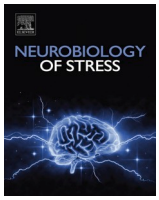
Neurobiology of Stress 30 (2024) 100631

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr



CRF binding protein activity in the hypothalamic paraventricular nucleus is essential for stress adaptations and normal maternal behaviour in lactating rats

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ARTICLE INFO

Keywords:
Corticotropin-releasing factor
CRF binding protein
Maternal behaviour
PVN
Stress

ABSTRACT

To ensure the unrestricted expression of maternal behaviour peripartum, activity of the corticotropin-releasing factor (CRF) system needs to be minimised. CRF binding protein (CRF-BP) might be crucial for this adaptation, as its primary function is to sequester freely available CRF and urocortin1, thereby dampening CRF receptor (CRF-R) signalling. So far, the role of CRF-BP in the maternal brain has barely been studied, and a potential role in curtailing activation of the stress axis is unknown.

We studied gene expression for CRF-BP and both CRF-R within the paraventricular nucleus (PVN) of the hypothalamus. In lactating rats, *Crh-bp* expression in the parvocellular PVN was significantly higher and *Crh-r1* expression in the PVN significantly lower compared to virgin rats. Acute CRF-BP inhibition in the PVN with infusion of CRF(6–33) increased basal plasma corticosterone concentrations under unstressed conditions in dams. Furthermore, while acute intra-PVN infusion of CRF increased corticosterone secretion in virgin rats, it was ineffective in vehicle (VEH)-pre-treated lactating rats, probably due to a buffering effect of CRF-BP. Indeed, pre-treatment with CRF(6–33) reinstated a corticosterone response to CRF in lactating rats, highlighting the critical role of CRF-BP in maintaining attenuated stress reactivity in lactation. To our knowledge, this is the first study linking hypothalamic CRF-BP activity to hypothalamic-pituitary-adrenal axis regulation in lactation. In terms of behaviour, acute CRF-BP inhibition in the PVN under non-stress conditions reduced blanket nursing 60 min and licking/grooming 90 min after infusion compared to VEH-treated rats, while increasing maternal aggression towards an intruder. Lastly, chronic intra-PVN inhibition of CRF-BP strongly reduced maternal aggression, with modest effects on maternal motivation and care.

Taken together, intact activity of the CRF-BP in the PVN during the postpartum period is essential for the dampened responsiveness of the stress axis, as well as for the full expression of appropriate maternal behaviour.

1. Introduction

Stress is a common experience in everyday life. However, prolonged stress exposure can have severe consequences on overall health (Marin et al., 2011; McEwen, 2007), especially if it occurs during sensitive time windows, such as the peripartum period (Dickens and Pawluski, 2018; Hillerer et al., 2012). Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, promoting the physiological adaptations to respond to such a challenge. In this context, corticotropin-releasing factor (protein: CRF; gene: *Crh*), a neuropeptide consisting of 41 amino acids, is the primary driver of the HPA axis in the brain (Deussing and Chen, 2018; Smith and Vale, 2006). CRF is produced by the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus and is released into the hypophyseal portal system following stress, where it stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. In turn, ACTH stimulates the release of glucocorticoids (cortisol in humans and corticosterone in rats) from the adrenal cortex. In addition to its action in stimulating the HPA axis, CRF is released centrally and can act within the brain, thereby controlling complex behavioural responses, such as

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https://doi.org/10.1016/j.ynstr.2024.100631
Received 13 December 2023; Received in revised form 15 March 2024; Accepted 21 March 2024
Available online 27 March 2024

social and stress-induced behaviours (Deussing and Chen, 2018; Hostetler and Ryabinin, 2013).

The CRF system includes two receptor subtypes (CRF-R1 and -R2), four ligands (CRF and urocortins – UCN1, 2, 3), and a regulatory factor, CRF binding protein (CRF-BP). CRF-BP is a 37 kDa glycoprotein, which does not bind to CRF-R (Deussing and Chen, 2018; Ketchesin et al., 2017), but sequesters freely available CRF and UCN1, preventing them from binding to and activating mainly CRF-R1, but also to a 40x lower extent (CRF), CRF-R2. Thus, CRF-BP indirectly reduces CRF-R activation (Ketchesin et al., 2017; Westphal and Seasholtz, 2006) and is considered a crucial regulator of the CRF system, contributing to shutting down or limiting the stress response cascade.

The activity of the CRF system and, consequently, the HPA axis, changes during specific life periods. The transition to motherhood is one such time, characterized by dramatic neuroendocrine changes (Dickens and Pawluski, 2018; Hillerer et al., 2012; Brunton et al., 2008). Indeed, during pregnancy and lactation, there are adaptations in basal and stress-induced ACTH and corticosterone secretion that promote offspring development and protect the developing fetus from the detrimental effects of prolonged stress exposure (Dickens and Pawluski, 2018; Brunton et al., 2008), such as developmental deficits and increased risk for psychopathologies (Creutzberg et al., 2021; Glover, 2015). In lactating rodents, central CRF signalling and HPA axis reactivity are generally dampened to facilitate the necessary brain adaptations for the transition to motherhood and to ensure the full display of maternal behaviour (Dickens and Pawluski, 2018; Brunton et al., 2008). However, these adaptations are brain region- and pregnancy/lactation stage dependent. For example, compared to virgin rats, basal *Crh* expression in the PVN is reduced during pregnancy and early-lactation (Johnstone et al., 2000; Klampfl et al., 2013; Lightman et al., 2001; Walker et al., 2001), but see also (da Costa et al., 2001). After stress exposure, PVN *Crh* mRNA levels are increased only in virgin rats (da Costa et al., 2001), ensuring a dampened response to stress during lactation. In early lactation, basal *Crh* expression can be increased in other regions, such as the bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) when compared to virgin or pregnant rats (Walker et al., 2001; da Costa et al., 2001). However, this does not necessarily reflect higher CRF transmission, but rather suggests a different mechanism that dampens the CRF response during lactation (Klampfl and Bosch, 2019a). Indeed, pharmacological experiments clearly show that hyperactivation of CRF-R, both throughout the brain and locally in the BNST and MPOA, impairs maternal behaviour (Klampfl and Bosch, 2019a, 2019b; Aguilera, 1998; Creutzberg et al., 2020; Klampfl et al., 2016a, 2018), indicating that fine-tuning of CRF-R transmission is an essential prerequisite for the expression of appropriate maternal behaviour.

Furthermore, restraining activity of the CRF system is important since experiencing chronic or severe stress and/or HPA axis dysregulation during the peripartum period can increase the vulnerability to develop perinatal mental illness (Dickens and Pawluski, 2018; Hillerer et al., 2012), which in turn can severely impair the quality of the mother-infant bond and even promote infant neglect (Klampfl and Bosch, 2019a; Sanson and Bosch, 2022).

To date, the role of CRF-BP in the maternal brain, especially in the context of maternal behaviour and the postpartum adaptation of attenuated HPA axis responses, has barely been investigated. In the present study, we hypothesized that CRF-BP in the PVN is necessary for the display of normal mother-pup interactions and also regulates the dampened stress response in lactating rats. We first assessed whether gene expression for *Crh-bp* and both the *Crh-r* within the PVN differs between primiparous and nulliparous rats. We then studied the effects of acute CRF-BP inhibition via intra-PVN infusion of CRF(6–33), a truncated version of CRF capable of selectively binding to and inhibiting CRF-BP with a similar affinity to endogenous CRF and UCN1, but without binding the CRF-R (Ketchesin et al., 2017; Sutton et al., 1995; Grigoriadis et al., 1996). CRF(6–33) displaces endogenous CRF/UCN1 from CRF-BP (Ketchesin et al., 2017), leading to the release of these ligands into the extracellular space, increasing their concentrations within physiological levels, and making them available for binding to CRF-R. We studied the impact of acute CRF-BP inhibition in the PVN on HPA axis (re-)activity. Moreover, we also investigated the effects of either acute or chronic intra-PVN infusion of CRF(6–33) on maternal care under non-stress conditions in the home cage as well as after acute stress exposure (maternal defence test), on maternal motivation in the pup retrieval test, on maternal aggression in the maternal defence test, on maternal emotionality, i.e., anxiety-related behaviour on the elevated plus maze (EPM) and passive stress-coping behaviour in the forced swim test (FST).

2. Materials and methods

2.1. Animals

Female Sprague-Dawley rats (weighing 230g–250g on arrival; Charles River Laboratories, Sulzfeld, Germany) were used for all procedures. Animals were kept under controlled standard laboratory conditions (12:12 h light/dark cycle; lights on at 7:00 a.m.; room temperature 22 ± 2 °C; 55 % relative humidity), with *ad libitum* access to water and standard rat chow (ssniff-Spezialdiäten GmbH, Soest, Germany). To obtain lactating rats, virgin females were mated with sexually experienced male rats in Eurostandard type IV cages (40×60 × 20cm) until pregnancy was confirmed by the presence of sperm in vaginal smears (pregnancy day, PD1). After mating, pregnant females were housed together in groups of 3–4 until PD18, when they were single housed in plexiglass observational cages (38×22 × 35cm) thereafter to ensure undisturbed delivery. On the day of birth (lactation day, LD0), litters were culled to 8 pups of mixed sexes, and half of the bedding was renewed.

Virgin females (weighing 230g–250g on arrival; Charles River Laboratories) used in experiments 1 and 2C were housed in groups of 3–4 in Eurostandard type IV cages until surgical procedures (experiment 2C). Afterwards, they were single housed in observational cages. All rats were handled daily to familiarize them with the experimenters, the procedures and to reduce non-specific stress responses.

Naïve virgin female Sprague-Dawley rats (weighing 200g–220g on arrival; Charles River Laboratories) at random stages of the estrous cycle were used as intruders for the maternal defence test (Bosch, 2013). All intruders were housed in a separate room to avoid olfactory recognition, which might influence the aggressive responses of the lactating residents (Klampfl et al., 2013).

All studies were conducted in accordance with the European regulations of animal experimentation (European Directive, 2010/63/EU) and were approved by the local Government of Unterfranken (Bavaria, Germany). Following the 3Rs principles, all efforts were made to minimize the number of animals used and their suffering.

2.2. Surgical procedures

Surgery was performed for rats used in experiments 2–4 (see section 2.3). All surgery was performed under inhalation anaesthesia (Isoflurane; Baxter Germany GmbH, Unterschleißheim, Germany) and semi-sterile conditions, as previously described (Bosch et al., 2010).

Experiment 2: On LD1 in lactating rats (experiments 2A and 2B), or 5 days prior to the experiment in virgin females (experiment 2C), stereotaxic surgery was performed to bilaterally implant 23G guide cannulae 2 mm above the PVN (−1.4 mm posterior; +1.8mm/−2.1 mm lateral; +6.0 mm ventral to bregma at ±10° angle). In addition, while still under anaesthesia, all rats from experiment 2 were also implanted with a chronic jugular vein catheter as previously described (Neumann et al., 1998) to enable undisturbed repeated blood sampling.

Experiment 3: On PD18, guide cannulae targeting the PVN were implanted bilaterally, as described for experiment 2.

Experiment 4: On LD2, surgery was performed to implant osmotic minipumps (Model 1007D, flowrate 0.5 µL/h; Alzet Osmotic Pumps, Cupertino, CA, USA) subcutaneously in the intra-scapular space. The minipumps were attached to infusion cannulae stereotaxically targeting both the left and right PVN (27G; −1.4 mm posterior, +1.8mm/−2.1 mm lateral, +8.3 mm ventral to bregma at ±10° angle) as described previously (Bosch and Neumann, 2008). The osmotic minipumps were filled with either vehicle (VEH; Ringer's solution, pH = 7.4; B. Braun Melsungen, Melsungen, Germany) or CRF(6–33) (0.05 µg/µL, Bachem, Bubendorf, Switzerland) shortly before surgery and placed in sterile Ringer's solution until implantation. The minipumps delivered the treatment solution into the PVN continuously for a period of 7 days, as certified by the manufacturer. The dose administered was calculated based on previous studies where peptides were chronically infused (Bosch and Neumann, 2008; Slattery and Neumann, 2010; Winter et al., 2021).

2.3. Experimental designs

All pharmacological experiments were performed during the first

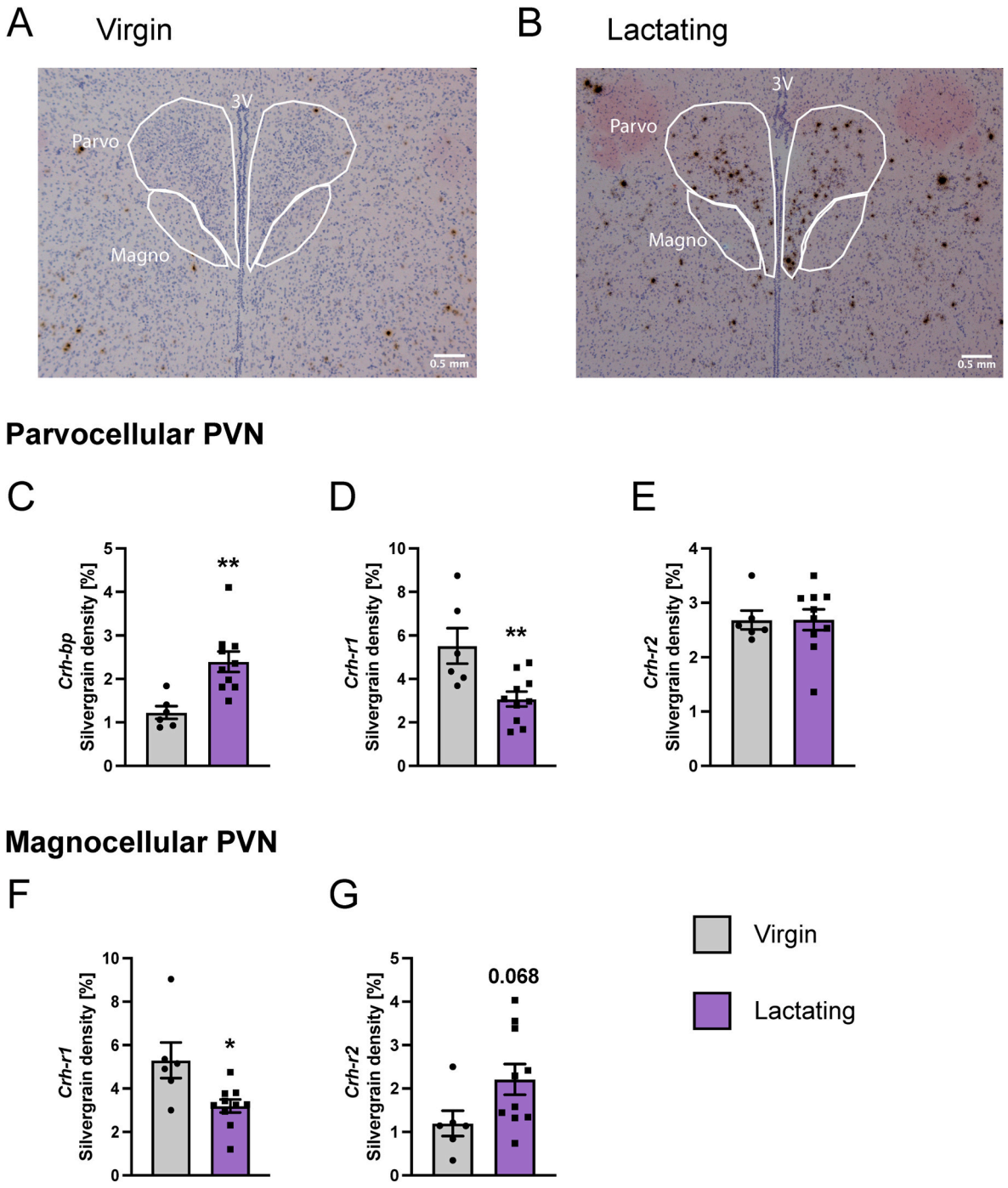

Fig. 1. ***Crh-bp, Crh-r1 and Crh-r2*** **mRNA expression in the PVN**
Representative anatomical localization of *Crh-bp* in (A) virgin and (B) lactating rats. Quantification of (C) *Crh-bp*, (D) *Crh-r1*, (E) *Crh-r2* mRNA expression in the parvocellular PVN, and of (F) *Crh-r1* and (G) *Crh-r2* mRNA in the magnocellular PVN. Virgin n = 6; lactating n = 10; *p ≤ 0.05, **p < 0.01 versus virgin (unpaired t-test or Welch's t-test).

week of lactation between 8:00 a.m. and 3:00 p.m.

2.3.1. Experiment 1: Crh-bp and Crh-r mRNA expression in the PVN

To determine the distribution and expression of *Crh-bp* and *Crh-r* mRNA within the PVN, and to assess changes due to motherhood, brains of lactating (LD9) and virgin female rats were collected. Rats were decapitated following brief exposure to CO_2. Brains were rapidly removed, flash-frozen in n-methylbutane and stored at −80 °C until sectioning. 16 μm coronal sections containing the hypothalamus were cut with a cryostat and mounted on Superfrost® microscope slides and stored frozen, until processing by *in situ* hybridization (ISH).

2.3.1.1. In situ hybridisation. Riboprobe ISH was performed according to an established protocol (Brunton et al., 2005, 2009). To detect *Crh-bp* mRNA, ^{35}S-radiolabelled sense and antisense CRF-BP riboprobes were synthesized from the plasmid rBP550 cloned from rat CRF-BP (Cortright et al., 1997; Speert et al., 2002). To detect *Crh-r1* and *Crh-r2* mRNA, ^{35}S-radiolabelled cRNA probes were used as previously described (Brunton et al., 2009, 2011). Following ISH and exposure to autoradiographic emulsion, the sections were developed (Kodak D-19; Sigma), fixed (Kodak Fixer P6557; Sigma) and counter-stained, as previously described (Brunton et al., 2009, 2011). Photomicrographs of the PVN (×5 magnification) were examined with ImageJ version 1.5i (Schneider et al., 2012). The area of each region of interest and the silver grain area overlying the region were measured and presented as grain density (% area). *Crh-bp* is expressed only in the parvocellular PVN (Henry et al., 2005), therefore it was not measured in the magnocellular division. Moreover, since *Crh-r1* and *Crh-r2* mRNA expression was mostly undetectable in the posterior part of the PVN, the analysis for these two genes focused only on the anterior and medial PVN, whereas all 3 subdivisions of the parvocellular PVN were included for the analysis of *Crh-bp* mRNA expression.

2.3.2. Experiment 2: effects of acute inhibition of CRF-BP in the PVN on HPA axis activity

2.3.2.1. Blood sampling. Blood sampling was performed 5 days after surgery (LD6 in lactating rats). Two hours prior to collection of the first

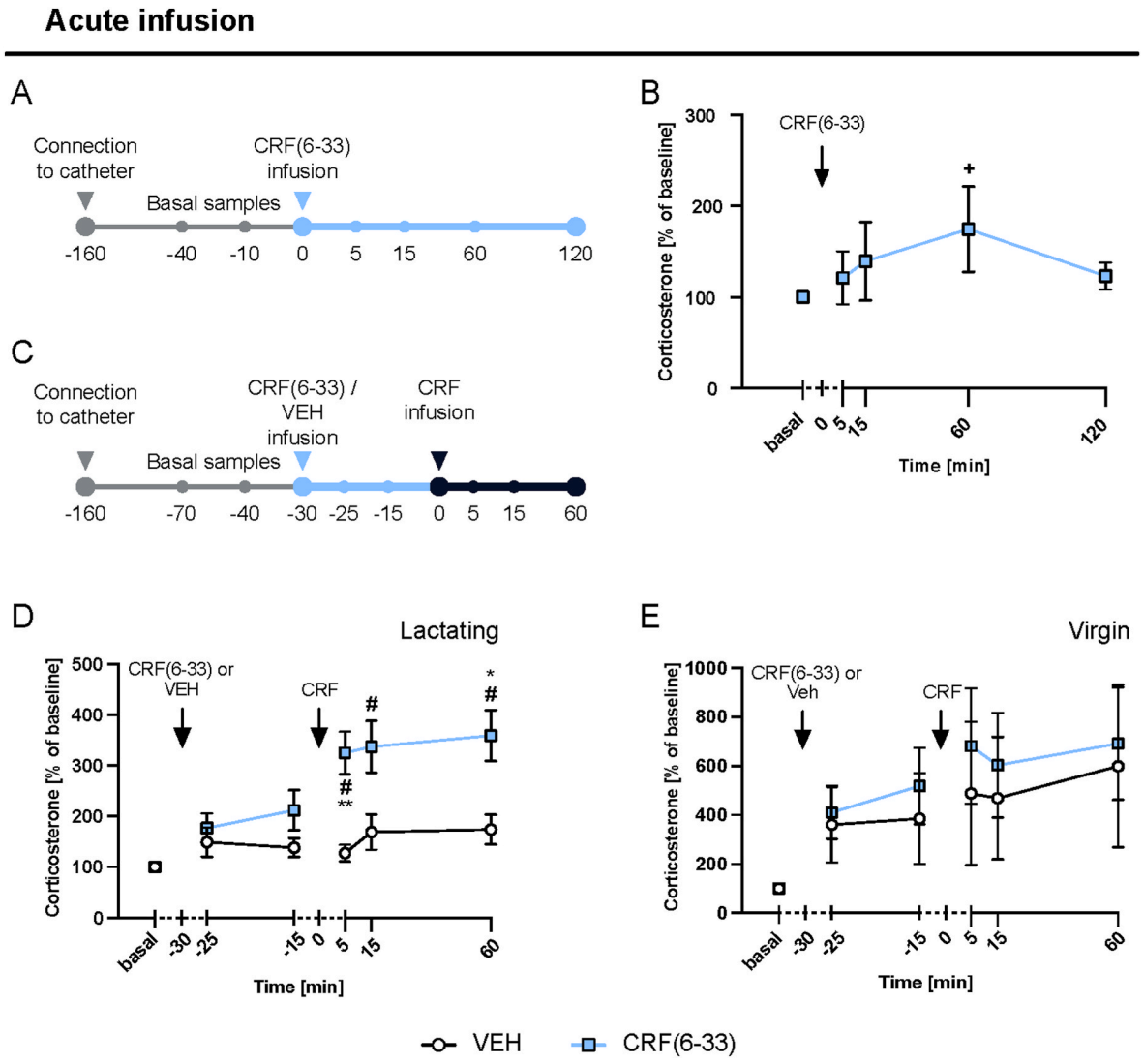

Fig. 2. Corticosterone secretion following acute intra-PVN CRF-BP inhibition alone or in combination with an acute CRF challenge in lactating rats. (A) Experimental timeline and (B) plasma corticosterone secretion following acute intra-PVN CRF(6-33) infusion under basal conditions in lactating rats (LD6; n = 6; $^{+}p \leq 0.05$ versus time point 5 min; Friedman test for repeated measures followed by Dunn's multiple comparisons). (C) Experimental timeline and plasma corticosterone secretion after acute intra-PVN CRF(6-33) infusion followed by an acute CRF challenge in (D) lactating rats (LD6; VEH n = 7; CRF(6-33) n = 9; $^{*}p \leq 0.05$, $^{**}p < 0.01$ versus VEH-treated rats; $^{\#}p \leq 0.05$ versus baseline; 2-way repeated measures ANOVA followed by Bonferroni post hoc comparisons) and (E) virgin rats (VEH n = 4; CRF(6-33) n = 8). Plasma corticosterone concentrations are presented as % change from baseline (set as 100%) and are expressed as mean ± SEM.

blood sample, the catheter was connected to a syringe via tubing filled with sterile heparinized saline. 300 µL of blood was collected for each measurement and transferred into EDTA-coated tubes (Sarstedt AG & Co, Nürnbrecht, Germany), and the volume was replaced by 300 µL of sterile saline. In experiment 2A (Fig. 2A), two basal samples were collected 30 min apart, followed by bilateral intra-PVN infusions of 0.5 µL/per side of the CRF-BP inhibitor (CRF(6–33), 10 µg/µL, Bachem). Further blood samples were collected 5, 15, 60 and 120 min after the infusion.

In two separate experiments (Fig. 2C; experiment 2B: lactating rats; experiment 2C: virgin rats), the collection of two basal samples 30 min apart, was followed by pre-treatment with 0.5 µL/per side of CRF(6–33) into the PVN (see experiment 2A) or an equivalent volume of VEH (Ringer's solution, pH = 7.4). Blood samples were collected 5 and 15 min after the first infusion. Then, all rats received bilateral intra-PVN infusions of 0.5 µL/per side of CRF (2 µg/µL, Tocris Bioscience, Bristol, UK; dose based on (Klampfl et al., 2016b)) and further blood samples were collected 5, 15 and 60 min after the second injection. At the end of the experiments, rats were killed by CO_2 inhalation and brains were collected for histological analysis of cannula placement (see 2.5).

All blood samples were centrifuged at 2823g (5000 rpm) at 4 °C for 15 min. Plasma was collected and stored at −20 °C until further processing. Plasma corticosterone concentrations were measured using a commercially available ELISA kit according to the manufacturers

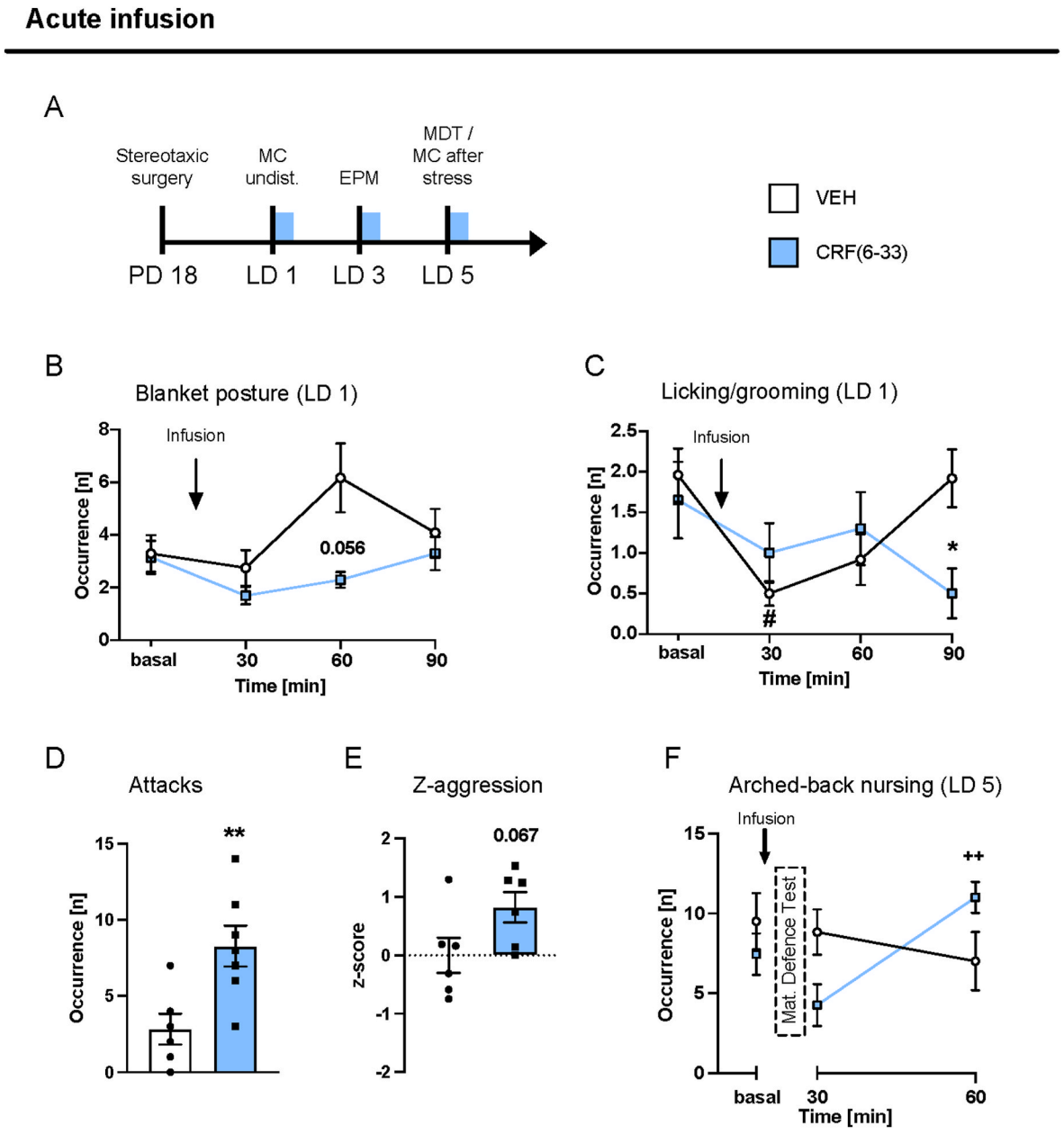

Fig. 3. Altered maternal behaviour following acute CRF-BP inhibition within the PVN

(A) Experimental timeline to assess the effects of acute CRF(6-33) infusions on aspects of maternal behaviour. (B) Blanket nursing posture and (C) licking/grooming under non-stress conditions on lactating day (LD) 1 (VEH n = 12; CRF(6-33) n = 10; $^{\#}p \leq 0.05$ versus basal values; $^{*}p \leq 0.05$ versus VEH-treated rats; 2-way repeated measures ANOVA followed by Bonferroni post hoc comparisons). (D) Number of attacks and (E) overall Z-aggression during the maternal defence test (MDT) on LD5 (VEH n = 6; CRF(6-33) n = 7; $^{**}p < 0.01$; Unpaired t-test). (F) Arched-back nursing following the MDT (VEH n = 6; CRF(6-33) n = 8; $^{++}p < 0.01$ versus time point 30 min; 2-way repeated measures ANOVA followed by Bonferroni post hoc comparisons). Data are presented as mean ± SEM. Other abbreviations: EPM, elevated plus maze test; MC, maternal care.

protocol (IBL international GmbH, Hamburg, Germany).

2.3.3. Experiment 3: behavioural effects of acute CRF-BP inhibition in the PVN

2.3.3.1. Behavioural assessment. On each day of behavioural testing, experimental rats received acute, bilateral infusions of 0.5 μL/per side of VEH (Ringer's solution, pH = 7.4) or of the CRF-BP inhibitor CRF(6–33) (10 μg/μL; Bachem; dose based on (Klampfl et al., 2016b)). Each rat received the same treatment on each of the different testing days. After drug infusions, dams were returned to their home cage and their behaviour was monitored after a lag-time of 20 min (Klampfl et al., 2016b; Zorrilla et al., 2001). As summarized in Fig. 3A, on LD1, maternal care (section 2.4.1) under non-stress conditions was monitored before and after drug infusion for a total of 90 min. On LD3, anxiety-like behaviour was assessed on the EPM (section 2.4.3). On LD5, care was

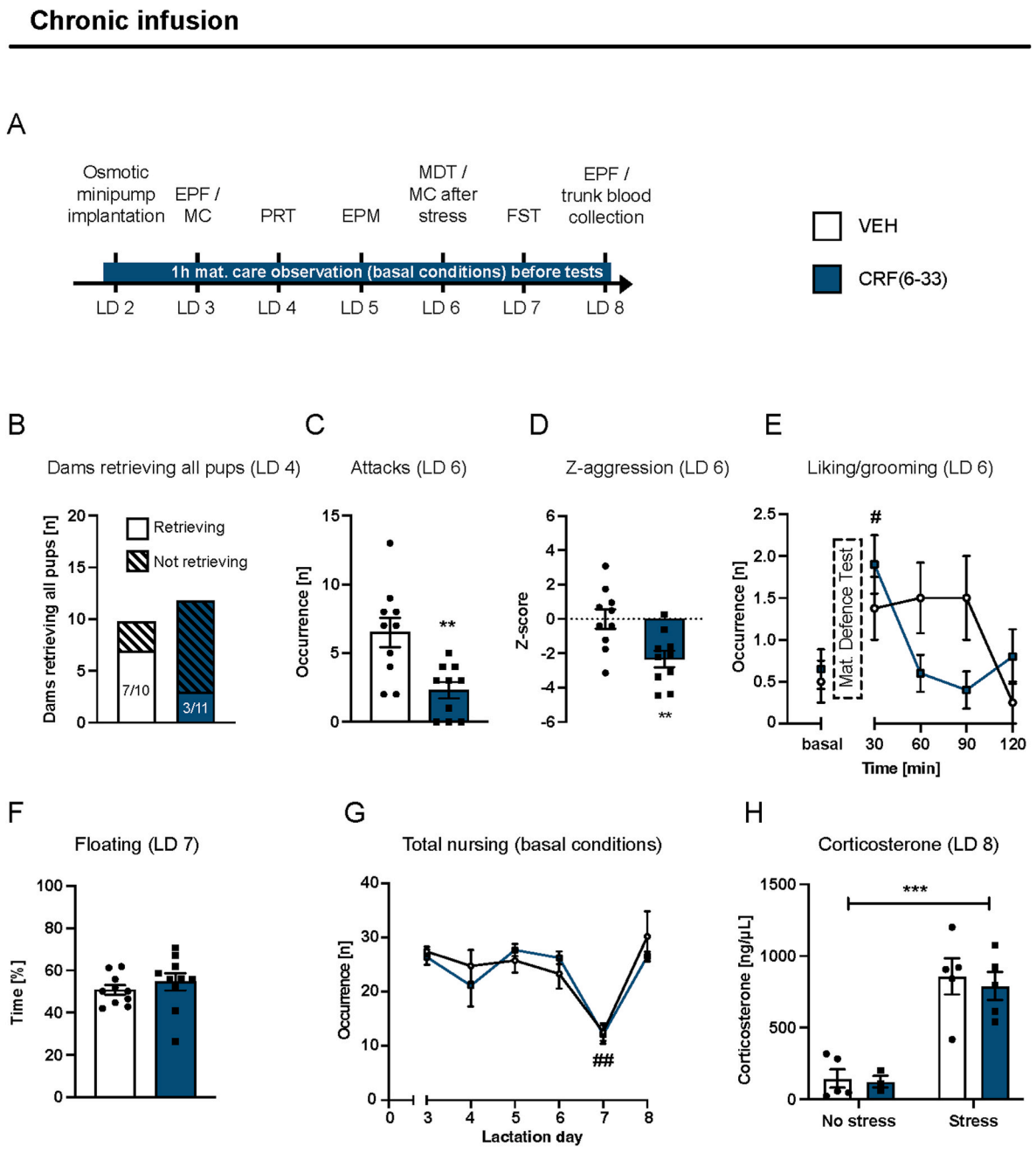

Fig. 4. Effects of chronic intra-PVN CRF-BP inhibition during lactation

(A) Experimental timeline to assess the effects of chronic intra-PVN CRF(6-33) infusions on aspects of maternal behaviour; (B) percentage of dams retrieving all pups in the pup retrieval test (PRT) on lactation day (LD) 4 (VEH: n = 10; CRF(6-33): n = 11); (C) number of attacks and (D) overall Z-aggression during the maternal defence test (MDT) on LD6 (VEH: n = 10; CRF (6-33): n = 10; $^{**}p < 0.01$; unpaired t-test); (E) licking/grooming behaviour following the MDT (VEH: n = 8; CRF(6-33): n = 10; $^{\#}p \leq 0.05$ versus respective basal; 2-way repeated measures ANOVA followed by Bonferroni post hoc comparisons); (F) percentage of time spent on floating in the forced swim test (FST) on LD7 (VEH: n = 10; CRF-BP(6-33): n = 10); (G) total nursing under basal conditions over LD3-8 (VEH: n = 9; CRF(6-33): n = 7; $^{\#\#}p < 0.01$ versus LD3 within same group; 2-way repeated measures ANOVA followed by Bonferroni post hoc comparisons); (H) plasma corticosterone concentrations under non-stress or acute stress conditions (5-min exposure to the elevated platform, EPF); VEH/no stress: n = 5; VEH/stress: n = 5; CRF(6-33)/no stress: n = 3; CRF(6-33)/stress: n = 5; $^{***}p < 0.001$ main effect of stress; 2-way ANOVA followed by Bonferroni post hoc comparisons). Data are presented as mean ± SEM. Other abbreviations: MC, maternal care.

monitored for 60 min before and after the maternal defence test (section 2.4.4), which was used to assess maternal aggression. Immediately after the end of the last test, rats were killed by CO_2 inhalation, and brains were collected for histological analysis of cannula placement (see 2.5).

2.3.4. Experiment 4: effects of chronic CRF-BP inhibition in the PVN in lactating rats

2.3.4.1. Behavioural assessment. Either VEH (Ringer's solution, pH = 7.4) or CRF(6–33) was continuously infused into the PVN for 7 days from LD2. As shown in Fig. 4A, from LD3 to LD8, undisturbed maternal care (section 2.4.1) was monitored every morning for 60 min prior to conducting the other behavioural tests. On LD3, rats were exposed to the elevated platform (a mild emotional stressor (Neumann et al., 2000)) for 5 min to acutely stimulate the HPA axis, followed by another maternal care observation period for 180 min (between 9:00 a.m.–12:00 p.m.) and additionally at 4:00 p.m. for 60 min. On LD4, maternal motivation was assessed using the pup retrieval test (section 2.4.2). On LD5, anxiety-like behaviour was monitored on the EPM; section 2.4.3). On LD6, the maternal defence test (section 2.4.4) was performed, and care observed over the following 120 min. On LD7, stress-coping behaviour was monitored using the FST (section 2.4.5). Lastly, on LD8, dams were killed by conscious decapitation under non-stress conditions (home cage) or 10 min after a 5 min exposure to the elevated platform. Trunk blood was collected and processed as described in 2.3.2. for determination of plasma corticosterone concentrations, while brains were collected for histological analysis of cannula placement (see 2.5).

2.4. Behavioural tests

2.4.1. Maternal care

Maternal care was monitored before and after drug infusion with or without exposure to the maternal defence test (for observation durations, see section 2.3) following an established protocol (Bosch and Neumann, 2008). Briefly, observations were made for 10s, every 2nd minute in 30-min blocks (i.e., 15 behavioral observations in each 30 min block). We quantified licking and grooming of pups and arched-back nursing, which are an index of the quality of maternal care (Klampfl and Bosch, 2019b; Bosch, 2011). The frequency of blanket and other nursing postures was recorded as a measure for the quantity of care (together with arched-back nursing, these nursing postures accounted for total nursing). Moreover, the time the mother was not in contact with her pups was also scored. The data presented are the total number of times a specific behaviour occurred in each 30 min block.

2.4.2. Pup retrieval test

Maternal motivation was assessed using the modified pup retrieval test as previously described (Bayerl et al., 2016). The day before the test, mothers were habituated to a red Perspex house (13 × x17 × 11cm, opening 6 × 8.5 cm) for use as a dimly lit potential nesting site for 150 min (Bayerl et al., 2016). On the testing day, pups were separated from the mother for 60 min and the red house was re-introduced into the mother's cage. Then, both the mother and the house were placed in a plastic box (54 × 34 × 60cm; novel environment) that contained a handful of bedding and the pups, distributed across the floor (Bayerl et al., 2016). The behaviour was recorded for 15 min and the percentage of pups retrieved by each mother, as well as the percentage of mothers retrieving all the pups was quantified.

2.4.3. Anxiety-related behaviour

Anxiety-related behaviour was assessed on the EPM as previously described (Klampfl et al., 2018). Briefly, 20 min after drug infusion, the rats were placed in the central zone facing one of the closed arms and were free to explore the EPM for 5 min while being video-recorded. The videos were analysed by an experienced observer blind to the treatments, and the following parameters were scored: time spent in the open and closed arms and the number of entries in each arm. A full open arm entry was scored when the rat was crossing the open arms with the whole body (all four paws), excluding the tail; an arm entry was scored when the rat was entering only with the front paws and shoulders. The percentage of time spent on the open versus all arms and the number of full open arm entries were parameters for anxiety-related behaviour, while the number of closed arm entries was an index of locomotor activity (Klampfl et al., 2018).

2.4.4. Maternal defence test

To assess maternal aggression in the lactating dams, the maternal defence test was performed in the presence of the litter using a modified resident-intruder test. The lactating dam was the "resident" and an unfamiliar virgin female rat served as the "intruder", as previously described (Klampfl et al., 2018). Since the maternal defence test represents a psychosocial stressor for the mother (Bosch, 2013), it also facilitates monitoring potential changes in maternal care after stress exposure. The dams were moved and habituated to the experimental room 60 min before the testing began. 20 min after drug infusion, the intruder was placed into the mother's home cage and the test was video-recorded for 10 min. The dam's behaviour was analysed by an experienced observer blind to the treatment using JWatcher (http://www.jwatcher.ucla.edu). The following parameters were analysed: number of attacks, pinning down (mother holding down the intruder with her front paws, also termed "keep down"), aggressive grooming (excessive grooming or dam nose-to-intruder fur sniffing, mostly directed to the neck region), lateral threat (mother approaching the intruder from the side to force the intruder aside), offensive upright (mother standing in upright posture against the intruder), aggressive sniffing (excessive sniffing mostly focused on the anogenital region) (Bosch, 2013) and non-aggressive behaviours. To define an aggression score and enhance behavioural phenotyping (Begni et al., 2020; Guilloux et al., 2011), Z-score normalization was performed (see 2.6).

2.4.5. Forced swim test

The FST was performed as previously described to assess passive stress-coping behaviour (Ebner et al., 2005). Briefly, 20 min after drug infusion dams were placed in a cylindrical tank (50 cm height; 30 cm diameter) filled with tap water (23 ± 1 °C) for 10 min. The tanks were filled to a depth that rats could not escape nor touch the bottom with their hind paws or tail. Trials were recorded for later analysis by an experienced observer blind to the treatment using JWatcher. The total time spent floating was analysed as an index of passive stress-coping behaviour, indicative of a trait of depressive-like phenotype (Slattery and Cryan, 2012).

2.5. Histology

For histological verification of correct cannula placements, brains were collected, flash-frozen in n-methylbutane, cut into 40 μm coronal sections with a cryostat, mounted on slides and Nissl stained. Stained brain sections were viewed under a light microscope and only animals with correctly implanted cannulas were included in the statistical analysis.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software) and SPSS Statistics Version 26 (IBM Corp.). Normality of the data was tested with Shapiro-Wilk test and homogeneity of variance with F-test. Where normality was violated, non-parametric tests were used; if homogeneity was violated, we used the corresponding corrections. Statistical outliers were calculated based on the double standard deviation method and were removed from analysis. Due to health problems, some animals had to be removed from the

experiment and the analysis. Data from the ISH, maternal defence test, EPM and FST studies were analysed using an unpaired *t*-test, non-parametric Mann-Whitney test, or unpaired *t*-test with Welch's correction. The occurrence of maternal behaviours over time or days was analysed using 2-way repeated measures ANOVA (factors: time x treatment). The percentage of mothers retrieving all pups was analysed with Fisher exact test. Single plasma corticosterone samples obtained after exposure to the elevated platform were analysed using a 2-way ANOVA (factors: stress x treatment). Corticosterone secretion patterns, calculated as percentage change from baseline, were analysed with paired Friedman test for repeated measures in experiment 2A and 2-way repeated measures ANOVA in experiments 2B–C. Where appropriate, Bonferroni post hoc comparisons were performed. Maternal aggression data were normalized using the Z-score method as previously described (Guilloux et al., 2011). In detail, Z-score for maternal aggression was calculated as follows (x: individual value; μ: mean of control group; σ: standard deviation of control group):

$$Z-aggression=\frac{\left(\frac{x-\mu}{\sigma}\right)attack+\left(\frac{x-\mu}{\sigma}\right)pinning\ down+\left(\frac{x-\mu}{\sigma}\right)aggr.\ grooming+\left(\frac{x-\mu}{\sigma}\right)lateral\ threat+\left(\frac{x-\mu}{\sigma}\right)off.\ upright+\left(\frac{x-\mu}{\sigma}\right)aggr.\ sniffing}{number\ of\ parameters}$$

In each case, p values of ≤0.05 were considered significant and a trend was accepted for $p < 0.08$.

3. Results

3.1. Crh-bp and Crh-r mRNA expression in the PVN is altered in lactating rats

The anatomical localization of *Crh-bp* in virgin (Fig. 1A) and lactating rats (Fig. 1B) indicates that *Crh-bp* is mostly localized in the parvocellular PVN. Expression of *Crh-bp* mRNA was significantly higher (unpaired *t*-test; $t(14) = 3.585$; $p = 0.003$; Fig. 1C), and *Crh-r1* was significantly lower ($t(14) = 3.187$; $p = 0.007$; Fig. 1D) in the parvocellular PVN of lactating rats compared to virgin rats. No differences were observed between the groups for *Crh-r2* mRNA expression (Fig. 1E).

In the magnocellular PVN, lactating rats showed significantly lower expression of *Crh-r1* mRNA ($t(6.3) = 2.412$, $p = 0.050$; Welch's *t*-test; Fig. 1F), while *Crh-r2* levels tended to be higher ($t(14) = 1.975$, $p = 0.068$; Fig. 1G) in lactating versus virgin rats. *Crh-bp* mRNA was not detectable in this region.

3.2. Acute inhibition of CRF-BP in the PVN activates the HPA axis

Next, we assessed a role for CRF-BP within the PVN in regulating the HPA axis in lactation. We first measured the effects of acute inhibition of CRF-BP in the PVN (using intra-PVN CRF(6–33) infusion) on corticosterone secretion under non-stress conditions in lactating rats (Fig. 2A and B and Table 1). Friedman test for repeated measures revealed a significant difference over time ($p = 0.015$). Specifically, plasma corticosterone concentrations were significantly increased 60 min compared to 5 min after infusion ($p < 0.05$, Dunn's multiple comparisons), suggesting that local CRF-BP inhibition increases basal activity of the HPA axis, presumably by increasing endogenous CRF availability.

To further assess a role for CRF-BP in stimulated HPA axis activity in lactation, we pre-treated lactating rats with either CRF(6–33) or VEH followed by acute intra-PVN CRF infusion and measured changes in corticosterone secretion (Fig. 2C and D and Table 2). 2-way repeated measures ANOVA revealed a significant interaction between time × treatment ($F[5, 70] = 6.28$, $p < 0.0001$). Interestingly, lactating rats pre-treated with the CRF-BP inhibitor showed significantly higher plasma corticosterone concentrations both 5 min ($p < 0.01$) and 60 min ($p < 0.05$) after the CRF challenge compared to dams pre-treated with VEH. Indeed, the corticosterone response to the CRF challenge was 3.6-fold greater than baseline in the CRF-BP inhibitor-treated rats, but only 1.7-fold greater in the VEH-treated rats, at the 60 min time-point. Moreover, in the CRF(6–33) pre-treated group, all measurements after CRF infusion were significantly higher compared to baseline levels ($p < 0.05$), while there was no significant effect on corticosterone secretion in response to CRF stimulation in the VEH-pre-treated lactating rats. When the same experiment was repeated in virgin rats (Fig. 2E), there was a significant main effect of time ($F[1, 11] = 6.107$; $p = 0.029$; 2-way repeated measures ANOVA), but post hoc comparisons did not reveal any significant differences between the groups, suggesting that PVN CRF-BP does not play a substantive role in regulating HPA axis activity in virgin rats. The VEH- and CRF(6–33)-treated virgin rats showed a 6.0- and 6.9-fold increase at the 60 min time-point compared to baseline, respectively, indicating a similar response regardless of treatment, but also indicating a greater corticosterone response to CRF in virgin compared to lactating rats.

3.3. Acute inhibition of CRF-BP in the PVN alters maternal behaviour

Acute inhibition of CRF-BP had a mild effect on maternal care under undisturbed conditions. In detail, a 2-way repeated measures ANOVA revealed a trend towards significance for time × treatment interaction (F

Table 1
Absolute plasma corticosterone concentrations in lactating rats treated with CRF (6–33)
CRF(6–33) was administered at t = 0 min. Data are expressed as group mean ± SEM.

Time [min]	Basal	0	5	15	60	120
Corticosterone [ng/mL]	695 ± 121	**CRF(6–33) INFUSION**	664 ± 49	714 ± 35	944 ± 71	781 ± 98

Table 2
Absolute plasma corticosterone concentrations in virgin and lactating rats pre-treated with VEH or CRF(6–33) and acutely infused with CRF into the PVN.
VEH or CRF(6–33) was administered at t = −30 min. All rats were administered CRF into the PVN at t = 0 min. Data are expressed as group means ± SEM (#p ≤ 0.05, ##p < 0.01 vs respective basal; 2-way repeated measures ANOVA followed by Bonferroni post hoc comparisons).

Corticosterone [ng/mL]				
Time [min]	Virgin		Lactating	
	VEH	(CRF6-33)	VEH	CRF(6–33)
Basal	548 ± 237	415 ± 168	685 ± 184	263 ± 63
−30	**CRF(6–33) or VEH INFUSION**			
−25	1002 ± 93	890 ± 58#	796 ± 114	385 ± 47
−15	999 ± 92	1075 ± 61##	755 ± 114	432 ± 55
0	**CRF INFUSION**			
5	1187 ± 249	1265 ± 106#	718 ± 112	684 ± 44##
15	1198 ± 204	1127 ± 115#	834 ± 82	704 ± 51##
60	1482 ± 245#	1294 ± 124#	928 ± 132	766 ± 67##

[3, 60] = 2.7; p = 0.053; Fig. 3B) for blanket nursing. A Bonferroni multiple comparisons test revealed a trend towards reduced blanket nursing 60 min after CRF(6–33) infusion compared to VEH-treated rats (p = 0.056). Analysis of licking/grooming behaviour revealed a significant effect of time × treatment interaction (F[3,60] = 3.24; p = 0.028; Fig. 3C). Indeed, licking/grooming was significantly reduced in VEH-treated rats 30 min after the infusion compared to their basal level (p < 0.05), but was restored to pre-infusion levels within 90 min. Whereas, in CRF(6–33)-treated dams, licking/grooming was significantly lower 90 min after the infusion compared to VEH-treated rats (p < 0.05; Fig. 3C). No significant differences in the other parameters of maternal care measured on LD1 were detected (Supplementary Table 1).

With respect to anxiety-like behaviour, no treatment effects were observed (% of time spent on the open arms: VEH: 21.2 ± 6.2%; CRF (6–33): 22.3 ± 4.2%; % open arm/total arm entries: VEH: 31.9 ± 17.0%; CRF(6–33): 40.7 ± 10.8%). Moreover, no differences were found for locomotor activity (number of closed arm entries: VEH: 14.3 ± 4.8; CRF(6–33): 14.4 ± 4.1).

In the maternal defence test, dams infused with CRF(6–33) displayed increased maternal aggression compared to VEH-treated rats (Fig. 3D and E). Indeed, CRF(6–33)-treated dams showed more attacks (t(11) = 3.153; p = 0.009; Fig. 3D), and the overall Z-aggression score tended to be increased (t(10) = 2.056; p = 0.067; Fig. 3E). Regarding post-defence test behaviour, analysis of arched-back nursing revealed a significant time × treatment interaction (F[2, 24] = 5.71; p = 0.009, Fig. 3F). Indeed, CRF(6–33)-treated dams showed a significant increase in arched-back nursing at 60 min compared to 30 min after the maternal defence test (p < 0.01; Bonferroni post hoc comparison), reaching pre-test levels. There were no significant differences in the other parameters of maternal care measured after the maternal defence test on LD5 (Supplementary Table 2).

3.4. Chronic intra-PVN CRF-BP inhibition impairs maternal aggression

To further characterize the role of the CRF-BP in the postpartum period, we assessed the effects of chronic CRF-BP inhibition on maternal behaviour (Fig. 4A).

As reported in Supplementary Table 3, there were no major effects of continuous CRF(6–33) infusion on any parameter of maternal care after acute stress (elevated platform) exposure on LD3. Indeed, over time, we detected similar behavioural fluctuations (in arched-back nursing, total nursing, time spent on pups) between groups.

In the pup retrieval test on LD4 (Fig. 4B), only 3 out of 11 CRF(6–33)-treated dams retrieved all their pups, compared with 7 out of 10 of the VEH-treated dams; however, this effect was not significant (Fisher's exact test; p = 0.086). Furthermore, the % of pups retrieved over time was not different between groups (data not shown).

Chronic CRF-BP inhibition strongly impaired maternal aggression on LD6. In detail, the number of attacks (Fig. 4C) as well as the overall Z-aggression score (Fig. 4D) were both significantly lower compared to VEH-treated rats (unpaired *t*-test; attacks: t(18) = 3.43; p = 0.003; Z-aggression: t(18) = 3.13; p = 0.006). Furthermore, after termination of the maternal defence test, the occurrence of licking/grooming was altered, with a significant interaction of time × treatment (F[4, 64] = 3.2; p = 0.018; 2-way repeated measures ANOVA, Fig. 4E). Bonferroni post hoc comparison revealed a significant increase in licking/grooming at 30 min compared to basal levels in rats treated with CRF(6–33), although there was no significant difference between the treatment groups. No other changes in maternal behaviour were observed after stress (see Supplementary Table 4).

Chronic intra-PVN CRF-BP inhibition did not affect anxiety-related behaviour (% time spent in open arm: VEH: 24.45 ± 4.3%; CRF (6–33): 20.70 ± 5.8%; % open arm/total arm entries: VEH: 33.1 ± 3.9%; CRF(6–33): 26.3 ± 6.0%), nor locomotor activity (number of closed arm entries: VEH: 13.6 ± 1.8; CRF(6–33): 12.6 ± 0.9). Neither was passive-stress coping behaviour affected, as measured by the % time spent floating (Fig. 4F).

To further assess the potentially long-lasting effects of chronic CRF-BP inhibition, we scored maternal care daily under undisturbed conditions in the 60 min period prior to behavioural testing (Fig. 4G). A 2-way repeated measures ANOVA revealed a significant main effect of time (F [3, 40] = 12.31; p < 0.0001), but not of treatment, nor any significant interaction. On LD7, total nursing dropped similarly in both groups and was significantly lower compared to LD3 (p < 0.01; Bonferroni multiple comparisons; for further comparisons see Supplementary Table 5).

Lastly, on LD8 we measured plasma corticosterone concentrations in trunk blood with or without acute exposure to the elevated platform (Fig. 4H). Statistical analysis showed a main effect of stress (2-way ANOVA; F[1, 18] = 51.1; p < 0.0001), but not of CRF(6–33) treatment. Indeed, both stressed groups had significantly higher plasma corticosterone concentrations, independent of treatment, when compared to their non-stressed counterparts (p < 0.001).

4. Discussion

In the present study, we identified CRF-BP activity in the PVN of lactating rats as a critical regulator of the HPA axis in lactation. Specifically, we found increased *Crh-bp* expression in the parvocellular PVN and decreased *Crh-r1* expression in both the parvo- and magnocellular PVN of lactating rats, compared with virgin rats. Acute CRF-BP inhibition within the PVN reinstated stimulated HPA axis activity that is usually inhibited during lactation (Dickens and Pawluski, 2018; Brunton et al., 2008). Furthermore, both acute and chronic intra-PVN CRF-BP inhibition altered aspects of maternal care and aggression.

Stress exposure during the sensitive peripartum period can have detrimental consequences for maternal health since it can predispose the mother to perinatal mental illness, as well as impair bonding with the newborn (Dickens and Pawluski, 2018; Hillerer et al., 2012). Thus, complex adaptations need to occur during pregnancy and persist through lactation in order to ensure dampened reactivity of the maternal CRF system and maintain reduced stress responses (Brunton et al., 2008; Slattery and Neumann, 2008). Despite increased basal *Crh* mRNA levels observed in early-lactating rats compared to virgins in some regions (e.g. the BNST and MPOA) (Walker et al., 2001; da Costa et al., 2001), research has demonstrated that local activation of CRF-R transmission impairs maternal behaviour (Klampfl and Bosch, 2019a, 2019b; Creutzberg et al., 2020; Klampfl et al., 2014, 2016a, 2018). Thus, it is known that dysregulation of the central CRF system can have adverse consequences for mother-offspring bonding, often leading to maternal neglect, as shown in animal models (Klampfl and Bosch, 2019a; Pedersen et al., 1991; D'Anna et al., 2005; Gammie et al., 2004). In humans, neglect is the most prevalent form of child maltreatment, accounting for 78% of cases where children report experiencing maltreatment (Brown et al., 2023). Despite this alarming incidence, the neurobiological basis of neglect is still not fully understood, and potential biomarkers to assess the risk of neglecting behaviours are still not available. As reactivity of the CRF system needs to be dampened in the peripartum period, a crucial - and potential regulatory - factor of this system is the CRF-BP, which sequesters freely available CRF and UCN1, thereby decreasing signalling through CRF-R (Ketchesin et al., 2017; Westphal and Seasholtz, 2006). However, the precise role of CRF-BP during lactation on mediating HPA axis adaptations and for the display of proper maternal behaviour has not been investigated, until now.

Interestingly, we found that, during lactation, expression of *Crh-bp* in the PVN is up-regulated, while *Crh-r1* expression is downregulated (Fig. 1C, D, F), which might be necessary for the display of proper maternal responses towards the newborns. Indeed, reduced expression of *Crh-r1* in the PVN might represent an adaptation at the apex of the HPA axis that contributes to reduced stress sensitivity in the postpartum period (Brunton et al., 2008; Slattery and Neumann, 2008). Consistent with this hypothesis, reduced CRF-R1 protein expression in the PVN was recently found in lactating mice when compared to virgin mice (De

Guzman et al., 2021, 2023), measured on LD1, LD7 and LD14 (De Guzman et al., 2023). However, an early study reported that *Crh-r1* mRNA levels were not different in early-lactating rats (LD3) compared to virgin rats (da Costa et al., 2001). This suggests complex adaptations across the different lactation stages, as well as species-specific modulations, as previously reported between mice and rats with respect to the HPA axis and stress responsiveness postpartum (Douglas et al., 2003). Furthermore, when CRF levels in the PVN increase, for instance after stress exposure (Deussing and Chen, 2018; Vandael and Gounko, 2019), the CRF system of a lactating animal needs to be kept in check to prevent an overactivated stress system and minimize the associated negative consequences on maternal physiology and behaviour (Klampfl and Bosch, 2019a). Hence, we speculate that the significant increase in *Crh-bp* mRNA expression in the parvocellular PVN during lactation represents an essential protective mechanism to minimize CRF-R signalling by sequestering any freely available CRF and UCN1. While we acknowledge that mRNA levels do not necessarily correlate with protein expression (Greenbaum et al., 2003; Liu et al., 2016), this is an interesting concept that warrants further research.

To further investigate a role for CRF-BP in facilitating reduced HPA axis responses in lactation, we acutely inhibited CRF-BP in the PVN and analysed plasma corticosterone concentrations. Interestingly, when studied under non-stress conditions, acute inhibition of CRF-BP induced a transient increase in corticosterone secretion 60 min after the infusion (Fig. 2B). This peak observed at 60 min may be due to the overall kinetics of our manipulation. CRF(6–33) first binds to CRF-BP, displacing endogenous CRF, which in turn, then binds to its receptors to activate the HPA axis cascade. We have previously shown that intracerebral bilateral infusions to lactating rats induces only a transient increase in corticosterone secretion 10 min after the infusion, with levels returning to baseline within 30 min of the infusion (Klampfl et al., 2016a). Therefore, it is unlikely that the increase in corticosterone observed at 60 min is due to the handling and injection procedures. Our finding is in line with the inhibitory function that CRF-BP has on CRF-induced ACTH secretion from the anterior pituitary gland *in vitro* (Ketchesin et al., 2017; Sutton et al., 1995; Behan et al., 1995; Cortright et al., 1995), and, consequently, corticosterone secretion. Hence, these data further support the role of CRF-BP in the PVN in modulating CRF availability in general (Ketchesin et al., 2017), as well as this novel role in the lactating brain with respect to HPA axis regulation and maternal behaviour (Klampfl and Bosch, 2019a), as discussed below. It should be noted that CRF-BP can undergo spontaneous cleavage, producing a 27 kDa and a 10 kDa fragment (Woods et al., 1999). The larger fragment can sequester freely available CRF, while the smaller fragment is thought to potentiate CRF-R2α signalling (Behan et al., 1995; Vasconcelos et al., 2019), suggesting that CRF-BP actively modulates CRF signalling in a complex manner (Chan et al., 2000). Moreover, CRF-BP may interact with CRF-R2, increasing its presence on the cell surface of certain neuronal populations (Curley et al., 2021; Haass-Koffler and Bartlett, 2012; Slater et al., 2016). Such different mechanisms of action depend on the cell type, signalling pathway, or brain region (Ketchesin et al., 2017). Thus, we cannot exclude that CRF(6–33) - in addition to releasing bound CRF from CRF-BP - might indirectly activate both CRF-R within the PVN, or on the contrary reduce the membrane expression of CRF-R2, thus dampening the transmission mediated by this specific subtype.

Next, we investigated the buffering capacity of CRF-BP within the PVN when the HPA axis is stimulated by an acute CRF challenge, mimicking an acute exposure to stress (Aguilera, 1998). In contrast to the virgin rats, CRF infusion failed to increase corticosterone secretion in the VEH-pre-treated lactating rats. This is probably a result of increased *Crh-bp* and reduced *Crh-r1* mRNA expression in the PVN in lactation (Fig. 1) and provides further support for "buffered" HPA axis responses to stress or CRF stimulation in lactation (Slattery and Neumann, 2008). Importantly, corticosterone responses to CRF stimulation were restored in lactating rats when they were pre-treated with the CRF-BP inhibitor (Fig. 2D), providing the first evidence that CRF-BP acts as regulatory factor within the PVN, attenuating CRF system transmission whenever CRF levels strongly increase, and hence minimising HPA axis activity. However, a significant portion of increased corticosterone secretion might be due to the initial pre-treatment with CRF(6–33), as also seen in experiment 2A at 60 min post-injection (Fig. 2B). Interestingly, CRF-BP inhibition did not impact the corticosterone response to CRF stimulation in virgin rats (Fig. 2E), probably due to the low *Crh-bp* mRNA levels observed in experiment 1 (Fig. 1C). This suggests that the regulatory role of CRF-BP only arises when dampened activity of the HPA axis is essential, for example, during lactation, but possibly also during pregnancy (Brunton et al., 2008; Slattery and Neumann, 2008), though this needs to be further investigated. However, one limitation of this study is that the estrous cycle stage of the virgin rats was not determined, which could provide additional information regarding *Crh-bp* expression across the cycle. To our knowledge, this is the first study demonstrating a role for CRF-BP in the PVN in regulating HPA axis responses in lactation.

Given the increased *Crh-bp* expression in the PVN together with its modulatory effect on the HPA axis under basal and stimulated conditions, we hypothesized that CRF-BP may also play a protective role in motherhood. To test this hypothesis, we monitored maternal behaviour after pharmacological inhibition of intra-PVN CRF-BP activity via local CRF(6–33) infusion. Previous studies from our lab determined that intracerebrally infused substances diffuse up to 1 mm^3 from the infusion site (Klampfl et al., 2014). Since no *Crh-bp* mRNA expression was observed in close proximity to the PVN (Fig. 1B), we are confident that the pharmacological manipulations specifically targeted intra-PVN CRF-BP. Acute CRF-BP inhibition caused a modest reduction in maternal care, specifically blanket nursing (60 min after infusion) and pup licking/grooming (90 min after infusion; Fig. 3B and C). This suggests that, under non-stress conditions, increased endogenous CRF levels due to the unavailability of the CRF-BP, lead to activation of the CRF system, thereby reducing maternal care, in line with previous research (Klampfl and Bosch, 2019a; Klampfl et al., 2016a). In addition, we found a significant increase in maternal aggression (Fig. 3D and E), consistent with previous studies showing that acute infusion of CRF(6–33) either centrally or locally into the bed nucleus of the stria terminalis (BNST) increases aggression in lactating rats (Klampfl et al., 2016b). However, other paradigms, such as acute restraint stress in lactating mice impairs maternal aggression, as well as other maternal behaviours (Gammie and Stevenson, 2006). Similarly, acute corticosterone infusion impairs maternal care and motivation (Pereira et al., 2015). Together these data suggest divergent behavioural effects following HPA axis stimulation by stress versus HPA axis activation following CRF-BP inhibition. 30 min after the maternal defence test, we observed a non-significant reduction in arched-back nursing in the CRF(6–33)-treated rats (Fig. 3F), which recovered to basal levels by 60 min (significant increase at 60 min versus 30 min after stress exposure). In contrast, no changes were observed in the control group. This suggests that CRF-BP activity is necessary to maintain constant levels of maternal care following stress exposure.

Chronic infusion of CRF(6–33) into the PVN had little impact on any nursing behaviour after exposure to the elevated platform (Supplementary Table 3). We hypothesize that the continuous CRF-BP inhibition, which started 24h in advance, might promote other physiological adaptations early on to counteract the increased availability of CRF and to minimize the impact on maternal care. Indeed, we previously described increased release of the pro-maternal peptide, oxytocin, within the MPOA following CRF-R1 activation, which is thought to provide a buffering mechanism facilitating a rapid return of the mother to her offspring (Klampfl et al., 2018). Furthermore, there is evidence that PVN *Crh* levels are not altered by acute stress exposure during early lactation (LD3) (da Costa et al., 2001), thus exposure to the elevated platform might not be a sufficient stimulus to induce different responses in VEH- or CRF(6–33)-treated rats. Our findings on maternal care contrast with studies in rats using chronic postpartum scarcity stress or postpartum corticosterone infusions, which induce deficits in maternal behaviour (Brummelte and Galea, 2010; Rincon-Cortes and Grace,

2022). That said, chronic daily restraint stress does not alter maternal behaviour in mice (Gammie and Stevenson, 2006), suggesting there are species-specific modulations.

Despite the lack of effects on maternal care, chronic inhibition of CRF-BP affected other aspects of maternal behaviour, which may be considered more 'active' behaviours. In particular, CRF(6–33)-treated rats appeared to display less maternal motivation compared to VEH-treated rats, despite not reaching statistical significance (Fig. 4B). Moreover, in contrast to acute inhibition, chronic CRF(6–33) infusion strongly impaired maternal aggression, as seen by the reduced number of attacks (Fig. 4C) and overall Z-aggression (Fig. 4D). This is consistent with a study in CRF-BP deficient mice showing reduced maternal aggression (Gammie et al., 2008), suggesting that long-term inhibition of CRF-BP activity, presumably leading to persistently increased CRF levels, can impair protective behaviours. Conversely, an acute manipulation might induce or aid an active response to the psychosocial stressor. Indeed, maternal aggression is related to anxiety-like behaviour (Bosch, 2013), thus it is plausible that a certain amount of CRF-R activation is required to respond to an acute social stress, while excessive CRF signalling might impair this behaviour. Furthermore, since we did not find any effect on anxiety-like or passive-stress coping behaviour, we propose that the CRF-BP might play a more prominent role in a psychosocial stress context. Indeed, a previous study described improved social interaction in socially stressed male rats after CRF-BP inhibition in the BNST (Vasconcelos et al., 2019), further highlighting the complex role of the CRF-BP in regulating social stress responses.

A transient increase in licking/grooming 30 min after the maternal defence test was observed in both groups compared to their respective pre-test levels, although this was only significant in animals receiving CRF(6–33) (Fig. 4E). It is interesting to note that enhanced self-grooming behaviour is often observed after CRF infusion (Dunn et al., 1987; Wiersielis et al., 2016), indicating arousal responses following the activation of the stress axis. Furthermore, increased licking/grooming was also observed after the maternal defence test in combination with increased CRF signalling within the MPOA (Klampfl et al., 2018). Since licking/grooming has been linked to oxytocinergic activity (Champagne et al., 2001; Pedersen and Boccia, 2002, 2003) and the CRF and oxytocinergic systems can modulate each other (Klampfl et al., 2018; Bosch et al., 2016; Winter and Jurek, 2019), we speculate that the brief increase in licking/grooming might arise from transiently increased local oxytocin levels, which might also represent a mechanism to alleviate the stress response. Interestingly, oxytocinergic magnocellular neurons in the PVN express CRF-R1 (Ugartemendia et al., 2022) and CRF-R2 (Dabrowska et al., 2011), suggesting local interaction of the two systems, which, during lactation, might help to restore maternal behaviour due to changes in oxytocinergic transmission. On the other hand, we found a significant reduction in baseline nursing (Fig. 4G) the day after the maternal defence test in both groups, whereas no changes were observed following the other stressors (elevated platform, EPM and FST), suggesting that psychosocial stressors might have longer-lasting effects on social behaviours like nursing, than stressors that do not involve a social component. Altogether, these findings suggest that chronic CRF-BP inhibition followed by an exposure to social stress can acutely impair protective behaviours (maternal aggression) and have a modest negative impact on maternal care.

Anxiety levels are generally dampened during lactation (Neumann, 2001; Pereira et al., 2005; Lonstein, 2007). Our data indicates that CRF-BP in the PVN does not appear to modulate anxiety-like behaviour in lactating rats, consistent with a previous study in lactating CRF-BP knockout mice (Gammie et al., 2008). However, there is evidence that CRF-BP deficient mice of both sexes show increased anxiety-like behaviour on the EPM and in the defensive withdrawal test (Karolyi et al., 1999). This suggests that if there is a role for CRF-BP in mediating reduced anxiety-like behaviour in lactating rats, it is likely acting in a brain region other than the PVN.

Exposure to the elevated platform on LD8 resulted in a similar increase in plasma corticosterone secretion in rats given CRF(6–33) chronically and those given VEH (Fig. 4H). We speculate that the experimental protocol used, involving multiple testing and stress exposure, might represent a model of mild chronic stress, which may have induced changes at the level of the PVN. Indeed, a switch from CRF to arginine vasopressin as the primary driver of the HPA axis has been reported for several models of chronic stress (Aguilera and Rabadan-Diehl, 2000; Keeney et al., 2006). If such a shift occurred here, then inhibiting CRF-BP would be unlikely to impact corticosterone secretion. Alternatively, our experimental protocol may have induced a ceiling effect in corticosterone secretion, which could potentially mask differential responses to the acute stress exposure on LD8. However, another interpretation of the current findings is simply that CRF-BP in the PVN of lactating rats is effective in restraining HPA axis activity under acute, but not chronic stress conditions. Lastly, to our knowledge, there is no information available on the peptide stability and activity under our experimental conditions. Thus, the data from the chronic infusion study should be interpreted with caution, though the significant impact on aggressive behaviour suggests the peptide is still active on day 6.

5. Conclusions

Our study provides novel insights into the role of the CRF-BP in the PVN during the sensitive lactation period. Furthermore, we provide the first evidence for the involvement of CRF-BP in the PVN in controlling HPA axis activity during lactation, highlighting a novel mechanism that contributes to the reduced responsiveness of the HPA axis to acute stress in lactating rats (Brunton et al., 2008; Slattery and Neumann, 2008). We found that gene expression for members of the CRF family in the PVN change during lactation compared to the nulliparous state, possibly contributing to the behavioural adaptations to motherhood. Moreover, both acute and chronic inhibition of CRF-BP activity can impair different aspects of maternal behaviour, while only acute inhibition evidently modulates HPA axis responsiveness, both under basal conditions and in response to an acute challenge. Hence, while a chronically dysregulated brain CRF system can severely alter postpartum adaptations like maternal-infant bond and maternal emotionality, plasma corticosterone measurements might not necessarily be a reliable indicator. Since postpartum stress is a crucial risk factor for the development of psychopathologies and/or for impaired maternal behaviour, further studies are needed to identify potential biomarkers of stress-induced neglect.

Taken together, this study suggests that intact activity of the CRF-BP in the PVN is necessary to maintain dampened HPA axis activity in lactation and to induce adequate maternal and active responses to a psychosocial stressor, such as during the maternal defence test.

Funding

This study was supported by the Deutsche Forschungsgemeinschaft (DFG; BO1958/8-1, BO1958/8-2 to O. J. B.) and the Biotechnology and Biological Sciences Research Council (BBSRC; BB/J004332/1 to P. J. B.).

CRediT authorship contribution statement

Alice Sanson: Writing – original draft, Visualization, Formal analysis. **Paula Krieg:** Writing – review & editing, Investigation, Formal analysis. **Milena M. Schramm:** Writing – review & editing, Investigation, Formal analysis. **Kerstin Kellner:** Writing – review & editing, Investigation. **Rodrigue Maloumby:** Investigation. **Stefanie M. Klampfl:** Writing – review & editing, Supervision, Investigation, Formal analysis. **Paula J. Brunton:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition. **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.

Data availability

Data will be made available on request.

Acknowledgements

We thank M. Fuchs, A. Havasi and M. Zanicolo for excellent technical support. We thank L. Demarchi for scientific discussion.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2024.100631.

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