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

Luisa Demarchi[1,2], Alice Sanson[1], Anna-Lena Boos[1], Oliver J. Bosch[1]*

[1]Department of Behavioural and Molecular Neurobiology, Regensburg Center for Neuroscience, University of Regensburg, 93053 Regensburg, Germany

[2]Current address: Department of Biological Sciences, Fordham University, Bronx, NY, USA

Email	Luisa Demarchi	ldemarchi@fordham.edu
	Alice Sanson	alice.sanson@ur.de
	Anna-Lena Boos	anna-lenaboos@gmx.de
	Oliver J. Bosch	oliver.bosch@ur.de

*** Corresponding author** Prof. Dr. Oliver Bosch
Department of Behavioural and Molecular Neurobiology,
Regensburg Center of Neuroscience
University of Regensburg
Universitaetsstr. 31
93053 Regensburg
Germany

Running title Neurobiological consequences of pup loss in rats

Abstract

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 examined two groups of lactating mothers: (I) a control group, in which dams remained with their pups until natural weaning, and (II) a separated group, in which all offspring were removed on lactation day 1 and the mothers experienced offspring loss until the time corresponding to weaning (19 days).

Our results reveal that pup removal increased OXT receptor binding and reduced dendritic spine density in limbic brain regions, without altering estrogen receptor α or calbindin cell expression. Separated mothers additionally showed elevated plasma corticosterone levels and increased passive stress-coping behaviors in the forced swim test. Remarkably, passive stress-coping behavior was rescued by central CRF receptor blockade but not by OXT treatment, indicating that the CRF system plays a central role in the distress response to offspring loss. These findings establish the rat as a novel animal model for maternal distress, provide new insights into the complex neurobiology of grief, and suggests potential directions for future studies.

Introduction

The maternal-infant bond is among the strongest social bonds in mammals[1]. In humans, disruption of this bond such as through offspring loss, elicits grief-like reactions in bereaved mothers, encompassing a range of emotional, cognitive, and behavioral

53 responses, including depression and altered stress response. These dysregulations can
54 progress to prolonged grief disorder (PGD)[2-4]. Despite the clinical relevance, the
55 neurobiological consequences of offspring loss on the maternal brain -particularly in
56 relation to neuroplasticity and the oxytocin (OXT) and corticotropin-releasing factor
57 (CRF) systems- remain poorly understood. Rodent studies have been instrumental in
58 characterizing maternal bonding. Reduced maternal care in rats negatively impacts the
59 offspring development and acts as emotional trauma (for review see[5]). Importantly,
60 repeated offspring separation has been shown to induce significant behavioral
61 consequences in mothers[6-,9]. However, repeated separations do not fully model the
62 experience of complete offspring loss -a scenario more analogous to human maternal
63 grief. Only a limited number of studies have specifically investigated permanent offspring
64 loss in rat mothers[10,11] (for review see[12]). Our previous work demonstrates that offspring
65 loss immediately after birth alters maternal brain function and stress-coping behavior
66 during the first postpartum week, including heightened passive stress-coping, altered
67 neuronal activity, and decreased OXT receptor (OXT-R) binding in the central amygdala
68 (CeA)[13]. Building on these findings, we hypothesized that prolonged offspring loss over 19
69 days in rats could cause enduring neurobiological changes paralleling aspects of human
70 grief[12], with particular emphasis on the CRF and OXT systems. Disruption of social bonds
71 in rodents affects neurotransmitter systems, neuroplasticity and behavior[14,15]. For example,
72 in the monogamous and biparental prairie vole (*Microtus ochrogaster*) partner loss
73 enhances CRF system signaling, reduces OXT signaling specifically in the nucleus
74 accumbens (NAcc), and alters microglial activity and morphology in a brain region- and
75 sex-specific manner[16-18].
76 The CRF system, a key mediator of stress and depression, and has been extensively
77 studied in both humans and rodents. Dysregulated CRF system activity is strongly linked
78 to mood disorders[19-24]. CRF ligands (CRF, Urocortin 1-3) and their receptors (CRF-R1 and

79 CRF-R2) are widely expressed both peripherally and centrally, and regulate
80 physiological, autonomic, and behavioral responses to stress[20,25]. Elevated CRF levels in
81 the cerebrospinal fluid and increased CRF-R binding have been reported in depressed
82 patients[26], consistent with upregulated CRF system activity[27,28]. In rats, CRF
83 overexpression induces depressive-like symptoms, altered hypothalamic-pituitary-
84 adrenal (HPA) axis function, and behavioral changes[29], while chronic stress can lead to
85 CRF system sensitization[30]. OXT, another key neuropeptide in social bonding, is
86 essential for maternal bonding[31-33], and other forms of social affiliation[34]. OXT-R are widely
87 distributed throughout the brain, where OXT signaling modulates stress responses and
88 facilitates social behavior[35]. OXT is thought to have antidepressant-like properties, partly
89 by dampening HPA axis reactivity[36-38], making it a candidate target for grief- and
90 depression-related disorders[34,35] including postpartum depression[39].
91 Neuroplasticity is another important factor in grief and depression. Alterations in dendritic
92 spine density, a key structural marker of plasticity, have been implicated in depression[40],
93 though the mechanisms remain incompletely understood[41]. Neuroplasticity and
94 emotional processing are also regulated by estrogen receptor ESR1 by influencing
95 dendritic spine formation and OXT-R expression[42-44]. ESR1 is expressed at high density
96 in calbindin-immunoreactive (ir+) cells of the ventromedial hypothalamus (VMH)[45], and
97 calbindin-ir+ cells are reduced in the occipital cortex of post-mortem depressed patients[46].
98 We hypothesized that early loss of offspring would lead to enduring changes in the
99 maternal stress-response, neuroendocrine systems and neuroplasticity. To test this, we
100 studied lactating female Sprague-Dawley rats that experienced one day of motherhood
101 followed by 19 days of offspring loss. We examined stress-coping behavior, HPA axis
102 reactivity, OXT-R binding, dendritic spine density, and ESR1 and calbindin ir+ cells
103 expression in key limbic and maternal brain regions. This work provides novel insights

104 into the neurobiology of maternal distress and highlights the potential therapeutic
105 relevance of targeting central CRF-R signaling.
106
107 **Materials and Methods**
108 **Animals**
109 Sprague-Dawley rats were obtained from Charles River Laboratories (Sulzfeld,
110 Germany) and kept under standard laboratory conditions (12:12h light/dark cycle, lights
111 on at 7 a.m., room temperature 22 ± 1°C, relative humidity 55 ± 5°C) with access to
112 standard rat chow (ssniff-Spezialdiäten GmbH, Soest, Germany) and water *ad libitum*.
113 After 7 days of habituation, females were mated with sexually experienced male
114 Sprague-Dawley rats in standard laboratory cages (Eurostandard Type IV, 60 cm x 40 cm
115 x 20 cm) for 10 days. From pregnancy day 18 on, pregnant females were single-housed
116 for undisturbed delivery in observational cages (plexiglass, 38 cm x 22 cm x 35 cm); all
117 rats delivered 8-15 pups within 3-4 days after being single-housed. The studies were
118 conducted in accordance with the ARRIVE guidelines, the European regulations of
119 animal experimentation (European Directive 2010/63/EU) and were approved by the local
120 government of Unterfranken (Bavaria, Germany). According to the 3-Rs principles, all
121 efforts were made to minimize the number of animals and their distress or suffering.
122
123 **Study design**
124 We investigated two primary groups of lactating females: (I) control mothers, which
125 remained with their pups until natural weaning, and (II) separated mothers, which had all
126 offspring removed on lactation day 1 and experienced offspring loss for 19 days (**Fig. 1**).
127 The 19-day separation period was selected because it corresponds to the pre-weaning
128 stage, a critical time when the mother–offspring bond remains strong in rats[47]. Our study
129 followed a stepwise approach. First, we assessed the effects of prolonged offspring loss

130 on OXT-R binding, ESR1 and calbindin-immunoreactive (ir$^+$) cells in brain regions
131 implicated in maternal behavior. This analysis provided an initial characterization of
132 neuroendocrine adaptations within the maternal brain and helped identify target regions
133 for subsequent investigation. Next, we evaluated dendritic spine density and behavioral
134 outcomes, incorporating an additional group of *virgin* females to distinguish experience-
135 dependent plasticity in maternal neural circuits.
136

137 **Experimental groups**

138 Rats were randomly assigned to one of three experimental groups: (I) control lactating
139 mothers (primiparous) that remained with their pups until weaning (lactation day 20,
140 *LD20* group), (II) separated mothers (*LD1+19* group; primiparous) whose litters were
141 removed on lactation day 1 (LD1), and (III) *virgin* females (*virgin* group; nulliparous). To
142 standardize litter size, each litter was adjusted to eight pups of mixed sexes on the day
143 of delivery (LD0). In the *LD1+19* group, all pups were removed on LD1 at 10:00 a.m.,
144 after which the mothers were left undisturbed in their home cages. To control for the
145 effects of single housing, *virgin* females were housed individually for the same duration
146 as separated mothers.
147

148 **Experimental design**

149 *Experiment 1*: a first cohort of rats followed the protocol described above and was
150 sacrificed on lactation day 20 (LD20) without further manipulations (**Fig. 1**). Brains were
151 collected for: (i) OXT-R binding analysis (*LD20* = 6; *LD1+19* = 6), (ii) immunofluorescence
152 analysis (*LD20* = 7; *LD1+19* = 5), and (iii) dendritic spine analysis (*LD20* = 4;
153 *LD1+19* = 4; *virgin* = 5).

154 *Experiment 2:* a second cohort (*LD20* = 15; *LD1+19* = 15; *virgin* = 9) was tested for
155 locomotor and anxiety-like behavior in the light–dark box (LDB) on LD19 and in the open
156 field (OF) on LD20. Immediately following the final behavioral test, rats were sacrificed.
157 *Experiment 3*: a third cohort (*LD20* = 13; *LD1+19* = 14; *virgin* = 8) was evaluated for
158 stress-coping behavior in the single-session forced swim test (FST) on LD20. Within 5
159 minutes of test completion, rats were sacrificed, and trunk blood was collected for plasma
160 corticosterone (CORT) analysis.
161 *Experiments 4 and 5*: two additional cohorts were prepared following the same protocol.
162 On LD15, all rats underwent stereotaxic implantation of an intracerebroventricular (icv)
163 guide cannula for acute substance infusion. On LD19, they completed the 15-min pre-
164 test session of the two-session forced swim test (FST). On LD20, they were tested in a
165 10-min session following infusion of either vehicle (VEH) or a pharmacological treatment.
166 In Experiment 4, the CRF-R1/2 antagonist D-Phe was administered (*LD20 VEH* = 10;
167 *LD1+19 VEH* = 8; *LD1+19 D-Phe* = 9), whereas in Experiment 5, synthetic OXT was
168 infused (*LD20 VEH* = 8; *LD1+19 VEH* = 8; *LD1+19 OXT* = 7). In both experiments, all
169 rats were sacrificed immediately after the final behavioral test and brains taken to verify
170 cannula placement.
171
172 **Brain analyses**
173 *OXT-R autoradiography.*
174 Rats were anesthetized and underwent cardiac perfusion with ice-cold 1x PBS and were
175 decapitated. Brains were flash-frozen in n-methylbutane, and stored at -20 °C until
176 cutting into coronal sections of 16 µm using a cryostat (CM3050S, Leica Microsystem
177 GmbH). For each brain region of interest (bed nucleus of the stria terminalis (BNST),
178 nucleus accumbens shell (NAcc shell), medial preoptic area (MPOA), accessory olfactory
179 bulbs (AOB), central amygdala (CeA), agranular insular cortex (AIP), ventromedial

180 hypothalamus (VMH), lateral septum (LS), prelimbic cortex (PL)) six slices per rat were
181 collected on SUPERFROST microscope slides and stored at -20 °C until further
182 processing. The OXT-R autoradiography was performed following an established
183 protocol[48]. Briefly, the ornithin-vasotocin analogue [125I]- OVTA
184 [d(CH2)5[Tyr(Me)2,Thr4,Orn8,[125I]Tyr9-NH2]; Perkin Elmer, USA) was used as a
185 tracer. First, the slides were thawed and allowed to dry thoroughly at room temperature.
186 The tissue was shortly fixed via 0.1 % PFA, washed 2 x in Tris (50 mM, pH 7.4), covered
187 with the tracer solution (50 mM tracer, 10 mM MgCl2, 0.1 % BSA) for 60 min, washed 3
188 x in Tris / MgCl2 buffer for 7 min, each, followed by 30 min spinning in Tris / MgCl2.
189 Finally, slides were dipped into water and air dried before being exposed to Biomax MR
190 films (Kodak, Cedex, France) for 15 days. The films were scanned using the EPSON
191 Perfection V800 Scanner (Epson GmbH, Munich, Germany), and the optical density of
192 each region of interest was analyzed using ImageJ[49] by subtracting the background
193 activity as previously described[48,50]. The analyses were performed simultaneously for 6
194 slices per rat and per region.
195 *Immunofluorescence staining.*
196 Rats were anesthetized and perfused with ice-cold 1x PBS and subsequently with 4 %
197 paraformaldehyde (PFA) dissolved in 1x PBS. Brains were removed, fixed overnight in 4
198 % PFA, and subsequently incubated in 30 % sucrose in 1x PBS until the brain sank. After
199 fixation and cryoprotection in sucrose, brains were flash-frozen in n-methylbutane, and
200 stored at -20 °C until cutting into coronal sections of 16 µm using a cryostat (CM3050S,
201 Leica Microsystem GmbH, Nussloch, Germany). Six consecutive slices containing the
202 VMH region were collected per rat on SUPERFROST micro- scope slides and stored at
203 -20 °C until further processing. Slides were washed in 1x PBS, permeabilized with 0.3 %
204 Triton, blocked with 5 % normal goat serum (Vector Laboratories, Newark, USA), and
205 incubated overnight at 4 °C with primary antibodies (Merck Millipore, rabbit anti ER alpha

206 1:2000; Merck, mouse anti-NeuN 1:1000; Synaptic Systems, chicken anti-calbindin
207 1:2000). The next day, slides went through 1 h incubation with secondary antibodies
208 (Alexa Fluor 488-conjugated anti-rabbit IgG 1:800, Alexa Fluor 594-conjugated anti-
209 mouse IgG 1:800 and Alexa Fluor 649-conjugated anti-chicken IgG 1:800) at room
210 temperature. All slides were finally mounted using ROTI®Mount FluorCare DAPI (Carl
211 Roth, Karlsruhe, Germany).

212 *Golgi staining for spine density visualization.*

213 The spine density was assessed in *virgin*, *LD20* and *LD1+19* groups to investigate
214 neuronal plasticity. Firstly, rats were sacrificed with CO_2 and brains were collected. The
215 Rapid GolgiStainTM Kit (FD NeuroTechnologies, Columbia, USA) was used and applied
216 according to the manufacturer's protocol. Brains were cut into 100 µm slices using a
217 cryostat (CM3050S, Leica Microsystem GmbH) at -28° and placed on gelatin-coated
218 microscope slides (FD NeuroTechnologies, Columbia, USA). After complete drying,
219 Golgi staining was performed, and sections were stored at room temperature in the dark
220 before acquiring images using a brightfield microscope (Leica Thunder DM6 B, Camera
221 Leica DFC9000 GT). ImageJ[49] was used to count the spines of secondary dendrites in
222 the PL, AIP, BLA and VMH. Secondary dendrites from different neurons (between 4 and
223 12, depending on the brain region) were chosen from 10X overview preview images
224 followed by acquiring 40X focus images for analysis. The ROI of each dendrite was
225 selected, brightness and sharpening function were adjusted. Spines were counted on a
226 25-30 μm^2 section of the dendrite (each dendrite belonging to a different neuron) and the
227 absolute spine density was calculated by using the following formula[51]: (n = number of
228 spines; µm = dendritic length): $\frac{n-1}{\mu m}$.

229

230 **Behavioral experiments**

231 *Light dark box (LDB).*

232 On LD19, between 9:00 a.m. and 12:00 p.m., rats from experiment 2 were tested in the LDB
233 to assess anxiety-like behavior[52]. The apparatus consisted of a light compartment (40 × 50
234 cm, 180 lux) and a dark compartment (40 × 30 cm, 0 lux) connected by a 7.5 × 7.5 cm
235 opening. At the start of the test, rats were placed in the center of the light box, and behavior
236 was recorded for 10 min using EthoVision XT (Noldus, Wageningen, the Netherlands) by an
237 experimenter blind to group assignment. The following parameters were analyzed: (a)
238 percentage of time spent in the light box, (b) latency to re-enter the light compartment, and
239 (c) locomotor activity. The arena was cleaned with tap water and dried thoroughly between
240 trials.

241 *Open-field (OF).*

242 On LD20, between 9:00 a.m. and 12:00 p.m., rats from experiment 2 were tested in the OF
243 to evaluate locomotor activity and anxiety-like behavior[53]. The apparatus was an empty
244 rectangular arena (80 × 80 cm). Rats were placed in one corner and allowed to explore freely
245 for 10 min. Behavior was recorded using EthoVision XT, with analysis performed by an
246 experimenter blind to treatment. The following measures were scored: (a) locomotion, (b)
247 velocity, and (c) number of center entries. The arena was cleaned with tap water and dried
248 between trials.

249 *Forced swim test (FST).*

250 Two versions of the FST were employed to assess passive stress-coping behavior.
251 *Rationale:* we used a single-session protocol to capture baseline group differences
252 without pre-exposure, whereas the two-session protocol provided higher sensitivity to
253 drug effects -particularly relevant because animals in experiment 4-5 underwent surgery
254 (see below).

255 *Single-session FST.* On LD20, between 9:00 a.m. and 12:00 p.m., rats from experiment
256 3 were tested in a single 10-min FST session. Each rat was placed in a cylindrical tank

257 (50 cm high, 30 cm diameter) filled with tap water (23 ± 1 °C) at a depth preventing
258 contact with the bottom. Behavior was recorded and analyzed using JWatcher
259 (https://www.jwatcher.ucla.edu) by an experimenter blind to group assignment. The total
260 time spent immobile (floating) was quantified as an index of passive stress-coping, which
261 is reminiscent of depressive-like behavior[54].
262 *Two-session FST.* In experiments 4 and 5, rats were tested using the two-day Porsolt
263 FST, the standard paradigm for evaluating antidepressant treatments[54]. These animals
264 had previously undergone stereotaxic surgery (see details below) and were tested 5 days
265 postoperatively. On LD19, rats underwent a 15-min pre-test session. On LD20, they were
266 exposed to a 10-min test session following intracerebroventricular infusion of vehicle or
267 a pharmacological agent. The two-session design was chosen to stabilize baseline
268 immobility at the pre-test and to maximize the dynamic range for detecting drug-induced
269 reductions at the test while controlling for post-surgical variability. All other experimental
270 conditions and behavioral analyses were identical to those described for the single-
271 session FST.
272
273 **Stereotaxic surgery**
274 On LD15, rats of experiments 4 and 5 were implanted with a stainless-steel guide cannula
275 (21-G, length 12 mm), above the right ventricle (1.0 mm posterior, 1.6 mm lateral to
276 bregma, 1.8 mm ventral)[55]. Surgeries were performed under isoflurane anesthesia,
277 following previously described procedures[56]. The guide cannula was closed using a 25-
278 G stainless steel stylet to prevent occlusion. To minimize post-surgical discomfort, all rats
279 received a subcutaneous injection of buprenorphine (0.05 mg/kg, Bayer Vital GmbH,
280 Leverkusen, Germany) prior to surgery. Following surgery, rats were monitored
281 continuously until recovery from anesthesia and then returned to their home cages. Rats

282 were observed daily for general health, body weight, and signs of discomfort or infection
283 throughout the recovery period.
284
285 **Drug administration**
286 On LD20, rats of experiment 4 and 5 received a single acute icv infusion 10 min prior to
287 the second session of the two-day forced swim test. Depending on the treatment group,
288 animals were infused with vehicle (VEH; 5 µL sterile Ringer's solution, pH adjusted to
289 7.4; Braun, Melsungen, Germany), the human/rat CRF-R1/2 antagonist D-Phe [(D-Phe^{12},
290 Nle$^{21–38}$, α-Me-Leu^{37})-CRF (12–41); 10 µg/5 µL; Bachem, Bubendorf, Switzerland], or
291 synthetic OXT (1 µg/5 µL; Tocris, Nordenstadt, Germany). All doses were selected based
292 on previous studies[56]. Infusions were performed using a 25 G stainless-steel infusion
293 cannula (14 mm length) connected to a 10 µL Hamilton micro syringe via PE-50 tubing
294 (50 cm). The infusion cannula was inserted into the pre-implanted guide cannula and
295 held in place with a piece of silicon tubing. Substances were delivered over 30s, after
296 which the cannula was left in position for an additional 30s to minimize reflux before
297 removal, as previously described[38].
298
299 **Verification of cannula placement**
300 Rats of experiment 4 and 5 were sacrificed immediately following the last behavioral test
301 with CO_2 and blue dye was injected via the infusion system into the guide cannula.
302 Verification of the infusion site of the icv cannula was observed by dye spread throughout
303 the ventricular system. Only animals with correct verified infusion sites were included in
304 the statistical analyses.
305
306 **ELISA for plasma CORT**

307 In experiment 3, rats were sacrificed within 5 min after the single-session FST, and
308 approximately 1 mL of trunk blood was collected in EDTA-coated tubes on ice (Sarstedt,
309 Numbrecht, Germany). Samples were centrifuged at 4 °C (10,000 rpm/12298 rcf) for 10
310 min. Plasma was aliquoted and stored at −20 °C until analysis. CORT concentrations
311 were quantified using a commercially available ELISA kit (Tecan IBL International GmbH,
312 Hamburg, Germany) following the manufacturer's instructions. All samples were
313 measured in duplicate, and mean values were used for statistical analysis.
314

315 **Statistical analysis**

316 All statistical analyses were performed with GraphPad PRISM 9 (GraphPad Software,
317 San Diego, USA). Data were first tested for normality (Shapiro-Wilk or Kolmogorov–
318 Smirnov test) and homogeneity of variance (Brown-Forsythe test). Outliers were
319 identified using the ROUT method.
320 Group comparisons of OXT-R binding, ESR1 and calbindin-ir+ cell counts were
321 performed using two-sample Student's *t*-test. Dendritic spine density, plasma CORT
322 concentration, anxiety-like behavior, locomotor activity and stress-coping behavior were
323 analyzed by one-way ANOVA followed by Sidak's post hoc multiple comparisons. Data
324 are expressed as mean ± SEM; and as significance was set at $p \leq 0.05$.
325

326 ## Results

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

328 To determine whether offspring loss alters OXT-R binding within maternal and limbic brain
329 regions, we performed receptor autoradiography in *LD20* and *LD1+19* mothers. *LD1+19*
330 dams exhibited significantly higher OXT-R binding in the ventromedial hypothalamus
331 (VMH; $M = 0.257$, $SD = 0.077$) compared to *LD20* dams ($M = 0.147$,
332 $SD = 0.083$; $t(10) = 2.366$, $p = 0.039$; **Fig. 2A**). A similar finding was observed in the

333 prelimbic cortex (PL), where OXT-R binding was slightly elevated in *LD1+19*
334 (M = 0.070, SD = 0.005) relative to *LD20* dams (M = 0.066, SD = 0.002,
335 $t(10) = 2.203$, $p = 0.052$; **Fig. 2B**).
336 No significant group differences were detected in the other regions analyzed (**Fig. 2C**):
337 MPOA ($t(10) = 0.215$, $p = 0.834$); BNST ($t(10) = 0.828$, $p = .427$); CeA ($t(10) = 0.061$,
338 $p = 0.952$); OB ($t(10) = 0.595$, $p < 0.565$); NAcc shell ($t(10) = 1.440$, $p = 0.180$);
339 AIP ($t(10) = 1.212$, $p = 0.254$); LS ($t(10) = 0.613$, $p = 0.554$)].
340
341 **ESR1 and calbindin ir+ cells in the VMH and PL did not differ between groups**
342 To investigate whether the increased OXT-R binding in the VMH and PL (see above) was
343 associated with changes in ESR1 expression, we quantified ESR1- and calbindin-
344 immunoreactive (ir+) cells in the VMH and PL of *LD20* and *LD1+19* mothers. In the VMH,
345 no significant group differences were detected in the proportion of ESR1-ir+ cells
346 ($t(10) = 0.456$, $p = 0.658$; **Fig. 3A, B**) or of calbindin ir+ cells ($t(10) = 0.706$, $p = 0.496$;
347 **Fig. 3A, C**). ESR1-ir+ cells were not detectable in the PL (data not shown).
348
349 **Reduced secondary dendritic spine density in the VMH after offspring loss**
350 To assess the impact of offspring loss on neuroplasticity, we quantified secondary
351 dendritic spine density in the VMH, PL, BLA and AIP of *virgin*, *LD20* and *LD1+19* females
352 (**Fig. 4A**), based on regions identified in our previous study[13]. Statistical analysis revealed
353 a significant group effect in the VMH (one-way ANOVA: $F(2, 10) = 8.734$; $p = 0.006$;
354 **Fig. 4B**). Post hoc analyses showed that *LD1+19* dams exhibited significantly lower
355 spine density compared to *virgin* ($p = 0.024$) and *LD20* mothers ($p = 0.009$). In contrast,
356 no significant differences were observed in the other regions examined. No differences
357 in spine density were found for the PL (*virgin*: 0.64 ± 0.07; *LD20*: 0.53 ± 0.04;
358 *LD1+19* : 0.62 ± 0.04; one-way ANOVA: $F(2,15) = 0.590$, $p = 0.356$), the BLA

359 (*virgin*: 0.61 ± 0.03; *LD20*: 0.64 ± 0.009; *LD1+19*: 0.61 ± 0.02; one-way ANOVA: $F(2,16)$
360 = 0.158, p = 0.855), and the AIP (*virgin*: 0.62 ± 0.05; *LD20*: 0.69 ± 0.04;
361 *LD1+19*: 0.68 ± 0.04; one-way ANOVA: $F(2,15) = 0.599$, $p = 0.562$).
362
363 **Anxiety-like behavior was not affected by offspring loss**
364 To examine whether offspring loss altered anxiety-like behavior, *virgin*, *LD20*, and
365 *LD1+19* rats were tested in the LDB on LD19. Groups differed in latency to re-enter the
366 light compartment (Kruskal–Wallis test, $p = 0.045$), with *LD20* dams tending to be faster
367 (48.6 s ± 15.9 s) than *virgin* females (213.5 s ± 77.5 s; Dunn's test, $p = 0.058$). *LD1+19*
368 dams did not differ from either group (121.9 s ± 31.9 s).
369 No group differences were observed in the time spent in the light compartment (*virgin*:
370 34.0 s ± 6.3 s; *LD20*: 36.6 s ± 3.6 s; *LD1+19*: 42.0 s ± 5.1 s; one-way ANOVA:
371 $F(2,36) = 0.676$, $p = 0.515$) or in locomotor activity within the LDB
372 (*virgin*: 3778 cm ± 326 cm; *LD20*: 3733 cm ± 194 cm; *LD1+19*: 3851 cm ± 312 cm;
373 one-way ANOVA: $F(2,36) = 0.054$, $p = 0.948$). Thus, *LD1+19* mothers did not differ from
374 either *virgin* or *LD20* mothers in any measure of anxiety-like behavior.
375
376 **Locomotor activity was not affected by offspring loss**
377 The same cohort of rats was tested in the OF on LD20. Groups differed significantly in
378 distance travelled (one-way ANOVA: $F(2,36) = 3.773$, $p = 0.032$; **Fig. 5A**) and in
379 velocity (one-way ANOVA; [$F(2,36) = 3.788$], $p = 0.032$; **Fig. 5B**). Post hoc comparisons
380 revealed that *virgin* females showed greater locomotion ($p = 0.029$) and velocity ($p =$
381 0.028) compared with *LD20* dams. *LD1+19* mothers did not differ from either group.
382 No significant differences were found in the number of center entries (*virgin*: 21.4 ± 2.7;
383 *LD20*: 16.3 ± 1.5; *LD1+19*: 20.5 ± 2.0; one-way ANOVA: $F(2,36) = 1.858$, $p = 0.170$).
384 Overall, offspring loss did not alter locomotor activity.

385

386 **Offspring loss increased passive stress-coping behavior and induced a virgin-like**
387 **stress response**

388 A different cohort of *virgin*, *LD20* and *LD1+19* rats was tested for passive stress-coping
389 behavior in the single-session FST on LD20. Groups differed significantly in time spent
390 floating (one-way ANOVA: $F(2,32) = 6.233$, $p = 0.005$; **Fig. 5C**). *LD1+19* dams spent
391 more time floating compared with both *virgin* ($p = 0.019$) and *LD20* ($p = 0.014$) rats.
392 Stress-induced plasma CORT levels, which were collected 15 min after the start of the
393 FST, also differed across groups ($F(2,28) = 13.50$, $p = 0.0001$; **Fig. 5D**). Post hoc
394 comparisons revealed significantly lower CORT concentrations in *LD20* compared with
395 *virgin* ($p = 0.002$) and *LD1+19* ($p = 0.0001$) rats. *LD1+19* mothers did not differ from *virgin*
396 females ($p > 0.05$), suggesting that offspring loss abolished the lactation-induced blunting
397 of HPA axis activity.

398

399 **Central CRF-R1/2 blockade normalized passive-stress coping behavior in *LD1+19***
400 **mothers**

401 Following a pre-test on LD19, *LD20* and *LD1+19* rats were tested in the FST on LD20
402 after ICV infusion of VEH or the CRF-R1/2 antagonist D-Phe. Groups differed
403 significantly in floating behavior (one-way ANOVA: $F(2,23) = 5.828$, $p = 0.013$; **Fig. 6A**).
404 *LD1+19 VEH* mothers spent more time floating than *LD20 VEH* rats ($p = 0.047$),
405 replicating the findings from the single-session FST (**Fig. 5C**). Importantly, floating time
406 was significantly lower in *LD1+19 D-Phe* rats compared to *LD1+19 VEH* ($p = 0.014$),
407 reaching levels indistinguishable from *LD20 VEH* ($p > 0.05$).

408

409 **Central OXT infusion did not alter passive-stress coping behavior in *LD1+19***
410 **mothers**

411 In a different cohort, *LD20* and *LD1+19* rats were tested in the FST after infusion of VEH
412 or synthetic OXT. Groups differed in floating behavior (one-way ANOVA: $F(2,20) = 5.273$,
413 $p = 0.014$; **Fig. 6B**). Consistent with previous experiments, *LD1+19 VEH* rats floated
414 more than *LD20 VEH* ($p = 0.014$). However, infusion of OXT in *LD1+19* mothers was not
415 sufficient to alter floating when compared with *LD1+19 VEH* ($p = 0.08$).
416

417 ## Discussion

418 The loss of offspring represents a profound life event with strong effects on physiology
419 and behavior. In humans, such experiences are associated with marked changes in
420 emotional and physical health, including a higher risk for prolonged grief
421 disorders[4,57–59]. To investigate the underlying mechanisms and to identify potential
422 therapeutic targets, appropriate animal models are required. In this study, we build upon
423 our previous work on consequences of short-term separation on rat mothers[13]. We
424 examined the long-term consequences of offspring loss and demonstrate that extended
425 separation manifests in distinct neurobiological and behavioral adaptations. After 19 days
426 of pup removal, mothers exhibited increased OXT-R binding specifically in the VMH and
427 PL, had reduced dendritic spine density in the VMH, and heightened HPA axis reactivity
428 together with increased passive stress-coping behavior. Importantly, central
429 pharmacological blockade of CRF-R1/2 normalized the impaired behavior, whereas OXT
430 infusion did not, highlighting the prominent role of CRF system activity in mediating
431 maternal distress responses due to pup loss.

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

433 During the peripartum period, the maternal brain undergoes dynamic adaptations in OXT
434 signaling that support the initiation and maintenance of maternal behaviors[60,61]. In our
435 study, OXT-R binding was significantly elevated in the VMH and PL following offspring

436 loss, contrary to our initial hypothesis that separation would reduce OXT signaling. The
437 VMH, which contains a high density of OXT-Rs and plays a role in maternal care,
438 aggression, and defensive behaviors[62-66], has not been studied in the context of pup
439 removal. The PL, a region critical for cognitive and emotional regulation[67,68], has also
440 been implicated in maternal care, as OXT-R blockade in this area disrupts caregiving
441 behavior[69]. Thus, the upregulation of OXT-Rs in both the VMH and PL may reflect
442 adaptive—yet potentially insufficient—neurobiological adjustments triggered by
443 permanent offspring removal. Previous studies investigating repeated maternal
444 separation also reported changes in OXT-R binding in the PL and MPOA[6,70,71], consistent
445 with the sensitivity of OXT signaling to maternal experience. Reduced maternal
446 interactions, including fewer opportunities for the milk ejection reflex, likely decrease
447 endogenous OXT release[72], which has been associated with reduced activity of OXT-
448 positive neurons in the PVN[73]. Based on this evidence, we propose that permanent
449 offspring loss alters OXT dynamics, with potentially less OXT release leading to the
450 detected OXT-R upregulation thereby serving as a compensatory adaptation. Future
451 studies should examine brain region-specific OXT release and transcriptional regulation
452 to provide a more complete picture of system-wide neuroendocrine adaptations.

453 **ESR1 and calbindin do not mediate OXT-R changes**

454 Estrogen has been shown to regulate OXT-R expression via ESR1 and to interact with
455 calbindin[44,74-76]. To assess whether the OXT-R upregulation observed in our study could
456 be explained by alterations of these factors, we quantified ESR1- and calbindin-
457 immunoreactive (ir$^+$) cells in the VMH and PL. No differences were detected in the
458 proportion of ESR1- or calbindin-ir$^+$ cells in the VMH between groups, and ESR1 was not
459 detectable in the PL. These results indicate that the increase in OXT-R binding following
460 offspring loss is unlikely to be mediated by changes in ESR1 or calbindin expression.
461 Instead, other neuroendocrine pathways may contribute to the observed receptor

462 alterations. In particular, CRF signaling represents a likely candidate, given its known
463 interactions with the OXT system and its role in stress-related neuroadaptations[77].

464 **Reduced spine density in the VMH after offspring loss**

465 Neuroplasticity is a central component of maternal adaptations, with dendritic spine
466 remodeling occurring across pregnancy and the postpartum period[78]. In our study, we
467 observed a selective reduction in secondary dendritic spine density within the VMH after
468 offspring loss, whereas no significant changes were detected in other regions examined.
469 Alterations in dendritic spine density have been widely reported in response to stress
470 and depression in both humans and rodents[79-89]. Given the VMH's established role in the
471 regulation of stress reactivity, social interactions, and aggression[90-92], the observed
472 decrease in spine density may contribute to the stress-response dysregulation observed
473 in separated mothers. Importantly, these structural changes were accompanied by
474 increased OXT-R binding in the VMH, indicating that both cellular and receptor-level
475 adaptations converge in this region. A plausible interpretation is that reduced spine
476 density may reflect altered excitatory connectivity within VMH circuits, while the
477 concurrent increase in OXT-R binding could shift the balance of neuromodulatory input,
478 thereby reshaping local network responsiveness. Rather than acting as a simple
479 compensatory mechanism, these parallel adaptations may represent a reorganization of
480 VMH function following offspring loss, potentially influencing how this region integrates
481 social and stress-related signals. A limitation of the present work is that estrous cycle
482 phases of *virgin* females were not monitored, which could have influenced spine density
483 measurements. Future studies should therefore control for reproductive state and directly
484 test the functional link between OXT-R signaling and structural remodeling in the VMH
485 to clarify how these adaptations interact to shape maternal behavioral responses.

486 **Anxiety-like and locomotor behaviors were unchanged**

487 Lactation is often associated with reduced anxiety-like behavior, largely mediated by
488 elevated central OXT signaling[93,94]. Consistent with this direction, *LD20* dams showed a
489 trend toward faster re-entry versus *virgin* females, but this did not generalize to other
490 LDB parameters. Crucially, separated mothers (*LD1+19*) did not differ from *LD20* or *virgin*
491 females on any measure, indicating that offspring loss did not produce robust changes
492 in anxiety-related behavior. The absence of locomotor differences across groups further
493 supports that the FST effects were not secondary to altered mobility or general activity.
494 While the lack of pup-derived sensory stimulation in separated mothers could diminish
495 central OXT release -and thereby remove a potential anxiolytic mechanism[95] -the present
496 data indicates no measurable anxiety-like phenotype in the LDB. Subtle behavioral
497 differences observed in *virgin* females may reflect their period of social isolation, a known
498 driver of heightened anxiety/hyperactivity in rodents.[96]

499 **Offspring loss increased passive stress-coping and HPA axis reactivity**

500 Our results show that permanent offspring loss exerted a sustained impact on maternal
501 emotional regulation. In the FST, separated mothers consistently exhibited elevated
502 passive stress-coping, as indicated by increased floating time, compared with both *virgin*
503 and *LD20* dams. This outcome replicates and extends previous findings that prolonged
504 maternal separation enhances passive stress-coping in rat dams[10,11,13]. The consistent
505 emergence of this phenotype across independent cohorts emphasizes its robustness
506 and supports the validity of this model for probing maternal adaptations to offspring loss.
507 Importantly, locomotor activity did not differ between *LD1+19* and *LD20* dams, confirming
508 that the increase in floating reflects a shift in stress-coping strategy rather than reduced
509 motor capacity or energy availability. In addition, physiological analyses aligned with
510 these behavioral findings. *LD20* mothers displayed the known dampened HPA axis
511 response, consistent with reduced stress reactivity during the postpartum period[97]. By
512 contrast, *LD1+19* mothers exhibited CORT levels similar to *virgin* females, indicating that

offspring loss disrupts the typical stress-buffering adaptations of lactation. This reversion to a virgin-like endocrine profile suggests both heightened vulnerability to acute stressors and the absence of maternal neuroendocrine mechanisms that normally confer resilience during caregiving. Whether this shift reflects the acute impact of pup loss on HPA axis regulation or the lack of sustained maternal experience remains an open question. Together, these findings demonstrate that offspring loss induces a dual effect: an increase in passive stress-coping behavior and the reactivation of heightened HPA axis activity. This behavioral–endocrine combination provides a mechanistic framework for understanding how the maternal brain adapts—or fails to adapt—after loss and reinforces the translational relevance of this model.

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

During lactation, activity of the brain CRF system is normally dampened, providing resilience against stress and allowing mothers to maintain adaptive caregiving responses[98]. However, chronic or severe stress around parturition can disrupt this regulation, leading to heightened CRF signaling and increased vulnerability to stress[99,100]. Furthermore, the CRF system has been implicated in behavioral adaptations following social bond disruption, as shown in prairie voles where partner separation activates CRF-dependent stress responses[101]. In line with this, our results demonstrate that icv administration of the CRF-R1/2 antagonist D-Phe normalized the increased passive stress-coping observed in separated mothers during the FST. These findings indicate that offspring loss induces hyperactivity of the CRF system and that CRF receptor blockade can effectively restore adaptive stress-coping. This interpretation aligns with prior work showing that D-Phe alleviates separation-induced behavioral alterations in both lactating[101] and male prairie voles[16]. Given the known functional

538 interactions between CRF and OXT systems[18,22], we also investigated whether
539 enhancing OXT signaling could counteract the behavioral phenotype. Previous studies
540 in prairie voles demonstrated that local OXT infusion into the NAcc shell rescues
541 separation-induced behavioral deficits[18], and work in lactating rats highlights OXT's
542 regulatory role in stress responses. However, in our study central OXT infusion did not
543 modify passive stress-coping in separated mothers. This lack of effect does not exclude
544 the OXT system as a relevant modulatory pathway; rather, it suggests that global central
545 infusion may not capture the region-specific actions of OXT. Indeed, targeted
546 manipulations in regions such as the PVN[102] or NAcc have produced stronger behavioral
547 effects in other models. Together, our data highlight CRF hyperactivity as a central
548 mechanism in the stress-related consequences of offspring loss, while suggesting that
549 OXT-based interventions may require more precise spatial targeting to be effective.

550 Conclusions

551 This study establishes a translational rat model to investigate the neurobiological
552 consequences of permanent offspring loss. Separated mothers displayed altered stress-
553 coping strategies, elevated HPA axis activity, and structural as well as receptor-level
554 adaptations in limbic brain regions. Specifically, increased OXT-R binding and reduced
555 dendritic spine density were observed in the VMH, identifying this region as a key locus
556 of maternal adaptation to loss. Importantly, pharmacological blockade of CRF-R1/2
557 signaling restored normal coping behavior, whereas OXT infusion did not, underscoring
558 the CRF system as a primary driver of these outcomes. These findings provide novel
559 insights into the interplay between neuroendocrine signaling, neuroplasticity, and
560 behavioral regulation in the maternal brain. They also suggest that interventions targeting
561 CRF signaling hold particular promise for mitigating the long-term consequences of
562 disrupted maternal experience.

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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, performed the experiments 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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Figure 1: Schematic timelines summarizing the maternal separation paradigm. Abbreviations: LD, lactation day.

Figure 2: OXT-R binding in brain regions of the limbic system and maternal network. (**A,B**) Representative coronal brain sections of the VMH and PL showing differences in OXT-R binding. Optical density (arbitrary units) for OXT-R binding in the (**A**) VMH and (**B**) PL. (**C**) Overview heatmap of gray density (arbitrary units) for OXT-R binding in the analyzed brain areas (* $p \leq .05$ versus *LD20*). 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. * $p \leq .05$ versus *LD20*.

Figure 3: ESR1 and calbindin ir+ cells in the VMH. (**A**) Representative fluorescent images showing DAPI (blue), ESR1 cells (red), calbindin (green) 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 and schematic illustration of the neuronal dendritic arbor (Scale bar 10 μm). (**B**) Absolute spine density / mm^2 across experimental groups. (**C**) One-way ANOVA. Data are expressed as mean ± SEM. * $p < .05$ versus all other groups.

Figure 5: (**A)** Locomotor behavior and **(B**) velocity in the OF, (**C**) passive stress-coping behavior in the classic FST, and (**D**) corresponding plasma CORT concentration. (**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 15 min after start of the FST. One-way ANOVA. Data are expressed as mean ± SEM. # $p < .05$ versus *LD20 VEH*; * $p < .05$ versus all other groups.

Figure 6: (**A**) Passive stress-coping behavior in the FST following central pharmacological manipulation of the brain CRF and (**B**) OXT systems. (**A**) Percentage of floating during the 10-min modified FST following icv infusion of 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 *LD20 VEH*.

Figure 1

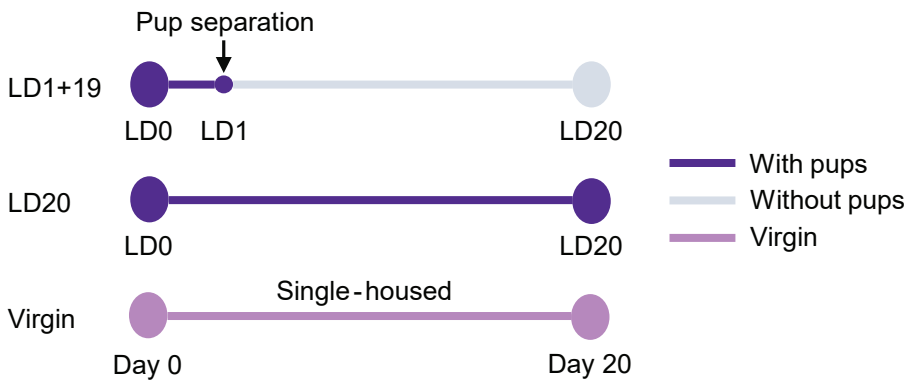

Pup separation
LD1+19
LD0
LD1
LD20
LD20
LD0
LD20
Virgin
Single-housed
Day 0
Day 20
With pups
Without pups
Virgin

Figure 2

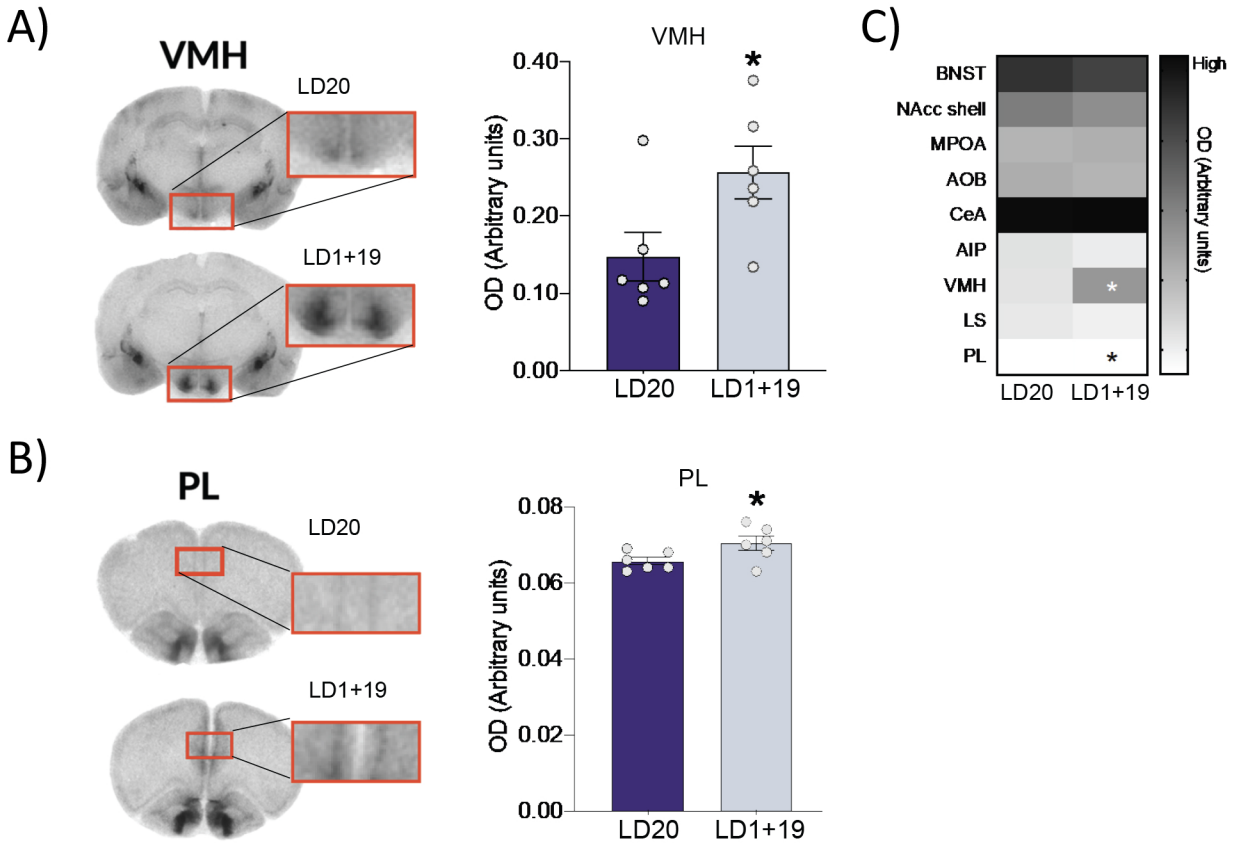

A)
VMH
LD20
LD1+19
VMH
OD (Arbitrary units)
0.40
0.30
0.20
0.10
0.00
*
LD20
LD1+19
C)
BNST
NAcc shell
MPOA
AOB
CeA
AIP
VMH
LS
PL
*
*
LD20
LD1+19
High
OD (Arbitrary units)
B)
PL
LD20
LD1+19
PL
OD (Arbitrary units)
0.08
0.06
0.04
0.02
0.00
*
LD20
LD1+19

Figure 3

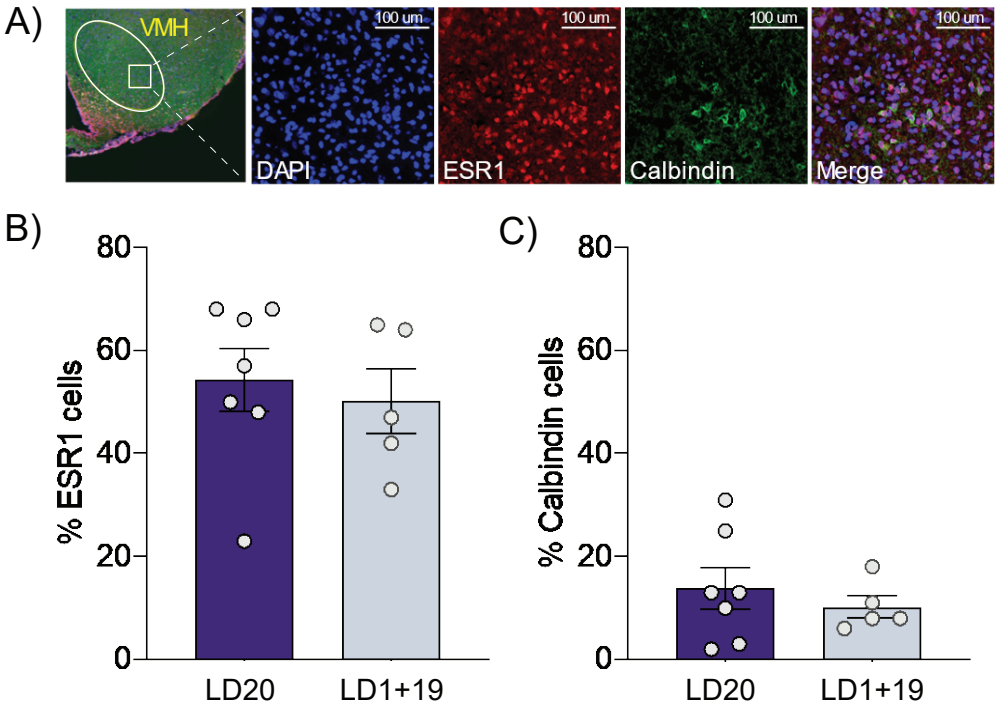

A)
VMH
100 um
DAPI
ESR1
Calbindin
Merge
B)
% ESR1 cells
80
60
40
20
0
LD20
LD1+19
C)
% Calbindin cells
80
60
40
20
0
LD20
LD1+19

Figure 4

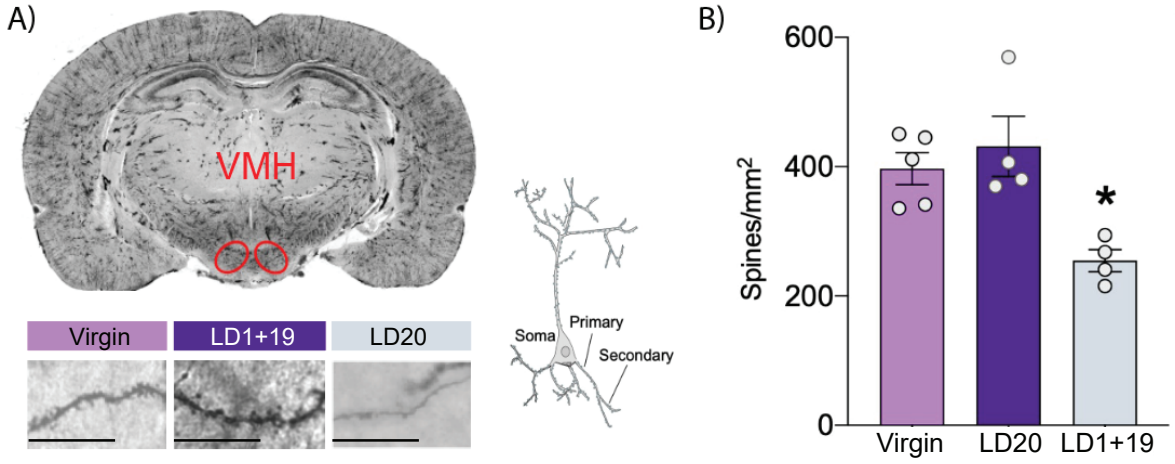

A)
VMH
Virgin
LD1+19
LD20
Soma
Primary
Secondary
B)
Spines/mm2
600
400
200
0
*
Virgin
LD20
LD1+19

Figure 5

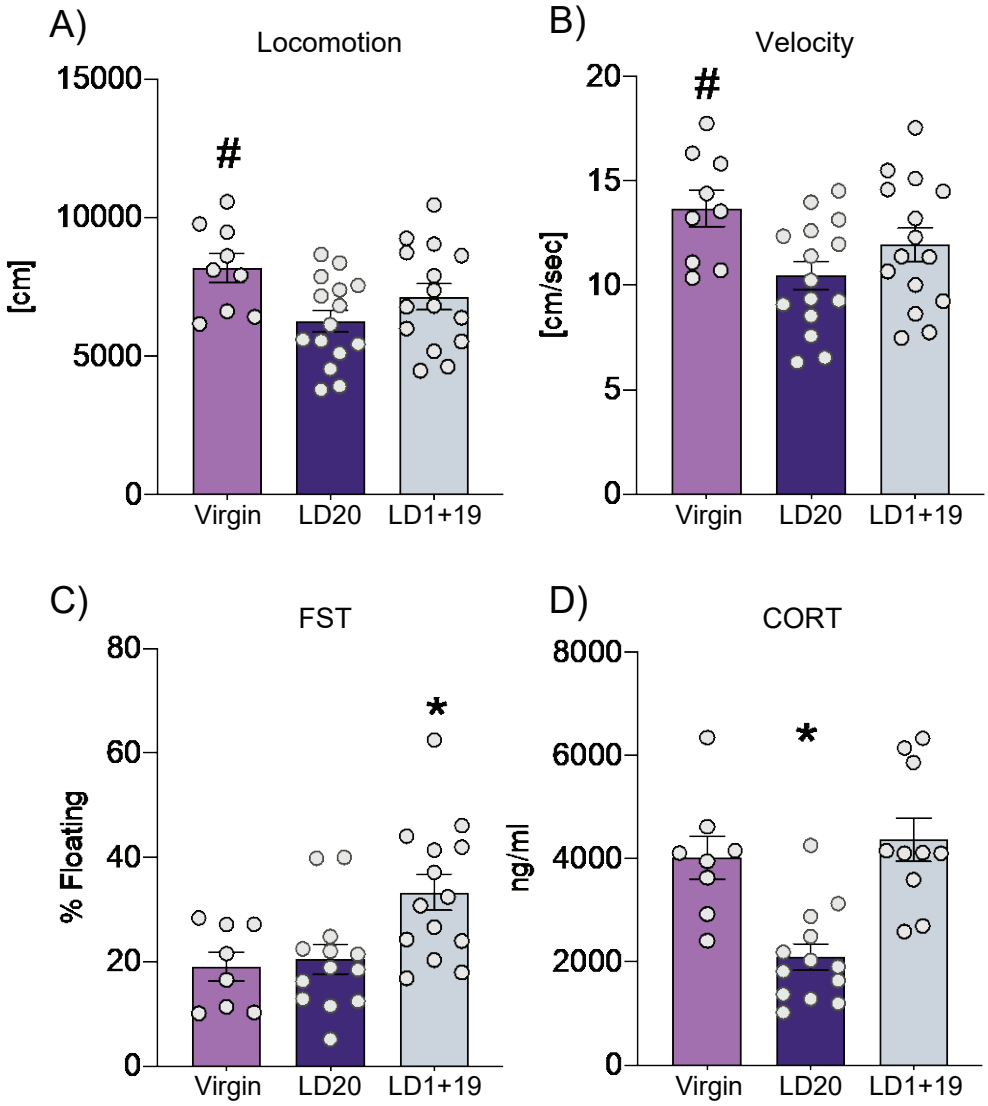
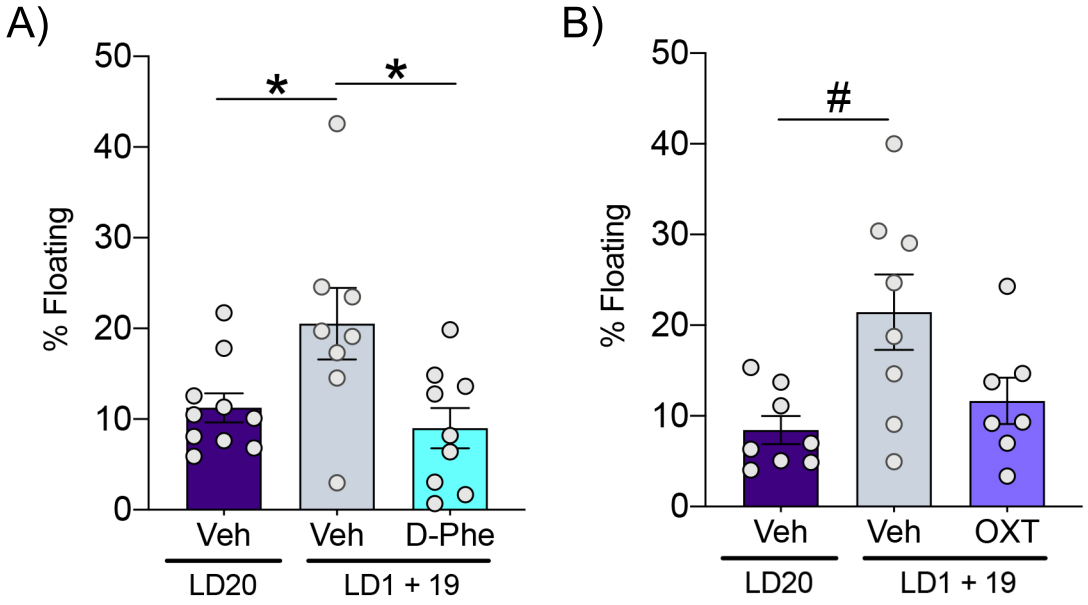

A)
Locomotion
15000
10000
5000
0
[cm]
#
Virgin
LD20
LD1+19
B)
Velocity
20
15
10
5
0
[cm/sec]
#
Virgin
LD20
LD1+19
C)
FST
80
60
40
20
0
% Floating
*
Virgin
LD20
LD1+19
D)
CORT
8000
6000
4000
2000
0
ng/ml
*
Virgin
LD20
LD1+19

Figure 6


A)
50
40
30
20
10
0
% Floating
*
*
Veh
Veh
D-Phe
LD20
LD1 + 19
B)
50
40
30
20
10
0
% Floating
#
Veh
Veh
OXT
LD20
LD1 + 19