a part in the adverse effects of offspring loss on maternal well-being. Further research is needed to investigate the functional implications of reduced spine density and its potential behavioral consequences. Additionally, we acknowledge a limitation of this study: we did not monitor the estrous cycle of the Virgin rats, so the observed changes could be influenced by the reproductive state.

Anxiety-like and locomotor behaviors were not altered after offspring loss

While we confirmed decreased anxiety-like behavior in lactation (for review see: [94,95]) when comparing NMS20 to Virgin, this postpartum adaptation was not apparent anymore in separated mothers (**Fig. 5**). In fact, the total absence of the nursing and caring experience in LD1+19 and, thus, the possible lack of high OXT brain levels might account for the return of their anxiety-like behavior to virgin levels as OXT is facilitating reduced anxiety levels in lactation[96]. In a similar study, permanent removal of offspring for 4 weeks did not alter anxiety-like behavior in separated mothers compared to Virgin and lactating dams[10]. This is different to repeated prolonged maternal separation, which can result in increased anxiety-like behavior[97,98,99]. It is worth noting that in the present study, the phenotype of the Virgin group could have been influenced by the experimental design. Indeed, Virgin rats underwent a period of social isolation to match this group with the pup-loss group, and previous research in male rats has linked such stress to increased anxiety and a hyperactive phenotype[100].

Overall, our results indicate that the separated mothers did not exhibit significant impairment in anxiety-like behavior or locomotion, while the Virgin group displayed distinct behavioral characteristics likely influenced by their social isolation.

Increased passive stress-coping behavior and stress response after offspring loss

To examine the potential impact of offspring loss on emotional behavior, we conducted the classic FST. Notably, the separated mothers displayed increased duration of floating compared to both the control Virgin group and the NMS20 lactating dams, which were not different from each other (**Fig. 5c**). Importantly, we were able to confirm the increased passive stress-coping after offspring loss twice in the following experiments 4 and 5 where we aimed to manipulate the behavior with pharmacological interventions (see below). Our findings support and expand

previous work demonstrating that mother-offspring separation leads to increased depressive-like behaviors, i.e., 24-h of maternal experience followed by 3 weeks[11] and 4 weeks[10] of offspring loss increases floating behavior in the FST and extend our recent work where one week post loss induced increase passive stress-coping[13]. Hence, offspring loss has a strong and long-lasting emotional impact on the mother leading to increased passive stress-coping behavior, which is reminiscent of depressive-like behavior[55]. Remarkably, we found no differences in locomotor activity between the control NMS20 mothers and LD1+19 mothers in the OF test, which endorses that any observed changes in the FST were likely due to emotional causes rather than altered body energy or locomotion impairments.

The analysis of plasma CORT concentrations following exposure to the classic FST (**Fig. 5d**) not only confirmed the dampened stress response in lactation when comparing Virgin with NMS20 but, more importantly, revealed that the CORT levels of LD1+19 mothers were back to the level of Virgins. On one hand, this suggests that the offspring loss experience disrupts the dampened stress response in lactation[101] implying that separated mothers are more susceptible to an acute stressor (FST) compared to lactating dams. On the other hand, one cannot exclude that the stress physiology of the separated mothers came back to virgin levels due to the long separation from the offspring and, thus, the lack of mothering experience.

Altogether, those results indicate that the offspring loss experience increases passive-stress responses hinting to a depressive-like phenotype in mothers. Furthermore, offspring loss disinhibits the lactation-associated dampened HPA axis activation, thereby being no longer different from the Virgin group.

Central CRF-R1/2 antagonism, but not OXT-R agonism, reversed passive stress-coping behavior after offspring loss

The activity of the brain CRF system is downregulated in lactation to enable the mother to adequately respond to a stressor[102]. However, exposure to, e.g., severe chronic stress before / during parturition can lead to increased CRF system signaling with strong impact on the emotionality[103,104]. In fact, the CRF system is hypothesized to be a potential neuromodulator following the break of a bond as shown in, e.g., lactating prairie vole mothers following separation from the pair-bonded male partner[105]. In the present study, central administration of the CRF-R1/2 antagonist D-Phe effectively reversed the offspring loss-associated high levels of passive stress-coping in the mFST (**Fig. 6**), which is recommended to assess antidepressant drug effects[55]. Our finding suggests that permanent separation from the pups leads to increased activity of the brain CRF system. This is in line with previous studies demonstrating that acute central infusion of D-Phe rescues the increased emotionality after the loss of a social bond, e.g., in lactating[105] and male prairie voles[16] after losing the partner.

The brain CRF system can interact with the OXT system as has been shown in, e.g., lactating rats[22] and male prairie voles[18]. In the latter, when separated from their female partner, local OXT infusion into the NAcc shell rescues their passive phenotype[18]. This led us to hypothesized that the high levels of passive-stress coping behavior of separated mothers could implicate a lack of central OXT release due to the loss of the pups' stimuli. However, central OXT infusion did not alter the passive phenotype in separated mothers. The lack of an effect does not necessarily exclude the OXT system as a potential target. The central infusion, which acts in almost all brain regions at the same time, might mask positive effects happening in certain brain regions as seen, e.g., the PVN of lactating rats[106] or the NAcc shell in male prairie voles[18] versus other brain regions.

Therefore, central CRF signaling plays a prominent role in the heightened passive stress-coping behavior resulting from offspring loss, and increased central OXT signaling may not be sufficient to restore the impaired phenotype.

Conclusions

The present study advances our knowledge on the neurobiological basis of a translational animal model to study the long-term impact of offspring loss in lactating rats and, thereby, provides significant insights into the complex nature of maternal grief.

Like maternal grief in humans, which is often paralleled with depressive episodes to various degrees, rat mothers developed increased emotionality after offspring loss. At the same time, rat mothers had increased OXT-R binding and decreased neuroplasticity specifically within the VMH, which suggest this brain region as a potential target region for future studies. Those neurobiological changes likely contribute to the emotional and behavioral manifestations of "grief" observed in the rat mothers, underscoring the intricate interplay between neurochemistry and emotional processing. Importantly, central blockade of CRF system signaling proved to be a promising intervention to reverse the passive phenotype. Thus, we provide further evidence for the involvement of the CRF system in the grieving process. At the same time, our findings suggest possibilities for targeted pharmacological interventions to support individuals experiencing grief-related disorders.

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Author contributions

L.D. designed the study, performed the experiments, analyzed data, and wrote the first draft of the manuscript. A.S. designed the study and revised the manuscript. A.B. performed the experiments. O.J.B. designed the study, secured funding, supervised the project, and revised the manuscript. All authors contributed to and have approved the final manuscript.

Competing interest statement

No potential conflict of interest was reported by the authors.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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