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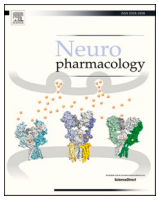

# Increased CRF-R1 transmission in the nucleus accumbens shell facilitates maternal neglect in lactating rats and mediates anxiety-like behaviour in a sex-specific manner

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ABSTRACT

During the transition to motherhood, complex brain adaptations occur to ensure adequate maternal responses to offspring' needs accompanied by reduced anxiety. Among others, the corticotropin-releasing factor (CRF) and oxytocin (OXT) systems have emerged as crucial regulators of these essential postpartum adaptations. Here, we investigated their roles within the nucleus accumbens shell (NAcSh), a central region of the reward and maternal circuits, in maternal neglect of lactating rats. Furthermore, we assessed the contribution of the local CRF system to anxiety-like behaviour, comparing lactating female, virgin female and male rats to evaluate potential sex-differences.

Increasing CRF receptor (CRF-R) 1 transmission via local CRF infusion in the NAcSh led to maternal neglect, reducing nursing and increasing self-directed behaviours. In turn, local CRF-R1 inhibition impaired maternal motivation. Intra-NAcSh Urocortin3 infusion did not promote maternal neglect but increased anxiety-like behaviour in lactating and virgin female rats, whereas CRF infusion had anxiogenic effects only in male rats. *Crh-r1* mRNA expression was higher in male and lactating rats compared to virgin females; furthermore, male rats had increased *Crh-bp* mRNA expression compared to virgin female rats, only. Lastly, pharmacological manipulations of the OXT system did not affect maternal responses.

In conclusion, finely balanced CRF-R1 signalling in the NAcSh is required for the proper expression of maternal behaviours. Dampened CRF-R2 signalling prevents the onset of anxiety-like behaviour in female rats, whereas CRF-R1 plays a more prominent role in males, highlighting complex sex-differences of the CRF system's regulation of anxiety within the NAcSh.

## 1. Introduction

The adequate expression of maternal behaviour is crucial for the development and survival of offspring. To ensure the onset of maternal responses, complex (neuro-)hormonal and functional/structural changes occur within the maternal brain, beginning during pregnancy and persisting throughout lactation (Dickens and Pawluski, 2018; Keller et al., 2019; Kinsley and Amory-Meyer, 2011; Navarro-Moreno et al., 2022; Servin-Barthet et al., 2023). Among these adaptations are fine-tuned changes in neuropeptidergic systems, as we recently reviewed (Sanson et al., 2024a). For instance, the activity of the "pro--maternal" oxytocin (OXT) system needs to increase, paralleled by a generally reduced reactivity of "anti-maternal" mediators, such as the members of the corticotropin-releasing factor family (protein, CRF; gene, *Crh*) (Klampfl and Bosch, 2019a; Sanson and Bosch, 2022; Sanson et al., 2024a). Indeed, stress responsiveness during the peripartum period is usually dampened (Brunton et al., 2008; Dickens and Pawluski, 2018), and any perturbation of this state might carry severe consequences for both the mother and her infant, impairing the quality of their bond.

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The CRF family includes four ligands (CRF and Urocortins, UCN1, 2 and 3), which show different affinities for the two receptor subtypes, CRF receptor (CRF-R) 1 and 2 (Deussing and Chen, 2018). Furthermore, the CRF binding protein (CRF-BP) is a regulatory glycoprotein that binds and sequesters freely available CRF and UCN1, thereby preventing them from binding and activating CRF-R (Deussing and Chen, 2018; Ketchesin et al., 2017). The primary function of CRF is to activate the physiological responses to stress by initiating the hypothalamic-pituitary adrenal (HPA) axis cascade, thus acting as an anxiogenic and pro-depressive neuropeptide (Deussing and Chen, 2018). Furthermore, CRF-R and their ligands are widely distributed throughout the brain, including the nucleus accumbens (NAc) (Bangasser, 2013; Deussing and Chen, 2018). Interestingly, the activity of the CRF family is sexually dimorphic and can be influenced by estrogens (Bangasser, 2013; Bangasser and Valentino, 2012; Weathington et al., 2014; Wiersielis et al., 2016), highlighting the complexity of this system. During the postpartum period, increased signalling through brain CRF-R impairs aspects of maternal behaviour (for reviews, see (Klampfl and Bosch, 2019a; Sanson et al., 2024a)). In contrast, as recently demonstrated by our group (Sanson et al., 2024c), hypothalamic CRF-BP contributes to the dampened HPA axis responsiveness typical of lactation (Brunton et al., 2008; Dickens and Pawluski, 2018; Slattery and Neumann, 2008). When the postpartum CRF system is defective, this can potentially culminate in maternal neglect, one of the most prevalent forms of child maltreatment (Brown et al., 2023). Neglect is known to alter the cognitive, emotional, and social development of the infant, and increase vulnerability to mental disorders (Kisely et al., 2018; Nemeroff, 2016). Despite these alarming consequences for the infant, the neurobiological bases promoting neglect are still not fully understood, and intervention strategies are still missing.

OXT is mainly produced in the supraoptic and paraventricular nucleus (PVN) of the hypothalamus. By binding to its receptor (OXT-R) in the periphery, OXT controls physiological activities related to reproduction (Jurek and Neumann, 2018). Centrally, OXT modulates complex behavioural activities, such as affiliative, social and maternal behaviours, as well as stress responses (Menon and Neumann, 2023; Sanson and Bosch, 2022).

The NAc is part of the ventral striatum and plays a prominent role within the brain reward circuit due to strong dopaminergic inputs (Floresco, 2015; Salgado and Kaplitt, 2015). Indeed, the NAc acts as an interface by integrating memory and emotional signals from the limbic system and translating them into motivated behaviours via the activation of motor effector sites (Floresco, 2015; Salgado and Kaplitt, 2015). Furthermore, the NAc is part of a complex neural network that modulates the display of pup-directed responses and maternal behaviour (Numan et al., 2005; Stolzenberg et al., 2007; Dulac et al., 2014; Kuroda et al., 2020; Servin-Barthet et al., 2023; Smiley et al., 2019). Anatomically and biochemically heterogeneous, the NAc can be divided into a central core flanked by an outer shell. The two subregions show differential connectivity, suggesting that they may mediate different behavioural responses (Brog et al., 1993; Floresco, 2015; Salgado and Kaplitt, 2015). The NAc shell (NAcSh), specifically, mediates the reinforcing properties of novelty and rewards (Floresco, 2015; Salgado and Kaplitt, 2015) and plays a role in maternal care, memory, and motivation (Numan, 2007; D'Cunha et al., 2011; Li and Fleming, 2003a, b; Witchey et al., 2024).

In this study, we hypothesised that altered neuropeptidergic transmission in the NAcSh of lactating rats might impair maternal behaviour and increase anxiety-like behaviour. Thus, we studied the specific function of the CRF and OXT systems in the NAcSh in relation to these behaviours. First, we assessed the effects of acute bilateral modulation of these systems on maternal care and motivation. Additionally, we examined the involvement of the CRF and OXT systems on anxiety-like behaviour in lactating rats. To evaluate any sex-specific effect of the CRF system, we also studied anxiety-like behaviour in virgin female and male rats, alongside investigating potential sex differences in gene expression of CRF family members in the entire NAc.

## 2. Materials and methods

### 2.1. Animals

Virgin female Sprague-Dawley rats (230g–250g at arrival; Charles River Laboratories, Sulzfeld, Germany) were housed in groups of 3–4 under standard laboratory conditions (12:12 h light/dark cycle; lights on at 07:00 a.m.; room temperature 22 ± 2 °C, 55 ± 5% relative humidity), with *ad libitum* access to water and standard rat chow (ssniff-Spezialdiäten GmbH, Soest, Germany). To obtain lactating subjects, two virgin female rats were mated with one sexually experienced male Sprague-Dawley rat in Eurostandard type IV cages (40 × 60× 20 cm) until pregnancy was confirmed by the presence of sperm in vaginal smears (pregnancy day, PD1). Pregnant females were housed in groups of 3–4 until PD18, when they underwent stereotaxic surgery and were single housed in observational cages (Plexiglass; 38 × 22 × 35 cm) for undisturbed recovery and delivery. On the day of birth (lactation day, LD0), litters were culled to 8 pups with balanced sexes. To study anxiety-like behaviour, we included virgin females (in the pro-estrous phase, see 2.5.3 for details; 230g–250g at arrival; Charles River Laboratories) and males (240g–260g at arrival; Charles River Laboratories), which were housed in groups of 3–4 of the same sex until stereotaxic surgery, after which they were single housed in observational cages. All rats were handled daily to familiarize them with the experimenters and the procedures, thereby reducing non-specific stress responses. To study gene expression of members of the CRF family, separate cohorts of lactating (LD5), virgin female (in the pro-estrous phase) and male rats were included in the studies.

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 minimise the number of animals used and to reduce their distress or suffering.

### 2.2. Experimental design

Before the behavioural experiments, each rat was randomly assigned to one of the treatment groups and received the same drug on each testing day. After acute treatment infusion, dams were returned to their home cage, and their behaviour was monitored after the corresponding lag time (see 2.4). In the first experiment (Fig. 1A), maternal care was assessed in the home cage on LD1 (see 2.5.1), maternal motivation was monitored on LD3 (2.5.2), and anxiety-like behaviour was assessed in the light-dark box (LDB) test on LD5 (2.5.3). The latter was complemented by a follow-up experiment (Fig. 1B) that included only virgin female and male rats. In a separate cohort of rats (Fig. 1C), brains of lactating females (LD5), virgin females (pro-estrous phase) and male rats were collected for subsequent gene expression analysis with qPCR. Fig. 1D shows a representative histological image of a correct implantation site (assessed as described in 2.6).

### 2.3. Surgical procedures

Rats underwent surgical procedures (pregnant rats on PD18 ± 1) under inhalation anaesthesia in semi-sterile conditions as previously described (Bosch et al., 2010). Stainless steel 23 G guide cannulas (length: 12 mm) were implanted 2 mm above the NAcSh (coordinates: anterior-posterior +1 mm, lateral ±3 mm, ventral −5.3 mm from the skull surface, angle 17.5°) (Paxinos and Watson, 2013).

### 2.4. Pharmacological manipulations

For acute bilateral local infusion, stainless steel 30 G infusion

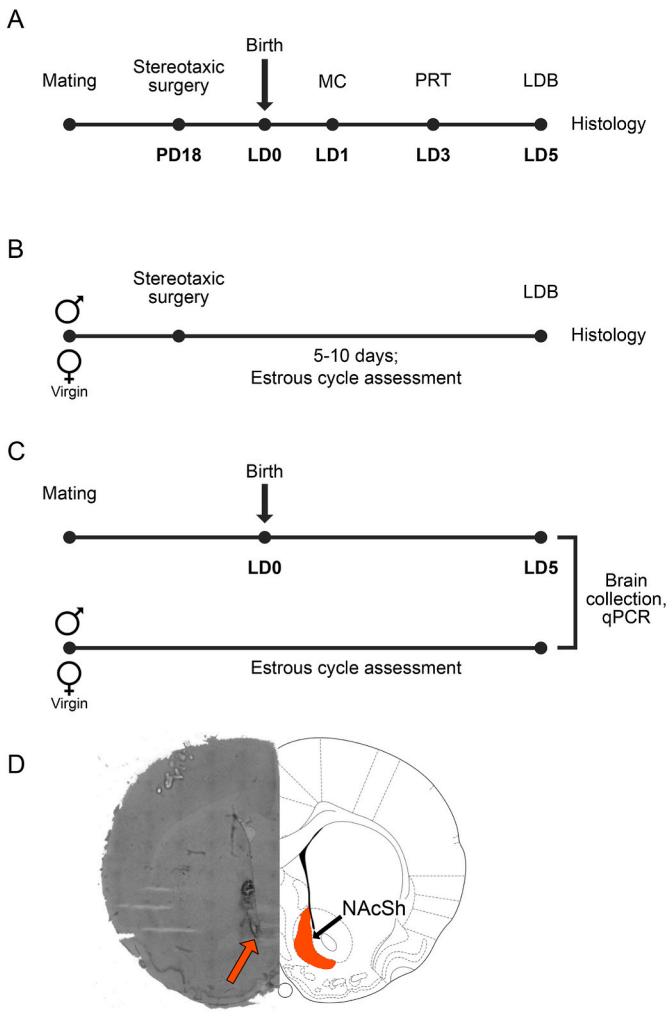


**Fig. 1.** Experimental designs and representative histological picture of cannula placement
Behavioural experimental timeline in (A) lactating rats and (B) virgin female and male rats. (C) Experimental timeline for brain collection in lactating female (upper line), virgin female and male (bottom line) rats. (D) Representative histological picture of cannula placement; coronal scheme from (Paxinos and Watson, 2013). Abbreviations: LD: lactation day, LDB: light-dark box, MC: maternal care, PD: pregnancy day, PRT: pup retrieval test.

cannulas (length: 14 mm) were connected via PE-50 tubing to 10 μL Hamilton syringes. The infusion cannula was lowered into the guide cannula and kept in place for approximately 30s during drug infusion. Per side, 0.5 μL of one of the following substances was infused (see Table 1 for concentrations, lag-times and suppliers).

- vehicle (VEH; when used for OXT-R antagonist (OXT-A): sterile Ringer's solution; pH adjusted to 7.4; B. Braun, Melsungen, Germany; when used for drugs targeting the CRF system: sterile Ringer's solution with 4% DMSO; pH adjusted to 7.4)
- OXT-A ($(d(CH_2)^1_5$, $Tyr(Me)^2$, $Thr^4$, $Orn^8$, des-Gly-$NH^9_2$)-vasotocin)
- human/rat CRF (primarily CRF-R1 agonist)
- CP-154,526 (selective CRF-R1 antagonist)

While CRF can bind to both CRF-R, it shows a 40X higher affinity for CRF-R1, thus, it is considered to act primarily on this subtype (Deussing and Chen, 2018; Hauger et al., 2003). Doses and lag-times between administration and behavioural experiments were based on previous studies (D'Anna and Gammie, 2009; Klampfl et al., 2016; Klampfl et al., 2018; Lukas et al., 2013).

### 2.5. Behavioural assessment

#### 2.5.1. Maternal care

Following an established protocol, maternal care was monitored on LD1 before and after drug infusion for 10s every 2nd min in 30-min blocks (Bosch and Neumann, 2008). The quality of maternal behaviour was measured by the occurrence of arched-back nursing (ABN) and licking and grooming (LG) (Bosch, 2011; Klampfl and Bosch, 2019b). Other nursing parameters included blanket posture, nursing while lying on the back or side, and hovering over the pups. These positions were summed up as "total nursing". In addition, non-maternal behaviours, including self-grooming, were quantified as "off-nest" behaviour.

#### 2.5.2. Maternal motivation

Maternal motivation was assessed using the modified pup retrieval test (PRT) following an established protocol (Bayerl et al., 2016). On the afternoon of LD2, a red plexiglass house (13 × 17 × 11 cm, opening 6 × 8.5 cm) was introduced to the mother's cage overnight for habituation. On LD3, the mother was moved to the testing room, and the red house and pups were removed 60 min prior to the test. Pups were kept as a whole litter in a box containing bedding from their home cage on a heating pad set at 32 °C. Immediately before the PRT, all pups were distributed in a new arena (54 × 34 × 31 cm) containing home cage bedding, and the house was placed at one of the short-edged walls. Following drug infusion, the mother was placed in the arena, and the behaviour was recorded for 15 min. The videos were manually analysed by an experienced observer blind to the treatments. The % of retrieved pups, the latencies to retrieve the first and last pups, and the % of dams retrieving all pups were analysed.

#### 2.5.3. Anxiety-like behaviour

Anxiety-like behaviour of lactating rats (tested at LD5) as well as of virgin female and male rats (tested 5–10 days post-surgery, depending on the estrous cycle stage of the virgin females) was assessed in the LDB test as previously described (Crawley and Goodwin, 1980; Demarchi et al., 2023). Following drug infusion, each rat was placed in the centre of the light box, and behaviour was recorded for 10 min for later analysis by an experimenter blind to the treatment using EthoVision XT (Noldus, Wageningen, The Netherlands). The following parameters were analysed: % of time spent in the light box, number of transitions from the dark to the light box, and locomotor activity. All virgin female rats were tested in the pro-estrous phase, characterized by lower anxiety-like behaviour (Lovick and Zangrossi, 2021; Zuluaga et al., 2005). The estrous cycle stage of female rats was determined using vaginal smears over 7 days before surgery and again starting 2 days after surgical procedures. Phase predictions for the day of testing were confirmed immediately after the behavioural experiment.

**Table 1**
Details of administered drugs.

| Biological activity | Substance | Concentration | Lag-time | Company | Cat. # |
|---|---|---|---|---|---|
| *OXT-R antagonist* | OXT-A | 0.1 μg/0.5 μL/side | 10 min | Provided by Dr. Manning | / |
| *CRF-R1 agonist* | CRF | 1 μg/0.5 μL/side | 10 min | Tocris Bioscience | 1151 |
| *CRF-R1 antagonist* | CP-154,526 | 0.4 μg/0.5 μL/side | 10 min | Tocris Bioscience | 2779 |
| *CRF-R2 agonist* | UCN3 | 3 μg/0.5 μL/side | 25 min | Phoenix Pharmaceuticals | 019–26 |

### 2.6. Histology

At the end of the behavioural experiments, all animals were euthanised by an overdose of $CO_2$ inhalation. For histological evaluation of cannula placements, 0.5 μL of ink was infused post-mortem via the cannulas. Brains were sectioned in 40 μm coronal sections using a Cryostat (CM3050S Leica Microsystem GmbH, Nussloch, Germany), slide-mounted, and Nissl-stained to identify the implantation sites. Rats with incorrect cannula placements were excluded from the statistical analyses.

### 2.7. Real-time qPCR

A separate cohort of lactating female (at LD5), virgin female (in the pro-estrous phase of the estrous cycle) and male rats were euthanised by decapitation after brief anaesthesia with $CO_2$. Brains were removed and cut with a cryostat into coronal sections (1.70–0.70 mm from bregma (Paxinos and Watson, 2013),) of 250 μm containing the NAc, which was harvested using a 1 mm-diameter puncher and stored at −80 °C until further processed. Due to technical limitations, a distinction between core and shell was not feasible; thus, the whole NAc was collected and analysed.

Total RNA was isolated using peqGold Trifast (VWR International, Radnor, USA) according to the manufacturer's protocol. For mRNA analysis, 500 ng of total RNA per sample was reverse transcribed using Ultra Script 2.0 (PCR Biosystems, London, UK). Relative quantification of RNA levels was performed using PowerUp SYBR Green Master Mix (Thermo Fischer, Waltham, USA), with Glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) used as the housekeeping gene. The targets and the sequences of all genes are listed in Table 2. Data were analysed following the 2^ΔCt method.

### 2.8. Statistical analysis

Statistical analysis was conducted using GraphPad Prism10 (GraphPad Software, Boston, USA). Normality and homogeneity of variance were tested using Shapiro-Wilk test and F-test, respectively. When normality was violated, non-parametric tests were used; if homogeneity of variance was violated, appropriate corrections were used. Statistical outliers were identified with Grubbs' method and removed from analysis. Data were analysed using two-way ANOVA for repeated measures (factors: time, treatment), 1-way ANOVA, Kruskal-Wallis test, Brown-Forsythe ANOVA test, unpaired *t*-test, non-parametric Mann-Whitney test, or unpaired *t*-test with Welch's correction. Where appropriate, post hoc comparisons were performed using Bonferroni or Dunnett's T3 correction. Differences in the distribution of PRT latencies were analysed with Log-rank Mantel-Cox survival analysis (Salais-Lopez et al., 2021). Size effects were calculated with Cohen's *d* coefficient and eta squared $\eta^2$. Statistical significance was set at $p \leq 0.05$, and a trend was accepted up to $p = 0.07$.

**Table 2**
Primers forward and reverse sequences.

| Gene | Forward | Reverse |
|---|---|---|
| Crh-r1 | TCC ACT ACA TCT GAG ACC ATT CAG TAC A | TCC TGC CAC CGG CGC CAC CTC TTC CGG A |
| Crh-r2 | ACA TCC GAG ACC CAG TA | GGA CTG CAG GAA AGA GTT GA |
| Crh-bp | CTG CAG CTT TTC CAT CAT TT | CAT CTT GGA GGT GTC CAG TC |
| Gapdh | TGA TGA CAT CAA GAA GGT GG | CAT TGT CAT ACC AGG AAA TGA G |

## 3. Results

### 3.1. CRF-R1 activation in the NAcSh impaired maternal care

We first studied the effects of CRF-R1, CRF-R2 and OXT-R manipulations on maternal care. When analysing the effects of CRF infusion on total nursing (Fig. 2A), a 2-way repeated measures ANOVA revealed a significant main effect of time ($F[4, 73] = 5.01$; $p < 0.01$, $\eta^2 = 0.21$) and treatment, with CRF reducing the occurrence of nursing (VEH = 10.4 ± 0.14, CRF = 7.1 ± 0.24; $F[1, 18] = 8.38$; $p < 0.01$, $\eta^2 = 0.32$), while time × treatment interaction was not significant ($F[6, 108] = 2.0$; $p = 0.072$, $\eta^2 = 0.1$). Interestingly, the CRF-treated dams showed a significant increase in self-grooming (time: $F[4, 67] = 5.16$, $p < 0.01$, $\eta^2 = 0.23$; treatment: $F[1, 18] = 6.24$, $p < 0.05$, $\eta^2 = 0.26$; interaction: $F[6, 108] = 4.39$, $p < 0.001$, $\eta^2 = 0.2$; Fig. 2B). Specifically, the infusion of CRF significantly increased self-grooming at t30 compared to baseline ($p < 0.05$); however, when compared to VEH-treated rats at the same time point, this increase did not reach statistical significance ($p = 0.064$). Remarkably, infusion of CP-154,526 or UCN3 did not affect any aspects of maternal care (Fig. 2C–F and Table 3).

When analysing the nursing behaviour of OXT-A-treated dams (Fig. 2G), we found significant main effects of time ($F[4, 87] = 5.5$, $p = 0.0004$, $\eta^2 = 0.2$) and treatment ($F[1, 21] = 5.2$, $p = 0.034$, $\eta^2 = 0.2$), but not of their interaction ($F[6, 126] = 0.76$, $p = 0.598$, $\eta^2 = 0.03$). No changes in self-grooming or other behaviours were observed in OXT-A-treated dams (Fig. 2H and Table 3).

### 3.2. Reduced CRF-R1 signalling in the NAcSh reduced maternal motivation

The effects of treatments on maternal motivation were assessed using the modified PRT. Only the infusion of CP-154,526 (CRF-R1 antagonist) impaired the onset of maternal motivation, as dams were slower to retrieve the first pup. The survival distributions of latency-to-first-pup retrieval for VEH- and CP-154,526-treated dams were significantly different ($\chi^2$ [1] = 4.1, $p = 0.043$; Fig. 3A), indicating that a lower percentage of CP-154,526-treated mothers (57%) retrieved the first pup. Furthermore, the latency to retrieve the first pup was increased ($t[6] = 2.8$; $p = 0.029$, Unpaired *t*-test with Welch's correction, Cohen's $d = 1.5$; Fig. 3B). No differences were found in the % of pups retrieved over time (data not shown). No further changes were observed with any other drug (data not shown).

### 3.3. CRF-R activation in the NAcSh modulated anxiety-like behaviour in a sex-specific manner

In lactating rats, the infusion of CRF (Fig. 4A, E) as well as OXT-A (Fig. 4D, H) had no effect on the time spent in the light compartment, or on the number of transitions from the dark to the light compartment (Student's *t*-test, $p > 0.05$). The infusion of CP-154,526 reduced the number of transitions from the dark to the light box compared to VEH-treated dams ($t[6] = 2.7$, $p = 0.035$, Unpaired *t*-test with Welch's correction, Cohen's $d = 1.6$; Fig. 4F) but did not affect the time spent in the light compartment (Fig. 4B; $p > 0.05$). In contrast, UCN3 infusion significantly reduced the time spent in the light compartment compared to VEH-treated dams (Unpaired *t*-test, $t[11] = 2.3$, $p = 0.039$, $d = 1.3$; Fig. 4C, I), without influencing the number of transitions (Fig. 4G). Total locomotor activity was not affected by any treatments (data not shown).

To further investigate whether the effect of UCN3 infusion on anxiety-like behaviour is influenced by the reproductive state or depending on the sex, we tested CRF and UCN3 infusion in virgin female and male rats. Interestingly, in virgin female rats tested during the pro-estrous phase, the effect on anxiety-like behaviour (% time spent in the light compartment; 1-way ANOVA: $F[2, 25] = 3.6$, $p = 0.043$, $\eta^2 = 0.22$) was similar to lactating rats. Acute activation of CRF-R2 via UCN3 infusion significantly reduced the % of time spent in the light

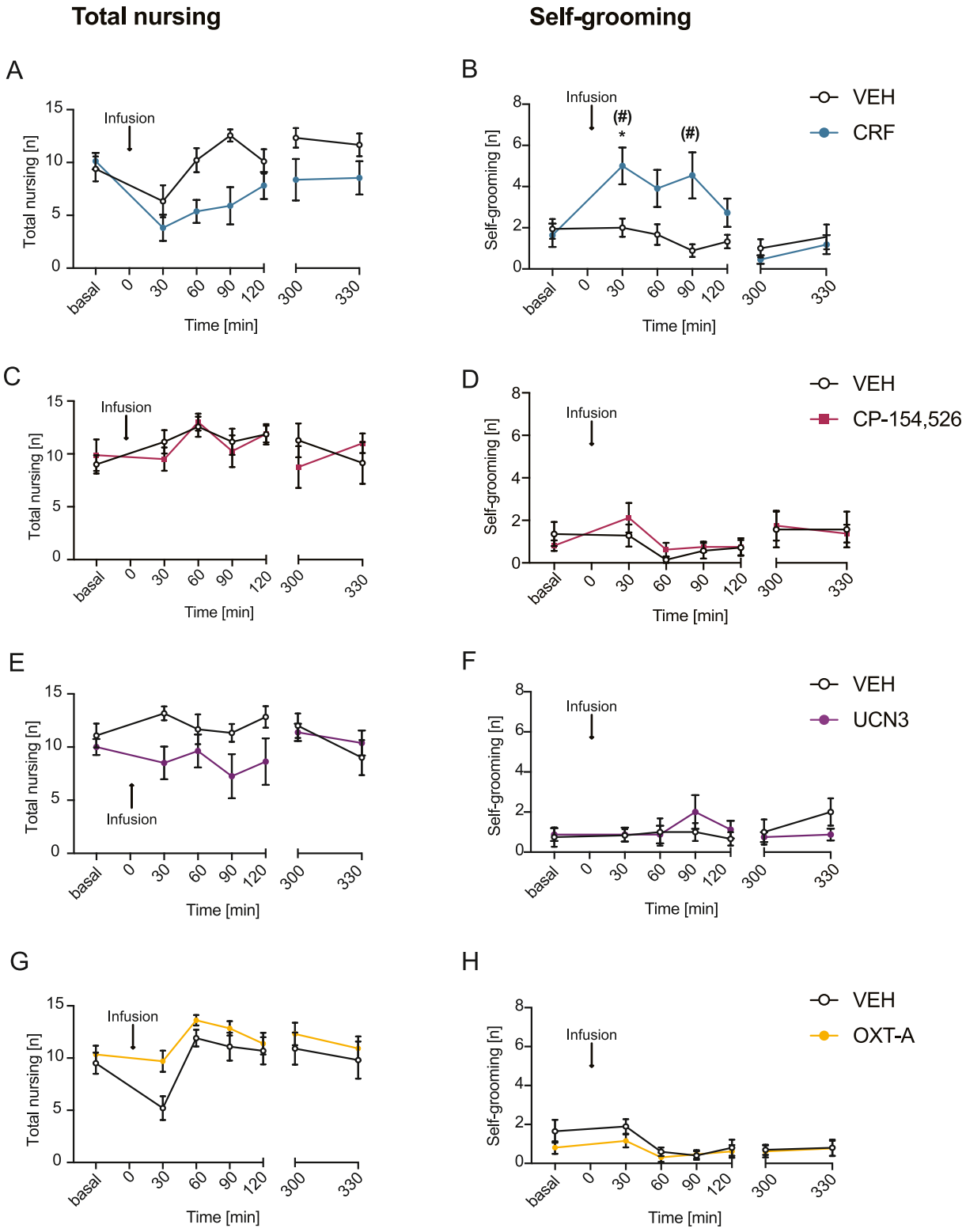


**Fig. 2.** Altered nursing and self-grooming following acute CRF-R1 activation within the NAcSh
Total nursing (A, C, E, G) and self-grooming (B, D, F, H) behaviour were analysed in dams treated with (A, B) CRF (VEH n = 9; CRF n = 11), (C, D) CP-154,526 (VEH n = 7; CP-154,526 n = 9), (E, F) UCN3 (VEH n = 6; UCN3 n = 8), and (G, H) OXT-A (VEH n = 10; OXT-A n = 13). Data are presented as mean ± SEM. *p ≤ 0.05 vs respective basal; (#) p ≤ 0.07 vs VEH-treated rats, two-way repeated measures ANOVA followed by Bonferroni post hoc comparisons.

compartment compared to VEH-treated rats ($p < 0.05$, Bonferroni's multiple comparisons; Fig. 5A, C), while the number of transitions did not differ between groups (Kruskal-Wallis test; $H = 3.4$, $p > 0.05$; Fig. 5B). Infusion of either CRF or UCN3 reduced locomotor activity compared to VEH-treated female rats (1-way ANOVA: $F[2, 26] = 9.12$, $p = 0.001$, $\eta^2 = 0.41$; $p < 0.01$ for both drugs vs VEH, Bonferroni post hoc comparisons; VEH = 4025 cm ± 267 cm; CRF-R1 ago = 2463 cm ± 317 cm; CRF-R2 ago = 2476 cm ± 284 cm).

In male rats, 1-way ANOVA revealed a significant effect of the treatment on the % of time spent in the light compartment ($F[2, 26] =$

**Table 3**
Behavioural profile before (basal) and after acute treatment infusion into the NAcSh on LD1.

| Behaviour | Group | Time [min] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Basal | +30 | +60 | +90 | +120 | +300 | +330 |
| *Arched-back nursing* | Vehicle | 0.4 ± 0.2 | 0.2 ± 0.2 | 1 ± 0.4 | 1 ± 0.4 | 1 ± 0.3 | 0.6 ± 0.2 | 0.8 ± 0.2 |
| | CRF | 0.8 ± 0.3 | 0.1 ± 0.1 | 0.5 ± 0.2 | 0.5 ± 0.4 | 0.4 ± 0.1 | 1 ± 0.4 | 0.6 ± 0.3 |
| | Vehicle | 1 ± 0.4 | 0.1 ± 0.1 | 0.3 ± 0.3 | 0.4 ± 0.3 | 0.4 ± 0.2 | 0.4 ± 0.3 | 0.3 ± 0.3 |
| | CP-154,526 | 0.6 ± 0.2 | 0.2 ± 0.2 | 0.1 ± 0.1 | 0.2 ± 0.2 | 0.4 ± 0.4 | 0.5 ± 0.3 | 0.6 ± 0.3 |
| | Vehicle | 2 ± 0.3 | 2 ± 1 | 2 ± 0.5 | 1 ± 1 | 2 ± 0.4 | 3 ± 0.4 | 2 ± 0.5 |
| | UCN3 | 2 ± 1 | 2 ± 0.4 | 1 ± 0.2 | 0.4 ± 0.2 | 1 ± 0.3 | 3 ± 1 | 2 ± 1 |
| | Vehicle | 2 ± 0.5 | 1 ± 0.4 | 1 ± 0.3 | 1 ± 0.3 | 2 ± 1 | 2 ± 1 | 2 ± 1 |
| | OXT-A | 2 ± 0.5 | 2 ± 1 | 2 ± 1 | 2 ± 0.6 | 2 ± 1 | 3 ± 1 | 2 ± 0.5 |
| *Blanket posture* | Vehicle | 9 ± 1 | 6 ± 1 | 9 ± 1 | 11 ± 1 | 9 ± 1 | 11 ± 1 | 10 ± 1 |
| | CRF | 8 ± 1 | 3 ± 1 | 5 ± 1 | 5 ± 2 | 7 ± 1 | 6 ± 2 | 8 ± 1 |
| | Vehicle | 8 ± 1 | 11 ± 1 | 12 ± 1 | 11 ± 1 | 11 ± 1 | 10 ± 2 | 8 ± 2 |
| | CP-154,526 | 8 ± 2 | 9 ± 1 | 13 ± 1 | 10 ± 1 | 10 ± 1 | 8 ± 2 | 10 ± 1 |
| | Vehicle | 8 ± 1 | 11 ± 1 | 10 ± 1 | 10 ± 1 | 11 ± 1 | 9 ± 1 | 7 ± 1 |
| | UCN3 | 8 ± 1 | 7 ± 1 | 9 ± 1 | 7 ± 2 | 8 ± 2 | 9 ± 1 | 8 ± 1 |
| | Vehicle | 6 ± 1 | 4 ± 1 | 10 ± 1 | 9 ± 2 | 8 ± 1 | 9 ± 1 | 8 ± 2 |
| | OXT-A | 9 ± 1 | 8 ± 1 | 10 ± 1 | 10 ± 1 | 9 ± 1 | 9 ± 1 | 9 ± 1 |
| *Licking/grooming* | Vehicle | 2 ± 0.3 | 1 ± 0.2 | 2 ± 0.4 | 1 ± 0.3 | 2 ± 1 | 1 ± 0.2 | 1 ± 0.4 |
| | CRF | 2 ± 0.4 | 1 ± 0.4 | 3 ± 0.4 | 1 ± 0.3 | 2 ± 0.5 | 1 ± 0.5 | 2 ± 0.5 |
| | Vehicle | 2 ± 0.3 | 1 ± 0.3 | 1 ± 0.5 | 2 ± 1 | 1 ± 0.5 | 1 ± 1 | 1 ± 0.4 |
| | CP-154,526 | 2 ± 0.5 | 2 ± 0.4 | 1 ± 0.5 | 2 ± 0.5 | 2 ± 0.3 | 1 ± 0.4 | 2 ± 1 |
| | Vehicle | 2 ± 0.3 | 1 ± 0.3 | 1 ± 0.4 | 2 ± 0.4 | 1 ± 0.4 | 1 ± 1 | 1 ± 1 |
| | UCN3 | 2 ± 0.2 | 1 ± 0.5 | 1 ± 0.3 | 1 ± 0.4 | 1 ± 0.5 | 2 ± 0.5 | 2 ± 0.4 |
| | Vehicle | 1 ± 0.2 | 1 ± 1 | 2 ± 0.5 | 1 ± 0.4 | 2 ± 0.5 | 1 ± 0.4 | 1 ± 0.3 |
| | OXT-A | 2 ± 0.2 | 1 ± 0.4 | 1 ± 0.2 | 1 ± 0.2 | 1 ± 0.4 | 1 ± 0.4 | 2 ± 0.4 |
| *Off-nest* | Vehicle | 4 ± 1 | 6 ± 1 | 2 ± 1 | 1 ± 0.4 | 3 ± 1 | 1 ± 1 | 2 ± 1 |
| | CRF | 3 ± 1 | 8 ± 1 | 5 ± 1 | 6 ± 2 | 4 ± 1 | 5 ± 2 | 5 ± 2 |
| | Vehicle | 3 ± 1 | 2 ± 1 | 1 ± 1 | 1 ± 1 | 1 ± 0.5 | 2 ± 1 | 5 ± 2 |
| | CP-154,526 | 3 ± 1 | 3 ± 1 | 1 ± 1 | 2 ± 1 | 1 ± 0.5 | 4 ± 2 | 3 ± 1 |
| | Vehicle | 2 ± 1 | 1 ± 0.4 | 2 ± 1 | 1 ± 1 | 1 ± 1 | 1 ± 1 | 4 ± 1 |
| | UCN3 | 2 ± 1 | 5 ± 1 | 4 ± 2 | 6 ± 2 | 5 ± 2 | 1 ± 0.4 | 2 ± 1 |
| | Vehicle | 4 ± 1 | 8 ± 1 | 1 ± 0.6 | 3 ± 1 | 2 ± 1 | 3 ± 1 | 4 ± 2 |
| | OXT-A | 2 ± 1 | 3 ± 1 | 0.5 ± 0.3 | 1 ± 0.3 | 2 ± 1 | 1 ± 1 | 2 ± 1 |

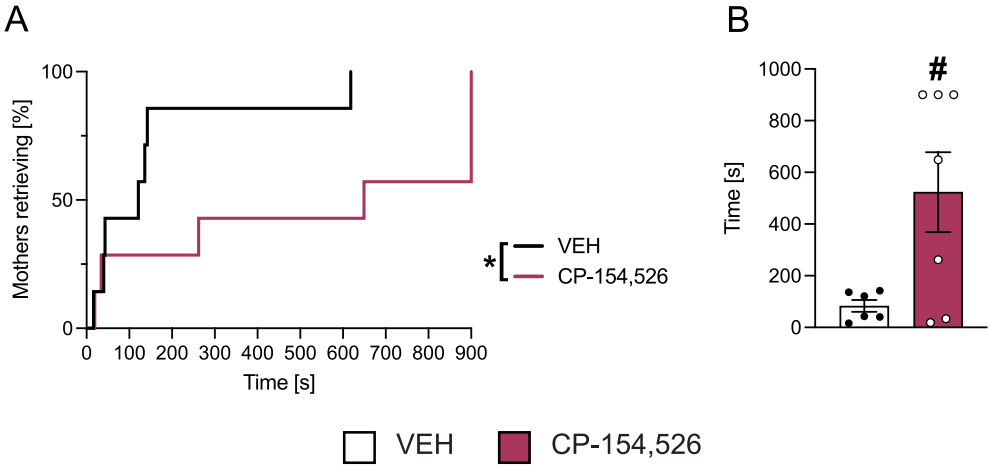


**Fig. 3.** Reduced maternal motivation following acute CRF-R1 inhibition within the NAcSh
(A) Survival curve indicating the cumulative percentage of dams retrieving the first pup and (B) latency to retrieve the first pup. Data are presented as mean ± SEM. *p ≤ 0.05, Log-rank Mantel-Cox test; #p ≤ 0.05 vs VEH-treated rats, Unpaired *t*-test with Welch's correction.

5.46, p = 0.011, $\eta^2$ = 0.29). In detail, CRF infusion induced a strong reduction of the % of time spent in the light compartment (p < 0.05 vs VEH group, Bonferroni post-hoc comparisons; Fig. 5D, F), while UCN3 administration only tended to increase anxiety-like behaviour (p = 0.06). No differences were observed for the number of transitions (F[2, 27] = 3.3; p = 0.053, $\eta^2$ = 0.2 Fig. 5E), but 1-way ANOVA showed a significant effect of the treatment on locomotion (F[2, 27] = 7.2, p = 0.003, $\eta^2$ = 0.35). Infusion of both CRF and UCN3 induced a significant

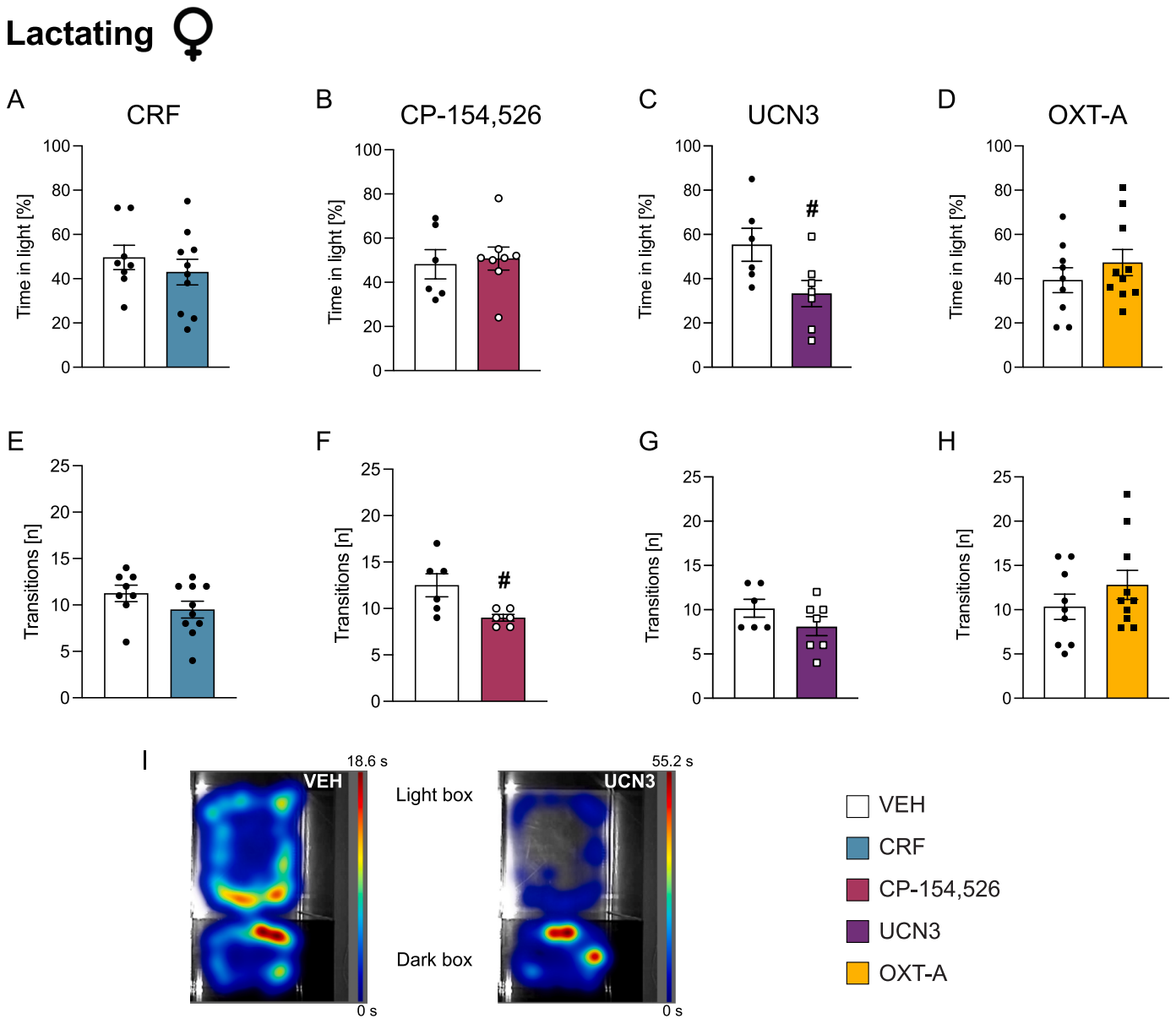


**Fig. 4.** Increased maternal anxiety-like behaviour following acute CRF-R2 activation within the NAcSh
(A–D) Percentage of time spent in the light compartment of the LDB and (E–H) number of transitions from the dark to the light compartment in dams treated with (A, E) CRF; (B, F) CP-154,526; (C, G) UCN3; (D, H) OXT-A. (I) Representative heatmaps of the time spent in both compartments of the LDB in lactating rats treated with vehicle (VEH) or UCN3. Data are presented as mean ± SEM. #p ≤ 0.05 vs VEH-treated rats, Unpaired *t*-test with or without Welch's correction.

reduction in locomotor activity compared to VEH-treated rats ($p < 0.05$ and $p < 0.01$, respectively; VEH = 3221 cm ± 314 cm; CRF-R1 ago = 1961 cm ± 203 cm; CRF-R2 ago = 2041 cm ± 261 cm).

### 3.4. *mRNA expression of CRF family members in the NAc was sexually dimorphic*

We measured the expression levels of *Crh-r1*, *Crh-r2* and *Crh-bp* in the entire NAc, comparing lactating, virgin female, and male rats. *Crh-r1* showed differential expression among the three groups (Brown-Forsythe ANOVA test: $F[2, 16] = 9.69$, $p = 0.002$, $\eta^2 = 0.55$; Fig. 6A). Specifically, both lactating female and male rats showed higher *Crh-r1* mRNA levels in the NAc compared to virgin female rats in the pro-estrus phase (lactating: $p < 0.05$; males: $p < 0.001$; Dunnett's T3 multiple comparisons). While the expression levels of *Crh-r2* mRNA did not differ significantly ($H = 5.3$, $p = 0.067$, Kruskal-Wallis test; Fig. 6B), mRNA levels of *Crh-bp* differed among the tested groups (1-way ANOVA; $F[2, 21] = 6.8$, $p = 0.005$, $\eta^2 = 0.39$; Fig. 6C). Specifically, male rats had significantly higher *Crh-bp* mRNA levels compared to virgin female rats ($p < 0.01$, Bonferroni's multiple comparisons), while no differences were observed when compared to lactating rats ($p > 0.05$, Bonferroni's multiple comparisons).

## 4. Discussion

Mothers neglecting their infant is one of the most prevalent forms of child maltreatment, which can drastically affect the overall development and mental health of the child (Brown et al., 2023; Kisely et al., 2018). In recent years, animal research has provided evidence that brain alterations in neuropeptide signalling may contribute to the onset of neglectful behaviours (as we recently reviewed in (Sanson et al., 2024a)). In the present study, we aimed to advance our understanding of the consequences of altered neuropeptidergic transmission on pup neglect, focusing on the NAcSh as a central region of maternal and

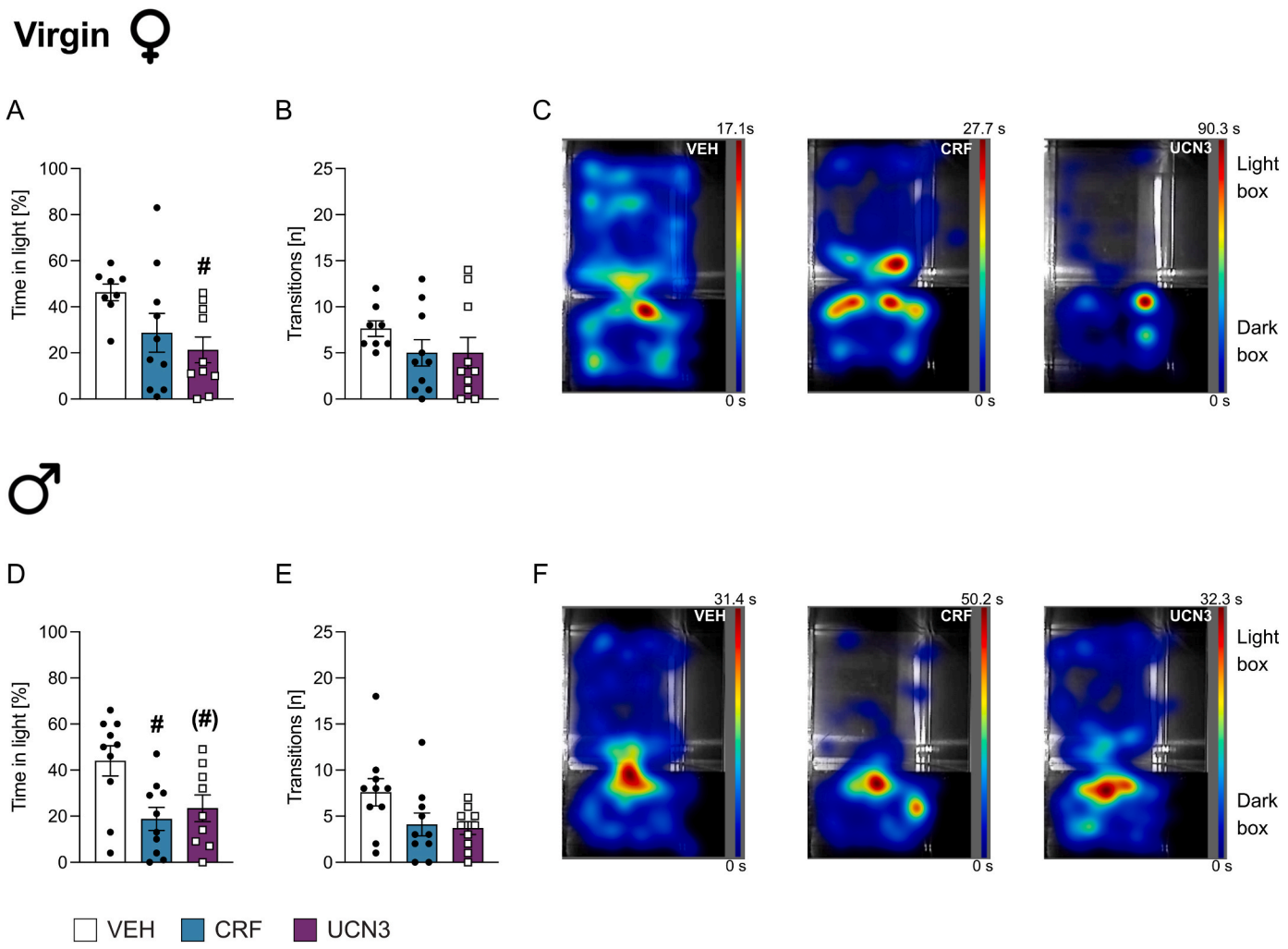


**Fig. 5.** Sex-specific effects of acute CRF-R activation in the NAcSh on anxiety-like behaviour
(A, D) Percentage of time spent in the light compartment of the LDB, (B, E) number of transitions from the dark to the light compartment of the LDB in (A, B) virgin female and (D, E) male rats following infusion of vehicle (VEH), CRF or UCN3. Representative heatmaps of the time spent in both compartments of the LDB in (C) virgin female and (F) male rats following infusion of VEH, CRF or UCN3. Data are presented as mean ± SEM. (#) $p \leq 0.07$; #$p \leq 0.05$ vs VEH-treated rats, 1-way ANOVA followed by Bonferroni's post hoc comparisons.

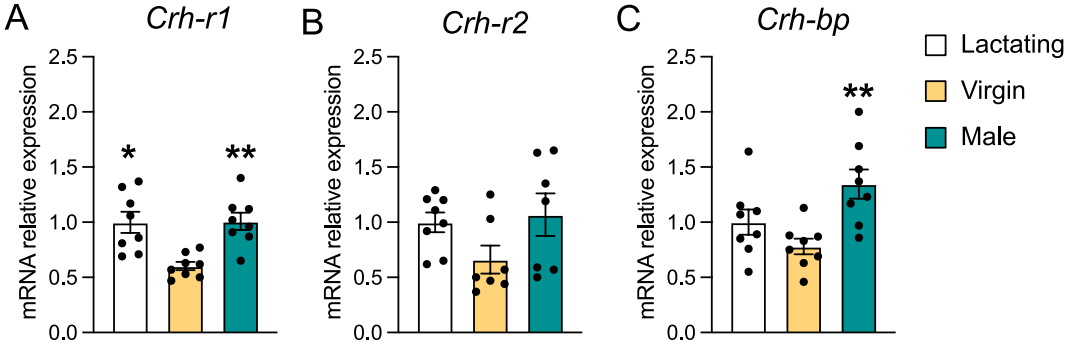


**Fig. 6.** Reduced *Crh-r1* and *Crh-bp* mRNA expression in the NAc of virgin female rats
Relative mRNA levels of (A) *Crh-r1*, (B) *Crh-r2* and (C) *Crh-bp* in the whole NAc of lactating, virgin female and male rats. Data are expressed as relative levels vs lactating animals, and are presented as mean ± SEM.
*$p \leq 0.05$, **$p \leq 0.01$ vs virgin female group; Brown-Forsythe ANOVA test followed by Dunnett's T3 multiple comparisons (A) or 1-way ANOVA followed by Bonferroni's multiple comparisons (C).

reward circuits (Numan et al., 2005; Stolzenberg et al., 2007; Dulac et al., 2014; Floresco, 2015; Salgado and Kaplitt, 2015; Servin-Barthet et al., 2023). Specifically, we identified CRF-R1 as a crucial modulator of maternal care and motivation, while CRF-R2 predominantly regulated anxiety-like behaviour in females, regardless of the reproductive state. Interestingly, local CRF infusion had anxiogenic effects in males, only.

This sex difference was paralleled by higher *Crh-r1* mRNA expression in the NAc of male and lactating female rats compared to virgin females, as well as higher *Crh-bp* mRNA expression in males versus virgins. In contrast to the modulatory actions of the CRF system, our data suggests that the OXT system in the NAcSh plays a marginal role in the context of maternal care and anxiety.

Subtle brain adaptations occur during the peripartum period as prerequisites for becoming maternal. In fact, maternal behaviour is a strong, deep-rooted behaviour in females (Bridges, 2015). The present data suggest that this firm behaviour can be impaired by CRF infusion within the NAcSh, promoting pup neglect as shown by reduced nursing (Fig. 2A, main effect of treatment) and increased self-grooming behaviour (Fig. 2B), as we hypothesised. These findings regarding the anti-maternal properties of the brain CRF system align with previous research demonstrating that central (Almeida et al., 1994) or local infusion of CRF in brain regions such as the bed nucleus of the stria terminalis (BNST) (Creutzberg et al., 2020; Klampfl et al., 2016) or the medial preoptic area (MPOA) (Klampfl et al., 2018) facilitates pup neglect. Moreover, CRF administration in the NAcSh increased self-grooming behaviour, as previously demonstrated in male rats (Holahan et al., 1997). A comparable effect was observed in rat mothers after acute infusion of CRF either centrally (Almeida et al., 1994) or locally in the MPOA (Klampfl et al., 2018). Enhanced self-grooming behaviour might represent a stereotypic response to heightened stress system activation (Holahan et al., 1997; Kalueff et al., 2016), thus our data imply that the presence of pups is insufficient to overcome this stereotypical response to heightened stress system activation. Furthermore, it may indicate that maternal behaviour shifts from pup care to self-care, as observed following central administration of an OXT-R antagonist (Pedersen and Boccia, 2003), suggesting that (hyper-)activation of CRF-R in the NAcSh reduces the overall engagement in pup-directed activities. In fact, cues and stimuli from pups that are perceived as rewarding during early lactation (Ferris et al., 2005; Lee et al., 2000) may be disrupted by the acute hyperactivation of CRF-R1 signalling within the NAcSh.

Infusion of CP-154,526 (a selective CRF-R1 antagonist) did not affect the expression of any pup care parameter (Fig. 2C–D and Table 3). Since CRF system activity is generally dampened during lactation (Klampfl and Bosch, 2019a), further reduction of its signalling under undisturbed conditions via the receptor antagonist infusion does not translate into any discernible effects on pup care. However, when CRF-R1 are unavailable for binding due to the presence of the CRF-R1 antagonist, the endogenous CRF could potentially bind to and activate CRF-R2 (Deussing and Chen, 2018). We can exclude this is influencing pup care, as even infusion of a selective CRF-R2 agonist did not alter maternal care (Fig. 2E–F and Table 3). Additionally, we demonstrated that intra-NAcSh acute inhibition of CRF-R1 followed by CRF administration has distinct effects on maternal aggression and pup care after stress compared to CRF administration alone (Sanson et al., 2024b), thus proving that CRF effects in the NAcSh of lactating rats are driven solely by CRF-R1 activation. To comply with the 3-Rs principles, we did not assess the effects of a CRF-R2 antagonist under non-stress conditions, considering that CRF-R2 primarily mediates late adaptive responses to stressors (Dedic et al., 2018) by dampening CRF-R1 activity (Bale et al., 2002), and that CRF signalling is generally reduced during lactation (Klampfl and Bosch, 2019a). Together, these findings suggest that intra-NAcSh activity of CRF-R is suppressed under undisturbed, non-stressed conditions in lactation, as previously described for other brain regions (Klampfl et al., 2013, 2016, 2018). Although we infused a high concentration of CRF to reveal behavioural phenotypes during the postpartum period, dose-dependent effects of CRF on maternal behaviour could be investigated in future studies.

The behaviour of dams infused with OXT-A did not differ from VEH-treated mothers (Fig. 2G–H, Table 3). This indicates that undisturbed OXT-R signalling is not required for maintaining established maternal care, as already discussed for the MPOA and BNST (Bosch et al., 2010). However, activation of the local OXT-R in the NAcSh is necessary for the onset of maternal behaviour in lactating mice (Witchey et al., 2024). Furthermore, disrupted OXT-R signalling within the NAcSh of lactating rats can impair maternal memory consolidation, delaying the re-establishment of maternal behaviour upon exposure to foster pups after 10 days of pup isolation (D'Cunha et al., 2011). Previous studies reported the involvement of the OXT and OXT-R within the NAc in social behaviour and cognition in male mice (Dolen et al., 2013; Dolen and Malenka, 2014), as well as in male and female mandarin voles (*Microtus mandarinus*) (Yu et al., 2016). Together with the present findings in lactating rats, it appears that the OXT-R transmission in the NAc primarily drives affiliative behaviours between conspecifics, while playing a marginal role in pup-directed activities once maternal behaviour is established.

Blocking intra-NAcSh CRF-R1 impaired mothers' motivation to retrieve their pups (Fig. 3). This not only confirms the general involvement of the NAcSh in maternal motivation (Numan et al., 2005; Li and Fleming, 2003b), but, to our knowledge, provides the first evidence for significant involvement of CRF-R1 signalling in pup retrieval behaviour. Interestingly, Lemos et al. demonstrated that acute CRF infusions in the NAc represent an appetitive stimulus for male mice linked to dopamine release (Lemos et al., 2012) while Pecina et al. showed that intra-NAcSh CRF infusions can enhance motivation to seek rewards in male rats (Pecina et al., 2006). Similarly, optogenetic stimulation of CRF+ neurons in the NAc increases reward-related motivation in male and female rats (Baumgartner et al., 2021). Hence, in a novel and potentially challenging environment, a certain amount of NAcSh CRF-R1 signalling seems necessary for the correct interpretation of pups' cues as appetitive and for the consequent initiation of motivated retrieval behaviour. In support, *Crh-r1* mRNA levels in the NAc were increased during lactation compared to a nulliparous state (Fig. 6), and local CRF-R activation induces dopamine release in lactating rats (Sanson et al., 2024b). Thus, the arousal induced by increased CRF-R1 signalling during exposure to this context might be rewarding and facilitate maternal motivation, which differs from the observed effects on maternal care in the home cage, a familiar and non-challenging environment. Furthermore, the NAc sends inhibitory projections to the ventral pallidum (Floresco, 2015; Salgado and Kaplitt, 2015), which, among other reward-related functions (Smith et al., 2009), is thought to translate motivational cues into goal-directed motor outputs (Mogenson et al., 1980; Mogenson and Yang, 1991). Interestingly, optogenetic stimulation of CRF+ neurons in the NAcSh increases Fos expression in regions linked to reward processing, including the ventral pallidum (Baumgartner et al., 2021). Additionally, NAc CRF+ neurons primarily project to the ventral pallidum and CRF release from NAc neurons is necessary for reward learning (Eckenwiler et al., 2024). Thus, it is feasible that reduced CRF-R1 transmission in the NAcSh might affect this downstream pathway, inhibiting the ventral pallidum and impairing the motivated motor response necessary to initiate pup retrieval (Numan et al., 2005). This hypothesis is further supported by the reduced number of transitions observed in the LDB (Fig. 4F), suggesting that acute CRF-R1 antagonist infusion (CP-154,526) reduced the initiation of innate exploratory behaviour, without affecting overall anxiety-related behaviour. However, further studies are needed to address the involvement of the CRF system in this circuitry in the context of maternal motivation. Altogether, the data highlight that finely tuned intra-NAcSh CRF-R1 transmission is essential for the correct display of different maternal behaviours.

In rats, the lactating period is characterized by reduced anxiety-like behaviour (Bosch, 2011; Lonstein, 2007; Neumann, 2001; Pereira et al., 2005). Evidence suggests that the presence of pups can reduce anxiety-like behaviour in the elevated plus maze in both lactating and sensitized, ovariectomized virgin rats (Pereira et al., 2005). In the present study, we identified a reproductive state-independent but sex-specific modulation of anxiety-like behaviour via the two CRF-R subtypes. Specifically, only CRF-R2 activation via UCN3 infusion acted

anxiogenic in female rats, regardless of the reproductive state (Figs. 4 and 5). All virgin female rats were tested in the pro-estrous phase, which is characterized by reduced anxiety-like behaviour (Lovick and Zangrossi, 2021; Zuluaga et al., 2005) allowing for distinct detection of potentially anxiogenic treatment properties. Conversely, in male rats CRF infusion in the NAcSh induced an anxiety-like phenotype confirming previous studies (Chen et al., 2012). To our knowledge, this is the first evidence for sex-specific modulation of an anxiety-like phenotype by CRF-R in the NAcSh. It is well established that the CRF system is sexually dimorphic, modulated by estrogens, and that different intracellular signalling pathways can be activated in male and female rats (Bangasser, 2013; Bangasser and Valentino, 2012; Weathington et al., 2014; Wiersielis et al., 2016), which could explain the differential effects of CRF-R activation on anxiety-like behaviour. To further investigate this aspect, we measured mRNA expression levels of *Crh-r1*, *Crh-r2* and *Crh-bp* in the entire NAc, comparing lactating, virgin female and male rats (Fig. 6). Interestingly, lactating female and male rats showed increased mRNA expression of *Crh-r1* compared to virgin females, while male rats also showed increased *Crh-bp* mRNA levels compared to virgin female rats. Since CRF-BP sequesters CRF and reduces CRF-R signalling (Ketchesin et al., 2017), the increased *Crh-bp* mRNA levels observed in male rats suggests that, under physiological conditions, CRF-R signalling is dampened. In the present experiment with acute, hyper-physiological infusion of CRF, CRF-BP might have been saturated, resulting in activation of CRF-R1 predominantly, which in turn induced anxiety-like behaviour. Additionally, in a novel and challenging environment like the LDB, release of CRF from stress-related brain regions (Itoga et al., 2019) might occur differently between male and female rats, potentially explaining the differential effects of CRF-R activation on anxiety-like behaviour. However further studies are needed to better characterize this aspect.

## 5. Conclusions

Our study provides new insights into the complex brain adaptations during the postpartum period and the impact of perturbations in finely balanced neuropeptide systems on infant neglect. Specifically, we demonstrated that the NAcSh, a reward-related region, is significantly involved in various aspects of maternal behaviour via the CRF system. CRF-R1 transmission plays a crucial role in maternal care and motivation but is distinct from anxiety-like behaviour. Anxiety appears to be controlled by CRF-R in a sex-dependent manner: CRF-R2 activation facilitated anxiety-like behaviour in female rats, independent of their reproductive state, whereas CRF-R1 mediated an anxious phenotype in males. Furthermore, mRNA expression of CRF family members was sexually dimorphic in the entire NAc, with lactating female and male rats showing increased *Crh-r1* mRNA expression compared to virgin females, while *Crh-bp* mRNA levels were elevated in male rats compared to virgin females only. Taken together, CRF-R1 and -R2 transmission in the NAcSh must be finely balanced in the postpartum brain to enable appropriate maternal caretaking and prevent neglect of the young.

## CRediT authorship contribution statement

**Alice Sanson:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Luisa Demarchi:** Writing – review & editing, Investigation. **Emma Rocaboy:** Writing – review & editing, Investigation, Formal analysis. **Oliver J. Bosch:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work submitted.

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## Data availability

Data will be made available on reasonable request to the corresponding author.

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